Novel *O*-glycan arrays to characterize human cancer-associated epithelial antigens

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by

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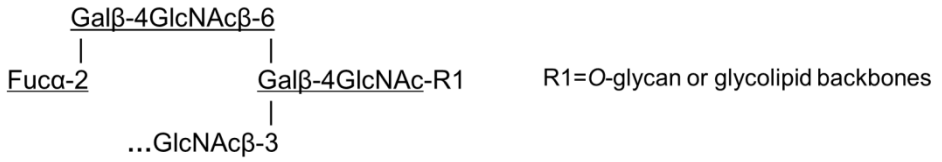
ABSTRACT

Aberrantly expressed carbohydrates occur on cancer cells and are antigenic. This thesis is focused on studies to elucidate two elusive hybridoma-defined, cancer-associated carbohydrate antigens: a prostate cancer-associated antigen F77 and a broadly distributed epithelial cancer-associated antigen AE3.

The key experiments performed are microarray analyses with mucin-type glycoproteins and generation of designer arrays, multidimensional chromatographies and mass spectrometry of *O*-glycomes. Antigen-positive sequences assigned are corroborated with focused arrays of natural and chemically synthetized oligosaccharides or glycolipids and products of glycosidase treatments.

The F77 antigen is assigned as blood group H type 2 on a 6-linked branch of a poly-*N*-acetyllactosamine backbone (structure a). This sequence is shown to occur on *O*-glycans and on glycolipids. The F77 antibody can bind, with lower intensities, to the blood group A and B analogues of structure a. The minimum F77 antigenic sequence is shown to be a pentasaccharide (underlined in structure a). The close association of F77 antigen with prostate cancer is proposed to be a consequence of up-regulation of branching enzymes together with persistent expression of H antigen. This may account for the prevalence of F77 antigen in prostate cancers irrespective of the patients' ABO blood group status.

a



There appear to be two distinct forms of AE3 antigen as evidenced by the ability of AE3 antibody to bind: (i) the *O*-glycan structure b, known as T antigen, which is joined by α-linkage to serine on mucin-type glycoproteins, and (ii) a sulphated glycolipid known as SM1a (structure c) in which "T" sequence is joined by β-linkage to galactose. An *O*-glycan analogue of structure c (structure d), has not been

reported so far. Chemo-enzymatic synthesis of structure d will shortly be attempted for antigenic analysis.

b Galβ-3GalNAcα-R2 R2=Serine, poly acrylamide, MUC1, MUC5Ac and aminopropyl spacer

c Galβ-3GalNAcβ-4Galβ-4Glcβ-Cer
|
SU-3

d Galβ-3GalNAcβ-4Galβ-4GlcNAcβ-3GalNAc
|
SU-3

With knowledge of details of these two antigens, the biosynthetic pathways, the biological functions and clinical applications can now be rationally pursued.

DECARATION OF ORIGINALITY

I, Chao Gao, hereby declare that the work presented in this thesis is my own work. Where I have used materials and results from other sources, this is clearly stated. This thesis has not been submitted for a degree to any other academic institution.

Most of the results in Chapter 3 of this thesis have been published in the two papers below.

Gao, C., Liu, Y., Zhang, H., Zhang, Y., Fukuda, M. N., Palma, A. S., Kozak, R. P., Childs, R. A., Nonaka, M., Li, Z., Siegel, D. L., Hanfland, P., Peehl, D. M., Chai, W., Greene, M. I., and Feizi, T. (2014) Carbohydrate Sequence of the Prostate Cancer-associated Antigen F77 Assigned by a Mucin *O*-Glycome Designer Array. *J Biol Chem* 289, 16462-16477

Nonaka, M., Fukuda, M. N., Gao, C., Li, Z., Zhang, H., Greene, M. I., Peehl, D. M., Feizi, T., and Fukuda, M. (2014) Determination of Carbohydrate Structure Recognized by Prostate-specific F77 Monoclonal Antibody through Expression Analysis of Glycosyltransferase Genes. *J Biol Chem* 289, 16478-16486

The work described in Section 3.8 has been presented at the 2014 Meeting of the Society for Glycobiology and the abstract has been published as below.

Gao, C., Imamura, A., Liu, Y., Zhang, H., Zhang, Y., Chai, W., Kiso, M., Greene, M., and Feizi, T. (2014) Clinching the elusive prostate cancer antigen F77. in 2014 *Joint Meeting of the Society for Glycobiology and the Japanese Society of Carbohydrate Research*, Glycobiology 24, 1195

Chao Gao

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ABBREVIATIONS

Abbreviations for carbohydrates

dHex	deoxyhexose
Fuc	fucose
Gal	galactose
GalNAc	*N*-acetylgalactosamine
GalNAcol	*N*-acetylgalactosaminitol
Glc	glucose
GlcA	glucuronic acid
GlcNAc	*N*-acetylglucosame
Hex	hexose
HexNAc	*N*-acetylhexosamine
HexNAcol	*N*-acetylhexosaminitol
LacNAc	*N*-acetyllactosamine
Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
poly-LacNAc	poly *N*-acetyllactosamine
SU	sulphate
SA	sialic acid
Xyl	xylose

Abbreviations for lectins

AAL	*Aleuria Aurantia* lectin
Con A	Concavalin A
HPA	*Helix pomatia* agglutinin
L-PHA	*L-phytohaemagglutinin*
LCA	*Lens culinaris* agglutinin
PNA	Peanut agglutinin
RCA_{120}	*Ricinus Communis* agglutinin I
UEA-I	*Ulex Europaeus* agglutinin I
WGA	*Wheat Germ* agglutinin

Abbreviations for amino acids

Asn	asparagine	N
Ser	serine	S
Thr	threonine	T
Pro	proline	P
Isoleucine	Ile	I

Other abbreviations

AFP	α-fetoprotein
ADCC	antibody dependent cell-mediated cytotoxicity
ADHP	*N*-aminoacetyl-*N*-(9-anthracenylmethyl)-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine
AOPE	aminooxy-functionalized DHPE
BSA	bovine serum albumin

CA 15-3	cancer antigen 15-3
CA 19-9	cancer antigen 19-9
CAR	chimeric antibody receptor
CCR	chimeric costimulatory receptor
CD	cluster of differentiation
CDC	complement dependent cytotoxicity
CEA	carcinoembryonic antigen
Cer	ceramide
CFG	Consortium of Functional Glycomics
CID	collision induced dissociation
CTCs	circulating tumour cells
CRD	carbohydrate-recognition domain
DAB	3,3'-diaminobenzidine
DC-SIGN	dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin
DHPE	1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinases
ES	electrospray
FACS	fluorescence activated cell sorter
Fc	constant region
Fv	variable fragment
GAG	glycosaminoglycan
GPI	glycosylphosphatidylinositol
HBS	HEPES buffered saline
HCA	human carcinoma antigen
HCC	hepatocellular carcinoma
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
Ig	immunoglobulin
mAb	monoclonal antibody
MS	mass spectrometry
MALDI	matrix-assisted laser desorption/ionization
MIC	micronemes
MICA	MHC class I-related chain A
MUC1	Mucin-1
MUC5Ac	Mucin-5Ac
NGL	neoglycolipid
NHS	*N*-hydroxysuccinimide
NKG2D	natural killer group 2 member D
PBS	phosphate buffered saline
PRRs	pattern recognition receptors
PSA	prostate-specific antigen
PSCA	prostate stem cell antigen
PSGL-1	P-selectin glycoprotein ligand 1
PSM	porcine stomach mucin
PSMA	prostate-specific membrane antigen

scFv	single chain variable region
Siglec	sialic acid-binding immunoglobulin-type lectins
SIGN-R	specific intercellular adhesion molecule 3-grabbing non-integrin-related
SPR	surface plasmon resonance
SSEA	stage specific embryonic antigen
SU	sulphate
TGF-β	transforming growth factor beta
TLC	thin-layer chromatography
TM	transmembrane domain
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor-2
VNTR	variable number of tandem repeat

Chapter 1
INTRODUCTION

1.1 General introduction to carbohydrates

As one of the major classes of molecules of a living organism, carbohydrates are ubiquitously distributed. Ranging in size from monosaccharide to polysaccharides of thousands of units (1,2), carbohydrates serve crucial biological roles. For instance, mammals utilize monosaccharide glucose as the major source of metabolic fuel and polysaccharide glycogen for energy storage. Carbohydrates coat most cells in the form of glycoconjugates, e.g. glycoproteins, glycolipids and proteoglycans. Many proteins, secreted or inside cells, have oligosaccharides that modulate their properties and activities. Apart from serving as structural components, these glycan chains also play key roles in almost every biological process and, directly or indirectly, in almost every major disease (3-5).

The numerous functions of carbohydrates are based on their structural diversity. Compared with nucleotides and amino acids, monosaccharides can be linked together in many ways. Each monosaccharide can theoretically be linked in either an α- or a β- linkage (anomers) to one of several positions on another monosaccharide or to another type of molecule. Thus carbohydrates have enormous structural complexity and heterogeneity in terms of, for example, monosaccharide composition, linkage position, branching pattern and anomeric configuration. Moreover, the structural diversity can be further expanded by modifications such as sulphation and phosphorylation (6,7).

Unlike the protein sequences, which are primary gene products, glycan structures are not encoded directly by the genome but are products of a cohort of sequentially acting and competing glycosyltransferases and glycosidases. Therefore, even with information in the expression levels of the relevant genes in a given cell type, the precise prediction of all the glycan structures is unachievable. Additionally, biosynthesis of the glycans is dynamically changed by metabolism, signal transduction and cellular status (8,9). This variable and dynamic nature of glycosylation potentially makes glycans more difficult to study than nucleic acids and proteins. It also results in a myriad of complex glycoconjugates of biological

importance. The structural characteristics of glycoconjugates and their biological functions are reviewed in the sections that follow.

1.1.1 Major classes of glycoconjugates

According to the type of carrier molecule (aglycone), the common classes of glycoconjuates found in or on eukaryotic cells are defined as glycoproteins, proteoglycans and glycosphingolipids as shown in Figure 1.1. The glycans of different classes have distinctive core regions whereby they are connected to the aglycones.

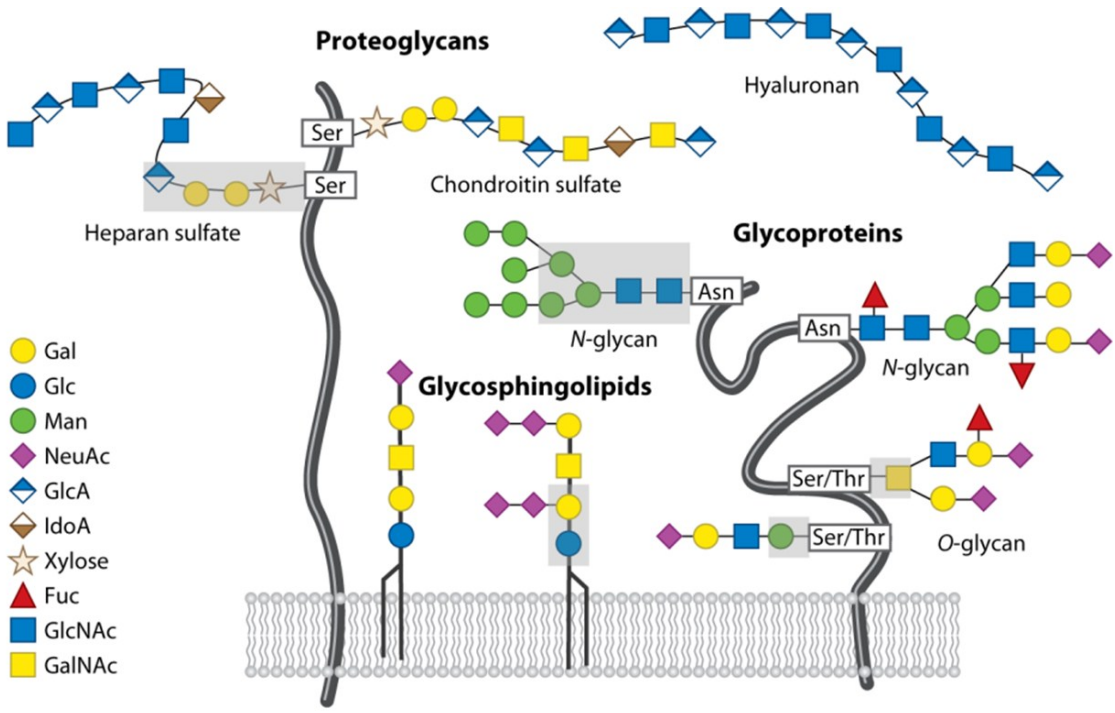


Figure 1.1 Common classes of mammalian glycans. The common core structures of each class are boxed in grey. Sequences are representative structural variations in mammalian cells. Taken from (10).

A glycoprotein is a glycoconjugate in which one or more glycans are covalently attached to a polypeptide backbone via *N*- or *O*-linkages. *N*-glycans are sugar chains covalently linked to asparagine (Asn) residues of a polypeptide chain. *N*-glycan is linked through an *N*-acetylglucosamine (GlcNAc) residue to peptide sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. The biosynthesis of *N*-glycans starts on the cytoplasmic face of the ER membrane and continues inside the endoplasmic reticulum (ER) and Golgi (Figure 1.2). *N*-glycans share a common

pentasaccharide core region and can be generally divided into three main classes: oligomannose (or high-mannose) type, complex type, and hybrid type (Figure 1.3). The most common *O*-glycans are linked to a serine or threonine residue on polypeptide backbones via core *N*-acetylgalactosamine (GalNAc). They are synthesized in the Golgi, initiated by addition of GalNAc by polypeptide-*N*-acetyl-galactosaminyltransferases (ppGalNAcTs). These *O*-glycans (also called *O*-GalNAc glycans) can be divided into many subtypes (core 1 to 8) based on their common core regions (Table 1.1). Some glycoproteins, such as mucins, carry many *O*-glycans in clustered form (2). There are also several other non mucin type *O*-glycans such as *O*-mannose, *O*-fucose and *O*-GlcNAc (3).

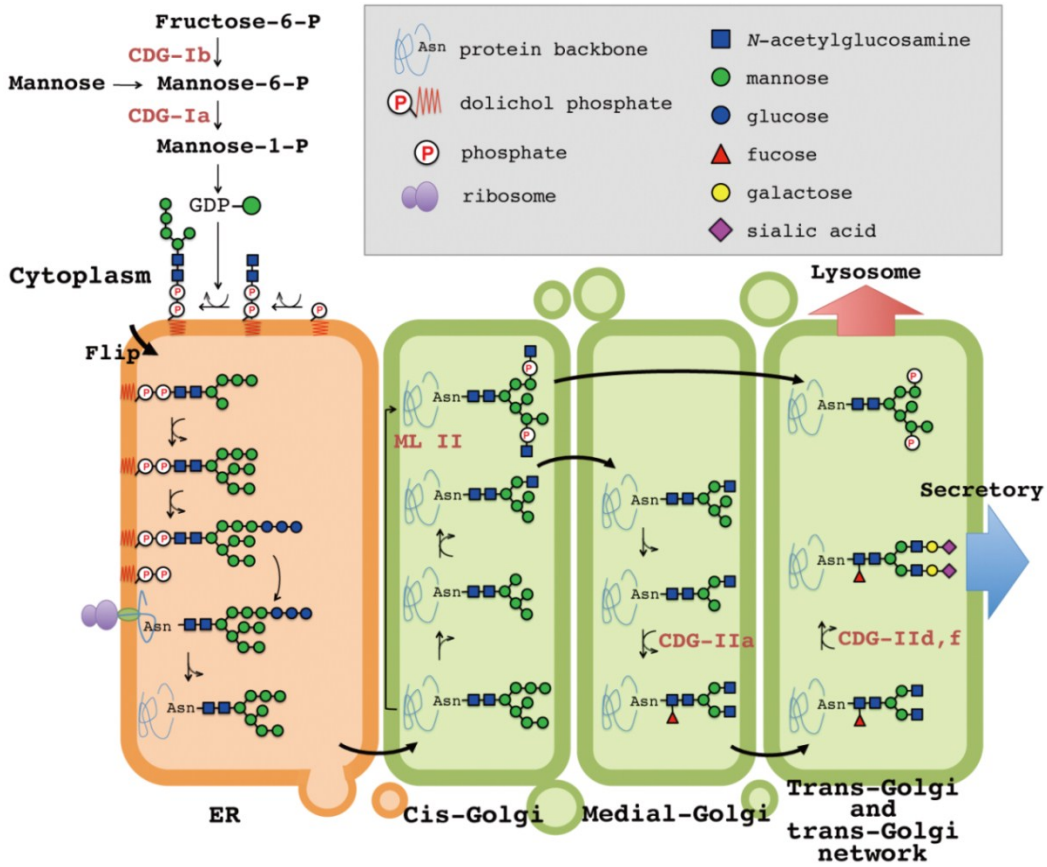


Figure 1.2 Biosynthesis and processing pathway of *N*-linked oligosaccharides. Taken from (11).

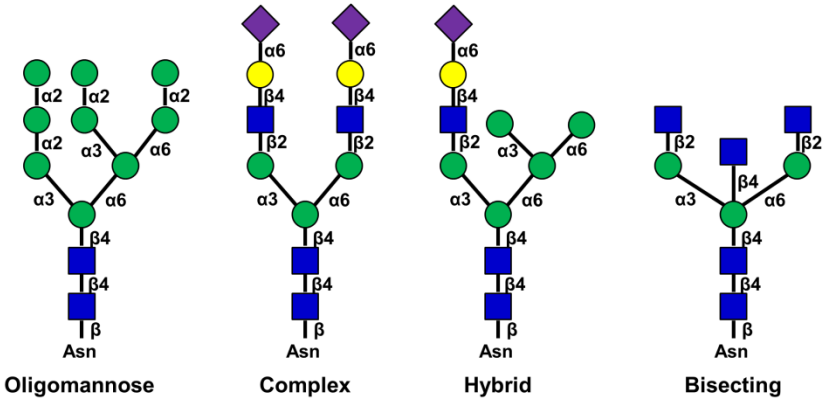


Figure 1.3 Sequences of the oligomannose, complex, hybrid and bisecting type of *N*-glycan. They all share the common core $Man_3GlcNAc_2Asn$. Figure is adapted from (12). Symbols are in Figure 1.1.

Table 1.1 Core sequences of common *O*-GalNAc glycans.

Core type	Sequence
Core 1	Galβ-3GalNAcα-Ser/Thr
Core 2	GlcNAcβ-6 | GalNAcα-Ser/Thr | Galβ-3
Core 3	GlcNAcβ-3GalNAcα-Ser/Thr
Core 4	GlcNAcβ-6 | GalNAcα-Ser/Thr | GlcNAcβ-3
Core 5	GalNAcα-3GalNAcα-Ser/Thr
Core 6	GlcNAcβ-6 | GalNAcα-Ser/Thr
Core 7	GalNAcα-6 | GalNAcα-Ser/Thr
Core 8	Galα-3GalNAcα-Ser/Thr

A proteoglycan is a glycoconjugate that has one or more glycosaminoglycan (GAG) chains attached to the hydroxyl group of a serine residue of a carrier protein. The typical core region of a proteoglycan is xylose-galactose-galactose-glucuronic acid (Xyl-Gal-Gal-GlcA). GAGs are linear polysaccharides with disaccharide repeating units normally consisting of an uronic acid and an amino sugar with sulphate substitution. Some proteoglycan polypeptides can carry both GAGs and *O*- and *N*-glycans.

Glycolipids can be classified into glycosphingolipids, glycosylphosphatidylinositols (GPIs) and glyco-glycerolipids distinguished by their lipid moieties. In vertebrates, the most abundant glycolipids by far are glycosphingolipids and are often called glycolipids for convenience. Glycosphingolipids consists of a glycan connected to the terminal hydroxyl group of the lipid moiety ceramide (Cer), which is composed of a long carbon chain base (sphingosine) and a fatty acid. Although ceramide also varies in length, hydroxylation and saturation of both sphingosine and fatty acid moieties, the major structural and functional classifications have been based on the glycans. Two types GlcCer and GalCer are distinguished based on the difference of the first sugar (Glc or Gal) linked to ceramide. Further extension of the former gives rise to a series of backbone structures (Table 1.2), ganglio-, lacto-, neolacto- and globo-series. Their patterns of expression are mostly tissue specific. Glycolipids can be neutral or anionic depending whether they have sialic acid or sulphate. Traditionally, glycosphingolipids containing one or more residues of sialic acid are called gangliosides, regardless of whether they are based on the ganglio-series backbone structure.

Table 1.2 Core sequences of major vertebrate glycosphingolipids.

Subfamily series	Sequence
Ganglio-	GalNAcβ1-4Galβ1-4Glcβ-Cer
	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-Cer
Lacto-	GlcNAcβ1-3Galβ1-4Glcβ-Cer
	Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-Cer
Neolacto-	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Cer
Globo-	Galα1-4Galβ1-4Glcβ-Cer
	GalNAcβ1-3Galα1-4Galβ1-4Glcβ-Cer

As the main subject matter of this thesis is glycoproteins and glycolipids, GAGs, GPIs and other types of glycoconjugates are not discussed further in this thesis.

1.1.2 Glycan sequences common to different glycoconjugates

Although different glycan classes have unique core regions by which they are attached to proteins, the backbone and peripheral regions are often shared.

1.1.2.1 Backbone regions

Two types of backbone regions are common to *N*- and *O*-glycans and some glycolipids. Presence of Gal in β1-3 linkage to the terminal GlcNAc residue forms a type 1 chain Galβ1-3GlcNAc (Figure 1.4), whereas its β1-4 linked analogue, Galβ1-4GlcNAc, constitutes a type 2 chain (also called *N*-acetyllactosamine, LacNAc, Figure 1.4). These backbone sequences are often further modified by addition of peripheral sequences such as fucose (Fuc), sialic acid and sulphate to form many antigenically or biologically active carbohydrate determinants such as the major blood group A, B, O (H) and Lewis antigens which are discussed below. In humans, expression of type 1 and type 2 chains is often regulated in a tissue- or lineage-specific manner. Type 1 backbone is said to be predominantly expressed in cells derived from the endoderm, whereas its type 2 counterpart is widely distributed in cells derived from the ectoderm and mesoderm (13). Indeed, type 1 backbone is mostly found in epithelial tissues of gastrointestinal or reproductive tracts but type 2 backbone is more broadly expressed (14). The regulation is the consequence of the differential expression and competition of seven β1-4 galactosyltransferases (β4GalT1-7) and five β1-3 galactosyltransferases (β3Gal-T1, -T2, -T5, -T8 and possibly -T4) in human beings. The occurrence of the multiple isoforms of the two types of enzymes suggests that the regulation of the two types of backbones is very complicated.

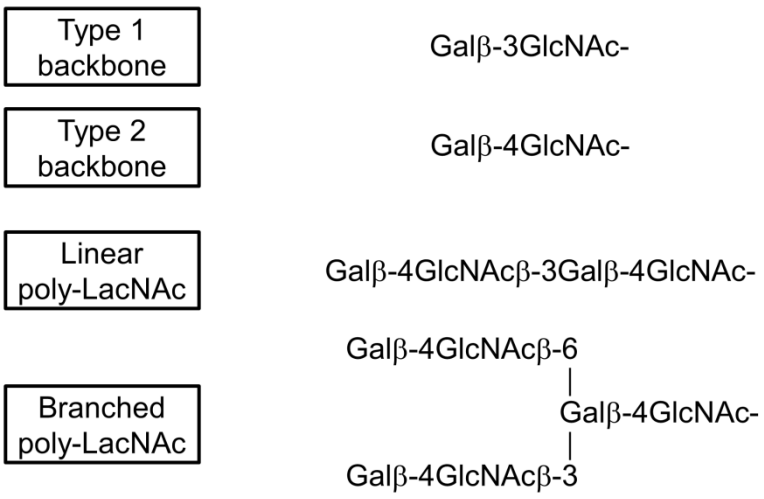


Figure 1.4 Sequences of type 1 and 2 backbones and the elongated linear and branched poly-LacNAc.

The LacNAc chain can be elongated sequentially by the addition of β3GlcNAc and β4Gal by the corresponding glycosyltransferases to form a linear poly-*N*-acetyllactosamine (poly-LacNAc) backbone (Figure 1.4). The linear poly-LacNAc backbone can become branched by addition of β1,6-linked GlcNAc to an internal Gal (Figure 1.4). Formation of the branched poly-LacNAc backbone is catalysed by β1,6-*N*-acetylglucosaminyltransferases GCNT2 (central I branching enzyme, cIGnT) or GCNT3 (distal I branching enzyme, dIGnT, Figure 1.5A); and the β1,6-linked GlcNAc branch allows for subsequent poly-LacNAc generation. In contrast to poly-LacNAc, tandem repeats of type 1 sequence are less common, although poly-LacNAc sequences are sometimes terminated with a type 1 unit in the peripheral region. The linear and branched poly-LacNAc backbones are antigenic structures that express the i and I antigens, respectively. They are found on *N*- and *O*-glycans and glycolipids. On *N*-glycans they are preferentially expressed on multi-antennary *N*-glycans, particularly on the β1-6GlcNAc branch of the pentasaccharide core. This process is controlled by mannosyl (alpha-1,6-)-glycoprotein beta-1,6-*N*-acetyl-glucosaminyltransferase (MGAT5, Figure 1.5B). On *O*-glycans, poly-LacNAc backbones often occur on the β1-6GlcNAc branch on the core GalNAc and are regulated by β1-6 glucosaminyl (*N*-acetyl) transferases (GCNT1, GCNT3 and GCNT4, Figure 1.5B). These poly-LacNAc sequences serve as scaffolds to present monosaccharides such as sialic acid, Fuc, Gal, GlcNAc at peripheral region, most of which are α-linked capping domains of the β-linked backbone. These poly-LacNAc sequences can be recognized as ligands of some endogenous carbohydrate-binding proteins, i.e. galectins (15-18). Changes in the expression of poly-LacNAc bearing i and I antigens occur during embryonic development and in carcinogenesis (19). A detailed discussion follows in Chapter 3.

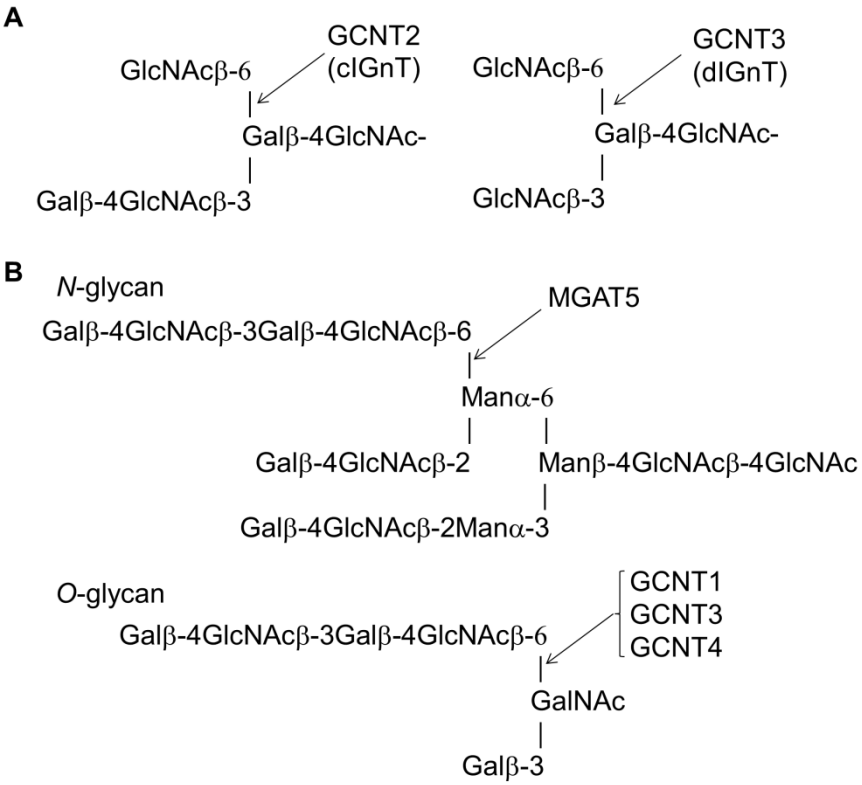


Figure 1.5 Key enzymes responsible for the biosynthesis of the I-antigens (A) and the β6-branches on *N*- and *O*-glycans (B).

1.1.2.2 Peripheral region

Type 1 and type 2 backbones are often decorated by α1-2 linked Fuc on the terminal Gal, with or without further additions of α1-3 linked GalNAc or Gal, forming the O(H), A and B blood group antigens, respectively (Figure 1.6). These antigens were discovered in the beginning of the twentieth century by Karl Landsteiner and colleagues (20,21) but it was not until 1950s that Morgan and Watkins, Kabat and their colleagues determined the structures of the carbohydrate epitopes of the A, B, O (H) blood groups (22-24). These antigens are not only expressed on the surface of red cells, but also on cells of many tissues including the vascular endothelium and a variety of epithelia and on some soluble, secreted glycoproteins and glycolipids. Therefore they are also known as histo-blood group antigens (25).

The A, B, and H antigens are formed by the concerted action of glycosyltransferases encoded by three genetic loci [the *ABO*, *H* and *Secretor* (*Se*) locus]. The synthesis starts from transfer of α1-2 Fuc to Gal catalysed by α1,2-fucosyltransferases (α1-2FucTs). Human genome encodes two α1-2FucTs, FUT1 and FUT2, encoded by *H*

and *Se* gene, respectively. FUT1 is predominantly expressed on red cells, vascular endothelium and primary sensory neurons (26) and it transfers Fuc to Galβ-4GlcNAcβ- or Galβ-3GalNAcβ- to form the H antigen. FUT2 transfers Fuc preferentially to Galβ-3GlcNAcβ- and Galβ-3GalNAcα- to form the H antigen (27) in epithelia lining the lumen of the gastrointestinal, respiratory, urinary and reproductive tracts, and some exocrine glands e.g. salivary glands (14). Individuals with an active *Se* gene express A, B, H antigens in their secretions such as saliva and are designated secretors. Individuals with an inactive *Se* gene do not express A, B, H antigens in their secretions but only the backbone sequences and are termed non-secretors. It has been found that the secretor status is associated with susceptibility to some diseases, i.e. Crohn's disease (28), and infections such as *Helicobacter pylori* and HIV. Rare humans that have inactive *H* and *Se* genes are called Bombay phenotype; their sera contain antibodies against red cells from all donors. A related phenotype is termed para-Bombay. People of para-Bombay type have an inactive *H* gene but one functional *Se* allele (29,30).

The *ABO* locus contains three alleles *A*, *B* and *O*. *A* allele encodes α1-3 *N*-acetylgalactosaminyltransferase (GTA) adding α1,3-linked GalNAc to Gal residue. *B* allele encodes α1-3 galactosyltransferase (GTB) that transfers α1,3-linked Gal to Gal residue to form blood group B determinant. *O* allele encodes a functionally inactive A/B glycosyltransferase that leaves the unmodified Fucα1-2Gal- epitope (Figure 1.6). The distribution of A and B antigens in tissues and their expression patterns during development has been reviewed in detail (13,25,31). Moreover, the expression of ABH antigens changes greatly during human oncogenesis. This is reviewed in Section 1.2.3. Other mammals such as pigs and cows have non-fucosylated αGal [so called B-like, (32)] which is synthesized by a different α1-3 galactosyltransferase from GTB. Humans lack the enzyme activity as the gene encoding this α1-3 galactosyltransferase is inactive (33). Human sera contain high titre anti-αGal antibodies which reject any nonhuman blood or organs. They are the major barrier to xenotransplantation of pig organs in humans (34).

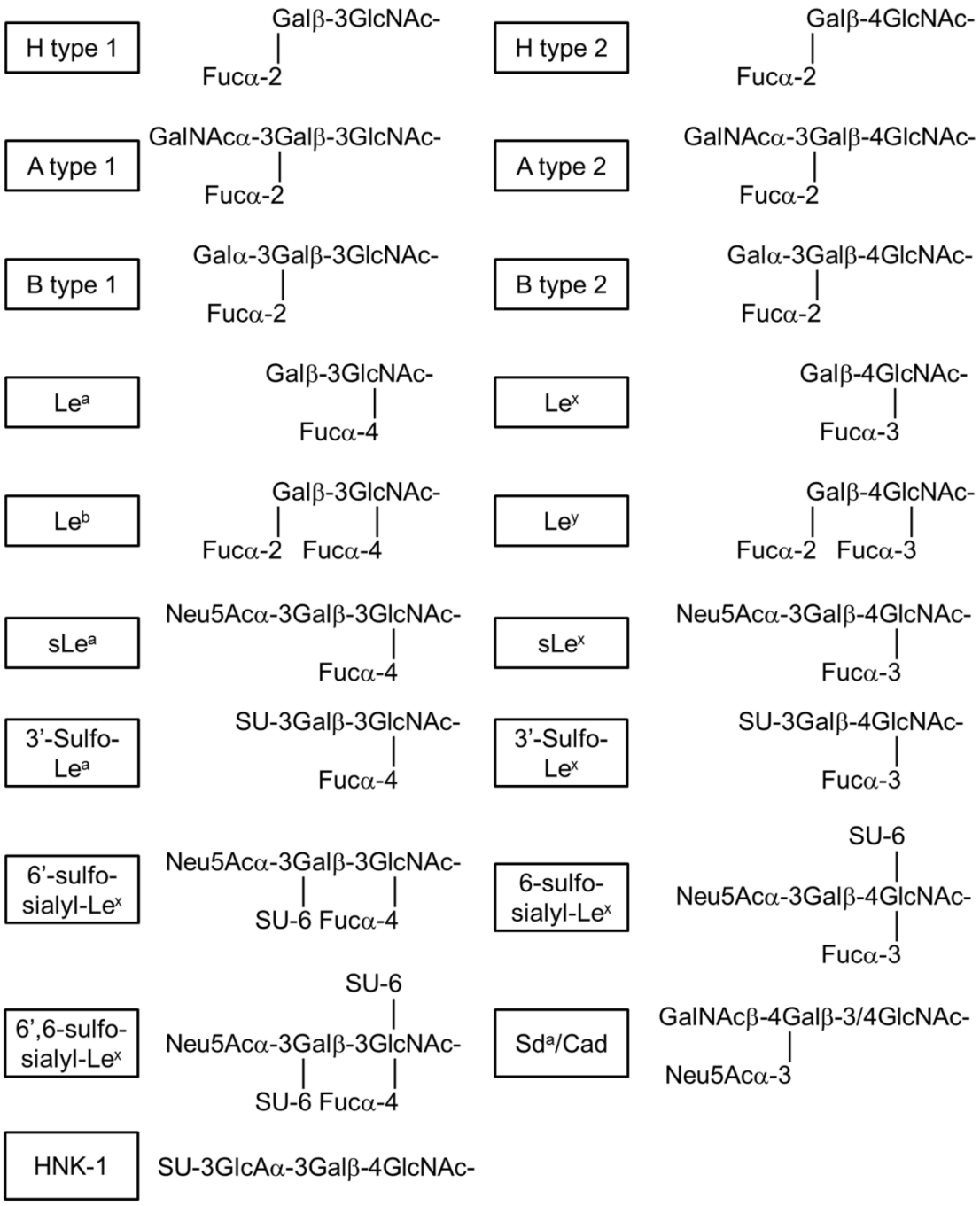


Figure 1.6 Major peripheral modifications common to different glycoconjugates.

Another common decoration in the peripheral region is the transfer of α1-3/α1-4 linked Fuc to the penultimate GlcNAc to form Lewis antigens. Combined with the backbone sequence and expression of α1-2 linked Fuc, in humans there are four Lewis antigen types named Le^a, Le^b, Le^x and Le^y (Figure 1.6), which are synthesized by eight α1-3/α1-4 fucosyltransferases (FUT3-7, FUT9-11). There also occurs sialylation or sulphation to these Lewis antigens forming 3'-sialyl $Lewis^a$ (sLe^a), 3'-sialyl $Lewis^x$ (sLe^x), 3'-sulpho-Le^a, 3'-sulpho-Le^x etc (Figure 1.6). Among them, only

two FUTs, FUT3 and FUT5 can generate the type 1 based sequences Le^a, Le^b, and sLe^a. In vivo these determinants seem to be controlled only by FUT3, which is encoded by *Lewis (Le)* locus. FUT5 is a silent enzyme which is not expressed in any tissues except in male spermatozoa (35-37). Thus, the concerted action of *Le* and *Se* loci determines the expression of type 1 based Lewis glycans synthesized in an individual. Indeed, expression of Le^a and Le^b is mainly restricted to the epithelia of gastrointestinal or reproductive tracts, where *Se* is also expressed. The type 2 based Le^x, Le^y and sLe^x determinants are synthesized by actions of α1-3 fucosyltransferases such as FUT3-7 and FUT9.

Sialic acid is a common capping modification of glycans. There are mainly three types of sialylated modifications, α2-3, α2-6 and α2-8. The α2-3 and α2-6 linked sialic acids can be found on *N*-glycans, *O*-glycans and glycolipids, but α2-3 sialylation is more ubiquitously distributed on glycans. In humans, only glycans with terminal Gal can be further modified by one of the six different α2-3 sialyltransferases (ST3Gal-I to -VI) to generate the α2-3 sialylated structures. These sequences are generally not further elongated or capped with α1-2Fuc, α1-3Gal or α1-3GalNAc. The α2-6 linked sialylated sequences are found on terminal Gal, terminal or penultimate GlcNAc, and internal GalNAc. These are the products of at least six α2-6 sialyltransferases (ST6Gal-I and -II, and ST6GalNAc-I to -IV). These sequences are normally not modified further except where there is poly α2-8 sialic acid capping. The α2-8 sialylated glycans are expressed in nervous system and in other tissues during embryogenesis, generated by at least six α2-8 sialyltransferasese (ST8Sia-I to VI). The α2-8 sialylation can occur on α2,3-linked sialic acid to form di- or trisialic acid sequences on gangliosides. The repeated α2-8 linked sialic acids of 200 or more occur on α2-3 or α2-6 sialylated *N*-glycans to form polysialic acid, specifically by ST8Sia-II (STX) or ST8Sia-IV (PST-1 or PST). Glycolipids carry single or multiple α2,8-linked sialic acid catalysed by ST8Sia-I, -III and -V. Apart from the linkage, modifications also occur to the neuraminic acid structure, such as *O*-acetylation, *O*-sulphation, *O*-methylation, de-*N*-acetylation (38). Sialic acids play important biological roles biologically and pathologically. These have been carefully reviewed by Varki and colleagues (39,40). The relevance to cancer is discussed in Section 1.2.5.

One well-known example of differential recognition of α2-3 or α2-6 sialylated sequences is by the human and avian influenza viruses. The haemagglutinin in the envelope of influenza viruses binds to sialic acid-bearing cellular receptors, and this binding is followed by release by neuraminidase, both of which are important for infection. Viruses infecting birds and pigs more commonly recognize α2,3-linked sialic acid, whereas those infecting humans prefer the α2,6-linked form. A single amino acid change to the haemagglutinin may change its specificity predisposed to a human influenza pandemic (41,42).

Another important peripheral sequence is Sd^a/Cad (Figure 1.6). It is expressed on glycoproteins of erythrocytes or in urine (i.e. Tamm-Horsfall glycoprotein) of many mammals, as well as on glycolipids of various gastrointestinal tissues (43,44). It shares the same precursor [NeuAcα-3Galβ-4(3)GlcNAc-] as sLe^a or sLe^x in its biosynthesis. The key glycosyltransferase for the generation of Sd^a/Cad is β1,4GalNAc-transferase that transfers GalNAc to the Gal of the 2,3-sialylated type 2 units. Expression of Sd^a is found in developing germ cells and spermatogonia in several species (45), suggesting this glycan sequence is a good marker for these cells. As both β1,4GalNAc-transferase and fucosyltransferase utilize the same acceptor, increased expression of Sd^a/Cad is often accompanied by reduction in expression of sLe^a or sLe^x, which are involved in cancer cell adhesion mediated by selectins. Restoration of Sd^a expression could markedly decrease the adhesion of cancer cells and suppress the metastatic potential (46).

Sulphation is usually found on Gal, GlcNAc, GlcA and GalNAc at internal or terminal positions in vertebrates. The most abundant sulphated glycans are GAGs i.e. heparin, heparan sulphate, chondroitin sulphate and keratan sulphate on proteoglycans - a detained discussion of which is beyond the scope of this thesis. Many sulphated sequences such as 3'-sulpho-Le^x, 6'-sulpho-sLe^x, 6-sulpho-sLe^x and 6,6'-sulpho-sLe^x (Figure 1.6) are expressed on *O*-glycans of high endothelial venules (Figure 1.6). They are ligands for L-selectin on lymphocytes. Homing of lymphocytes to HEV is mediated by binding of L-selectin to sulphated sialylated Lewis sequences (47,48). Another example of a sulphated glycan ligand found on *N*-glycans and *O*-glycans, as well as GAGs and glycolipids, is HNK-1 epitope, which is composed of a 3-O-sulphated GlcA attached in α1-3 linkage to a terminal LacNAc (Figure 1.6). The HNK-

1 epitope is expressed on a variety of neuronal cell glycoproteins in the vertebrate nervous system and serves as ligand for laminin, L-selectin, P-selectin, and a cerebellar adhesion protein termed amphoterin. The epitope is expressed on *O*-mannosyl-glycans among brain glycopeptides (49). Sulphation is normally catalysed by specific sulphotransferases. Dozens of sulphotransferases have been described with various substrate specificities. Among them, the sulphation of GlcA on HNK-1 is generated possibly by carbohydrate sulphotransferase 10 (CHST10).

Other capping modifications of glycan backbones such as Galα1-3Gal- and GalNAcβ1-4GlcNAc- (LacdiNAc) are not within the scope of this thesis and are not discussed further. But they are also common modifications and may play important roles in physiological and pathological settings.

1.1.3 Biological functions of glycans

The biological functions of carbohydrates are very diverse: some are crucial for growth, development or survival of human beings and other living things, but others are more subtle. Generally, these biological roles can be divided into two categories: (1) the structural and modulatory roles, which normally do not require binding by other molecules and (2) the specific recognition of epitopes that needs the binding by other molecules such as glycan-binding proteins (GBPs). In this regard, the structural information contained in the entire complement of glycans (glycome) is deciphered by these GBPs, resulting in various biological effects including host-pathogen interactions, cell trafficking and transmembrane signalling (50). According to the type of recognition, the second category can be further subdivided into two groups: (1) the endogenous recognition system (2) the exogenous recognition system of host-pathogen interactions. These aspects are discussed below.

1.1.3.1 Structural and modulatory roles of glycans

Many proteins have glycan chains attached to them. These glycans influence the glycoproteins through indirect mechanisms on their conformation, stability, oligomerization, folding, turnover and even recognition specificities (51).

Many glycans expressed on therapeutic proteins have modulatory functions. Alterations of these glycans by glycoengineering have a great impact on the biological activities and half-life of these glycoproteins. Erythropoietin (EPO), for instance, carries three sialylated complex forms of *N*-glycans and one sialylated *O*-glycan. Alpha2,3-sialylated EPOs have longer half-life than the non-sialylated analogues in the circulation. This is because in the liver, galactose-terminated glycoproteins can be bound by galactose-binding asialoglycoprotein receptor. The α2,3-linked sialic acid blocks this interaction and prevents the glycoprotein from rapid clearance from the circulation thereby prolonging the effective circulating time in vivo (52). Similar to EPO, optimization of glycosylation of many recombinant glycoproteins, e.g. recombinant monoclonal antibodies (mAb) and cytokines is desirable as glycan chains often have great impact on the stability, activity, antigenicity and pharmacodynamics.

To date, all licensed recombinant mAbs have been of the IgG class. Human IgG is a glycoprotein with *N*-glycans attached at Asn297 in the Fc region (Figure 1.7). There are, in total, 32 possible *N*-glycan sequences on each glycosylation site on the heavy chain, and the two heavy chains can be differently glycosylated (53). This gives rise to a very diverse profile of glycosylation for IgG. Most of the *N*-glycans are neutral, with <10% of structures being monosialylated or disialylated (53,54). Some of the immunological functions of antibodies are crucially dependent on these *N*-glycans on IgG Fc.

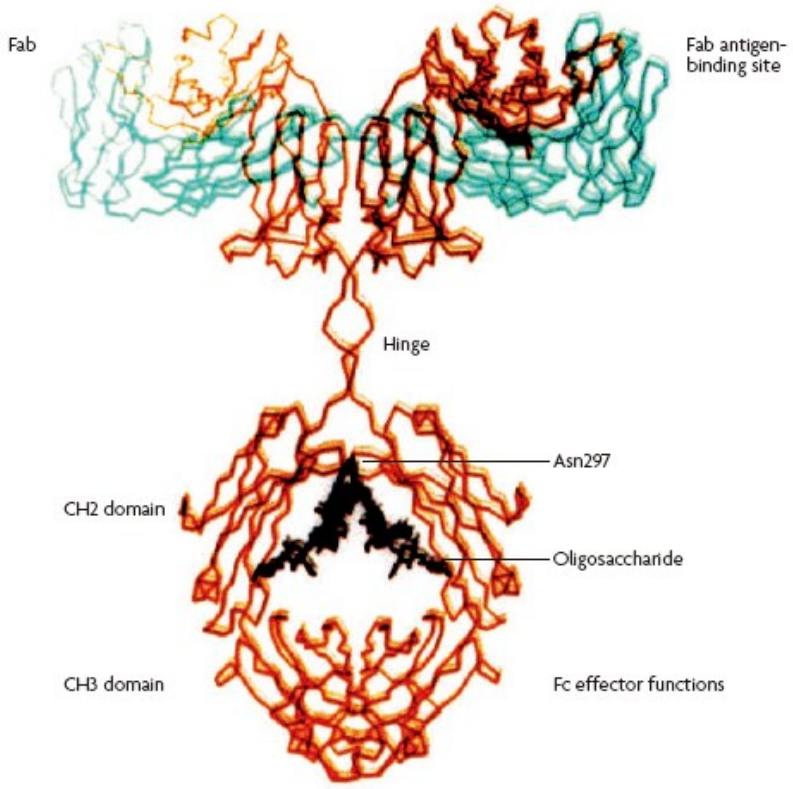


Figure 1.7 Modelling structure of the IgG molecule. The glycan chain is integral to the protein structure and has a defined conformation. CH, constant heavy; Fab, antigen-binding fragment; Fc, crystallizable fragment. Taken from (53) with permission.

Studies have shown that both antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) are dependent on appropriate glycosylation (55,56). In order to increase ADCC and CDC of these therapeutic antibodies, many researchers have tried to manipulate the glycan structures by using engineered host cells such as Chinese hamster ovary (CHO) and human embryonic kidney (HEK) cells. For example, recombinant mAbs with bisecting *N*-glycans (Figure 1.8) were generated by transfecting CHO cells with β-1,4-*N*-acetylglucosaminyltransferase III (GnTIII or MGAT3). The antibodies produced in these cells exhibited a substantial increase in ADCC and Fcγ receptor III (FcγRIII) binding, thus improving the efficacy for treatment of cancer such as neuroblastoma (57). Given that the formation of bisecting GlcNAc prevents the enzymatic functions of other GlcNAc-transferases and fucosyltransferase which leads to the decrease of mutiantennary *N*-glycans and core-fucosylation [(58), Figure 1.8], the relationship between non-fucosylated *N*-glycans and enhanced Fcγ receptor binding was further investigated. Some variants of CHO cells and rat hybridoma cells were used to generate non-fucosylated IgG and these antibodies showed enhanced ADCC (59-61).

Furthermore, reduction or removal of the α1-6 fucosyltransferase (FUT8) that is responsible for transferring the core Fuc in the host cells boosted ADCC (62,63). Although the molecular basis for the increased affinity of non-fucosylated IgG Fc for FcγRIII has not been clear, it was proposed that the Fuc on IgG Fc may affect the binding between the heavily glycosylated FcγRIII with Fc by steric inhibition (64).

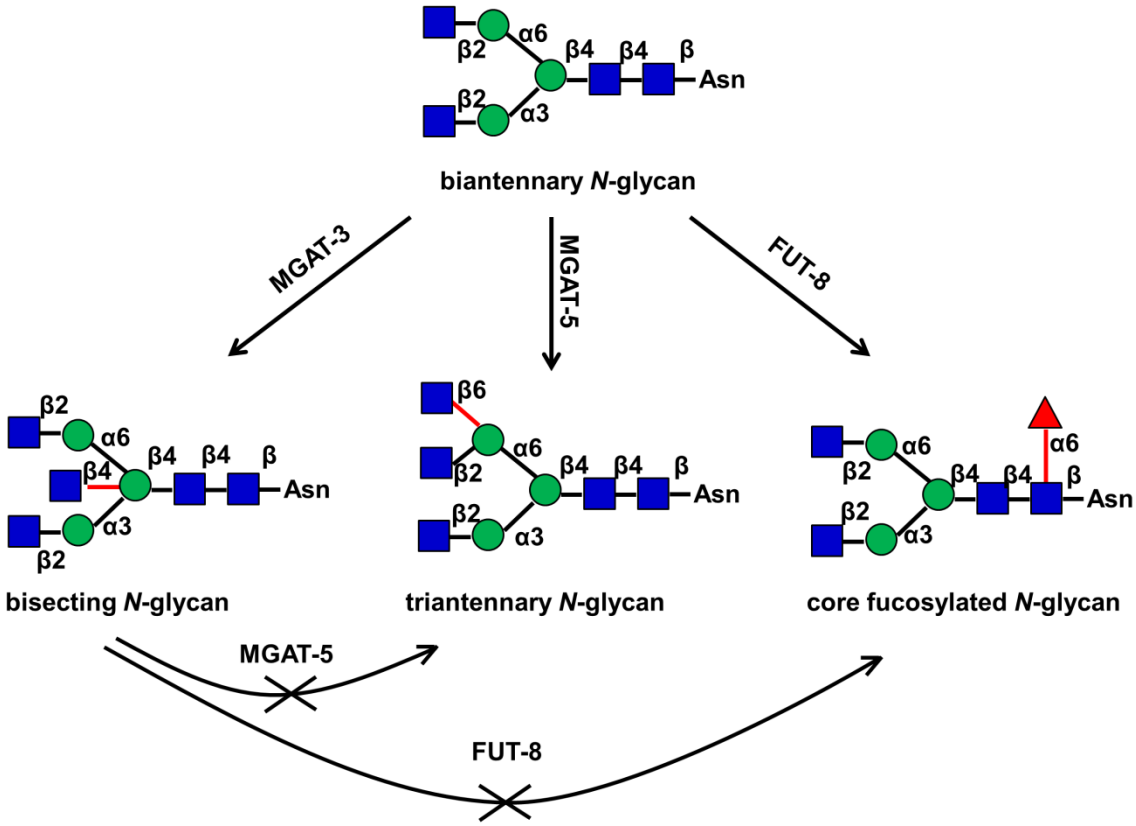


Figure 1.8 Scheme of further modification of biantennary *N*-glycan by MGAT-3, MGAT-5 and FUT-8 to form the bisecting, triantennary and core fucosylated *N*-glycan. The bisecting GlcNAc prevents the formation of triantenary and core fucosylated *N*-glycans. Highlighted in red are the glycosidic bonds that can be formed by the three glycosyltransferases. MGAT-5 and FUT-8 can only act in the absence of bisecting GlcNAc.

Distinct from the cytotoxic activity, the IgGs can also display anti-inflammatory activity through inhibitory Fc receptors such as FcγRIIB. Intravenous immunoglobulin (IVIG) has been widely used for decades to treat a variety of acute and chronic autoimmune diseases (65). High doses (1-2 g/kg) of IgG purified from sera of thousands of healthy donors were given to patients in order to treat auto-immune diseases such as immune thrombocytopenia, rheumatoid arthritis and systemic lupus erythematosus. The anti-inflammatory activity has been found to be associated with the *N*-glycans on IgG Fc fragment and particularly, a minor fraction containing α2-6 sialylated biantennary *N*-glycans. These sialic acid-containing antibodies showed decreased

affinity to the activating receptor FcγRIII but induced the expression of the inhibitory receptor FcγRIIB (66,67). The low percentage of sialylated IgG among the total pool IgGs explained the requirement of high dose for IVIG. This was further demonstrated using a recombinant human IgG1 Fc with a α2-6 sialylated biantennary glycan. The glycoengineered IgG Fc recapitulated the in vivo anti-inflammatory activity of intact IVIG, but a much lower dose was required (68).

Although existence of specific receptors for recognition of the IgG *N*-glycans cannot be ruled out, the crystallographic analysis of the IgG Fc region has shown that the *N*-glycans mainly display modulatory effects to the IgG glycoproteins. The *N*-glycans on Asn297 are located in a hydrophobic patch and form multiple non-covalent interactions with IgG Fc (Figure 1.7), with only the Manα1-3 branch protruding into the space between the Fc domains (69). These intramolecular interactions help to maintain the conformation of Fc, which is essential for binding by FcγRs. Some reports showed that binding sites of Fc for FcγRs only involves protein moiety. However, recognition of FcγRs is markedly influenced by *N*-glycans on Fc as different oligosaccharides show different modulatory abilities to the conformation of IgG Fc (53). There were reports on the binding to the sialylated Fc by receptors other than FcγRs, i.e. C-type lectin, specific intercellular adhesion molecule 3-grabbing non-integrin-related 1 (SIGN-R1) and dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN; also known as CD209) in mouse and human, respectively (70,71). It was proposed that binding of SIGN-R1 or DC-SIGN to the sialylated glycans on Fc triggered downstream signalling which resulted in the increased ratio of inhibitory- versus activating-FcγR on the vicinal effector cells, thereby attenuating autoimmunity (68). The conclusion, however, is based on cellular studies. Involvement of other molecules on the cell surface in the recognition cannot be excluded. SIGN-R1 or DC-SIGN showed strong binding to mannose-type and Lewis-type carbohydrates but weak or no binding to sialylated glycans (72-74). Therefore it would seem likely that other proteins or other mechanisms are involved in the recognition of IgG *N*-glycans.

1.1.3.2 Recognition in cell-cell interactions

At the centre of deciphering the 'glyco codes' are the interactions between glycan ligands and their receptors, i.e. glycan binding proteins (GBPs). The vast diversity of glycan structures provides recognition epitopes for GBPs which are involved in many biological activities including both cell-cell interactions and cell-microbe interactions. Among the best-characterized examples are the binding of the C-type lectins, selectins, in early cell adhesion events, e.g. tethering and rolling of leukocytes on vascular surfaces at the initial stages of the inflammation reaction.

Selectins are a family of three membrane proteins comprising E-, P- and L-selectins. The three selectins have different expression patterns: E-selectin is expressed on activated endothelial cells; P-selectin is additionally expressed on activated platelets; L-selectin constitutively expressed on leukocytes. Each selectin has a carbohydrate-recognition domain (CRD) at the amino terminus with a canonical calcium ligating elements that mediates carbohydrate binding. This is followed by an epidermal growth factor (EGF)-like domain and various numbers of short consensus repeats composed of sushi domains (also known as complement control protein modules). In the carboxyl terminus, the selectins have a cytoplasmic domain and a single transmembrane domain (TM) (75). Binding to carbohydrate is directed by the CRDs, which recognize sialylated or sulphated sequences with α1-3/4-linked fucose residues.

Based on the knowledge that E- and P-selectins bind granulocytes and monocytes which express Le^x and sLe^x, these two glycans, together with sLe^a (Figure 1.6), which is an isomer of sLe^x, were first examined as candidate carbohydrate ligands for the two selectins. Indeed the latter two were shown to be recognized by all three selectins (76,77). In addition, L- and P-selectins were shown to be able to interact with a second class of ligands with L-selectin binding to sulphatides (sulphated Gal-Cer) and sulphotyrosine (77,78), and P-selectin binding to sulphotyrosine (78,79).

A glycoprotein, called "P-selectin glycoprotein ligand-1" (PSGL-1, CD162) is the major counter-receptor on leukocytes for P- and L-selectin and is also an important ligand for E-selectin. It is a homodimeric, disulphide-bonded, *O*-glycosylated protein

containing many decapeptide repeating units in the ectodomain which is heavily glycosylated (80). The amino terminus of the ectodomain has been suggested to be vital for selectin binding. It contains three potentially sulphated tyrosine residues and an adjacent *O*-glycosylation site bearing an sLe^x motif (81-83). Synthetic glycosulphopeptides of *N*-terminal domain of PSGL-1 with sLe^x and tyrosine sulphation at the terminal region were bound by P-selectin with the same high affinity (~300 nM) as that of native PSGL-1. Crystallographic study of co-complexed P-selectin and the PSGL-1-derived glycosulphopeptide confirmed the complex and tight association between these two binding partners (84).

In addition to PSGL-1 as the major counter-receptor common for all the three selectins, several glycoproteins bearing sLe^x can also interact with E-selectin. These include a glycoprotein called "E-selectin ligand-1" and CD44. Another family of selectin ligands, namely, the 3'-sulpho-Le^a and 3'-sulpho-Le^x (Figure 1.6) were discovered (85). By NMR studies of the sialyl-Le^a and sulpho-Le^a in solution, it was found that the carboxyl group of the sialyl-Le^a and the sulphate of the sulpho-Le^a occupied the same position in their conformation (86) suggesting a similar role for the two acidic moieties.

L-selectin mediates lymphocyte homing through interaction with peripheral node addressins, which are a group of *O*-glycosylated proteins expressed on high endothelial venules (HEV) of lymph nodes. These addressins include CD34, GlyCAM-1 and MAdCAM-1. On these glycoproteins, the ligands for L-selectin are sulphated glycans, e.g. 6-sulpho-sialyl-Le^x (Figure 1.6). Initially 6'-sulpho-sialyl-Le^x, 6-sulpho-sialyl-Le^x, and 6',6-sulpho-sialyl-Le^x (Figure 1.6) were found on the L-selectin counter-receptor GlyCAM-1 but it was not possible to compare their relative potencies as ligands due to limited amounts of oligosaccharides that could be obtained from this glycoprotein (87). Chemical synthesis of lipid-linked forms of these glycans enabled a detailed comparison of their ability to support L-selectin binding (88). The results established that the sulphate at C6 of subterminal GlcNAc enhances the L-selectin binding elicited by sLe^x, whereas sulphate at C6 of terminal Gal markedly impaired the binding. Co-transfection of an α1-3 fucosyltransferase (FUT7) and one or other of two *N*-acetylglucosamine β6-sulphotransferases

(GlcNAc6ST-1 or GlcNAc6ST-2) into cell lines lacking L-selectin ligands led to the reconstruction of L-selectin ligands (89,90).

Many cancers overexpress sLe^x or sLe^a which may mediate metastasis through interactions with E- and L-selectin. This is discussed in Section 1.2.4. Other than the sialylated oligosaccharide epitopes, some heparin and heparan sulphate-containing proteoglycans are also found to be bound by P- and L-selectins (91,92). The physiological significance of this binding has not been clear but one possible explanation is that heparan sulphate and heparin display high densities of carboxylic acid groups and 6-*O*-sulphate, which resemble the sequence of 6-sulpho-sialyl-Le^x. Regardless of the physiological meaning, this has led to the exploitation of heparin, which is an approved therapeutic reagent by FDA to suppress selectin activities (93,94) in cancer metastasis.

1.1.3.3 Recognition in cell-microbe interactions

In addition to functions in endogenous recognition, glycans play a pivotal role in many microbial pathogen-host interactions.

As a prerequisite to colonization, many microorganisms use their surface carbohydrate-binding proteins to mediate adhesion to their host cells. Many viruses, for instance, have GBPs that bind to terminal sialylated sequences on the host cell surface. An example previously mentioned is the specific recognition of sialylated glycans by haemagglutinin on influenza virus. Many other viruses such as rotavirus, adenovirus, reovirus and polyomavirus also use sialylated glycans for adhering to host cells (95). Bacteria often express more than one type of adhesins that bind directly to cell surface glycoconjugates or matrix glycoproteins or mucins, thereby determining tropism. *Helicobacter pylori* bacteria, for example, express many adhesins including BabA and SabA that recognize fucosylated blood group H type 1 and Le^b (96,97), and sLe^x (98), respectively. The expression of blood group H type 1 and Le^b in gastric secretions is controlled by secretor phenotype which is a determinant of infection by *H. pylori* strains that express BabA (99). This tropism of the bacteria is dependent on glycosylation patterns in gastric mucosa. The bacteria are mainly found at the surface of epithelial cells where there is expression of fucose-

containing Lewis antigens, but not the deeper gastric glands where there is less fucosylation and glycans capped by α-4GlcNAc are highly expressed. This is because the terminal α-4GlcNAc could inhibit the synthesis of α-glucosylcholesterol, an important element of the bacterial cell wall, therefore suppressing bacterial growth (100). Recently, a new adhesin was discovered and designated as LabA which recognizes LacdiNAc motif (GalNAcβ1-4GlcNAc) (101). It was suggested the tissue tropism of these strains of *H. pylori* may be due to the restricted expression of LacdiNAc in gastric mucosa.

Parasites also utilize glycans as receptors for adhesion. Apicomplexan parasites such as *Toxoplasma gondii*, have unique organelles termed micronemes (MIC). They use MIC proteins to penetrate host cells. A novel fold of microneme adhesive repeat on the MIC1 was identified by crystallographic studies. With the help of carbohydrate microarray analysis, it was found that a wide range of sialylated oligosaccharides could be bound by this domain (102). This explained the capability of *T. gondii* to infect a wide range of warm blooded animals. This study was followed by the finding of a threonine residue crucial for sialic acid-binding in microneme adhesive repeat, which is not only found on MIC1, but also on other proteins of the same family in other apicomplexan parasites (103). These MIC proteins have different binding specificities toward sialylated glycans which correspond to the glycans expressed on the host cells of these parasites (102-104).

Most microorganisms are covered by glycoconjugates. In most cases, humans have immune responses, innate and acquired, towards these structures. Structures such as mannan and lipopolysaccharide (LPS) constitute pathogen-associated molecular patterns, which are recognized by pattern recognition receptors (PRRs). Some of the PRRs such as Toll-like receptors trigger the intracellular signalling leading to the secretion of pro-inflammatory cytokines. Other PRRs such as C-type lectins, e.g. DC-SIGN, Dectin-1 and the mannose receptor, can mediate endocytosis and phagocytosis or activate the complement system.

Some pathogens camouflage themselves as “self” to avoid immune recognition by presenting common host glycan structures, such as sialic acid. *Neisseria gonorrhoea*e, for instance, can utilize tissue CMP-sialic acid to generate poly sialic

acid-containing capsules, which help to evade immune surveillance. A possible explanation is by presenting these sialylated structures, the bacterium can recruit host factor H which binds to sialic acid so as to negatively regulate the alternative complement pathway (105,106). Alternatively, it has also been proposed that the terminal sialic acid on the pathogens interact with proteins of the host Siglec family, and thereby sending a negative signal to the innate immune cells (107). The host cells have also evolved to avoid being recognized by the pathogen GBPs. They secrete soluble sialylated mucins at the surface of intestinal mucosa to prevent the microorganisms from binding to the epithelial cell membrane. The microorganisms are thus removed with the secreted mucins.

1.2 Changes in expression of carbohydrate structures and glycosyltransferase activities in cancer

Ubiquitously expressed in humans, the diverse carbohydrate sequences are under dynamic changes. Altered or abnormal protein glycosylation is a universal feature of cancer cells (108,109), and many of such glycan structures are potential cancer biomarkers in both tissue and serum assays (110). Changes in glycans may occur in the core region, the backbone and the peripheral regions. These include but may not be confined to the following patterns: incomplete biosynthesis, abnormal elongation, aberrant branching of backbone regions and neosynthesis of glycan sequences that are inappropriate for the cell or tissue in question (111,112). Many of these aberrant structures reflect the altered expression or function of the corresponding glycosyltransferases.

1.2.1 Truncated *O*-glycans and associated antigens on mucins

Aberrant forms or amounts of mucins have long been described in epithelial cancer cells (113). Mucins are a family of high molecular weight proteins produced by various epithelial cells, either secreted or membrane-associated. The hallmark of mucin is variable number of tandem repeat (VNTR) domains, which are rich in serine and threonine that are heavily *O*-glycosylated. The compositions and biological functions of mucins, as well as the glycan chains have recently been reviewed in detail (114). In malignant cells, loss of cell polarity results in the incorrect topology of the transmembrane and secreted mucins (115). Overexpression of membrane-associated mucins can be exploited by malignant cells to modulate growth and survival signalling through various receptors such as receptor tyrosine kinase ERBB2. Some transmembrane mucins such as MUC1 and MUC16 can be released to blood plasma instead of to the epithelial lumen, and often, these contain glycan chains that are distinct from the normal form.

Abnormal glycosylation of mucins has been reported in many carcinomas such as those of breast, cervix, endometrium, ovary, bladder, prostate, lung, oesophagus, stomach, colon and pancreas (116-122), as detected by lectins or monoclonal

antibodies. These changes range from absence or truncation of *O*-glycans such that Tn, T and sialyl Tn antigens are expressed (Figure 1.9) (108,116). In fact, it is estimated that Tn and sialyl Tn are expressed in more than 80% of human carcinomas (123,124). The expression of these antigens on mucins has been associated with poor prognosis of breast, cervical, lung, stomach and colorectal cancers (125-131). Mucins containing truncated *O*-glycan sequences can elicit autoantibodies, which together with the aberrant glycoforms, have been extensively investigated as cancer biomarkers for diagnostic and prognostic purposes, and targets in cancer therapies. These are reviewed in Section 1.2.7.

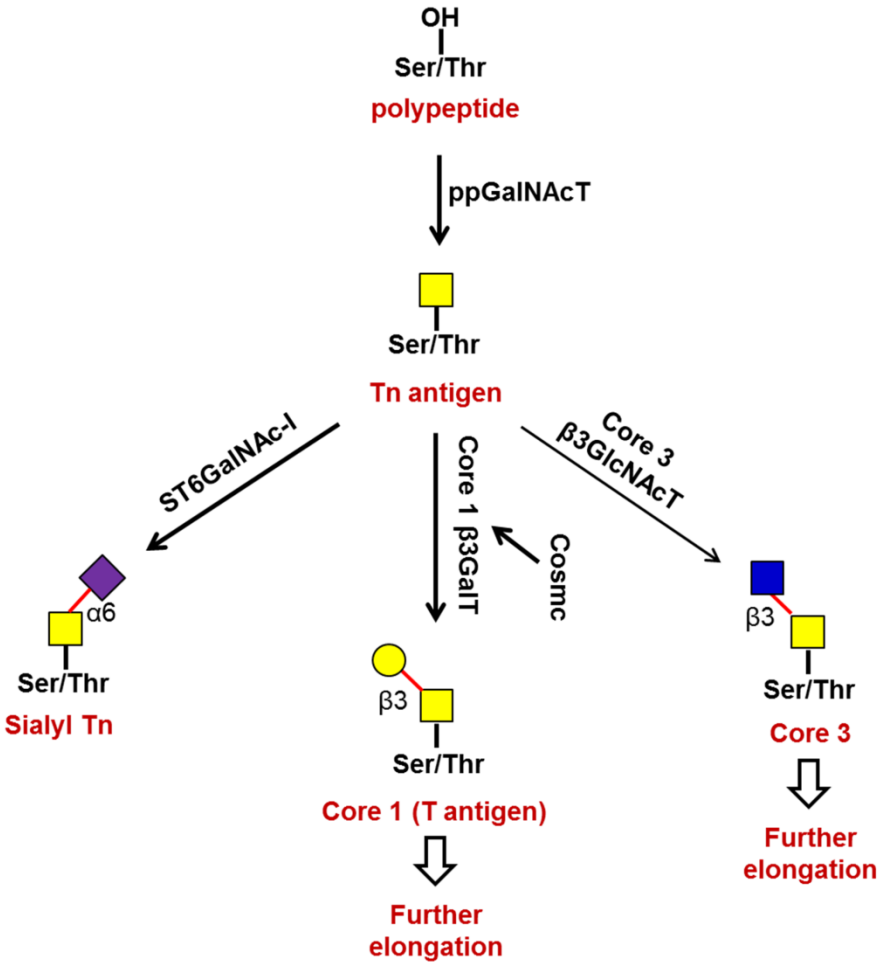


Figure 1.9 Biosynthesis pathways of *O*-glycans in the core region. The symbols are in Figure 1.1.

Simple though their structures are, the molecular mechanisms for regulation of expression of these *O*-glycans are complicated (Figure 1.9). Upon formation of the Tn antigen, i.e. the addition of core GalNAc by one of the 24 members of ppGalNAcT family, further modifications occur in three competitive ways (132): addition of β1,3-

linked Gal to generate core 1 (T-antigen), formation of core 3 with β1,3-linked GlcNAc and generation of sialyl-Tn by addition of α2,6-linked sialic acid. Key enzymes in these processes are core 1 β3GalT (T-synthase), core 3 β1,3-*N*-acetylglucosaminyltransferase (core 3 β3GlcNAcT) and α2,6-sialyltransferase (ST6GalNAc-I), respectively (Figure 1.9).

Among the three pathways, the core 3 synthase transcripts have restricted expression in gastrointestinal tract (133) and the activity is down-regulated in colon carcinoma (134). Core 1-based further elongation and formation of core 2 glycans occur in normal cells. However, in cancer cells, generation of core 1 is often abrogated, particularly due to the malfunction of core 1 β3-galactosyltransferase-specific molecular chaperone (Cosmc, Figure 1.9). This chaperone is required for the correct folding of the T-synthase (135). The turning-off of the other two biosynthesis pathways results in the accumulation of Tn and sialyl Tn, as the latter cannot be further modified. Overexpression of ST6GalNAc I in breast cancer cell line, MDA-MB-231, can induce the expression of sialyl Tn and the overall change of *O*-glycan profiles which results in the decreased adhesion and increased migration *in vitro* and increased tumour growth in a mouse model (136). The expression of core 3 synthase is associated with the suppression of the growth and metastasis of colon (134) and pancreatic cancer cells (137), partly by suppression of sialyl Tn expression.

1.2.2 Altered branching of *N*- and *O*-glycans and expression of I/i antigens

High expression of β1,6-linked branches on both *N*- and *O*-glycans has been associated with human cancers (138). MGAT5 synthesizes the β1,6-linked GlcNAc on β1,6-Man branch on the backbone of *N*-glycans (Figure 1.8) which can be immuno-stained with plant lectin *L-phytohemagglutinin* (L-PHA). Increased expression of β1,6-GlcNAc has been reported in many cancer specimens including those of breast, colon, melanomas, esophageal and stomach (139-143). Transfection of MGAT5 cDNA to immortalized lung epithelial cell line Mv1Lu resulted in a series of cellular transformations including loss of contact-inhibition and increased tumourigenicity in nude mice (144). Remarkably, knockout of MGAT5 in mice showed

reduced tumour growth and metastasis in a polyomavirus induced breast cancer model (145). A mouse model with overexpressed MGAT5 showed increased endothelial-mesenchymal transition and keratinocyte migration (146).

The increased activity of MGAT5 and its glycan products have been closely associated with epithelial cancer cell growth, decreased cell-cell and cell-matrix adhesion, increased cell motility, and increased invasiveness and metastasis, as reviewed by Taniguchi and Korekane (146). Many investigations have been performed toward understanding how phenotypic changes are caused by the β1,6-GlcNAc branch on *N*-glycans. Functions of the cell surface glycoproteins such as cytokine receptors, adhesion receptors and solute transporters are found to be regulated by their β1-6 branch of *N*-glycans through tuning the life cycles of these receptors. Examples include EGF and transforming growth factor beta (TGF-β) receptors (147) and T cell receptors (148). The increased β1-6 branching attenuates signalling from adhesion receptors such as integrins and cadherins (149-151).

The formation of β1,6-GlcNAc is often followed by subsequent elongation of poly-LacNAc and terminal decorations such as sLe^x. The poly-LacNAc and sLe^x sequences are potentially ligands of endogenous lectins, i.e. galectins and selectins. Many studies did not differentiate the β1,6-branching point *per se* from the subsequent modifications following the increased β1,6 branching. For instance, a recent study indicated galectin-1 engaged vascular endothelial growth factor receptor-2 (VEGFR2), leading to VEGF-like signalling and enhanced angiogenesis in tumours refractory to anti-VEGF treatment. It appeared that galectin-1 binding was associated with the expression of MGAT5 (152). In fact, rather than binding to the β1-6GlcNAcβ1-6Man- branching point, galectin-1 interacted with the poly-LacNAc that is subsequently added to the β1,6-GlcNAc. It has long been known that poly-LacNAc-rich glycoconjugates are good ligands for galectin-1, termed calf heart lectin (16). The branching point *per se*, which is a consequence of MGAT5, is an important prerequisite for the expression of poly-LacNAc.

As mentioned previously (Figure 1.8), there is an interesting competition for glycan acceptors in biosynthesis between MGAT5 and MGAT3. The formation of bisecting GlcNAc by MGAT3 prevents the enzymatic functions of other GlcNAc-transferases,

thus resulting in the decrease of β1,6-branched *N*-glycans. In many cancers, overexpression of MGAT3 has been found to suppress cancer metastasis (153). The bisecting GlcNAc branch and its glycosyltransferase were suggested to be associated with supressed cell adhesion and migration via adhesion molecules and extracellular matrix such as E-cadherin, laminin, and integrin. In one particular example of endometrioid ovarian carcinoma, MGAT5 transcripts were found upregulated by 2-3 fold, but only small amounts of β1-6 linked *N*-glycan products were detected by L-PHA binding. This was due to the competition by much larger (13-fold) increase of MGAT3, resulting in mainly the bisected *N*-glycans (154,155).

Other branched sequences such as core 2 on *O*-glycans, I-branching on both glycoproteins and glycolipids, and linear i antigen-related poly-LacNAc sequences are also aberrantly expressed in many cancer types. These are discussed in Chapter 3.

1.2.3 Loss or retention of ABO (H) blood group antigens

Expression of A, B, and O (H) and related antigens also undergoes changes during cellular differentiation, development and aging, as well as pathological progress, such as carcinogenesis (156,157).

It has long been noted that deletion or reduction of A and B antigen occurs in many types of human cancers and the decrease has been correlated with degree of malignancy and metastatic potential in gastrointestinal (158), lung (159), cervical cancers (160), oral (161), bladder carcinomas (162) and haematological malignancies (163,164). Transfection of cDNA encoding GTA or GTB into various H/Le^y-expressing human tumour cell lines significantly reduces their directional (haptotactic) motility (165). A follow-up experiment further separated blood group A positive from A negative clones among the GTA transfected colorectal carcinoma SW480 cells and HT29 cells. The blood group A negative clones of both cell lines showed much higher motility than the A positive clones (166). Furthermore, this phenotype change was suggested to be correlated with the A/B glycosylation of integrin chains α3, α6, and β1 (165,166). Interactions between the α and β subunits

may be affected by the A/B glycosylation, which in turn controls haptotactic motility of colonic tumour cells. As regards H antigen expression, loss of A and B in cancer reveals the precursor H antigen. But the transformation of H to Le^b and Le^y by adding α1-4/α1-3 linked Fuc also influences the level of H antigen detection in cancer. Thus factors determining the overall expression are complex. The mechanism underlying the suppressed expression of A, B, O (H) antigens is not clear although some evidence showed that the blood group ABO glycosylation change was regulated by *ABO* allelic expression at mRNA level (167,168).

People's blood group, A, B, O (H), has been linked with the individual's susceptibility to some carcinomas, such as gastric and pancreatic cancers. A cohort study showed patients of blood group A had a higher risk of gastric cancer (169). Wolpin *et al* systematically investigated the relationship between the susceptibility to pancreatic cancer and blood groups in genome-wide studies. The results showed that the ABH genotypes were significantly associated with pancreatic cancer risk; patients of blood groups A or B had an increased risk compared to group O patients (170-173).

1.2.4 Altered expression of Lewis-related antigens

The expression patterns of Lewis-related antigens in cancer tissues are very complicated. Although it is difficult to draw a general conclusion about the expression pattern of Lewis-related antigens in tumourigenesis, I highlight below a few examples where changes in expression of these antigens have had a clear impact.

As mentioned in Section 1.1.2.2, there are eight α1-3/α1-4 fucosyltransferases (FUT3-7, FUT9-11) in total with distinct tissue-specific expression patterns. The expressions of these α1-3/α1-4 fucosyltransferases, together with those of α1-2 fucosyltransferases, make a diverse combination of Lewis structures on the cell surface, not to mention the impact of other competing glycosyltransferases (e.g sialyltransferase), the availability of precursors and donors and the existence of glycosidases. According to a review by Le Pendu et al (156), Le^x and Le^y expressions are often increased in carcinomas with the exception of endometrial cancer for the former and endometrial and pancreas cancers for the latter; Le^a is

upregulated in many carcinomas but with more exceptions such as colon, breast and prostate; Le^b shows a similar pattern to that of A and B antigens. The biological roles of these antigens in tumour progression are still not well-understood.

Competition between H/Le^y generating α1,2-fucosyltransferase and sialyl Lewis forming α2,3-sialyltransferase has also been reported. Transfection of α1,2-fucosyltransferase, FUT1, induced the expression of H and Le^y antigens and suppressed sialyl Lewis antigen expression in human pancreatic cancer cell line BxPC-3, obviating E-selectin-mediated adhesion and decreasing gastrointestinal metastasis (174,175). This is because the type 1 or type 2 LacNAc backbone sequences are common substrates for α1,2-fucosyltransferases and α2,3-sialyltransferase. The expression of these competing enzymes results in the modulation of the glycans on tumour cell surface.

Overexpression of sLe^x and sLe^a on both glycoproteins and glycolipids has been confirmed in many adenocarcinomas, such as lung (176), colorectal (177), gastric (178) and pancreatic carcinomas (179). As mentioned in section 1.1.2.2, these glycans are involved in the adhesion events in the lymphoid and vascular system. Tumour cells hijack the interaction system between selectins and leukocytes to mediate cancer cell extravasation and metastasis.

Expression of sLe^x and sLe^a has been reported to be associated with the potency of adhesion of cancer cells to the vascular endothelium via E-selectin (180,181). Using either anti-E-selectin antibodies or anti-sLe^x antibodies, this adhesion was clearly inhibited (176). In a transgenic mouse model, metastasis of tumour cells expressing E-selectin ligands could be redirected to a tissue expressing E-selectin (182). Colon cancer cells transfected by antisense RNA directed at α1-3/4fucosyltransferase lost expression of sLe^x and sLe^a, and this in turn abolished colonization of the cancer cells to the liver (183,184).

In summary, the general changes in the expression of A, B, O (H) antigens, Lewis antigens and sialyl Lewis antigens in tumour progression from precancerous stage to metastatic stage were proposed by Le Pendu et al (156) and shown in Figure 1.10.

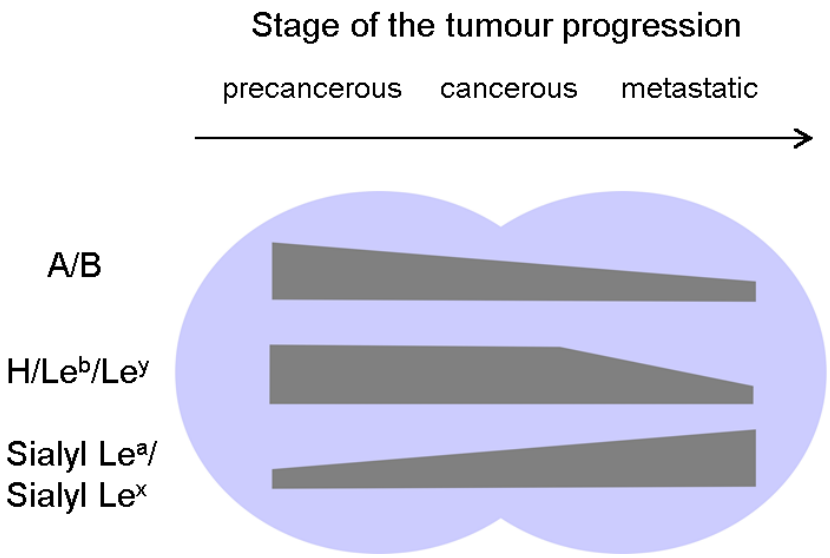


Figure 1.10 Schematic diagram of expressions of blood group A, B, H, Le^b, Le^y, sialyl Le^a and sialyl Le^x in different stages of cancer. Redrawn from reference (156) with permission.

1.2.5 Changes in expression of sialic acid forms

It has long been known that cell-surface sialic acid content is increased on tumour cells, as evidenced by the increased binding of wheat germ agglutinin (WGA) (185-187) which has specificity for sialic acid and GlcNAc terminating sequences. Increased sialylation is believed to be a mechanism whereby tumour cells have protection from activation of the alternative pathway of the complement system by recruiting factor H (188). Factor H could inhibit the activation of the alternative pathway by accelerating the decay of the key players, C3b convertase, and acting as cofactor for the factor I to inactivate C3b (189).

Modifications to the neuraminic acid structure have been reported, such as acetylation and de-*N*-acetylation on C9 and C5, respectively (Figure 1.11). In colon carcinomas, a decreased amount of acetylation was observed (190) and was proposed as an early sign of colon cancer progression (191), whereas in melanoma cells increased acetylation is found on neuraminic acid on gangliosides GM2 and GD3 [(192), see Figure 1.12 for sequences]. In addition to the modifications to neuraminic acid, the expression of α2,6-linked sialic acids on type 2 backbones is increased, as revealed by the staining with the plant lectin *Trichosanthes japonica* agglutinin I (193). It has been suggested that cell surface β1 integrin is involved as

the carrier of the aberrant sialylation (194). The biological effects of these modifications and overexpression are not clear at present.

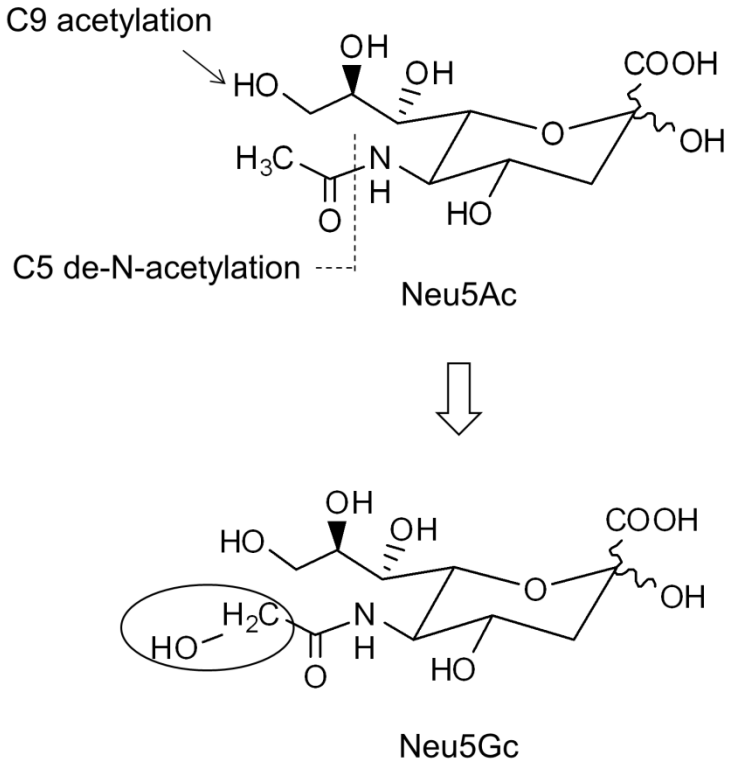


Figure 1.11 Modifications of sialic acid structure in cancer. Arrow indicates the position of C9 acetylation. Dashed line shows the C5 de-*N*-acetylation. Circle indicates the difference between Neu5Ac and Neu5Gc form of sialic acid.

There is an aberrant expression of the *N*-glycolyl form of neuraminic acid (Neu5Gc) in some human tumour cells [(195), Figure 1.11]. The accumulation of the Neu5Gc in tumours is thought to be due to uptake from dietary sources as the hydroxylase enzyme responsible for converting Neu5Ac to Neu5Gc is lacking (mutated) in humans (196). The expression of Neu5Gc in tumours may be an explanation for the presence of “Hanganutziu-Deicher” antibodies (antibodies to Neu5Gc gangliosides) in some patients (197).

1.2.6 Changes in expression of sialo-glycolipids (gangliosides) and globosides and biological effects

The sialic acid-containing glycolipids, gangliosides, are often aberrantly expressed by a range of tumours particularly those of neuroectodermal origin such as melanoma and neuoblastoma (198,199). They can be detected in sera of patients with

mammary carcinomas, neuroblastomas, sarcomas, melanomas, small-cell lung carcinomas and lymphomas (199). Gangliosides may influence tumour and host cell interactions through modulating cellular differentiation, adhesion, angiogenesis and invasion (108,200,201).

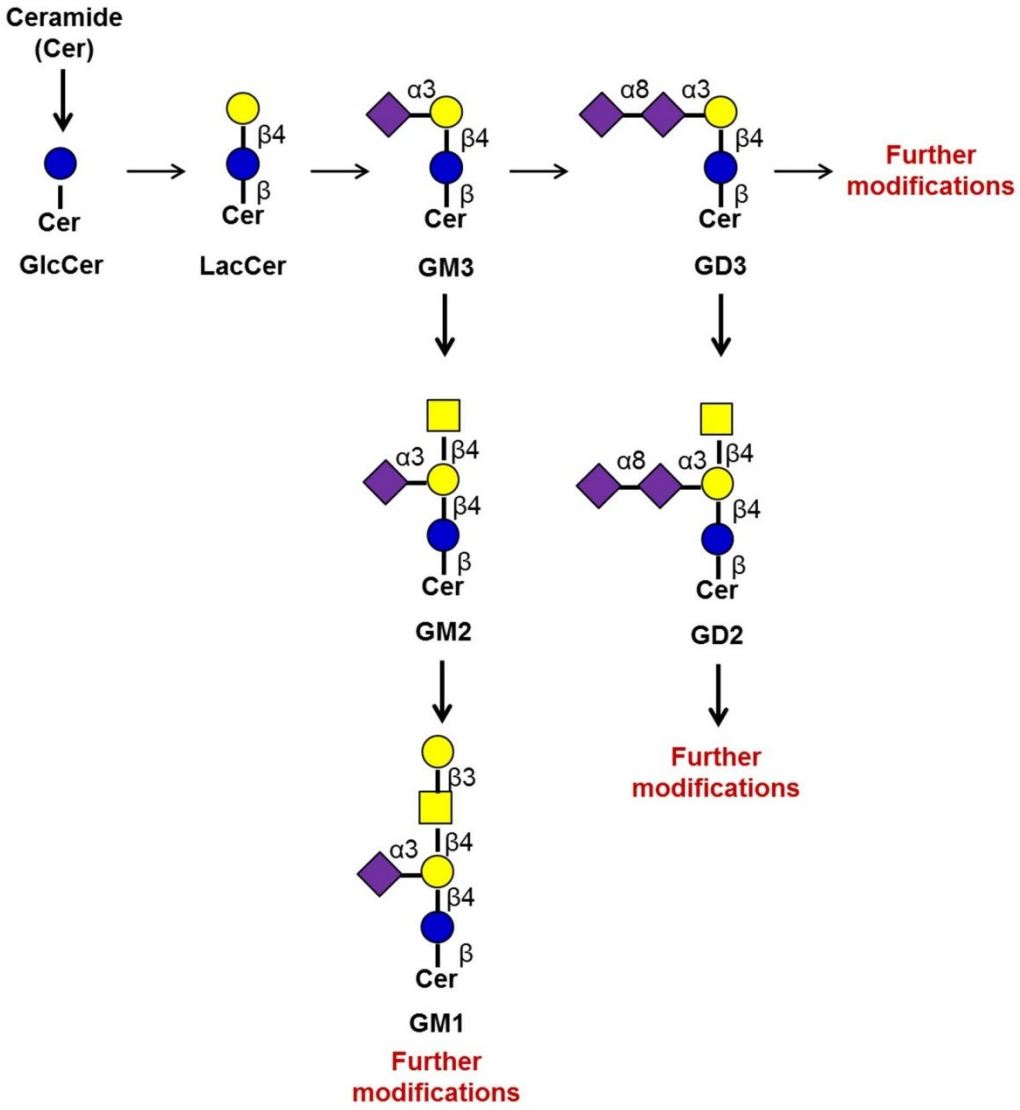


Figure 1.12 Sequences of ganglioside that are aberrantly expressed in cancer and their biosynthetic pathways. Cer is ceramide, the rest of the symbols are as in Figure 1.1.

A monosialoganglioside GM3 (Neu5Acα-3Galβ-4Glcβ-Cer, Figure 1.12) has been extensively studied for its tumour inhibitory activity. An initial study showed that the addition of GM3 inhibited the growth of human epidermoid carcinoma cell lines such as KB and A431 (202). Further studies indicated that GM3 expression was associated with enhanced apoptosis (203) and reduced cell motility (203,204). GM3 was suggested to be the key factor in attenuating metastasis of human hepatoma cells to lymph nodes; and this effect was suggested to be the result of inhibition of

EGFR phosphorylation and PI3K/AKT signalling (205). Addition of GM3 was found to be able to counteract the pro-angiogenic effects of VEGF and reduce the phosphorylation of the VEGFR2 (200). Other receptors have also been implicated in angiogenesis. These include those receptors of insulin-like growth factor-1, basic fibroblast growth factor, epidermal growth factor and platelet-growth factor, as reviewed in (206). Carbohydrate-carbohydrate interactions were suggested to be the underlying mechanism in the engagement between EGFR and GM3. There was some evidence showing that *N*-glycans on EGFR could interact with GM3, thereby inhibiting the activation of EGFR tyrosine kinase (207,208). Other mechanisms such as regulating metastasis suppressor gene products CD82 and CD9 were also suggested (203,209). A cell surface sialidase "Neu3" was found to be upregulated in human colon cancer, resulting in the hydrolysis of sialic acid in gangliosides including GM3. This in turn led to the accumulation of desialylated backbone lactosylceramide (Lac-Cer), which was suggested to inhibit apoptosis through increased *bcl-2* and decreased caspase expression (210).

In contrast to the above mentioned "simple" form, more complex gangliosides containing longer oligosaccharide chains than GM3 can often enhance tumour progression. It has long been noted that a mixture of glycolipids enhances xenograft tumour growth and metastasis (198). As reviewed in (211), the disialoganglioside GD3 (Figure 1.12) was found to be overexpressed in many types of cancer cells, not only of neuroectodermal origin, but also in epithelial carcinomas including breast, prostate and many others, also in acute T-cell lymphoblastic leukemia. Studies showed that when GD3 knockout cell line F-11 was injected into mice, they produced less VEGF and the vascularization was significantly reduced compared to the normal cell line, suggesting that GD3 could promote VEGF production and was involved in angiogenesis, thereby facilitating tumour progression (212). Interestingly, GM3:GD3 ratio is an important indicator of prognosis in human melanoma. Patients with higher GM3:GD3 ratio survive significantly longer (213). GD2 (elongated GD3, Figure 1.12) is uniformly expressed in neuroblastoma and most melanoma, gliomas, but little expressed in normal tissues. It has now been exploited as a target in immunotherapy (214). This is reviewed in Section 1.2.7.1 and Chapter 5.

Gangliosides are also important regulators in the formation of cell membrane lipid rafts, which facilitate the formation of EGFR complexes. In breast cancer, GM1 was found to promote the formation of heterodimer Erbb2-Erbb3 receptor and this oligomerization of tyrosine kinase receptor can in turn regulate the growth of tumour cells through EGFR-mediated signalling (215).

In addition to gangliosides, aberrant expression of globosides (Figure 1.13) has also been found in human cancers. Gb3Cer, also known as P^k antigen and CD77, is highly expressed on immature B-cells and Burkitt's lymphoma (216), pancreatic and colon adenocarcinomas (217), and ovarian carcinoma (218). Gb5Cer (also known as stage specific embryonic antigen-3 or SSEA-3 antigen) and its monosialylated and fucosylated forms, sialyl-Gb5Cer (also known as SSEA-4 antigen) and Globo H, respectively (Figure 1.13), are also expressed on many cancer cells including breast cancer cells and breast cancer stem cells (219,220). Recently, Chi-Huey Wong group performed a thorough survey of expressions of SSEA-3, SSEA-4 and Globo H in various cancer cell lines by flow cytometry (221). These include cancers of brain, lung, breast, mouse, oesophagus, stomach, liver, bile duct, pancreas, colon, kidney, cervix, ovary and prostate. SSEA-4 was broadly expressed in every type of cancer cell line, sharing most of them with SSEA-3. Globo H was less commonly distributed than SSEA-4 but was also widely detected (221). In addition, SSEA-4 was found on glioblastoma multiforme cell lines and human glioblastoma multiforme cancer specimens (221).

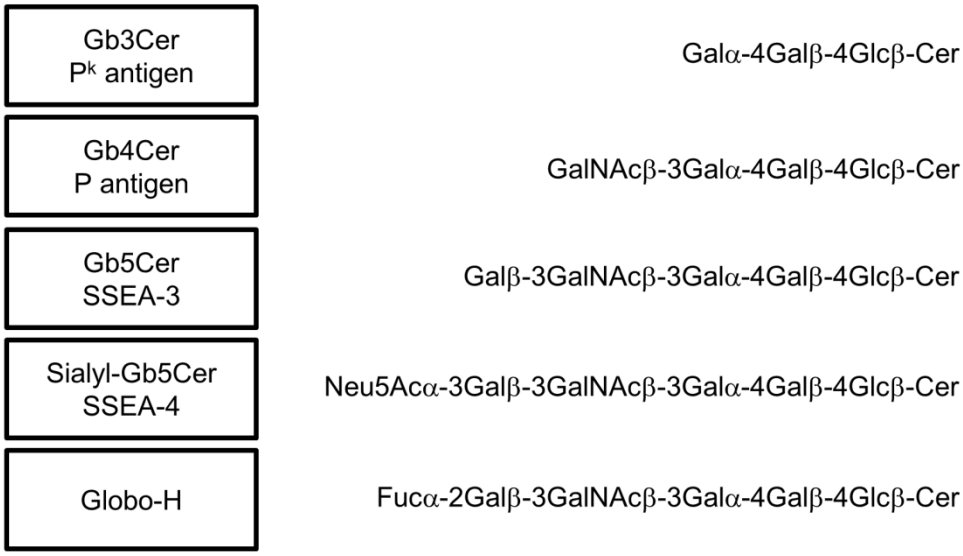


Figure 1.13 Sequences of globoside glycolipids.

Although the above mentioned globosides are widely distributed, understanding of their functions remains limited. Gb4Cer was reported to bind to another glycolipid nLc4 (Galβ-4GlcNAcβ-3Galβ-4Glcβ-Cer) through carbohydrate-carbohydrate interaction, which was suggested to promote the activity of transcription factors AP1 and CREB in cell adhesion processes (222). Another study indicated Gb4Cer promoted the ERK signalling through interaction with EGFR (223). A cytoplasmic protein, FKBP4 [FK-506 (Tacrolimus, an immunosuppressive drug) binding protein 4], which was detected in breast cancer cells, was also found to bind to SSEA-4 but the biological significance has not been clear (224).

1.2.7 Clinical relevance

1.2.7.1 Diagnosis and prognosis

The search for carbohydrates as potential human cancer-associated biomarkers has been ongoing for many years. This has been discussed in numerous reviews (225-227). However, the outcomes have been less fruitful than expected. Although there are numerous reports of inappropriate expression of certain carbohydrate structures for given cell types, they are almost always normal antigens in other cell types, which are referred to as “onco-foetal” or “onco-developmental” antigens (228). Therefore,

few of the candidate cancer-associated carbohydrate antigens have stood up to critical assessments for cancer diagnosis and prognosis.

Cancer antigen 19-9 (CA19-9) is a carbohydrate tumour-associated antigen sLe^a, which was originally isolated from a human colorectal cancer cell line by Koprowski *et al* (229) in 1979. CA 19-9 assay has been established to monitor colorectal, stomach, and particularly pancreatic cancers in clinical (206). A recent study showed high serum concentrations of both CA 19-9 and E-selectin significantly correlated with low 5-year survival rate of patients with colorectal cancer (230). One of the major limitations is that the expression of CA 19-9 is highly dependent on the enzyme FUT3, which is encoded by *Lewis* gene. About 10% of the Caucasians are Lewis-negative and cannot produce CA 19-9 antigen (231). In these populations, a sialylated precursor, Neu5Acα-3Galβ-3GlcNAc- is expressed and can be detected by antibody DU-PAN-2.

Many glycoproteins and glycopeptides have been exploited as cancer biomarkers, such as carcinoembryonic antigen (CEA) for colon cancer, prostate-specific antigen (PSA) for prostate cancer, cancer antigen 15-3 (CA 15-3, MUC1) for breast cancer, cancer antigen 125 (CA 125, MUC16) for ovarian cancer, and cancer antigen 72-4 (CA 72-4, tumour associated glycoprotein-72) for gastrointestinal cancer. Serological studies have been established to measure the quantity of these glycoconjugates. So far the most important application of these biomarkers has been monitoring tumour progression, regression and efficacy of treatment but not diagnosis. CA 15-3, for example, is an abnormally expressed mucin (MUC1) bearing truncated *O*-glycans such as Tn, T and sialyl Tn. The aberrantly glycosylated MUC1 is shed into circulation and can be detected in serum in a CA 15-3 assay. MUC1 level has been shown to be useful in monitoring cancer progression in some late stage patients with metastatic breast cancer (232,233); the role of this marker in decision-making for treatment is not clear, thus it was not recommended by American Society of Clinical Oncology for cancer screening and diagnosis (234). One exception, however, is CA 125, which is elevated in 50% of patients with stage I ovarian cancer and in 25% of serum samples collected 5 years before diagnosis of ovarian cancer (235). It has also been shown to be useful in evaluating and monitoring prognosis of ovarian cancer (236).

Most of these glycoconjugate biomarkers have both altered polypeptide and altered glycosylation. One can use specific glycoforms of these glycoproteins to improve the sensitivity and specificity of the biomarkers. Therefore, a new strategy trying to identify aberrations in glycoforms of these glycoproteins or glycopeptides highly specific to malignancy has been investigated. A glycoprotein α-Fetoprotein (AFP) has been used as a serum biomarker in clinical diagnosis of hepatocellular carcinoma (HCC) (237). A detailed study showed that the total AFP can be separated into three glycoforms AFP-L1,-L2 and -L3 according to the differential affinities to lectin *Lens culinaris* agglutinin (LCA). Among the three glycoforms, AFP-L3 with core-fucosylated *N*-glycan has been shown to be highly associated with hepatocellular carcinoma (238).

It should be noted that sometimes the aberrantly glycosylated proteins cannot be detected shed in the serum. For example, it is known that the truncated glycoforms such as Tn and T on MUC1 are clearly associated with many types of cancers. However, these glycans could not be detected on MUC1 in serum of patients with early stage breast cancer using antibody-based ELISA assay (239). It is thought that MUC1 carrying these glycoforms is cleared by autoantibodies that are elicited, as well as scanvenger lectin receptors. Therefore careful investigation is needed when any of these glycoforms is proposed to be used as biomarkers.

As the aberrant glycosylation is the reflection of the abnormally expressed glycosyltransferases and/or glycosidases, these enzymes themselves have been suggested to be able to serve as biomarkers (240). Good examples, as mentioned above in Section 1.2.2, are the overexpression of β1-6 branching enzymes MGAT5 and GCNT1, and the malfunction of the core 1 *O*-glycan synthase chaperone Cosmc.

Autoantibodies can be found prior to the manifestations of cancer symptoms (241), thereby are considered ideal cancer biomarkers for early cancer detection (242). Blixt *et al* (243) developed a platform to display large numbers of glycopeptides with various aberrant glycoforms based on chemoenzymatic synthesis. With this platform, autoantibodies to Tn and sTn glycosylated MUC1 and MUC4 can be detected in sera from patients with colon cancer but not in controls, which shows promise (244). They

later developed a new microarray containing Tn (GalNAcα-), STn (Neu5Acα2-6GalNAcα-), ST (NeuAcα2-3Galβ1-3GalNAcα-) and extended core 3 (GlcNAcβ1-3GalNAcα-) glycoforms based on two 20mer tandem repeat sequences of MUC1 glycopeptides. The array was used for a screen of sera from 395 breast cancer patients, 108 benign breast cancer patients and 99 healthy controls. The results showed that autoantibodies were present and in higher levels in sera of early stage breast cancer patients. There was also correlation between high levels of autoantibodies against core 3- and sTn-MUC1 and a better prognosis in terms of reduced rate and longer time to metastasis (245). However, a follow-up study showed no significant difference in binding activity between the sera from patients who subsequently developed invasive breast cancer and those from the control group (246). Although autoantibodies to truncated glycoforms on MUC1 could be indicative as prognostic marker, they would seem inappropriate at least for the early screening of cancers such as breast, ovarian, lung or pancreatic cancers.

1.2.7.2 Therapy

The aberrant glycosylation occurring during cancer progression has for long been considered a potential target for cancer therapy.

One application under investigation is to target cancer-associated carbohydrate antigens in both the active and passive immunotherapy (247). The disialoganglioside GD2 (Figure 1.12) is an excellent target for vaccine development and for cancer treatment using mAbs. It is uniformly expressed in tumours of neuroectodermal origin such as neuroblastoma, melanomas and osteosarcoma but only restrictedly expressed in normal tissues (248). Numerous anti-GD2 antibodies have been developed and now in clinical trials (249). Among them, a humanised antibody ch14.18 has been found very promising for cancer treatment. Results of phase III clinical trial indicated that adding ch14.18 to standard treatment improved survival in patients with neuroblastoma (214). Moreover, ch14.18 consolidation immunotherapy after the induction therapy prevented the relapse and prolonged long-term survival rate, compared to the oral maintenance chemotherapy and to no consolidation (250). Other anti-carbohydrate mAbs have been produced targeting gangliosides GD3,

GM2 and to other glycans such as Le^y and Le^x but have not been as successful as ch14.18.

Another application is to exploit the aberrantly expressed glycoconjugates in design of cancer vaccine. Although poor in immmunogenicity, many glycolipids have been used to elicit immune response in patients and some of them have resulted in promising results (251). But a lot of vaccines against GD2 and GD3 have yet to yield positive clinical results (252). Some vaccines have been developed based on conjugated oligosaccharides. A vaccine designated Theratope, based on sialyl Tn disaccharide in conjugation with the keyhole limpet hemocyanin has been on clinical trial but could not pass phase III (123). It did not show improved median time to disease progression nor overall survival in patients with metastatic breast cancer (253). More recently, advances in synthetic chemistry have made possible the use of fully synthetic glycopeptides as vaccines to generate immunoreactions against homogeneous targets (254). A good example under investigation is the MUC1-based glycopeptides with aberrant glycoforms such as Tn and sTn (255).

In conclusion, in spite of the great efforts being put into investigating carbohydrates and their derivatives as biomarkers or targets for cancer therapy, there has been limited success and the need for better cancer biomarkers is still very evident. Success requires a better understanding of the biological functions of the carbohydrates in the context of cancer, especially a better understanding of how these glycans could modulate key events of cancer cells such as growth, apoptosis, angiogenesis, adherence, through interactions with their binding proteins. Investigations of two cancer-associated carbohydrate antigens F77 and AE3, defined by two hybridoma monoclonal antibodies, are the subjects of Chapter 3 and Chapter 4. It is hoped that the study of the these two cancer-associated antigens would not only help in better understanding aberrant glycosylation in epithelial cancers and the biological relevance, but also provide potential cancer biomarkers and novel therapeutic tools.

1.3 Methods for investigating expression of carbohydrate antigens

From the discussions above, it is clear that the interactions between glycans and GBPs play important roles in numerous biological and pathological events, including cancer (256). It is desirable therefore to have effective methods to assign the carbohydrate ligands of GBPs. In this section I discuss the challenges in the identification of carbohydrate ligands/antigens and strategies applied to tackle these problems. Emphasis will be placed on the neoglycolipid (NGL)-based array technology developed in the Glycosciences Laboratory (257-259) in which the majority of the work within this thesis was performed.

1.3.1 Challenges

Structural heterogeneity and complexity is a big challenge to the investigation of GBP-glycan interactions. It is estimated that the human glycome contains ~3000 glycan species on glycoproteins and glycolipids, and ~4000 theoretical pentasaccharide sequences on GAGs (7) but only a small proportion of these sequences are currently available for bioactivity studies. Due to the complexity of their structures, many glycans cannot be synthesized in sufficient amounts within a short period of time, although big progress has been made (260-263).

Isolation of glycans from natural sources is an alternative choice. As glycans are very heterogeneous in biological systems and the quantities of each glycan that can be isolated for ligand analyses are rather small, there is a need for powerful separation, sequencing methods with high sensitivity. Recent progress has been made in both of these fields (264-268).

Another obstacle to overcome in studying carbohydrate ligands is the typically low affinity of the interaction between GBPs and glycans. In the natural state this interaction normally relies on multivalency, accomplished by clustered presentation of ligands and/or carbohydrate recognition domains to provide increased binding avidity. *In vitro*, the clustering of glycans can be achieved chemically by using scaffolds such

as proteins, peptides, lipids and other molecules and nanoparticles (269) and, as adopted by the neoglycolipid approach, in the form of liposomes (see below).

1.3.2 Historical aspects of carbohydrate binding assays

In 1931, Landsteiner and van der Scheer established the principles of inhibition assay (270) and this has been summarized by Watkins in her review (271): substances with a structure closely related to or identical with, the immunological determinant (haptenic) group of an antigen can combine with the antibody and thereby competitively inhibit the reaction between antigen and antibody. Shortly before 1931, the first study of quantitative precipitation was reported by Heidelberger and Kendall (272,273) and it was further improved by Heidelberger and Macpherson (274). These have become the basis of many immunological methods such as inhibition of precipitation (24) and haemagglutination inhibition (275) to uncover the structure of an unknown antigen or surface ligand.

The two approaches haemagglutination inhibition and inhibition of precipitation were used in the assignment of the major blood group A, B, O (H) epitopes by Morgan and Watkins, and Kabat and colleagues between 1950's and 1970's. Important reagents used in these assays included antisera, obtained by immunizing rabbits or humans with blood group expressing materials such as peptone and porcine gastric mucin (276,277). Lectins were also used (278).

Initially, monosaccharides Fuc and GalNAc were found by Morgan and Watkins to inhibit the haemagglutination of O cells by eel *Anguilla Anguilla* (279) and that of A cells by plant lectins (22), respectively. Independently, Kabat laboratory found that GalNAc could inhibit the precipitation between human anti-A antibody and A substance from pig stomach mucin (24). In addition, Gal was found to inhibit the precipitation of B substance from human saliva or horse B stomach by human anti-B reagents (24). With the help of acidic and alkaline degradation and chromatographies such as paper chromatography and charcoal-celite column chromatography, *O*-glycans were released from ovarian cyst glycoproteins, purified, and tested as inhibitors one by one. The blood group antigen epitopes A, B and O were

characterized on *O*-glycans (280-282). Thereafter, blood group-active glycolipids (283,284) and *N*-glycans (285) were also isolated.

Radioimmunoassays were first described in the 1950's. Feizi and colleagues later applied this technique to the investigation of the interactions between GBPs and carbohydrates. Materials under investigation were tested as inhibitors of the binding of the antibodies (antisera) and radio-iodinated antigen-positive glycoproteins (286,287). Compared with the conventional inhibition of precipitation, inhibition assays based on a radiolabelled reference improved sensitivity by several orders of magnitude and, therefore, smaller amounts of inhibitors could be used (288). This method could also be used to determine the epitopes on glycolipids when they were analysed in the form of liposomes complexed with carrier lipids cholesterol and lecithin (289,290). Feizi and colleagues determined epitopes for the anti-I and anti-i antibodies and the stage specific embryonic antigen-1 (SSEA-1) by this method.

Solid phase direct binding assay was then developed. Instead of labelling antigen-positive glycoproteins for inhibition studies, the direct binding assay relies on labelling the GBPs or secondary antibody, which recognizes certain structural features in the primary antibody or GBP (291). Originally, the labels used were radio-labels. This enabled the antigenic analysis of glycolipids by thin-layer chromatography (TLC)-binding assay. In early 1980s, the ganglioside GM1 was identified in total lipid extracts as the glycolipid ligand for the radio-labelled cholera toxin (292). Hakomori *et al* (293) corroborated the assignment of the antigenic determinant of SSEA-1 made by Feizi and colleagues (294). They did this by using glycolipids extracted from human blood cells (294). Since then, TLC-binding assay has been employed to study the antigenic activities of glycolipid extracts from human normal and cancer cell lines and tissues. Examples include the characterization of SSEA-3, SSEA-4 (295), and the glycolipid carriers of sLe^x (296), and the identification of carbohydrate receptor for Shiga toxin (297). For health and safety reasons radioimmunoassay technology has been superseded by the enzyme-linked immunosorbent assay (ELISA) which is widely used and is the basis of many biochemical detection methods. Binding is detected using an enzyme-conjugated protein (using an enzyme such as peroxidase) and colour development with enzyme-substrates instead of radioactivity. ELISA is also used in TLC-binding assays for antigenic analyses of glycolipids. In addition to

enzyme-conjugated proteins, fluorescence-labelled or RNA-labelled proteins are also used in a variety of binding assays including microarray analysis.

Label free technologies such as surface plasmon resonance (SPR) have been developed to study the molecular interactions (298). It can also be used in the kinetic studies to measure the association rate and dissociation rate. This technology has been used in the investigation of recognitions by the lectins such as Concavalin A (Con A) and jacalin (299,300).

1.3.3 Neoglycolipid (NGL) technology

In classical inhibition of binding studies performed by Morgan and Watkins, and Kabat and colleagues, milligram levels of purified free glycans per tube were used for inhibition of binding assays (288,301). Although these amounts could be achieved in the investigation of blood group related antigens, it is rare to obtain sufficient purified material for bioactivity studies and concomitant structural determination by conventional methods.

To address the requirement of a microscale technique to investigate directly interactions between GBPs and oligosaccharides, Feizi and colleagues developed the NGL technology (257,302). This involves microgram-scale conjugation of the oligosaccharides to a lipid tag, which renders oligosaccharides amphipathic. The hydrophobicity enables the oligosaccharides to be immobilized on solid matrices, for example, plastic microwells, silica thin layer chromatography plates, nitrocellose-coated glass slides, in a clustered mode, thereby enabling the detection of binding by GBPs. Furthermore, similar to natural glycolipids, NGL mixtures could be resolved by high performance TLC (HPTLC) (also referred to as *macro*array), which is a big advantage compared with the glycan-protein conjugates. In addition to generation of *macro*arrays of oligosaccharides released from *O*- and *N*-glycosylated proteins, the NGL technology makes possible to investigate the antigen or ligand activity of glycans of natural glycolipids after their release using endo-ceramidase or those of GAGs and polysaccharides derived after their partial depolymerisation.

Reducing oligosaccharides with free aldehyde group can be linked to an amino-phospholipid, such as 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE, Figure 1.14) by reductive amination. Reduced *O*-glycans released from antigen-positive glycoproteins can also be conjugated after mild periodate oxidation (303). With the fluorescent lipid tag N-aminoacetyl-N-(9-anthracenylmethyl)-1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine, [ADHP, (304)], the AD-NGLs (Figure 1.14) can be visualized directly under UV light or by a fluorescence detector in an HPLC system. In addition, the NGLs have excellent ionization properties, enabling sequencing *in situ* by mass spectrometry (MS) to reveal molecular mass, monosaccharide sequence, branching pattern and the presence of functional groups such as sulphate or phosphate (85,305,306).

The NGL-based techniques enable identification of antigen-positive oligosaccharides, or ligands, from highly heterogeneous *O*-glycome of epithelial mucins. A particularly important example was the study of the HNK-1 antigen (See Figure 1.6 for sequence). This antigen is a target of auto-antibodies that give rise to a demyelinating disease. Among the *O*-glycans released from glycopeptides of the brain, a minor population were shown to carry the HNK-1 epitope (Figure 1.6). These were unusual *O*-mannosyl sequences rather than the well-known, commonly occurring, *O*-GalNAc sequences (49). Thereafter, upon analysis of the antigen-negative *O*-glycans, it was found that the *O*-mannose species constitute about 30% of total *O*-glycans in the brain (307). In addition, the NGL technology has contributed to a better understanding of the binding specificities of endogenous GBPs of the immune system, such as selectins. This is highlighted by the discovery of a new family of E-selectin ligand, sulphated Le^a and Le^x (3'-sulpho-Le^a and 3'-sulpho-Le^x), isolated from an epithelial mucin and characterized by MS (85,308). Subsequent studies showed that these two structures are also recognized by L-selectin and more strongly bound than the sialylated analogues (48,309).

Generation of NGLs by reductive amination leads to the ring opening of the monosaccharide on the reducing terminal (DH-NGL and AD-NGL, Figure 1.14), limiting its application to the investigation of GBPs whose binding epitopes involve the reducing terminal core monosaccharide. An alternative conjugation method using aminooxy-functionalized DHPE (AOPE) by oxime ligation (AO-NGLs, Figure 1.14)

has been developed (310). A significant proportion of the AO-NGLs synthesized were in ring closed form, thereby enabling the binding of short-chain oligosaccharides and enhancing the applicabilities of the NGLs. Notably, binding of plant lectins *Pisum sativum*, which requires the fucosylated core sequence, can be detected with the AO form, but not with the conventional DH-NGLs (310).

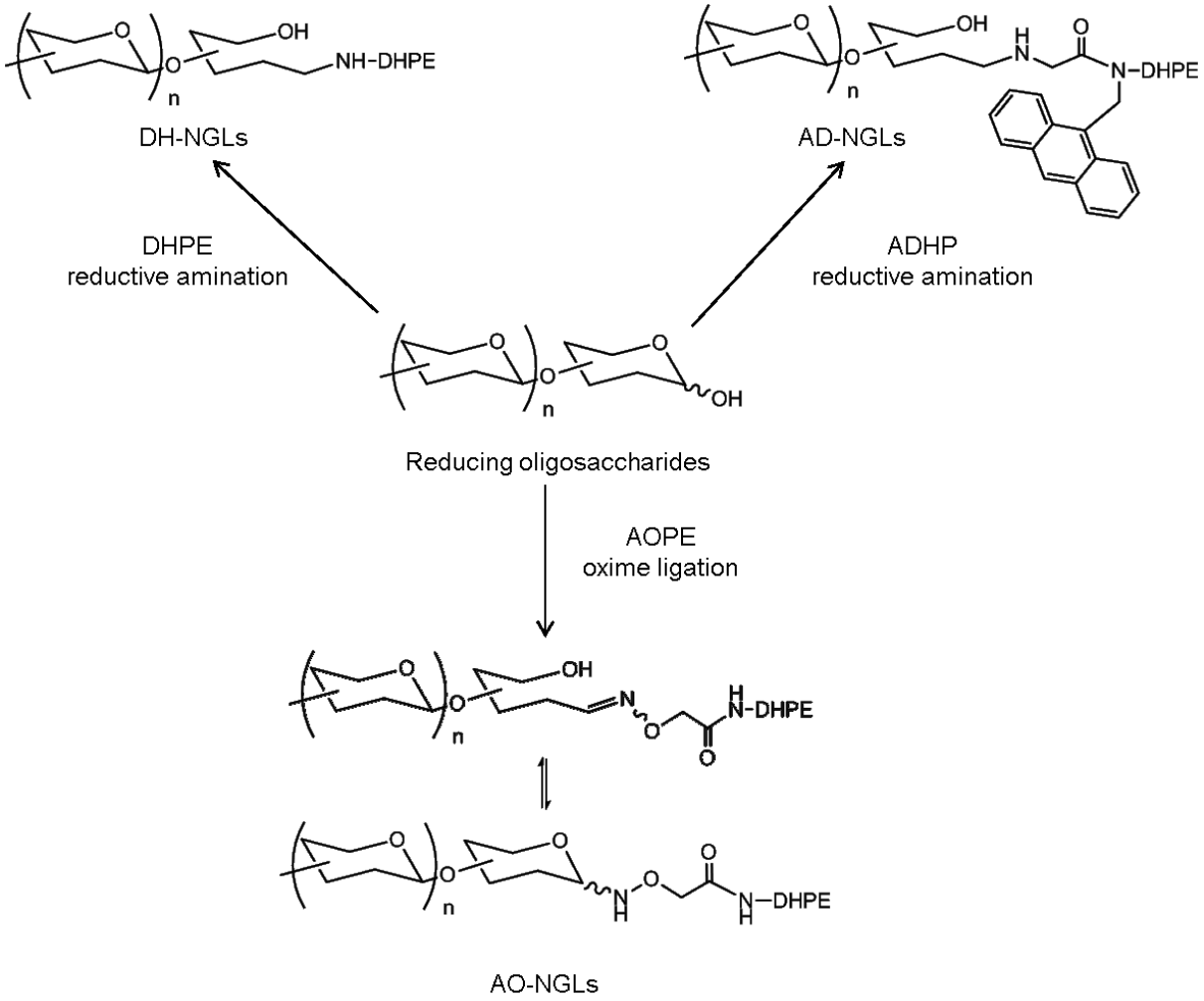


Figure 1.14 Reaction schemes for preparation of the DH-, AD- and AO-NGLs from reducing oligosaccharides by reductive amination and oxime ligation. Updated and redrawn from (310).

1.3.4 Microarray system based on the NGL technology

There was a need, however, to further miniaturize the platform to fulfil the increasing demand for smaller consumption and higher throughput of glycan samples during structural and ligand analyses.

In 2002, the first sequence-defined oligosaccharide microarray system based on NGL technology was introduced in the Glycosciences Laboratory (258). Each lipid-linked probe is incorporated in a liposome together with carrier lipids, cholesterol and lecithin, and is robotically arrayed at low fmol level, on nitrocellulose coated glass slides (Figure 1.15). This platform features noncovalent presentation of lipid-linked probes in clustered display and an element of probe mobility within the liposome. These characteristics confer high avidity and mimick to some extent the status of natural glycans on the cell surface (311). The library of probes has expanded to 800, including NGLs and glycolipids, naturally occurring and synthetic, comprising a variety of mammalian type *N*- (high mannose, complex and hybrid forms) and *O*-glycans (*O*-GalNAc, *O*-Fuc and *O*-Man), blood group-related sequences (A, B, H, Le^a, Le^b, Le^x and Le^y and their sialylated and sulphated forms), glycolipids (neutral, sialylated and sulphated), GAG-related sequences, as well as probes derived from oligosaccharides of fungal, bacterial and plant polysaccharides (259).

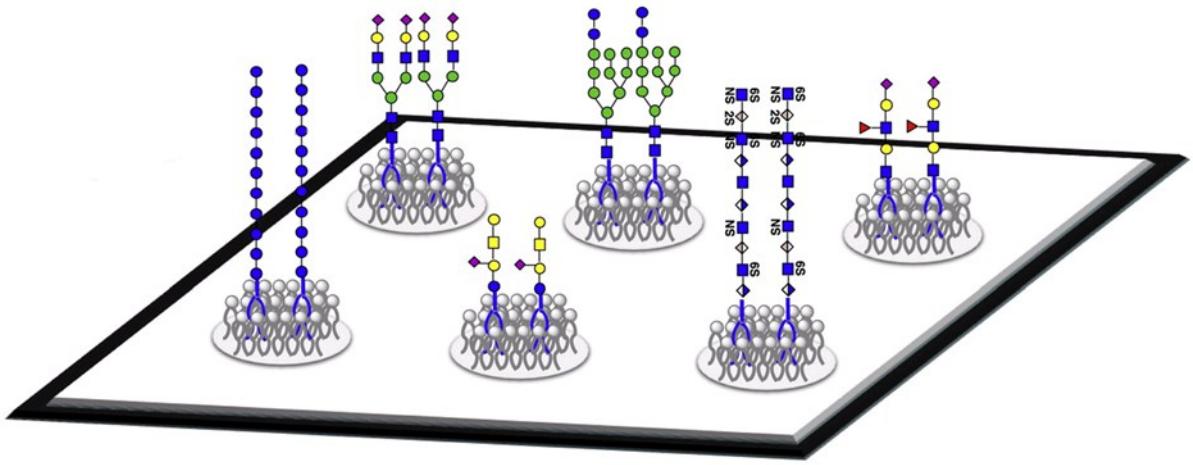


Figure 1.15 Schematic representation of the NGL-based microarray. NGLs of generated from *N*-glycans, *O*-glycans, depolymerized polysaccharides, GAGs and naturally occurring glycolipids were depicted. Symbols were in Figure 1.1. Taken from (259) with permission.

Applications of the microarray platform have included the study of the carbohydrate ligands for diverse GBPs of parasites (102,103,312,313), viruses (314-318) and antibodies (319-322). Of special interest were the analysis of pandemic human influenza A (H1N1) 2009 viruses, using a NGL-based microarray of 80 sialylated oligosaccharides and 6 neutral controls (323). The microarray encompassed sialylated sequences with different backbone types, chain lengths, branching patterns, and various combinations of sialylation, fucosylation and sulphation

patterns. Results showed that the 2009 pandemic human influenza viruses bound to α2,3-sialylated sequences in addition to the α2,6-sialylated sequences. In comparison, the seasonal human H1N1 viruses lacked binding to the α2,3-sialylated sequences (323). The lower respiratory tract expresses a larger proportion of α2,3-linked sialylated glycans than the α2,6-linked, which are mainly expressed in upper respiratory tract. Binding to α2,3-linked sialic acid receptors may therefore account, at least in part, for the higher virulence of the pandemic virus. A follow-up study showed that binding to the α2,3-sialylated sequences was increased by an spontaneous single mutation of the haemagglutinin (D222G) in viruses isolated from fatal or severe cases of infection (41).

One big advantage of the NGL technology is the so-called 'designer' array approach, by which the probes can be generated from a targeted ligand/antigen-containing source so that the oligosaccharide ligands can be revealed, isolated and characterized. Using this approach, Palma et al. (324) determined the carbohydrate ligand structure of the C-type lectin-like receptor Dectin-1, an important player in the innate immune system against fungal pathogens. The 'designer' probes were generated from partially depolymerized Dectin-1 ligand-positive and -negative glucan polysaccharides. Only β1,3-linked gluco-oligosaccharides longer than 10mer were bound, whereas the β1,6-linked analogues were not bound (324). The NGL-based carbohydrate microarray system is moreover a powerful platform which enables the generation of a comprehensive array of oligosaccharides from the glycomes of antigen- or ligand-positive cells, tissues or glycoconjugates to isolate and characterize the bioactive components (311). This is exemplified by the characterization of an elusive human prostate cancer-associated antigen F77, which is the subject of Chapter 3 in this thesis.

Of particular importance to any microarray platform is software for data presentation, storage and retrieval. Dr. Mark Stoll in the Glycosciences Laboratory has developed dedicated software to fulfil this need (325).

1.3.5 Other microarray platforms

Since 2002, carbohydrate microarray platforms have flourished with the need to rapidly probe glycan-GBP interactions. There are two glycan microarray platforms with the large coverage of glycan sequences. One is the Welcome Trust supported NGL-based carbohydrate microarray facilities in the Glycosciences Laboratory (259), the other is that of the Consortium of Functional Glycomics (CFG) built by National Institute of General Medical Sciences in the US (10,326). In addition numerous microarray systems containing small focused repertoires of glycan sequences have been set up.

Overall, the existing oligosaccharide microarrays can be divided into two categories based on immobilization strategies: non-covalent and covalent. The NGL-based microarray platform is an example of non-covalent immobilization strategy. The lipid moiety of NGLs forms hydrophobic interactions with the nitrocellulose-coated solid surface (Figure 1.16A). In addition to NGLs, biotin- and oligonucleotide-conjugated oligosaccharides can be immobilized to streptavidin- and oligonucleotide-coated surfaces, both through non-covalent interactions.

The other strategy is to immobilize the glycans via covalent bonds. This often requires a modified solid surface and glycan derivatives with special functional groups. These glycans are often obtained by chemical derivatization or by *de novo* chemical or chemo-enzymatic synthesis. Amine functionalized glycans, for example, can be immobilized to the *N*-hydroxysuccinimide (NHS) ester-derivatized glass slides. This is the basis of the CFG microarrays (Figure 1.16B) (327). So far many reactions have been explored to prepare glycan microarrays, including Diels-Alder reaction, Staudinger ligation, and click chemistry. These have been reviewed in reference (326).

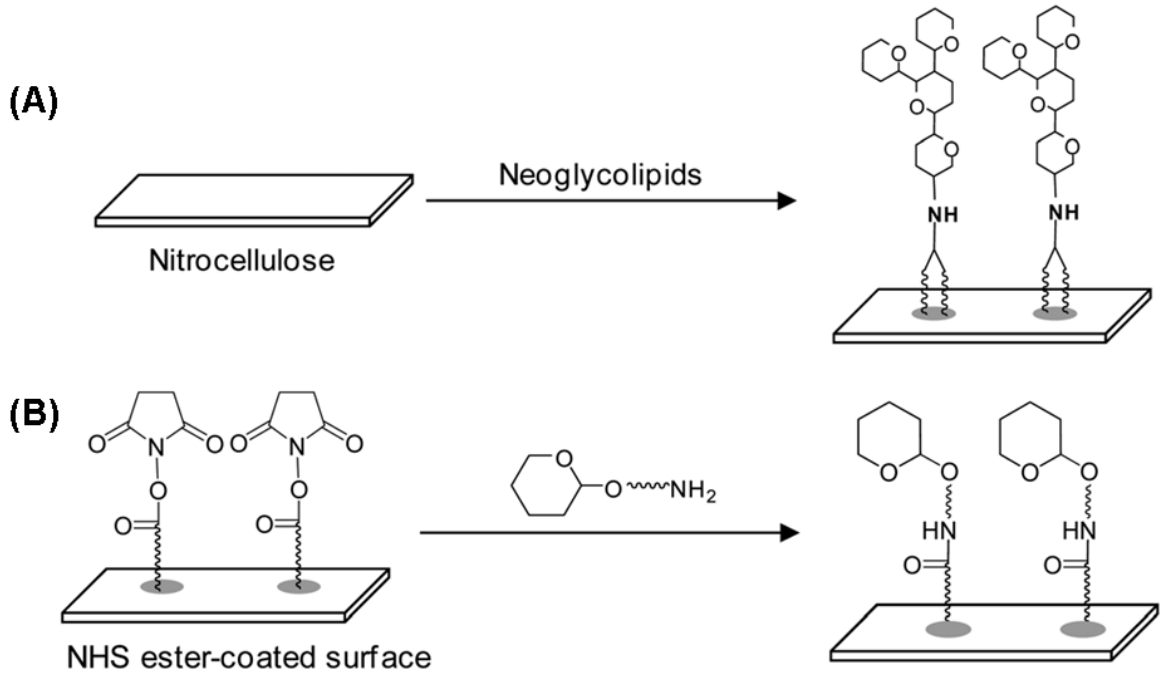


Figure 1.16 Examples of the two immobilization strategies, non-convalent (A) and covalent (B), used for generating carbohydrate microarrays. In (**A**) the neoglycolipids were immobilized on the hydrophobic surface via hydrophobic lipid chains. In (**B**) the amine functionalized glycans react to the *N*-hydroxysuccinimide (NHS) - coated surface by covalent bond. Taken from (326) with permission.

Presentation of the glycans in the microarrays is an important consideration for all the carbohydrate microarray platforms. There is consensus that features of presentation, such as glycan orientation and density, could have a great impact on the interactions between glycans and GBPs. One particular factor that influences the orientation is the linker which connects the glycans to the solid phase. Using molecular dynamics simulations, Wood et al suggested that the chain length, 3D structure and the potential interactions of the linker with the surface could have impact on the glycan orientation (328). This influence cannot be neglected as it could lead to false-negative results (329).

Glycoconjugates printed onto surfaces present glycans in a multivalent form and are valuable for screening GBPs for binding. These include natural glycoproteins, neoglycoproteins, neoglycopeptides and glycodendrimers. In the Glycosciences Laboratory, high molecular weight mucin-type glycoproteins are immobilized after enrichment from crude materials and being treated with proteases. These mucin arrays have become unique tools for screening for ligand- or antigen-positive sources, which open way to the “designer” array approach. Examples are discussed in Chapter 3 and Chapter 4.

A microarray strategy recently developed has been termed “shotgun array” (330), a term Cummings and colleagues refer to, for focusing structural analyses on functionally important glycans in the complex glycomes of animal cells and tissues. There are many analogies with designer arrays. In the “shotgun array” approach, oligosaccharides empirically from glycomes such as bovine brain gangliosides (330), human milk (331) and pig lung (332) have been resolved and arrayed, although the glycans may not be fully structurally characterized. These arrays can subsequently be probed with various GBPs. Those of interest are characterized using a combination of binding data obtained with a series of sequence-specific GBPs and other biochemical approaches including MS, MS^n and exoglycosidase treatments of the glycans. Song *et al* prepared a shotgun array of 40 fractions of glycolipids derived from bovine brain gangliosides. Using this ganglioside array, they found that the antibody profile of sera of patients with Lyme disease was different from those of control group. In particular, a ganglioside GD1b-lactone was found to be bound by sera of the patients but not of controls (330). The most recent example was the generation of pig lung shotgun array for the purpose of identification of receptors for a panel of influenza viruses isolated from human, birds and swine (332). The glycan library consisted of 96 *N*-glycan, 62 *O*-glycan and 36 glycolipid fractions. Interestingly, the viruses tested by this method showed binding to variously sialylated *N*-glycans, but binding to *O*-glycans and glycolipid-derived probes was not detected (332).

In sum carbohydrate microarrays now enable the studies of carbohydrate recognition systems at the glycome level. With accumulating knowledge on binding specificities of each GBP, it is anticipated that we can finally investigate GBP-glycan interaction at the interactome level. Comprehensive understanding of each GBP-glycan interaction will provide key information for further biological studies (311).

1.3.6 Cell transfection with glycosyltransferases

The last two decades have witnessed a boom in gene technology. In the field of glycobiology, the discovery and cloning of genes encoding glycosyltransferases and sulphotransferases enables cell transfection studies.

This approach has been well exemplified by the characterization of the antigenic determinant of HMOCC-1, which is recognized by humanized mAb HMOCC-1 raised against ovarian cancer RMG-I cells (333). Immunohistochemistry studies showed that the antibody stained 83.2% of ovarian carcinomas, also to a lesser extent some other epithelial tumours such as gastric, colonic, and pancreatic tumours. HMOCC-1 could attenuate tumour metastasis by inhibiting the attachment of RMG-I cells to mesothelial cells (334). The researchers first identified an antigen-negative cell line, which was HEK293T cell in this study. A mixture of glycosyltransferases and sulphotransferases were transferred into the HEK293T cells to generate a new cell line which expresses the HMOCC-1 antigen. By subtraction and various combinations of these enzymes, the key enzymes responsible for antigen generation finally emerged to be a sulphotransferase GAL3ST3 and a glycosyltransferase B3GNT7. Another sulphotransferase, CHST1, could enhance the antigen intensity. This was complemented by gene expression analysis and knockdown of the identified enzymes in the HMOCC-1-positive cells. Based on the specificities of these enzymes, the antigen was deduced as a sulphated poly-LacNAc sequence SU-3Galβ-4GlcNAcβ-(±SU-6)3Galβ-4GlcNAcβ-. Finally the deduction was confirmed by inhibition assays using four synthetic mono- and di-sulphated and non-sulphated oligosaccharides (333). Using the same strategy, another antigen recognized by a endometrial cancer-associated antibody HMMC-1 was characterized as a blood group H-related structure Fucα-2Galβ-4GlcNAcβ-3Galβ-3GalNAc- (335).

The transfection approach can be applicable to the characterization of carbohydrate antigens carried on many glycoconjugate species. It can be informative and is a good complement to the microarray binding analysis and other approaches mentioned above. It gives insights to the gene expression of the relevant glycosyltransferases and sulphotransferases, which link the sequences of carbohydrate ligand/antigen (glycome) to the biosynthesis pathways (genome). The application will be further discussed in Chapter 3.

Aims of this thesis

The aims of this thesis have been to characterize two cancer-associated carbohydrate antigens. The first is F77, which is a human prostate cancer-associated carbohydrate antigen, and the second is AE3, which is more broadly distributed and has been considered a promising serum biomarker for epithelial cancers. I investigate the sequences of these antigens by carbohydrate microarray approaches.

I have made two approaches to elucidate these antigens. The first is the 'designer' array approach whereby antigen-positive mucin-type glycoproteins are identified as sources. Glycans are released from them and converted to NGLs. Multidimensional chromatographies are used to isolate the antigen-positive components and mass spectrometric analyses are performed for sequence assignment. The second approach is the generation of focused microarrays of sequence-defined oligosaccharides (as NGLs) or glycolipids for close comparisons of the antigenic activities of the closely-related glycan sequences. The assignments of the F77 and AE3 antigens are corroborated using chemically synthesized oligosaccharides.

The sequences of the two antigens and their biological relevance are discussed, as well as the potential clinical applications of the antigens and the corresponding antibodies.

Chapter 2

MATERIALS AND METHODS

2.1 Reagents and solvents

2.1.1 Solvents for HPTLC and HPLC

Table 2.1 Mixed solvents for HPTLC and HPLC

Solvent*	Ratio (v/v)
$CHCl_3$/MeOH/H_2O	130/50/9
	60/35/8
	55/45/10
	105/100/28
	25/25/8
	50/55/18
	40/70/30
	25/70/30
	20/70/30
	15/70/30
	130/70/9
	10/20/8
$CHCl_3$/MeOH/0.05 M NH_4OAc	25/25/8
ACN/H_2O	80/20
	20/80

*storage of the solvents was at ambient temperature, except for the two ACN/H_2O solvents which are stored at 4 ˚C.

2.1.2 Reagents for HPTLC staining

Table 2.2 Reagents for HPTLC staining

Name	Preparation*
Orcinol spray reagent	Dissolve 0.9 g orcinol monohydrate (Sigma) in 25 ml H_2O, add 375 ml ethanol, 50 ml concentrated H_2SO_4 with stirring and cool to 4 ˚C
Resorcinol spray reagent	Dissolve resorcinol (Sigma) in H_2O to make 2 % resorcinol solution. Mix 5 ml of 2 % resorcinol solution with 45 ml 5 M HCl and 125 µl 0.1 M $CuSO_4$ and stay for 4 h
Primulin stock solution	Dissolve primulin (Sigma) in acetone/H_2O, 1:9 (v/v) to make 1 mg/ml solution
Primulin spray reagent	Dilute the primulin stock solution to 1/100 in acetone/H_2O, 4:1 (v/v)
Primulin TLC staining reagent	Dilute the primulin stock solution to 1/100 in PBS buffer
Ninhydrin spray reagent	Dissolve 0.2 g ninhydrin (Sigma) in 100 ml ethanol

*storage of the solvents was at 4 ˚C, except for the orcinol spray reagent and resorcinol spray reagent were stored at ambient temperature, and the primulin TLC staining reagent was freshly made.

2.1.3 Buffers and solutions for binding assays

Table 2.3 Buffers and solutions for binding assays

Name	Recipe	Preparation*
PBS buffer	10 mM phosphate buffer, 150 mM NaCl, pH 7.4	Dilute from 10X solution (Sigma)
HBS buffer	5 mM Hepes, 150 mM, 5 mM $CaCl_2$, pH 7.4	Add 2.5 ml 1 M Hepes (Fisher Scientific), 15 ml 5 M NaCl and 2.5 ml 1M $CaCl_2$ to 480 ml H_2O
3% BSA	3% bovine serum albumin in HBS buffer	Dilute from 30% BSA (Sigma, protease free) in HBS buffer
Casein	1% casein in 100 mM sodium phosphate, 150 mM NaCl, pH 7.4	Directly use (Pierce)
Plexigum TLC overlay solution	0.5% poly(ethyl methacrylate) in ether/hexane (1:5, v/v)	Dissolve poly(ethyl methacrylate) (Sigma) in ether/hexane (1:5, v/v)
Streptavidin-HRP	5 µg/ml in 3% BSA	Dilute the 1 mg/ml streptavidin-HRP solution (Sigma) in 3% BSA
3,3'-diaminobenzidine (DAB) liquid substrate system	N/A	Add 30 µl of the DAB liquid chromogen solution to 1 ml DAB liquid buffer solution (Sigma)
FAST OPD	0.4 mg/ml o-phenylenediamine dihydrochloride, 0.4 mg/ml urea hydrogen peroxide, 0.05 M phosphate-citrate, pH 5.0	Drop two tablets of the commercial *FAST OPD* (Sigma) into 20 ml H_2O

*These were all freshly prepared except for the casein.

2.2 Materials

2.2.1 Antibodies and lectins

Table 2.4 Antibodies

Name	Clone	Isotype	Source	Cat. No.	Dilution or concentration
F77	not known	mIgG3*	Hongtao Zhang	170712	10 µg/ml
AE3	culture supernatant	mIgM	Juthathip Mongkolsapaya	200711	1:3
Anti-H type 1	17-206	mIgG3	Abcam	ab3355	1:100
Anti-H type 2	BRIC-231	mIgG1	Abcam	ab33404	1:100
Anti-A	T36	mIgG3	Abcam	ab3353	1:100
Anti-B	89-F	mIgM	Santa Cruz	sc-52371	1 µg/ml
Anti-SSEA-1	MC480	mIgM	Developmental Studies Hybridoma Bank, Univ of Iowa	N/A	1:300
Anti-Le^y	C14	mIgM	Lindy Durrant	290513	Neat
Anti-Le^y	FG27/18	mIgG	Lindy Durrant	290513	20 µg/ml
Ma	anti-serum	hIgM**	Glycosciences Lab	4˚-Daniel-0	1:100
Biotin anti-mIgG (whole molecule)	polyclonal	not known	Sigma	B7264	1:200
Biotin anti-mIgG (Fc specific)	polyclonal	not known	Sigma	B7401	1:200
Biotin anti-mIgM (µ-chain specific)	polyclonal	not known	Sigma	B9265	1:200
Biotin anti-hIgM (µ-chain specific)	not known	not known	Vector	BA-3020	1:200

*m is mouse.
**h is human.

Table 2.5 Lectins

Name	Abbr.	Source	Cat. No.	Concentration (µg/ml)
Biotin *Ulex Europaeus* agglutinin I	UEA-I	Vector	B-1065	50
Biotin *Aleuria Aurantia* lectin	AAL		B-1395	2
Biotin Peanut agglutinin	PNA		B-1075	5
Biotin *Ricinus Communis* Agglutinin I	RCA_{120}		B-1085	5
Biotin *Wheat Germ* Agglutinin	WGA		B-1025	2
Biotin *Helix pomatia* lectin	HPA	Sigma	L-6512	10

2.2.2 Glycosidases

Table 2.6 Glycosidases

Name	Buffer	Source	Cat. No.
β1,4-galactosidase	50 mM sodium citrate buffer, containing 100 mM NaCl (pH 6.0)	New England BioLabs	P0730
β-*N*-acetylglucosaminidase	50 mM sodium citrate buffer, containing 1 µg/ml BSA (pH 6.0)		P0732
α1,2-fucosidase	50 mM sodium citrate buffer, containing 100 mM NaCl and 1 µg/ml BSA (pH 6.0)		P0724

2.2.3 Glycoproteins

Fifty-one preparations of mucin-rich glycoproteins were examined in this thesis (Table 2.7). These had been previously enriched or purified as described below and included in Mucin Array Set 1 (Table 3.1) and Mucin Array Set 2 (Table 4.2).

Samples 1 to 31 were derived from lyophilised ovarian cystadenoma fluids and were from collections of Winifred Watkins and Walter Morgan at the former Lister Institute. These were enriched for mucin type glycoproteins by treatment at 37 ºC for up to 16 h with pepsin, pronase or trypsin. The ratio of enzyme to dry weight was 1% w/w. After reaction, the samples were centrifuged (4,000 x g, 10-20 min), the supernatants were lyophilised and taken up in 3.5 mg/ml sodium acetate and precipitated with ethanol, 80% v/v at -20 ºC. After centrifugation (4,000 x g, 10-20 min), the pellets were washed with 80% (v/v) ethanol at -20 ºC and reconstituted in H_2O. Sample 31 had been further purified by phenol extractions (336). Samples 32 to 37 were purified ovarian cystadenoma glycoproteins from the Elvin A Kabat collection (Columba Medical Center, New York). These had been pepsin-treated and precipitated with various concentrations of ethanol (301,337). Samples 38-42 were from meconium and enriched after pronase digestion and ethanol precipitation (338). Sample 43, HCA, the so-called human carcinoma antigen (gift of Dr Joe Zhou, Egenix), had been extracted from human prostate cancer cell line PC3 by ammonium sulphate precipitation, followed by purification by gel filtration chromatography and affinity chromatography using antibody AE3 (8). Two mucin-rich milk preparations, batch #3 and #6, were also included as sample 44 and 45. Two mucin preparations, designated sheep stomach (S1+S10) and new sheep pool had been obtained from sheep gastric mucosal scrapings after pepsin digestion and ethanol precipitation (339), and were included as samples 46 and 47. Samples 48-51, porcine stomach mucin (PSM), bovine submaxillary mucin (BSM), transferrin and invertase were from Sigma.

Table 2.7 Mucin-type epithelial glycoproteins used in this thesis

Position	Designation	Donor red cell blood-group	Secretor status
	Enriched cystadenoma glycoproteins		
1	Cys 301 (Pro)*	weak A	Non-secretor
2	Cys 301 (Pep)	weak A	Non-secretor
3	Cys 350 (Trp)	A1	Non-secretor
4	Cys 379 (Pro)	A2B1	Non-secretor
5	Cys 379 (Pep)	A2B1	Non-secretor
6	Cys 444 (Trp)	Unknown	Non-secretor
7	Cys 444 (Pro)	Unknown	Non-secretor
8	Cys 444 (Pep)	Unknown	Non-secretor
9	Cys 446 (Pro)	B	Non-secretor
10	Cys 446 (Pep)	B	Non-secretor
11	Cys 461 (Trp)	Unknown	Non-secretor
12	Cys 461 (Pro)	Unknown	Non-secretor
13	Cys 461 (Pep)	Unknown	Non-secretor
14	Cys 654 (Pro)	B	Non-secretor
15	Cys 654 (Pep)	B	Non-secretor
16	Cys 705 (Pro)	Unknown	Non-secretor
17	Cys 717 (Pro)	A1	Non-secretor
18	Cys 717 (Pep)	A1	Non-secretor
19	Cys 733 (Pro)	A1	Secretor
20	Cys 745 (Pro)	A	Non-secretor
21	Cys 745 (Pep)	A	Non-secretor
22	Cys 754 (Pro)	Unknown	Non-secretor
23	Cys 754 (Pep)	Unknown	Non-secretor
24	Cys 756 (Trp)	A	Non-secretor
25	Cys 756 (Pro)	A	Non-secretor
26	Cys 756 (Pep)	A	Non-secretor
27	Cys 762 (Trp)	O	Non-secretor
28	Cys 762 (Pro)	O	Non-secretor
29	Cys 762 (Pep)	O	Non-secretor
30	Cys 765 (Pro)	O	Non-secretor
	Purified cystadenoma glycoproteins		
31	B substance (Pro)	B	Secretor
32	Og 10% 2x (Pep)	Unknown	Non-secretor
33	Og 10% from 20% (Pep)	Unknown	Non-secretor
34	Og 20% 2x (Pep)	Unknown	Non-secretor
35	N1 20% (Pep)	Unknown	Non-secretor
36	Tij 10% 2x (Pep)	B	Secretor
37	OC2	Unknown	Unknown
	Meconia		
38	Meconium B (Pro)	B	Unknown
39	Meconium Mo (Pro)	Unknown	Unknown
40	Meconium Wo (Pro)	Unknown	Unknown
41	Meconium He (Pro)	Unknown	Unknown
42	Meconium Pa (Pro)	Unknown	Unknown
	Other glycoproteins		
43	HCA	Unknown	Unknown
44	Milk batch #3	Unknown	Unknown
45	Milk batch #6	Unknown	Unknown
46	Sheep stomach (S1+S10) (Pep)	Unknown	Unknown
47	New sheep stomach pool (Pep)	Unknown	Unknown
48	Porcine gastric mucin	A+H	(Sigma)

49	Bovine submaxillary mucin	Unknown	(Sigma)
50	Transferrin	Unknown	Unknown
51	Invertase	Unknown	Unknown

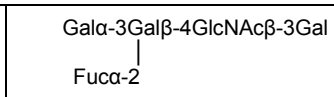
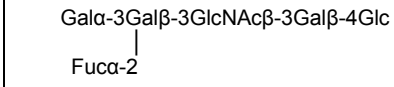
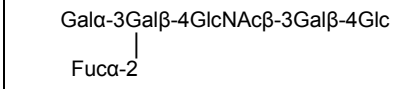
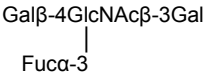
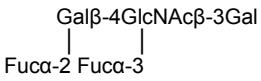
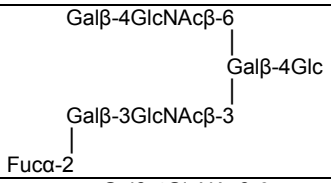
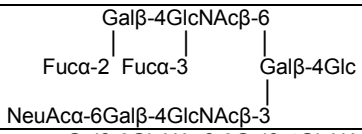
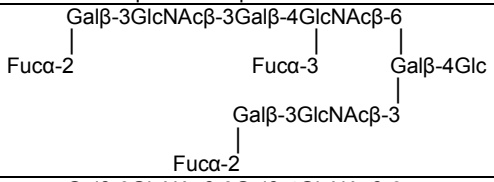
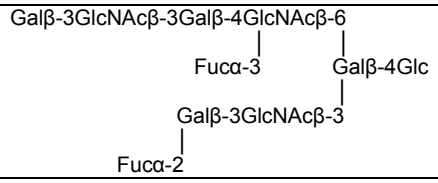
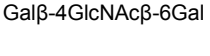
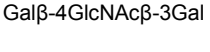
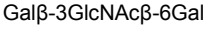
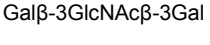
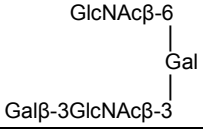
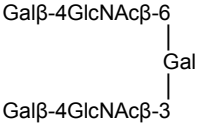
* Trp, Pro and Pep refer to pronase, trypsin or pepsin enzyme treatments for solubilizing mucin type glycoproteins.

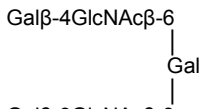
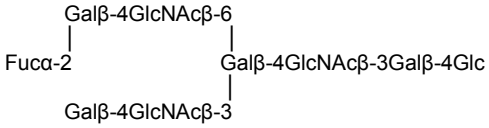
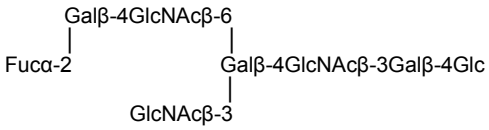
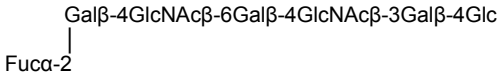
2.2.4 Oligosaccharides and a glyco-amino acid

The key oligosaccharides and a glyco-amino acid used in this thesis and their structural features are listed in Table 2.8. Ten oligosaccharides were from Elicityl and were purified by normal-phase HPLC using an XBridge amide column (Waters). The elution was with a solvent system of ACN/H_2O (Solvent A: ACN/H_2O, 80:20 and B: ACN/H_2O, 20:80), with a gradient from 25% to 33%B in 33 min at a flow rate of 0.7 ml/min. Detection was by UV at 196 nm. The oligosaccharide TFiLNO (1-2, -2, -3) was from Dextra and its sequence was confirmed by negative-ion ES-CID-MS/MS. A difucosylated decasaccharide was received from Dextra as an unknown impurity of TFiLNO(1-2,-2,-3), which was later characterized by ES-CID-MS/MS and renamed as DFiLNO. Oligosaccharides referred to as Orsay-1 to -7 were chemically synthesized in the laboratory of the late Professor S. David [cited in (340)]. GSC-915 is a nonasaccharide synthesized in Kiso Laboratory of Gifu University, Japan (unpublished). Four other oligosaccharides GSC-915-2 to -5 were generated by sequential glycosidase treatment (Section 2.10). A T disaccharide (Galβ-3GalNAc)-containing glyco-amino acid Galβ-3GalNAcα-Ser was also purchased from Dextra.

Table 2.8 Oligosaccharides and a glycoamino acid used in the thesis

Designation	Sequences	Source	Cat. No.	Structural features
H-tetra-T2	Galβ-4GlcNAcβ-3Gal | Fucα-2	Elicityl	GLY032-2	H type 2
LNFP-I	Galβ-3GlcNAcβ-3Galβ-4Glc | Fucα-2	Glycoscience Laboratory	N/A	H type 1
LnNFPI	Galβ-4GlcNAcβ-3Galβ-4Glc | Fucα-2	Elicityl	GLY033-2	H type 2
A-penta-T2	GalNAcα-3Galβ-4GlcNAcβ-3Gal | Fucα-2	Elicityl	GLY036-2	A type 2
A-Hexa-T1	GalNAcα-3Galβ-3GlcNAcβ-3Galβ-4Glc | Fucα-2	Elicityl	GLY037-1	A type 1
A-Hexa-T2	GalNAcα-3Galβ-4GlcNAcβ-3Galβ-4Glc | Fucα-2	Elicityl	GLY037-2	A type 2

B-penta-T2		Elicityl	GLY039-2	B type 2
B-Hexa-T1		Elicityl	GLY040-1	B type 1
B-Hexa-T2		Elicityl	GLY040-2	B type 2
Le^x-tetra		Elicityl	GLY050	Le^x
Le^y-penta		Elicityl	GLY052	Le^y
MFLNH-I		Dextra	L735	H type 1
MSDFLNnH		Dextra	SLN907	Le^y
TFiLNO (1-2,-2,-3)		Dextra	L1001	H type 1
DFiLNO		Dextra	N/A	H type 1
Orsay-1		Serge David	N/A	Type 2 backbone
Orsay-2		Serge David	N/A	Type 2 backbone
Orsay-3		Serge David	N/A	Type 1 backbone
Orsay-4		Serge David	N/A	Type 1 backbone
Orsay-5		Serge David	N/A	Type 1 backbone
Orsay-6		Serge David	N/A	Type 1 backbone

Orsay-7		Serge David	N/A	Type 2 & 1 backbone
GSC-915		Kiso	010514	H type 2
GSC-915-2		Enzymatic treatment	N/A	H type 2
GSC-915-3		Enzymatic treatment	N/A	H type 2
GSC-915-4	Galβ-4GlcNAcβ-6Galβ-4GlcNAcβ-3Galβ-4Glc	Enzymatic treatment	N/A	Type 2 backbone
GSC-915-5	GlcNAcβ-6Galβ-4GlcNAcβ-3Galβ-4Glc	Enzymatic treatment	N/A	N/A
pLNH	Galβ-3GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4Glc	Glycoscience Laboratory	N/A	Type 1 backbone
pLNnH	Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4Glc	Glycoscience Laboratory	N/A	Type 2 backbone
T-Ser	Galβ-3GalNAcα-Ser	Dextra	GS203	T antigen

2.2.5 Glycolipids

A glycolipid extract of prostate cancer cell line PC3 was prepared as described (341) and provided by our collaborator Dr. Hongtao Zhang in Professor Mark Greene's lab of University of Pennsylvania. Other glycolipids and their structural features are shown in Table 2.9. Four human red cell-derived blood group H and A-active glycolipids were from archival collections of Professor Michiko Fukuda of Sanford-Burnham Medical Research Institute. They were further purified by semi-preparative HPTLC as described in Section 2.7. The branched blood group A-active glycolipid Ad was de-fucosylated by α1,2-fucosidase as described in Section 2.10 to remove the minor H residue to ensure the absence of H-activity. Two branched blood group B-active glycolipids BIII and BIV, a B-like decaglycosylceramide and an I-active octaglycosylceramide were from Dr. Peter Hanfland of University of Bonn. Two sulphated glycolipids, SM1a and SB1a, were chemically synthesized in Professor Makoto Kiso Lab in Gifu University.

Table 2.9 Glycosylceramides used in this thesis

Designation	Sequences	Source	Ref	Structural features
H_2*	Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4Glc-Cer | Fucα-2	Michiko Fukuda	(342)	H type 2
H_3*	Galβ-4GlcNAcβ-6 | | Fucα-2 Galβ-4GlcNAcβ-3Galβ-4Glc-Cer | Galβ-4GlcNAcβ-3 | Fucα-2	Michiko Fukuda	(342)	H type 2
Ab*	GalNAcα-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4Glc-Cer | Fucα-2	Michiko Fukuda	(343)	A type 2
Ad*	GalNAcα-3Galβ-4GlcNAcβ-6 | | Fucα-2 Galβ-4GlcNAcβ-3Galβ-4Glc-Cer | GalNAcα-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3 | Fucα-2	Michiko Fukuda	(343)	A type 2
B-III dodeca	Galα-3Galβ-4GlcNAcβ-6 | | Fucα-2 Galβ-4GlcNAcβ-3Galβ-4Glc-Cer | Galα-3Galβ-4GlcNAcβ-3 | Fucα-2	Peter Hanfland	(344)	B type 2
B-IV tetradeca	Galα-3Galβ-4GlcNAcβ-6 | | Fucα-2 Galβ-4GlcNAcβ-3Galβ-4Glc-Cer | Galα-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3 | Fucα-2	Peter Hanfland	(344)	B type 2
B-like decaglycosylceramide	Galα-3Galβ-4GlcNAcβ-6 | Galβ-4GlcNAcβ-3Galβ-4Glc-Cer | Galα-3Galβ-4GlcNAcβ-3	Peter Hanfland	(345)	B-like
I-active octaglycosylceramide	Galβ-4GlcNAcβ-6 | Galβ-4GlcNAcβ-3Galβ-4Glc-Cer | Galβ-4GlcNAcβ-3	Peter Hanfland	(346, 347)	Type 2 backbone
SM1a	Galβ-3GalNAcβ-4Galβ-4Glc-Cer | SU-3	Kiso	(348)	sulphate/ T disaccharide
SB1a	Galβ-3GalNAcβ-4Galβ-4Glc-Cer | | SU-3 SU-3	Kiso	(349)	disulphate

*A minor component with an additional fucose residue was detected in H_2, Ab and Ad preparations and a minor component lacking one fucose residue was detected in H_3, by MALDI-MS analysis.

2.3 Release of *O*-glycans in reduced form from mucins

For typical experiments of reductive and non-reductive *O*-glycan release, 800-1,000 mg and 20-250 mg (dry weight) of mucin were referred to as large-scale and small-scale hydrolysis, respectively.

Reductive release was performed using the alkaline borohydride degradation method established by Carlson (350,351). Powder of lyophilised mucin was taken up in 0.05 M NaOH-1 M $NaIO_4$ at a final concentration of 25 mg/ml and heated at 45 °C for 16 h. After the reaction, AcOH/H_2O (1:1) was added to destroy borohydride and the mixture was then passed through a column of AG50W-X8 (H form, Bio-Rad) which was pre-equilibrated with H_2O. The boric acid generated was removed by repeated co-evaporation with methanol under N_2 stream, followed by lyophilisation. The products of PSM were reconstituted in H_2O for further analysis and fractionation. The products of crude ovarian cystadenoma materials (such as Cyst 756) were taken up with 0.05 M NH_4OAc and were further purified by size fractionation. The products were applied to a Bio-Gel P6 column (1.6 × 30 cm, Bio-Rad) equilibrated by 0.05 M NH_4OAc. Elution was with the same buffer so as to separate the oligosaccharide-enriched fraction in the included volume from residual glycoproteins or large glycopeptides eluted at void volume and salts eluted in the total volume. The oligosaccharide-enriched fraction was pooled, lyophilised, and reconstituted in 0.05 M NH_4OAc for further analysis.

2.4 Release of *O*-glycans in reducing form from mucins

Two non-reductive methods were applied in this study to release the oligosaccharides in reducing form.

Hydrazinolysis was performed by our collaborator Radoslaw Kozak at Ludger (Oxford) to release *O*-glycans from PSM as described (352). In brief, 50 mg (dry weight) PSM were dissolved in 10 ml of H_2O and divided into 1 ml aliquots. To each aliquot 1 ml hydrazine was added. Incubation was at 60 °C for 6 h. The products were re-*N*-acetylated and desalted by passing over LudgerClean CEX cartridges (cation exchange cartridge). One percent of the released products was labelled with 2-aminobenzamide (2-AB) using a Ludger 2-AB Glycan Labelling Kit as described (352) and analysed by HPLC and MALDI-MS. The rest were lyophilised and sent to us for further analysis.

Ethylamine was used to release the oligosaccharides in reducing form from ovarian cystadenoma material as described previously (308). Typically, ovarian Cyst 756 (250 mg, dry weight) was dissolved in 10 ml 70% (w/v) aqueous ethylamine and kept in the dark at either 22°C for 48 h or at 65°C for 6 h. These two conditions are referred to as E22 and E65, respectively. The bulk of ethylamine was evaporated under a stream of N_2 at 20°C and 60°C for the two reaction conditions, respectively, and the viscous yellow syrup was diluted with a small volume of H_2O before acidification to pH 4-5 by addition of AcOH/H_2O (1:1, v/v). The mixture was left at 4°C overnight and then passed through a cation-exchange column (AG50W-X8, H^+ form, 10 ml gel) equilibrated with H_2O. The column was washed with three bed-volumes of H_2O. The combined fall-through and washes were further purified using Bio-Gel P6 as described in Section 2.3, and the oligosaccharide-enriched fraction was freeze-dried and reconstituted in 0.05 M NH_4OAc for further analysis and fractionation.

2.5 Fractionation and purification of the oligosaccharides from reaction mixtures

2.5.1 Fractionation of *O*-glycans by charge

Two anion-exchange methods were used to fractionate the oligosaccharide mixtures released from PSM or ovarian cyst material.

For the reducing oligosaccharides released from PSM by hydrazinolosis and the reduced oligosaccharide alditols released from Cyst 756 by Carlson degradation, fractionation was performed with a column of DEAE-Sephadex A25 (OH form, GE). The oligosaccharide-enriched fraction was applied onto the column prewashed with 2 M NaCl, 500 mM NH_4OAc buffer (pH 5.0) and 10 mM NH_4OAc buffer (pH 5.0). The column was washed stepwise with 10 mM. 100 mM, 300 mM, and 500 mM NH_4OAc buffer (pH 5.0). After removal of the NH_4OAc by repeated co-evaporation with H_2O by lyophilisation, the fractions were ready for analysis or size fractionation. For the fractions of reducing *O*-glycans of PSM released by hydrazinolysis, further purification was performed. Each fraction was passed through a C18 Sep-Pak cartridge (Waters) and applied to a graphitised carbon SPE cartridge (Grace Davison Discovery Sciences) equilibrated with H_2O containing 0.1% (v/v) TFA. Elution was by 25% CH_3CN containing 0.1% (v/v) TFA. The purified fractions were lyophilised repeatedly and re-dissolved in H_2O for analysis and conjugation.

For the reducing oligosaccharides non-reductively released from Cyst 756 by ethylamine (E65), charge fractionation was performed using a column of AG1-X8 (acetate form, Bio-Rad). The column was washed with 300 mM pyridine/AcOH and equilibrated by 2 mM pyridine/AcOH. The reaction products were reconstituted in 2 mM pyridine/AcOH (pH 5.0) and applied on the column. Three fractions were eluted by 2 mM pyridine/AcOH (pH 5.0), 100 mM pyridine/AcOH (pH 5.0) and 300 mM Na_2SO_4, respectively. The former two fractions were repeatedly lyophilised to remove the pyridine/AcOH. The Na_2SO_4 eluent was desalted by Superdex peptide column (1 x 30cm, GE) with 0.05 M NH_4OAc as the solvent. All the fractions were lyophilised and reconstituted in H_2O for analysis and conjugation.

2.5.2 Fractionation of *O*-glycans by size

The oligosaccharide fractions obtained above and the *O*-glyan products from Carlson degradation of PSM were further fractionated by gel filtration chromatography on a Bio-Gel P4 column (1.6 × 90 cm, Bio-Rad). Samples containing mainly neutral oligosaccharides were dissolved in H_2O and were applied onto the column pre-equilibrated with H_2O. Samples with both neutral and acidic oligosaccharides were dissolved in 0.05 M NH_4OAc and were applied onto the column pre-equilibrated with 0.05 M NH_4OAc. Elution was at a flow rate of 15 ml/h with H_2O or 0.05 M NH_4OAc for the former or latter situations. Detection was by refractive index detector. The column was pre-calibrated using dextran and dextran hydrolysate under the same condition. Fractions were pooled according elution position. For refractionation of a pool of subfractions in the F77 study, a Bio-Gel P6 column (1.6 × 90 cm, Bio-Rad) was used and the conditions were the same as those used for Bio-Gel P4. Subfractions were combined and lyophilised. NH_4OAc was removed by repeated co-evaporation with H_2O. The dried fractions were re-dissolved in H_2O for further analysis or conjugation reaction.

2.5.3 Affinity chromatography for enrichment of antigen-positive *O*-glycans

Affinity chromatography was carried out using both UEA-I column and F77 antibody column for the purpose of enriching F77 antigen-positive *O*-glycan alditols.

The UEA-I absorbent (10 ml, Vector Labs) was packed in a syringe (1.6 × 5 cm) and prewashed with 10 mM PBS (pH 7.4). An *O*-glycan fraction, F3, obtained from size fractionation of the *O*-glycans released from PSM was dissolved in PBS and applied on top of the column. This was followed by washing with the same buffer until no materials appears in the effluent monitored by a refractive index detector. The bound material was then eluted by 40 ml of 10 mM, 30 mM and 50 mM Fuc in PBS. Fall-through and eluents were collected and combined according to the elution profile.

The obtained subfractions were purified by graphitised carbon SPE cartridge as described in Section 2.5.1 to remove the Fuc and salts.

The F77 affinity column was prepared by conjugating the F77 antibody to NHS-activated agarose (Thermo) in a spin column by our collaborators in Mark Greene Laboratory of University of Pennsylvania. An *O*-glycan fraction, F3, obtained from size fractionation of the *O*-glycans released from PSM was dissolved in PBS and applied onto the F77 affinity column equilibrated with the same buffer. The fall-through was collected and designated A1F. After a two-hour incubation at room temperature, the column was centrifuged (1000 × g) and washed with PBS three times. The washes were combined as A1W. Elution was with 100 mM glycine HCl (pH 2.5-3) and the eluents were neutralized by 1 M $(NH_4)_2CO_3$ and designated as A1E. The fraction A1F was then reapplied onto the column. The procedure was repeated and A2F, A2W and A2E were similarly obtained. All the five fractions were desalted by graphitised carbon SPE cartridge as mentioned in Section 2.5.1.

All the fractions obtained by affinity chromatographies were lyophilised and dissolved in H_2O for analysis and conjugation.

2.6 Preparation of NGLs

2.6.1 Lipid conjugation

2.6.1.1 Reducing oligosaccharides

Oligosaccharides listed in Table 2.8 and those *O*-glycans in reducing form in each fraction were converted into NGLs by reductive amination (for DH- and AD-NGLs) (353) or oxime ligation (for AO-NGLs) (310). Typically, 100 nmol of oligosaccharide was lyophilised and 80 µl DHPE, ADHP or AOPE solution (10 nmol/µl) in $CHCl_3$/MeOH, 1:1 (v/v) was added. For DH- and AD-NGL generation, 20 µl freshly prepared tetrabutylammonium cyanoborohydride (20 µg/µl in MeOH) were added to the mixture. Incubation of the reaction mixture was at 60 ˚C for 16 h. For AO-NGL generation, the solution was directly incubated at 60 ˚C for 16 h. When oligosaccharides larger than hexasacharides were used in both cases, a small amount of H_2O (~5%, w/v) was included at the beginning and the incubation time was prolonged to 72 h. After incubation, the solvent was evaporated and the residue was reconstituted in $CHCl_3$/MeOH/H_2O 25:25:8 for TLC analysis.

2.6.1.2 Reduced oligosaccharide alditols

Reductively released oligosaccharide alditols were conjugated to ADHP after mild periodate oxidation (303). Oligosaccharides in each fraction (~100 nmol) were lyophilised, followed by addition of 70 µl freshly prepared $NaIO_4$ (1.25 mg/ml) in imidazole buffer (40mM, pH 6.5). The mixture was incubated on ice in the dark for 5 min. Butane-2,3-diol (8 µl, 9 mg/ml in MeOH) was added and the mixture was incubated on ice for a further 40 min. Thereafter 200 µl ADHP solution (10 nmol/µl in $CHCl_3$/MeOH, 1:1) and 145 µl freshly prepared tetrabutylammonium cyanoborohydride solution (20 µg/µl in MeOH) was added followed by further incubation at 60 °C for 16 h. The solvent was evaporated and the mixture was dissolved in $CHCl_3$/MeOH/H_2O 25:25:8 for TLC analysis.

2.6.1.3 Glycoamino acid

The amino-terminating glyco-amino acid Galβ-3GalNAcα-Ser (Table 2.8) was converted to NGL using a novel lipid reagent (designated DHPA) with an aldehyde functionality linked to DHPE (unpublished) developed by Pengtao Zhang, Chunxia Li (Ocean University of China, Qingdao) and Wengang Chai (Glycosciences Laboratory). The details of preparation of the DHPA conjugate will be described elsewhere.

2.6.2 HPTLC analysis of conjugation products

Aliquots of the reaction mixtures (~1 nmol starting sugar) were analysed by HPTLC as described (353). The reaction product was applied onto aluminium-backed HPTLC plates (Merck) and developed in suitable solvents listed in Table 2.1. After development, the fluorescent NGLs and excess lipids were directly visualized under long-wave UV light. The non-fluorescent DH- and AO- NGLs were stained with primulin reagent (Table 2.2) before visualization under long-wave UV light. The same plate can be sprayed with orcinol reagent (Table 2.2) further and heated at 105 ˚C for detection of hexose.

2.7 Separation and purification of NGLs or glycolipids

For the purpose of removing excess lipid and isolating and purifying antigen-positive NGLs or glycolipids, silica cartridge, semi-prep HPTLC and HPLC were performed.

For normal-phase chromatography on silica cartridge, the reaction mixture was dried under N_2 stream, reconstituted in $CHCl_3$/MeOH/H_2O, 130:50:9 and applied onto a silica Sep-Pak cartridge containing 100 mg or 200 mg sorbents (Waters) preconditioned by $CHCl_3$. The column was then washed sequentially with $CHCl_3$/MeOH/H_2O, 130:50:9 (×3), 60:35:8 (×3), and 25:25:8 (×3). An aliquot of each fraction was analysed by HPTLC to monitor the separation as mentioned in Section 2.6.2. According to the mobility profile, washes were pooled into one or several fractions according to activity and interest and dried under N_2 stream for further investigation.

Alternatively, semi-prep HPTLC was performed to remove excess lipid and to purify the NGLs and glycolipids. The dried samples were reconstituted in $CHCl_3$/MeOH/H_2O, 25:25:8 and applied as long straits on HPTLC plates. The plates were then developed in a suitable solvent (Table 2.1). For fluorescent NGL fractions, after visualization under UV light, the TLC bands of interest were cut out and the silica gels were scraped off the plates. The NGLs were extracted from the silica gels with $CHCl_3$/MeOH/H_2O 25:25:8; alternatively a mini-column was packed with the scraped slilica gels and the NGLs were eluted off by the same solvent. Non-fluorescent NGLs and glycolipids can also be purified in the same way but they were localized by primulin staining of the two vertical edges (a few mm) cut from the plate.

Fluorescent NGLs can be fractionated and separated by HPLC (Gilson system) with a fluorescence detector at λ_{ex} 255 nm and λ_{em} 405 nm or a UV detector at λ_{max} 255 nm as described (304). An APS-2 column (5 µm Hypersil, 4.6 × 250 mm) was used with a gradient of $CHCl_3$/MeOH/H_2O 130:70:9 (solvent A) to 10:20:8 (solvent B) in 60 min at a flow rate of 0.5 ml/min. Fractions were pooled according to the chromatogram and were dried under N_2 stream for further investigation.

The NGL fractions obtained in silica cartridge, semi-prep TLC and HPLC were desalted by reverse-phase chromatography using C18 cartridge. Samples were reconstituted in a minimal amount of $CHCl_3$/MeOH/H_2O, 15:70:30 and applied onto a C18 Sep-Pak cartridge (100 mg or 200 mg sorbents, Waters) prewashed by $CHCl_3$/MeOH/H_2O, 15:70:30. After washing with MeOH/H_2O, 1:1, the NGLs were eluted by $CHCl_3$/MeOH/H_2O, 60:35:8, dried under N_2 stream and stored at -20 ˚C for further analysis.

2.8 Quantitation

2.8.1 Quantitation of oligosaccharides

Quantification of hexose in samples was carried out by dot orcinol-sulphuric acid method on TLC plates, using galactose as standard. Oligosaccharide fractions and serially diluted standard galactose were dotted on HPTLC plates. After spraying with orcinol reagent, the colour developed was measured by densitometry on a Shimadzu CS-9000 flying-spot scanner at 550 nm. Hexose content in samples was determined against the calibration curve recorded by the standard galactose samples. The molar quantities of the oligosaccharides in each fraction were estimated based on hexose content and molecular mass determined by MS analysis.

Quantification of sialic acid was carried out by resorcinol assay, using Neu5Ac as standard. The oligosaccharide fractions and standard Neu5Ac were dotted on HPTLC plates and sprayed with resorcinol reagent (Table 2.2). The silica surface of the plates was immediately sealed by clamping with two glass plates and heated at 140 ˚C for 10 min. Measurement was by densitometer at 580 nm. Content of sialic acid in samples was determined against the calibration curve recorded by the standard Neu5Ac samples.

2.8.2 Quantitation of NGLs

NGLs were generally quantified on HPTLC plates. The purified NGLs were dissolved in $CHCl_3$/MeOH/H_2O, 25:25:8 and applied on the HPTLC plate together with DH- or AD-NGL standards. After development the plate was sequentially immerged in PBS buffer, primulin solution, and PBS buffer for 10, 20 and 10 min, respectively, and air-dried. The fluorescence was measured by densitometer in linear reflectance fluorescence mode with excitation at 370 nm and detection through a filter with cut-off at 460 nm. The quantities of the NGLs in each fraction were then calculated by the calibration curve with the standard DH-NGLs. The NGLs were then adjusted to 50-100 pmol/μl as stock solution and stored at -20 ˚C.

2.9 Mass spectrometry of oligosaccharides, glycolipids and NGLs

MALDI-MS of the oligosaccharides and the derived NGLs was carried out on a TOF Spec-2E (Waters) or an AXIMA Assurance instrument (Shimadzu). Negative-ion MALDI-MS/MS was performed on an AXIMA Resonance instrument (Shimadzu). The oligosaccharides were dissolved in H_2O and the NGLs in $CHCl_3$/MeOH/H_2O, 25:25:8. The sample was deposited on the sample target together with ~1 µl of matrix of 2',4',6'-trihydroxyacetophenon. Laser energy was set at 20% (coarse) and 60% (fine) for TOF Spec-2E and at attenuation scale 80 for the AXIMA Assurance. For MS/MS, a collision gas Ar (2 bar) and collision energy set at 80-140 depending on specific samples were used for oligosaccharides and NGLs.

ES-MS and ES-CID-MS/MS of oligosaccharides were carried out on a Synapt G2-S instrument (Waters). Cone voltage was at 80 eV for the oligosaccharides while capillary voltage was maintained at 2.7 kV for conventional scale electrospray. The source temperature was at 120 °C and the desolvation temperature at 150 °C. Product-ion spectra were obtained using argon as the collision gas at a pressure of 1.7 bar. The collision energy was adjusted between 12 and 20 eV for optimal fragmentation of reducing oligosaccharides. A scan rate of 1.0 s/scan was used for both ES-MS and MS/MS experiments and the acquired spectra were summed for presentation. For analysis, oligosaccharides were dissolved in H_2O at a concentration of 10−20 pmol/µl, of which 1 µl was injected via HPLC injector. Solvent (ACN/H_2O 1:1) was delivered by a HPLC pump (Waters) at a flow rate of 10 µl/min.

2.10 Glycosidase treatment of oligosaccharides and NGLs

The synthetic nanosaccharide GSC-915 was treated sequentially with exoglycosidases, β1,4-galactosidase, β-*N*-acetylglucosaminidase, α1,2-fucosidase and β1,4-galactosidase to prepare a series of oligosaccharides GSC-915-2 to GSC-915-5 as shown in Table 2.8. The conditions were indicated in Table 2.6. Oligosaccharide substrates were lyophilised before reaction and dissolved in the reaction buffer to make 1 nmol/μl solution. The enzymes (3-20 U/nmol sugar) were added in the solution and incubated at 37 °C for 48 h. Thereafter, the reaction mixtures in each step were separated by HPLC on a Click-Mal column (5 μm, 4.6×150 mm) with a solvent system of A (ACN/H_2O, 80/20) and B (ACN/H_2O, 20/80). The gradient was from 20 to 45% B in 30 min at a flow rate of 1 ml/min and detection was by UV at 196 nm. The products were analysed by MALDI-MS, reconstituted in H_2O, quantified by dot-orcinol before converting to NGLs.

The NGLs and glycolipids were also treated with α1,2-fucosidase to remove minor component or to verify the carbohydrate sequences. Typically, 2 nmol of the NGL or glycolipids were taken up with 70 μl 50 mM sodium citrate buffer (pH 6.0), containing 100 mM NaCl, 1 μg/ml BSA and 0.5 mg/ml sodium cholate. The reaction mixture was sonicated briefly before addition of 200 U of α1-2 fucosidase, followed by incubation at 37 °C for 48 h. A standard LnNFPI AD-NGL was treated and analysed in parallel. The reaction process was monitored by MALDI-MS. After the reaction, the products were purified on a Sep-Pak C18 cartridge, reconstituted in $CHCl_3$/MeOH/H_2O, 25:25:8 and stored at -20 ˚C for further analysis.

2.11 Assays of antigen activities of glycoproteins, glycolipids, NGLs and oligosaccharides

Binding of mAb F77 was found to be temperature-dependent. Thus temperature of the binding experiments involving F77 was specified in the Chapter 3. Unless otherwise specified, the binding and inhibition experiments described below were performed at ambient temperature. The buffers and solutions used in the binding assays are listed in Table 2.3. The antibodies and lectins are listed in Table 2.4 and Table 2.5, respectively.

2.11.1 Chromatogram-binding and dot-blot assays

Mixtures of NGLs (~1 nmol of starting oligosaccharides) derived from oligosaccharides released from mucins, together with NGL standards (50-150 pmol) were applied onto a HPTLC plate. The plate was then developed in a suitable solvent (Table 2.1) and air-dried. The fluorescent AD-NGLs were visualized directly under UV light. If glycolipids or non-fluorescent DH- and AO-NGLs were included in the analysis, a duplicate plate was prepared for visualization by UV light after primulin staining of lipids. Thereafter, the plate was cut to a desired size (e.g. 3 × 3 cm) and immerged in plexigum solution for 1 min and air-dried. The plate was then soaked in 3% BSA or casein blocking solution for 1 h followed by overlay with antibodies or biotinylated lectins for 2 h. For antibody binding, appropriate, biotinylated secondary antibodies (typically at 3 μg/ml) were used. The incubation time was 1 h. This was followed by incubation of streptavidin-peroxidase at 5 μg/ml. Plates for probing with biotinylated lectins were overlaid with streptavidin-peroxidase directly after the lectin binding. The binding signals were detected by 3,3'-diaminobenzidine (DAB) liquid substrate system.

For dot-blot assays, dilutions of glycoproteins (1 μl) were spotted directly onto a nitrocellulose membrane using an Eppendorf pipette. Purified glycolipids or NGLs (50-150 pmol) were applied as a mini-band using a Linomat IV TLC applicator (CAMAG). The nitrocellulose sheet was blocked with 3% BSA and the remaining procedures for detection binding were similar to these mentioned above.

2.11.2 Microwell-binding and inhibition assays

PC3 cells were grown in 96-well microplates (Nunc MaxiSorp, Thermo Fisher Scientific). Thereafter the cells were fixed with 4% (w/v) paraformaldehyde in PBS and treated with MeOH containing 0.3% H_2O_2 overnight at 4 ˚C. Alternatively, the microwell plates were coated with PSM solution (0.5 µg/well) in PBS buffer by incubating overnight at 4 ˚C. After removal of the coating solutions, 3% BSA was added to each well and incubated for 1 h. This was followed by the addition of serial dilutions of mAb F77 or biotinylated lectins and further incubation for 2 h. To the wells with mAb F77, anti-mouse IgG (Fc specific) (1:2000) was added, followed by streptavidin-peroxidase polymer (2 µg/ml). To the wells with biotinylated lectins, streptavidin-peroxidase polymer was added directly. Colour development was using *FAST* OPD substrate (200 µl/well) and absorbance was read at 492 nm by plate reader.

Inhibition of F77 binding was performed similarly. PC3 cells or PSM were in the microwells as described above. The F77 antibody at the indicated dilution was pre-incubated with serially diluted oligosaccharides and the mixtures were added to each well after blocking with 3% BSA. The remaining procedures were the same as in the binding assays mentioned above. The results were expressed as percentage inhibition according to:

$$[OD_{(\text{without inhibitor})} - OD_{(\text{with inhibitor})} / OD_{(\text{without inhibitor})}] \times 100$$

2.11.3 Microarray analysis

Four microarrays were used in this thesis. Three of them were prepared previously. A microarray of 492 sequence-defined oligosaccharide probes (319), designated Glycosciences Array Set 32-39 (Appendix S1) was used for screening analysis of mAbs F77 and AE3. The probes included were from the collection assembled in the course of research in Glycosciences Laboratory. These include a variety of mammalian type sequences, representative of *N*-glycans (high-mannose-type and neutral and sialylated complex-type), peripheral regions of *O*-glycans; blood group

antigen-related sequences (A, B, H, Le^a, Le^b, Le^x, and Le^y) on linear or branched backbones and their sialylated and/or sulphated analogues; linear and branched poly-*N*-acetyllactosamine sequences; gangliosides, oligosaccharide fragments of GAGs and polysialic acid. They also include microbial and plant-derived homo-oligomers of glucose and of other monosaccharides. Two glycoprotein microarrays designated Mucin Array Set 1 and Mucin Array Set 2, (Table 3.1 and Table 4.2, respectively) were used for screening of mAb F77 and AE3 for binding. All the glycoproteins included are listed in Table 2.7 and the details of treatment are in Section 2.2.3. These glycoproteins were arrayed in duplicate at 30 and 150 pg per spot on nitrocellulose-coated glass slides (Grace Bio-Labs) using a noncontact robotic Piezorray arrayer (PerkinElmer LAS). Cy3 was included in each probe for spot localization and monitoring the arrayed spots. A new microarray (designated F77/Ii focused array) containing 54 lipid-linked oligosaccharide probes (Table 3.7) was generated during the studies of the F77 antigen. The probes were arrayed as described previously (354) at 2 and 5 fmol per spot in duplicate, with Cy3 included for localization and monitoring of arrayed spots.

Microarray analyses were performed essentially as described (354). The arrayed slides were placed in a 16 pad silicon gasket and inserted into a FAST frame. To each pad of the slide, 3% BSA or casein was added and blocking was for 1 h. Thereafter the blocking reagents were removed and each pad was overlaid with antibodies of interest for 2 h. Antibody binding was detected using biotinylated secondary antibodies (typically at 3 μg/ml) followed by an Alexa Fluor-647-labelled streptavidin (Molecular Probes, 1 μg/ml). Binding by plant lectins was detected directly by Alexa Fluor-647-labelled streptavidin. The slides were thereafter scanned at 633 nm using a ProScanArray scanner (PerkinElmer LAS). Signals quantified with the scanner software. Microarray data were stored, analysed and presented using dedicated glycan array software developed by Mark Stoll of the Glycosciences Laboratory (325). Binding signals were related to dose of probe spotted; results presented are at 150 pg per spot for glycoproteins and 5 fmol per spot for lipid-linked probes.

Chapter 3

CHARACTERIZATION OF THE PROSTATE CANCER-ASSOCIATED CARBOHYDRATE ANTIGEN F77

3.1 Introduction

Prostate cancer is the second most common cancer and the fifth leading cause of death from cancer in males worldwide. It is estimated that 1.1 million men were diagnosed with prostate cancer and more than 0.3 million died of prostate cancer in 2012 (355). In the UK, it is the most common cancer in men in 2011, accounting for 25% cases of cancer in males (356). Prostate cancer incidence has increased in the UK and worldwide (355,356). This is partially due to the introduction and increasing usage of prostate-specific antigen (PSA) test. The crude 5-year survival rate of prostate cancer is 84.1%, but the 5-year survival rate for metastatic prostate cancer is only around 30% (356,357).

As an existing serum biomarker, PSA has made great contributions to prostate cancer diagnosis and significantly reduced prostate cancer mortality because of better diagnosis (358). However, it has been found that the PSA test may give false-positive and false-negative results. On the one hand, serum PSA level is elevated in some benign diseases such as prostatic hyperplasia and subclinical prostatic inflammation (359). On the other hand, although PSA level of 4 ng/ml or less is considered normal, many 'normal' cases can still be determined as prostate cancer by biopsy detection (360). Reliable biomarkers for prostate cancer are therefore urgently needed for both diagnostic and prognostic purposes.

Originally generated in 1984, mAb F77 (IgG3) was raised against the human prostate cancer cell line PC3 in the laboratory of Mark Greene (361). Recently the F77 antigen was considered as a potential biomarker for human prostate cancer (341). It was detected on both androgen-independent (PC3-MM2, PC3 and Du145) and androgen-dependent (LNCap) human prostate cancer cell lines, with little or no detection on non-prostatic cell lines from lung, kidney or skin. The mAb F77 was found to label 112 of 116 primary and 29 of 34 metastatic cancer specimens but with only limited focal staining in benign prostate tissues. Minimal staining to a small fraction of small blood vessels in human brain was observed. No specific staining was detected on normal or tumour tissues of human colon, kidney, cervix, pancreas,

lung and skin (341). Thus it was concluded that the F77 antigen is highly restricted to the prostate and overexpressed in prostate cancer.

In a nontumourigenic human prostate epithelial cell line RWPE-1, F77 antigen expression was associated with a small malignant subpopulation which led to tumours after being transplanted in nude mice. Transfection of the oncogene *K-ras* to RWPE-1 cells resulted in a more tumourigenic cell line RWPE-2, which had a higher F77 antigen level, grew faster and displayed enhanced colony-forming activity.

In vitro studies showed that mAb F77 could directly promote PC3 cell death by apoptosis/necrosis process and it also mediated complement-dependent and antibody-dependent cell death of PC3 and Du145 cells. *In vivo*, treatment of mice bearing established prostate cancer xenografts with mAb F77 significantly inhibited the tumour growth, with no activity against an F77-negative xenograft. Altogether, these findings suggest that the F77 antigen is a novel, biologically important, clinically relevant prostate cancer-associated molecule.

Efforts have been made to identify the nature of the antigenic determinant of mAb F77. Immuno-precipitation of PC3 cell extracts with mAb F77 followed by gradient SDS/ polyacrylamide gel electrophoresis (4-16%) revealed a significant band with MW <5 KD, which was not stained by Coomassie blue but only by carbohydrate staining (Figure 3.1A). A dose-dependent decrease of F77 antigen expression was observed on surface of PC3 and Du145 cells after treatment with the glycolipid synthase inhibitor 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol, but not with the protein *N*- and *O*-glycosylation inhibitors, tunicamycin and benzyl-α-GalNAc, respectively (Figure 3.1B). Indeed, the lipid extracts of PC3 and Du145 cells were bound by mAb F77, whereas lipid extracts from A431 and HEK293 cells lacked the activity (Figure 3.1C). Taken together, these data indicated that glycolipids were the main carriers of F77 antigen in PC3 cells, but the carbohydrate structure was not determined.

This chapter is concerned with the characterization of the carbohydrate structure of the F77 antigen using a multifaceted approach.

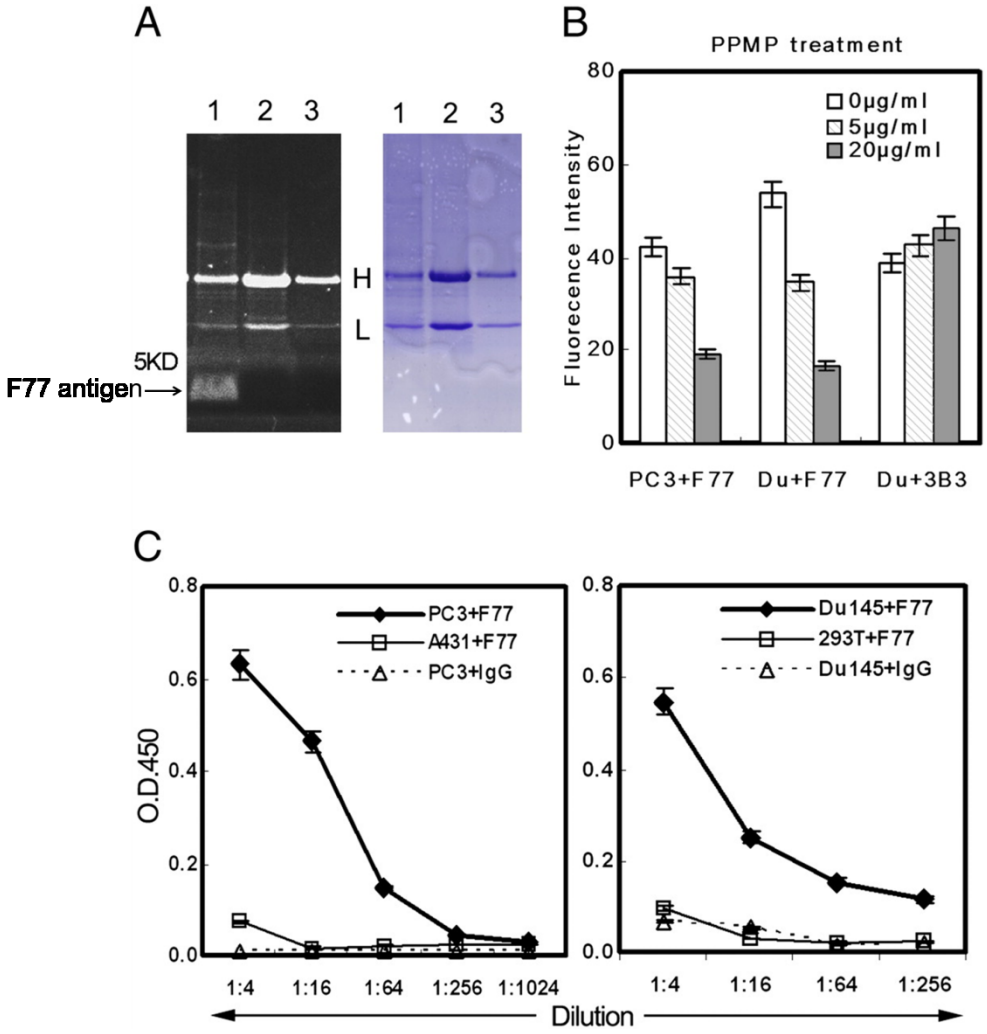


Figure 3.1 F77 binds to a glycolipid antigen. (**A**) F77 Immunoprecipitation. Lane 1: PC3 + F77. Lane 2: A431 + F77. Lane 3: PC3 + control IgG. Samples were separated by Tris SDS-PAGE (4-16%). Gel was first stained with Pro-Q Emerald 300 carbohydrate staining (left panel), and then stained with Coomassie blue (right panel). H and L indicate the position of heavy and light chains of mAb F77. (**B**) PPMP treatment. Anti-CD147 mAb 3B3 was used as a control. (**C**) Specific binding of mAb F77 to glycolipids extracted from PC3 and DU 145 but not from A431 or 293T cells. Glycolipids extracted by chloroform/methanol/water (5.4/10.8/6.5, v/v/v) were examined in ELISA assay with F77 antibody or a control antibody at 1 μg/ml. Taken from Zhang et al reference (341).

3.2 Microarray screening analysis of mAb F77 with sequence-defined oligosaccharide probes

Here in the Glycosciences Laboratory, my colleague Dr. Yan Liu explored the carbohydrate sequence of the F77 antigen in microarray screening analysis of mAb F77 using an existing array of 492 sequence-defined oligosaccharide probes (Glycosciences Array Set 32-39, Appendix S1). Only two branched, poly-*N*-acetyllactosamine-based, blood group B-related glycolipids BIII (dodecaglycosylceramide, probe 226) and BIV (tetradecaglycosylceramide, probe 227) were revealed, in a strikingly selective manner (Figure 3.2). These two probes were also strongly bound by anti-blood group B, which proved their identity. In contrast, the unbranched blood group B-related hexaglycosylceramide (probe 147), although bound by anti-blood group B, gave no detectable binding signal with mAb F77, nor were binding signals detected with the branched B-like analogues lacking α1,2-fucose (probe 209) and the polyLacNAc based I-octaosylceramide (probe 205) lacking both α1,2-fucose and α1,3-galactose. Though at that stage the possibility could not be excluded that a minor component exists in these two glycolipids which was bound very strongly by mAb F77, the microarray results suggested the α1,2-fucose present on the 6-branch is involved in the recognition of the antibody. As the branched blood group H and A analogues were not readily available for inclusion in the existing microarray, it was not possible to assign the epitope to a particular domain on the BIII and BIV glycolipids.

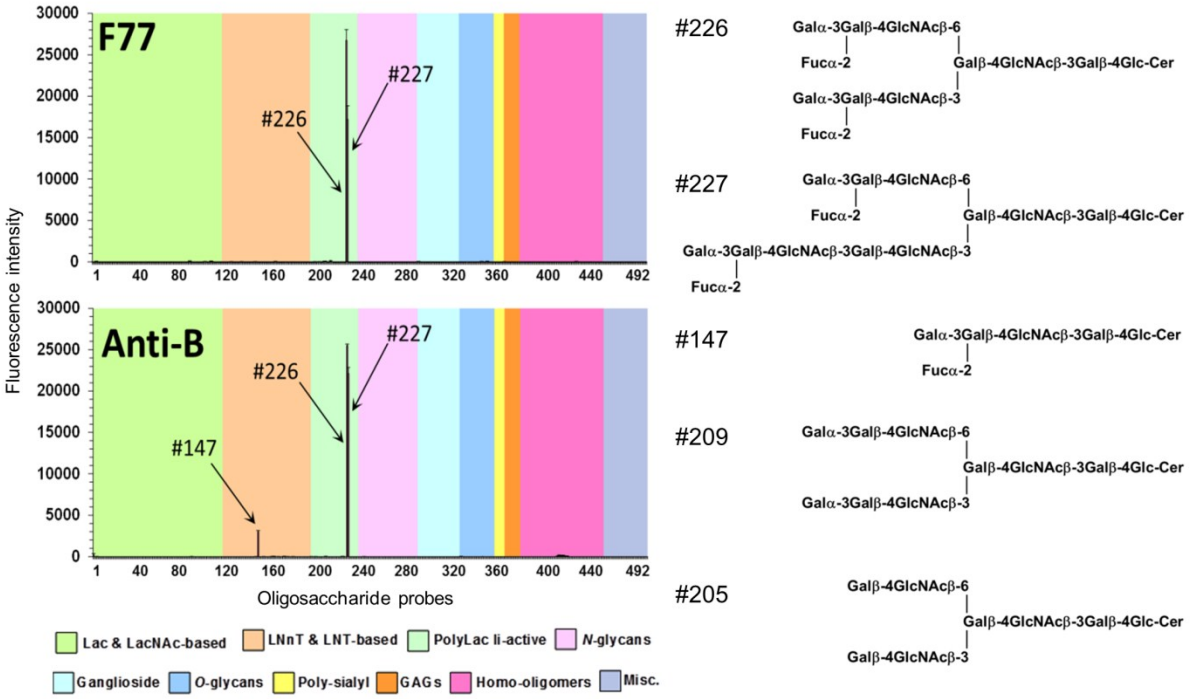


Figure 3.2 Microarray analyses of mAbs F77 and anti-B (89-F) with lipid-linked sequence-defined oligosaccharide probes. The results are the means of fluorescence intensities of duplicate spots, printed at 5 fmol with error bars representing half of the difference between the two values. These 492 lipid linked probes (Glycosciences Laboratory Array Set 32-39) are arranged according to their backbone sequences as annotated in the coloured panels below the figure. The binding intensities are listed together with the probe sequences in Appendix S1.

3.3 Initial mAb F77 binding studies with a glycolipid extract from PC3 cells

Initially, I analysed the antigenic activity of a glycolipid extract from PC3 cells (5×10^8). An aliquot of the extract was desalted using C18 cartridge, resolved by HPTLC and stained with primulin to detect the lipid moieties. Numerous primulin stained components were revealed (Figure 3.3). However, when mAb F77 was overlaid onto the PC3 glycolipid extract, only multiple minor components were detected and they did not correspond to the migration positions of any clearly primulin-stained components.

These results suggest that the F77 antigen is strongly expressed on a series of glycolipids that are extremely minor cellular components. As glycolipids from PC3 cell extracts are limited in amount and not readily amenable to detailed characterization, these were not investigated further. Rather, I searched for abundant sources of F77 antigen.

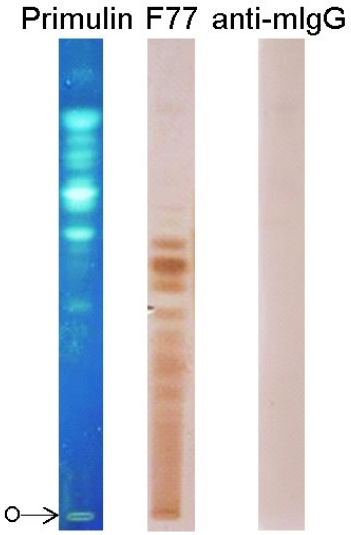


Figure 3.3 Binding of mAb F77 to PC3 glycolipid extract resolved by HPTLC. The PC3 glycolipid extract was chromatographed on a silica gel HPTLC plate using the solvent $CHCl_3$/MeOH/0.5M sodium acetate, 25:25:8 (v/v) and visualized with lipid stain primulin (left panel). Duplicate lanes were overlaid with mAb F77 (middle panel) or with the secondary antibody alone (right panel). Binding was visualized by colour development using the streptavidin-peroxidase followed by DAB liquid substrate system. O indicates the origin.

3.4 *O*-glycome designer array to detect and characterize the F77 antigen

3.4.1 Identification of porcine stomach mucin (PSM) as a F77 antigen-positive glycoprotein

Given that *O*-glycans and glycolipids have glycan sequences in common, I performed an exploratory microarray analysis of mAb F77 (Section 2.11.3) using an array of 30 preparations of mucin type glycoproteins (Mucin array Set 1, Table 3.1) in order to identify abundantly available F77 antigen-positive sources from which to isolate the antigen. Among the 30 mucin type glycoprotein preparations, blood group A, B or H antigen activities were detected by microarray analysis using anti-A, anti-B antibodies and the plant lectin UEA-I, respectively (Figure 3.4).

The strongest F77 binding activity was with PSM (glycoprotein 29). Little or no binding of mAb F77 was detected to the other human glycoproteins included in the array. As predicted from the classical work with PSM (362), strong binding signals were elicited with PSM using anti-A and UEA-I, corresponding to blood group A and H antigen activities, respectively. Blood group B activity was not detected on PSM. Four strongly B-active glycoproteins (glycoproteins 18, 22, 23 and 27) were only very weakly bound by mAb F77, indicating that the blood group B trisaccharide determinant *per se* is not an essential part of the F77 antigen. Two glycoproteins (10 and 21), which were strongly A-active and relatively weakly H-active compared with PSM, gave no binding signals with mAb F77. Collectively, these results suggested that mAb F77 bound to a blood group H-related structural motif present on PSM. Therefore, PSM was selected for further investigation by the glycome designer array approach.

Table 3.1 Mucin-type epithelial glycoproteins arrayed in Mucin array Set 1.

Position	Designation	Donor red cell blood-group	Secretor status
	Enriched cystadenoma glycoproteins		
1	Cys 350 (Trp)*	A1	Non-secretor
2	Cys 444 (Trp)	Unknown	Non-secretor
3	Cys 444 (Pro)	Unknown	Non-secretor
4	Cys 446 (Pro)	B	Non-secretor
5	Cys 461 (Trp)	Unknown	Non-secretor
6	Cys 461 (Pro)	Unknown	Non-secretor
7	Cys 654 (Pro)	B	Non-secretor
8	Cys 705 (Pro)	Unknown	Non-secretor
9	Cys 717 (Pro)	A1	Non-secretor
10	Cys 733 (Pro)	A1	Secretor
11	Cys 745 (Pro)	A	Non-secretor
12	Cys 754 (Pro)	Unknown	Non-secretor
13	Cys 756 (Trp)	A	Non-secretor
14	Cys 756 (Pro)	A	Non-secretor
15	Cys 762 (Trp)	O	Non-secretor
16	Cys 762 (Pro)	O	Non-secretor
17	Cys 765 (Pro)	O	Non-secretor
	Meconia		
18	Meconium B (Pro)	B	Unknown
19	Meconium Mo (Pro)	Unknown	Unknown
20	Meconium Wo (Pro)	Unknown	Unknown
21	Meconium He (Pro)	Unknown	Unknown
22	Meconium Pa (Pro)	Unknown	Unknown
	Purified cystadenoma glycoproteins		
23	B substance (Pro)	B	Secretor
24	Og 10% 2x (Pep)	Unknown	Non-secretor
25	Og 10% from 20% (Pep)	Unknown	Non-secretor
26	N1 20% (Pep)	Unknown	Non-secretor
27	Tij 10% 2x (Pep)	B	Secretor
	Other glycoproteins		
28	HCA	Unknown	Unknown
29	Porcine gastric mucin	A+H	(Sigma)
30	Bovine submaxillary mucin	Unknown	(Sigma)

Details for the enrichment procedures for the mucin preparations 1-27 from the lyophilised powders and of sample 28 are given in Section 2.2.3. Samples 29 to 30 were commercial samples.

*Trp, Pro and Pep refer to proteases, pronase, trypsin or pepsin, used in the process of solubilizing the mucins.

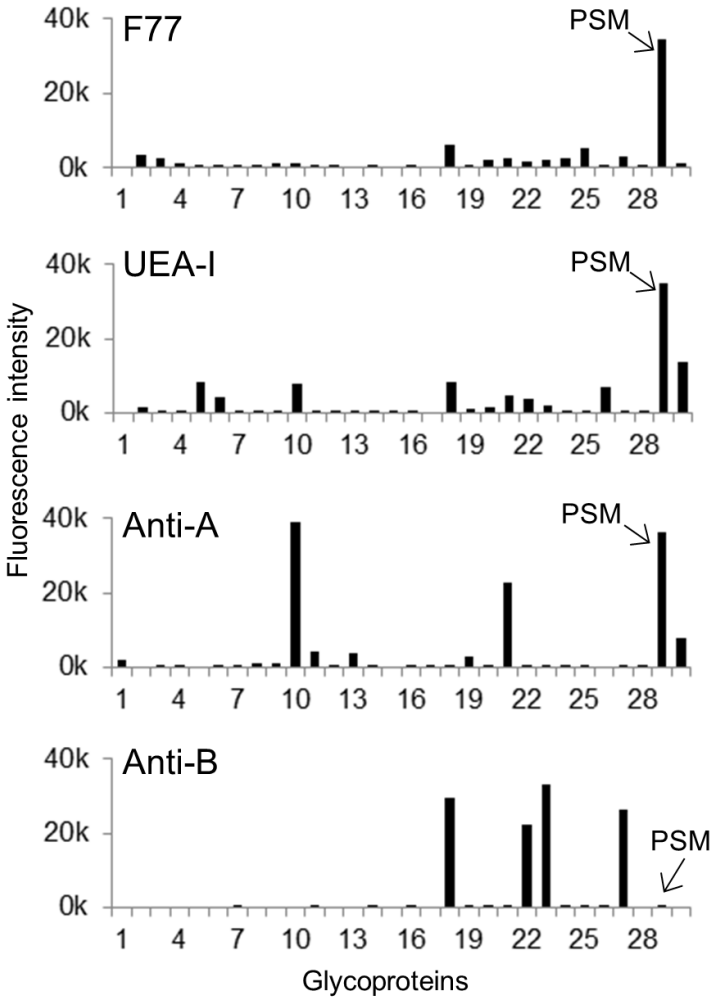


Figure 3.4 Microarray analyses of mAbs F77, anti-B (89-F), anti-A (T36), and UEA-I lectin with mucin type glycoproteins. The descriptions of the glycoproteins are in Table 3.1. Results are the means of fluorescence intensities of duplicate spots printed at 150 pg glycoprotein per spot. The error bars represent half of the difference between the two values.

3.4.2 Detection of F77 antigen activity among non-reductively released *O*-glycans from PSM

In order to investigate the F77 antigen sequence expressed on PSM, I performed antigenic analysis with the reducing *O*-glycans released from PSM. With the help of our collaborators at Ludger (Oxford), PSM (50 mg) was subjected to non-reductive *O*-glycan release by hydrazinolosis (Section 2.4).

With the products obtained from hydrazinolosis of PSM, I first performed anion-exchange chromatography to separate the neutral oligosaccharides from the acidic as described in Section 2.5.1. As the results of microarray screening analysis with

sequence-defined glycans suggested that the F77 antigen is expressed on neutral oligosaccharides, the neutral fraction was investigated further.

Fluorescent AD-NGLs were prepared with the neutral reducing *O*-glycans using ADHP as the lipid reagent (Section 2.6.1.1). After removal of excess lipid, the NGLs were analysed by MALDI-MS (Table 3.2). The monosaccharide compositions (in terms of dHex, Hex and HexNAc) of the major components were deduced based on their molecular ions. The results indicated that there are in total 25 glycan species corresponding to *O*-glycans ranging from di- to nona-saccharide. Three of these glycans (labelled ^{p}) in Table 3.2 are most likely products of alkaline catalysed degradation (peeling).

Table 3.2 MALDI-MS of the NGLs of *O*-glycans non-reductively released from PSM. Monosaccharide compositions of were deduced from MALDI-MS analysis. P refers to possible peeled *O*-glycan.

$[M-H]^-$	Monosaccharide Compositions
1219	dHex1.Hex1^{p}
1276	Hex1.HexNAc1
1318	HexNAc2
1479	Hex1.HexNAc2
1422	dHex1.Hex1.HexNAc1
1439	Hex2.HexNAc1^{p}
1585	dHex1.Hex2.HexNAc1
1627	dHex1.Hex1.HexNAc2
1642	Hex2.HexNAc2
1682	Hex1.HexNAc3
1723	HexNAc4^{p}
1787	dHex1.Hex2.HexNAc2
1828	dHex1.Hex1.HexNAc3
1845	Hex2.HexNAc3
1950	dHex1.Hex3.HexNAc2
1991	dHex1.Hex2.HexNAc3
2007	Hex3.HexNAc3
2048	Hex2.HexNAc4
2096	dHex2.Hex3.HexNAc2
2153	dHex1.Hex3.HexNAc3
2194	dHex1.Hex2.HexNAc4
2221	Hex3.HexNAc4
2299	dHex2.Hex3.HexNAc3

2356	dHex1.Hex3.HexNAc4
2502	dHex2.Hex3.HexNAc4

Abbreviations: dHex, deoxyhexose; Hex, hexose; HexNAc, *N*-acetylhexosamine

F77 antigenic analysis was carried out on HPTLC with mAb F77 using 1 nmol of the starting *O*-glycans. Although multiple fluorescent NGL bands were resolved on the plate and revealed by UV light (Figure 3.5A), no antigen-positive band was detected (not shown). The total NGL products were thereafter fractionated into four subfractions a-d (Figure 3.5A) by preparative HPTLC so that larger amounts of NGLs could be applied to detect any very minor antigen-positive components. Higher amounts of subfractions c and d, 10 and 20 nmols, respectively, were subjected to chromatogram binding assay with mAb F77 (Figure 3.5B). A smear of weak immunostaining was detected in faction d, in a slow migrating region. Similar to the glycolipid extracts of PC3 cells, the F77 antigen-positive oligosaccharides were extremely minor and heterogeneous.

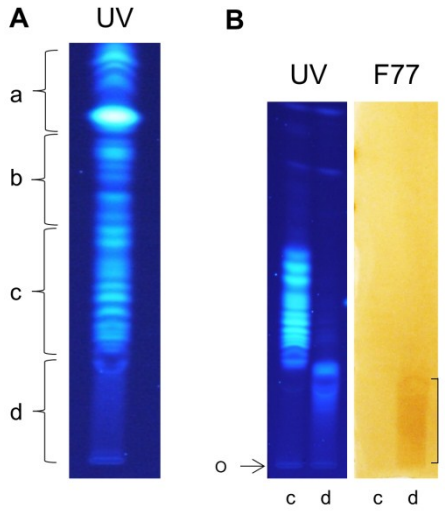


Figure 3.5 F77 antigenic analyses with NGLs derived from *O*-glycans non-reductively released from PSM. (**A**). HPTLC analysis of the total NGLs derived from *O*-glycans non-reductively released from PSM. a to d indicate the four subfractions collected from prep-TLC. (**B**). Binding analysis with mAb F77 of the NGLs in subfractions c and d. The TLC plates were developed using $CHCl_3$/MeOH/H_2O, 60:35:8 (v/v), as solvent. The fluorescent NGLs were visualized under UV light and binding by mAb F77 was detected as in the legend to Figure 3.3. *O* indicates the origin.

It would be extremely difficult to isolate the F77 antigen-positive components from this minor, and highly heterogeneous population of *O*-glycans. Therefore an alternative approach was made to identifying the F77 antigenic components as described in the sections that follow.

3.4.3 Detection of F77 antigen activity among reductively released *O*-glycans from PSM

O-glycans were released by reductive alkaline borohydride degradation from 800 mg PSM. The reduced oligosaccharides (alditols) were enriched from the reaction mixture as described in Section 2.3. These were quantified by hexose assay: total hexose content was 15 mg. The products were thereafter fractionated by size using Bio-Gel P4 (Figure 3.6A). Six fractions **a** to **f** were collected based on the elution profile. MALDI-MS analysis indicated the major components of fractions **b** to **d** were small oligosaccharides mono- to tri-saccharides. Orcinol assay showed fractions **e** to **f** did not contain oligosaccharides (not shown).

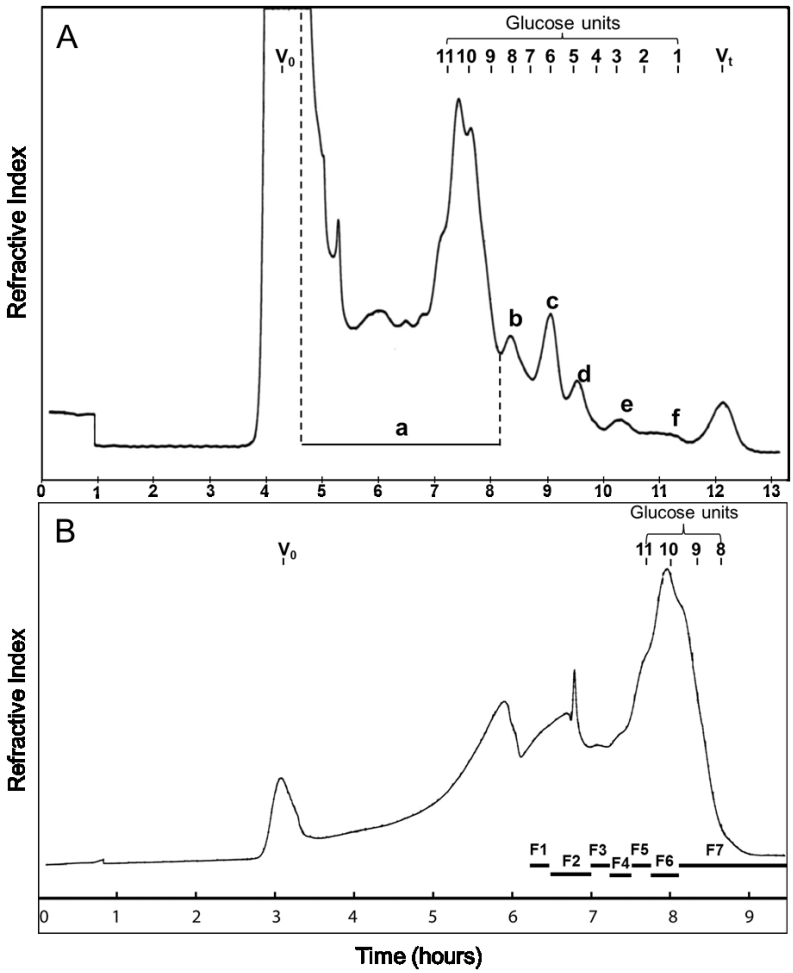


Figure 3.6 Gel filtration chromatography of the products of reductive alkaline hydrolysis from PSM. (**A**) Initial chromatography profile using a Bio-Gel P4 column (1.6×90cm) eluted with H_2O. (**B**) Rechromatogrph of fraction a in panel (A) using a Bio-Gel P6 column (1.6×90cm) eluted with H_2O. V_0 is the void volume of the column; V_t is the total volume; glucose units 1-11 indicate positions of elution of oligosaccharides with degrees of

polymerization 1-11 in an acid hydrolysate of dextran. a to f and F1 to F7 designate the pooled fractions in each preparation.

Elutions between 4.5-8 h on the Bio-Gel P4 column were pooled as fraction **a**. The major components in **a** ranged from tetra- to tridecasccharide. This fraction was subjected to further Bio-Gel P6 fractionation. Seven fractions (F1 to F7) were harvested from Bio-Gel P6 (Figure 3.6B) and analysed by MALDI-MS. The monosaccharide compositions of the major components in each fraction are deduced and listed in Table 3.3. There are in total 43 glycan species with various compositions. The two peaks to the left of F1 gave no detectable orcinol but strong ninhydrin staining signals, suggesting these peaks were dominated by peptides. F1 contained a small amount of oligosaccharides larger than undecasaccharides; fractions 2-5 contained hepta- to nona-saccharides; fractions 6-7 contained penta- to hexa-saccharides, as indicated by MALDI-MS profiling.

Table 3.3 Compositions of the reductively released *O*-glycan alditols in the Bio-Gel P6 fractions 1-7, deduced from MALDI-MS analysis.

Fractions	$[M+Na]^+$	Composition
7		
	814	Hex1, HexNAc2, HexNAcol1
	919	Fuc1, Hex2, HexNAc1, HexNAcol1
	935	Hex3, HexNAc1, HexNAcol1
	960	Fuc1, Hex1, HexNAc2, HexNAcol1
	976	Hex2, HexNAc2, HexNAcol1
	1017	Hex1, HexNAc3, HexNAcol1
	1065	Fuc2, Hex2, HexNAc1, HexNAcol1
	1081	Fuc1, Hex3, HexNAc1, HexNAcol1
	1122	Fuc1, Hex2, HexNAc2, HexNAcol1
	1138	Hex3, HexNAc2, HexNAcol1
	1179	Hex2, HexNAc3, HexNAcol1
6		
	1122	Fuc1, Hex2, HexNAc2, HexNAcol1
	1179	Hex2, HexNAc3, HexNAcol1
	1325	Fuc1, Hex2, HexNAc3, HexNAcol1
5		
	1122	Fuc1, Hex2, HexNAc2, HexNAcol1
	1179	Hex2, HexNAc3, HexNAcol1
	1284	Fuc1, Hex3, HexNAc2, HexNAcol1
	1325	Fuc1, Hex2, HexNAc3, HexNAcol1
	1430	Fuc2, Hex3, HexNAc2, HexNAcol1
4		
	1179	Hex2, HexNAc3, HexNAcol1
	1284	Fuc1, Hex3, HexNAc2, HexNAcol1
	1325	Fuc1, Hex2, HexNAc3, HexNAcol1
	1341	Hex3, HexNAc3, HexNAcol1
	1382	Hex2, HexNAc4, HexNAcol1
	1430	Fuc2, Hex3, HexNAc2, HexNAcol1
	1471	Fuc2, Hex2, HexNAc3, HexNAcol1
	1487	Fuc1, Hex3, HexNAc3, HexNAcol1
	1503	Hex4, HexNAc3, HexNAcol1
	1633	Fuc2, Hex3, HexNAc3, HexNAcol1
	1690	Fuc1, Hex3, HexNAc4, HexNAcol1
3		
	1325	Fuc1, Hex2, HexNAc3, HexNAcol1
	1382	Hex2, HexNAc4, HexNAcol1
	1430	Fuc2, Hex3, HexNAc2, HexNAcol1
	1471	Fuc2, Hex2, HexNAc3, HexNAcol1
	1487	Fuc1, Hex3, HexNAc3, HexNAcol1
	1544	Hex3, HexNAc4, HexNAcol1
	1633	Fuc2, Hex3, HexNAc3, HexNAcol1
	1649	Fuc1, Hex4, HexNAc3, HexNAcol1
	1690	Fuc1, Hex3, HexNAc4, HexNAcol1
	1795	Fuc2, Hex4, HexNAc3, HexNAcol1
2		
	1487	Fuc1, Hex3, HexNAc3, HexNAcol1
	1544	Hex3, HexNAc4, HexNAcol1
	1633	Fuc2, Hex3, HexNAc3, HexNAcol1
	1690	Fuc1, Hex3, HexNAc4, HexNAcol1
	1747	Hex3, HexNAc5, HexNAcol1
	1795	Fuc2, Hex4, HexNAc3, HexNAcol1
	1894	Fuc1, Hex3, HexNAc5, HexNAcol1
	1852	Fuc1, Hex4, HexNAc4, HexNAcol1
	1942	Fuc3, Hex4, HexNAc3, HexNAcol1
	1999	Fuc2, Hex4, HexNAc4, HexNAcol1
	2055	Fuc1, Hex4, HexNAc5, HexNAcol1
1		
	1747	Hex3, HexNAc5, HexNAcol1
	1836	Fuc2, Hex3, HexNAc4, HexNAcol1
	1852	Fuc1, Hex4, HexNAc4, HexNAcol1
	1894	Fuc1, Hex3, HexNAc5, HexNAcol1
	1909	Hex4, HexNAc5, HexNAcol1
	1998	Fuc2, Hex4, HexNAc4, HexNAcol1
	2055	Fuc1, Hex4, HexNAc5, HexNAcol1
	2112	Hex4, HexNAc6, HexNAcol1
	2160	Fuc2, Hex5, HexNAc4, HexNAcol1
	2201	Fuc2, Hex4, HexNAc5, HexNAcol1
	2258	Fuc1, Hex4, HexNAc6, HexNAcol1
	2306	Fuc3, Hex5, HexNAc4, HexNAcol1
	2364	Fuc2, Hex5, HexNAc5, HexNAcol1
	2405	Fuc2, Hex4, HexNAc6, HexNAcol1
	2421	Fuc1, Hex5, HexNAc6, HexNAcol1
	2510	Fuc3, Hex5, HexNAc5, HexNAcol1
	2567	Fuc2, Hex5, HexNAc6, HexNAcol1

Abbreviations: dHex, deoxyhexose; Hex, hexose; HexNAc, *N*-acetylhexosamine, HexNAcol, *N*-acetylhexosaminitol.

Fluorescent NGLs were prepared from fractions 1 to 7 after mild periodate oxidation using ADHP as the lipid reagent as in Section 2.6.1.2. The periodate oxidation results in the splitting of each *O*-glycan alditol at the core GalNAcol into two fragments and each is converted into an NGL (Figure 3.7). The NGLs derived from the 3-linked branches are designated –OX (OX: -OCH_2-CH(NHAc)-CH_2OH-CH_2-ADHP). Those derived from the 6-linked branches are designated –OY (OY: -OCH_2-CH_2-ADHP). Monosaccharide compositions of the resulting NGLs were deduced by MALDI-MS (Table 3.4). In total 29 NGL species with different compositions were detected in these seven fractions. Clearly a substantial number of species were shared in many fractions as different parent oligosaccharides can have the same 3- or 6-fragment after periodate oxidation.

As a result of the cleavage of the core GalNAcol, the glycans in the NGLs represented branches of the parent oligosaccharide. For example, the major components of fraction 4 among the parent oligosaccharides were hepta- to octa-saccharide alditols (Table 3.3), whereas the NGL components were di- to hepta-saccharides derived from the 3-linked branches (-OX) and mono- to tetra-saccharides from the 6-linked branches (-OY) as shown in Table 3.4.

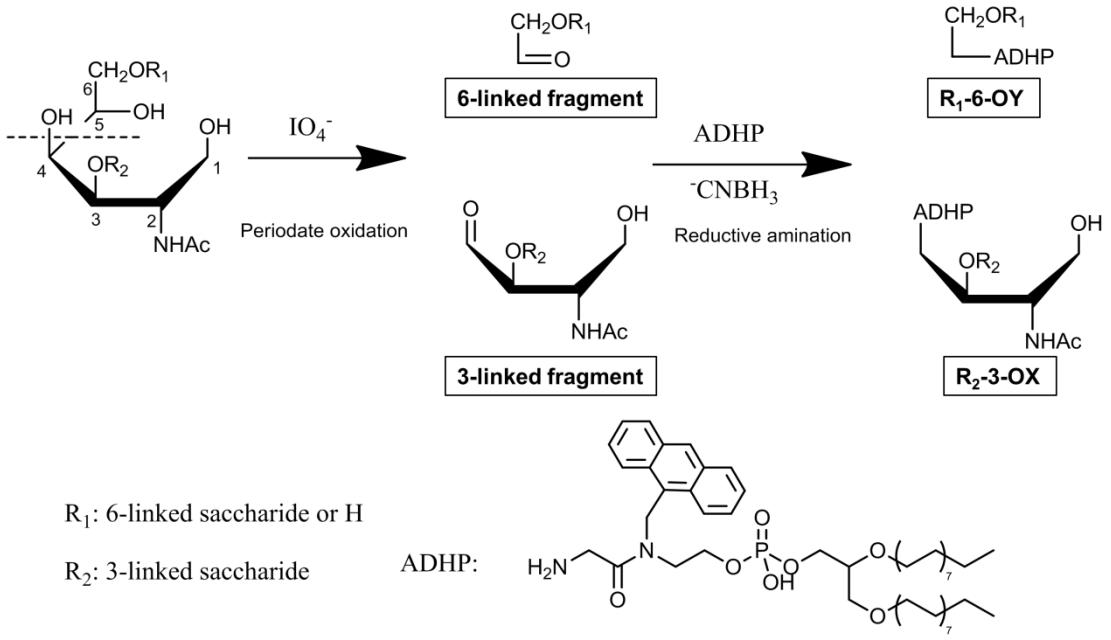


Figure 3.7 Reaction scheme of periodate oxidation cleavage and conjugation to the ADHP lipid (363). The sugar ring splits between C4 and C5 of the core GalNAc, yielding 3- and 6-linked fragments. The NGL that derives from the 3-linked fragment is designated 3-OX. The other, which derives from the 6-linked fragment, is designated 6-OY.

Table 3.4 Compositions of the NGLs derived from the *O*-glycan alditols in Bio-Gel P6 fractions 1-7, deduced from MALDI-MS analysis.

Fractions	[M+H]+	Composition	Fractions	[M+H]+	Composition	Fractions	[M+H]+	Composition
7				2298	Fuc1, Hex3, HexNAc3-OX		1991	Hex2, HexNAc3-OX
	1218	Hex1-OX		2355	Hex3, HexNAc4-OX		2035	Fuc1, Hex2, HexNAc3-OY
	1364	Fuc1, Hex1-OX					2095	Fuc1, Hex3, HexNAc2-OX
	1421	Hex1, HexNAc1-OX	3				2137	Fuc1, Hex2, HexNAc3-OX
	1466	Fuc1, Hex1, HexNAc1-OY		955	-OY		2153	Hex3, HexNAc3-OX
	1523	Hex1, HexNAc2-OY		1057	-OX		2241	Fuc2, Hex3, HexNAc2-OX
				1158	HexNAc1-OY		2298	Fuc1, Hex3, HexNAc3-OX
6				1321	Hex1, HexNAc1-OY		2355	Hex3, HexNAc4-OX
	1219	Hex1-OX		1364	Fuc1, Hex1-OX		2445	Fuc2, Hex3, HexNAc3-OX
	1365	Fuc1, Hex1-OX		1422	Hex1,HexNAc1-OX		2505	Fuc1, Hex3, HexNAc4-OX
	1422	Hex1, HexNAc1-OX		1467	Fuc1, Hex1, HexNAc1-OY		2648	Fuc2, Hex3, HexNAc4-OX
	1467	Fuc1, Hex1, HexNAc1-OY		1524	Hex1, HexNAc2-OY		2693	Fuc2, Hex2, HexNAc5-OX
	1524	Hex1, HexNAc2-OY		1568	Fuc1, Hex1, HexNAc1-OX			
				1670	Fuc1, Hex1, HexNAc2-OY	1		
5				1730	Fuc1, Hex2, HexNAc1-OX		955	-OY
	955	-OY		1787	Hex2, HexNAc2-OX		1158	HexNAc1-OY
	1158	HexNAc1-OY		1890	Hex2, HexNAc3-OY		1218	Hex1-OX
	1422	Hex1,HexNAc1-OX		1934	Fuc1, Hex2, HexNAc2-OX		1321	Hex1, HexNAc1-OY
	1524	Hex1, HexNAc2-OY		1991	Hex2, HexNAc3-OX		1422	Hex1 ,HexNAc1-OX
	1568	Fuc1, Hex1, HexNAc1-OX		2095	Fuc1, Hex3, HexNAc2-OX		1467	Fuc1, Hex1, HexNAc1-OY
	1670	Fuc1, Hex1, HexNAc2-OY		2241	Fuc2, Hex3, HexNAc2-OX		1524	Hex1, HexNAc2-OY
	1787	Hex2, HexNAc2-OX		2298	Fuc1, Hex3, HexNAc3-OX		1568	Fuc1, Hex1, HexNAc1-OX
	1934	Fuc1, Hex2, HexNAc2-OX					1670	Fuc1, Hex1, HexNAc2-OY
			2				1787	Hex2, HexNAc2-OX
4				955	-OY		1934	Fuc1 ,Hex2, HexNAc2-OX
	955	-OY		1057	-OX		1991	Hex2, HexNAc3-OX
	1158	HexNAc1-OY		1158	HexNAc1-OY		2137	Fuc1 ,Hex2, HexNAc3-OX
	1321	Hex1, HexNAc1-OY		1218	Hex1-OX		2298	Fuc1 ,Hex3, HexNAc3-OX
	1422	Hex1,HexNAc1-OX		1321	Hex1, HexNAc1-OY		2355	Hex3, HexNAc4-OX
	1524	Hex1, HexNAc2-OY		1364	Fuc1, Hex1-OX		2399	Hex2, HexNAc5-OX
	1568	Fuc1, Hex1, HexNAc1-OX		1422	Hex1, HexNAc1-OX		2445	Fuc2, Hex3, HexNAc3-OX
	1670	Fuc1, Hex1, HexNAc2-OY		1467	Fuc1, Hex1, HexNAc1-OY		2501	Fuc1, Hex3, HexNAc4-OX
	1730	Fuc1, Hex2, HexNAc1-OX		1524	Hex1, HexNAc2-OY		2545	Fuc1, Hex2, HexNAc5-OX
	1787	Hex2, HexNAc2-OX		1670	Fuc1, Hex1, HexNAc2-OY		2607	Fuc2, Hex4, HexNAc3-OX
	1934	Fuc1, Hex2, HexNAc2-OX		1730	Fuc1, Hex2, HexNAc1-OX		2648	Fuc2, Hex3, HexNAc4-OX
	1991	Hex2, HexNAc3-OX		1787	Hex2, HexNAc2-OX		2692	Fuc2, Hex2, HexNAc5-OX
	2095	Fuc1, Hex3, HexNAc2-OX		1890	Hex2, HexNAc3-OY		2749	Fuc1, Hex2, HexNAc6-OX
	2241	Fuc2, Hex3, HexNAc2-OX		1934	Fuc1, Hex2, HexNAc2-OX		2811	Fuc2, Hex4, HexNAc4-OX

Abbreviations: dHex, deoxyhexose; Hex, hexose; HexNAc, *N*-acetylhexosamine, HexNAcol, *N*-acetylhexosaminitol. –OX and –OY are the 3- and 6- linked fragments of core GalNAc after periodate oxidation as further explained in Figure 3.7.

The NGLs in each fraction were first resolved by HPTLC, visualized by UV light, and then probed for binding by mAb F77 (Figure 3.8). In each fraction, UV detection revealed numerous fluorescent bands. Many discrete F77-positive bands were detected in the middle migrating area in fractions 1, 3, 4 and 5. F77 binding was also detected to the slow migrating area in fractions 1-3. None of these were bound by IgG3 control, which revealed binding to the fast migrating region. The F77 antigen-positive bands did not correspond with the major fluorescent components, suggesting that mAb F77 binding was to minor components among the large number of heterogeneous components.

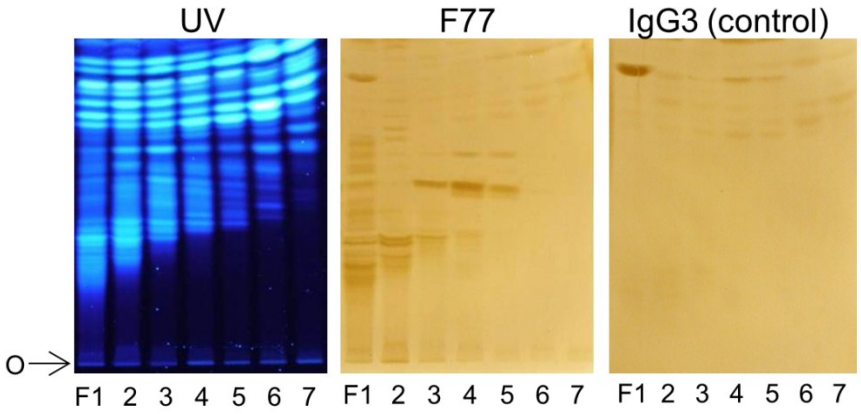


Figure 3.8 Chromatogram binding of mAb F77 to NGLs derived from the *O*-glycan alditols in fractions 1–7. The NGLs derived from the *O*-glycan alditols in fractions 1–7 were chromatographed on HPTLC plates, using $CHCl_3$/MeOH/H_2O, 60:35:8 (v/v), as solvent. The fluorescent NGLs were visualized under UV light (left panel). The same plate and a duplicate plate were incubated with mAb F77 (middle panel) and the isotype IgG3 control, MG3-35 (right panel), followed by biotinylated anti-mouse immunoglobulins. Binding was detected as in Figure 3.3. *O* indicates the origin.

3.4.4 Attempts to enrich the F77 antigen-positive *O*-glycan alditols by affinity chromatography

As results so far indicated that the F77 antigen is expressed only on minor components among *O*-glycans released from PSM, an attempt was made to enrich the F77 antigen-positive *O*-glycans in fraction 3 (PSMF3) by affinity chromatography using an anti-H specific lectin UEA-I and the F77 antibody as absorbents. Below I summarize the results.

UEA-I affinity chromatography was initially investigated. This is because the F77 antigen-positive motif on *O*-glycans of PSM was proposed to be a blood group H sequence from the results of the initial screening analysis with oligosaccharide microarray (Figure 3.2) and glycoprotein array (Figure 3.4). After application of the *O*-glycan alditols of fraction 3 (Figure 3.6) onto the column, a retarded peak was observed by refractive index detection (Figure 3.9A). Two fall-through fractions (a and b) and three retarded fractions (c to e) were obtained. Elution was with 10 mM, 30 mM and 50 mM Fuc stepwise and three fractions f to h, were collected. The two fall-through fractions were later combined; the oligosaccharides in these seven fractions (a+b, c to h) were converted to NGLs for probing with mAb F77.

By UV detection multiple fluorescent bands were revealed in fractions a+b and c to f, indicating the presence of heterogeneous NGLs in these NGL subfractions (Figure 3.9B). Very weak fluorescent bands were detected in the NGLs derived from fractions g and h, indicating that the amounts of oligosaccharides tightly bound and eluted from the UEA-I column were limited. F77 antigen activity was restricted mainly to the middle- and slow-migrating area of fractions e-g (Figure 3.9D) and correlated with blood group H type 2 activities, as shown by binding of UEA-I (Figure 3.9E). Clearly the column had a relatively low capacity as the H type 2 activity was also detected in the fall-through fraction a+b and the retarded fractions c and d. In addition, the separation of the retarded fractions from the fall-through was not complete. Therefore this approach was not pursued further.

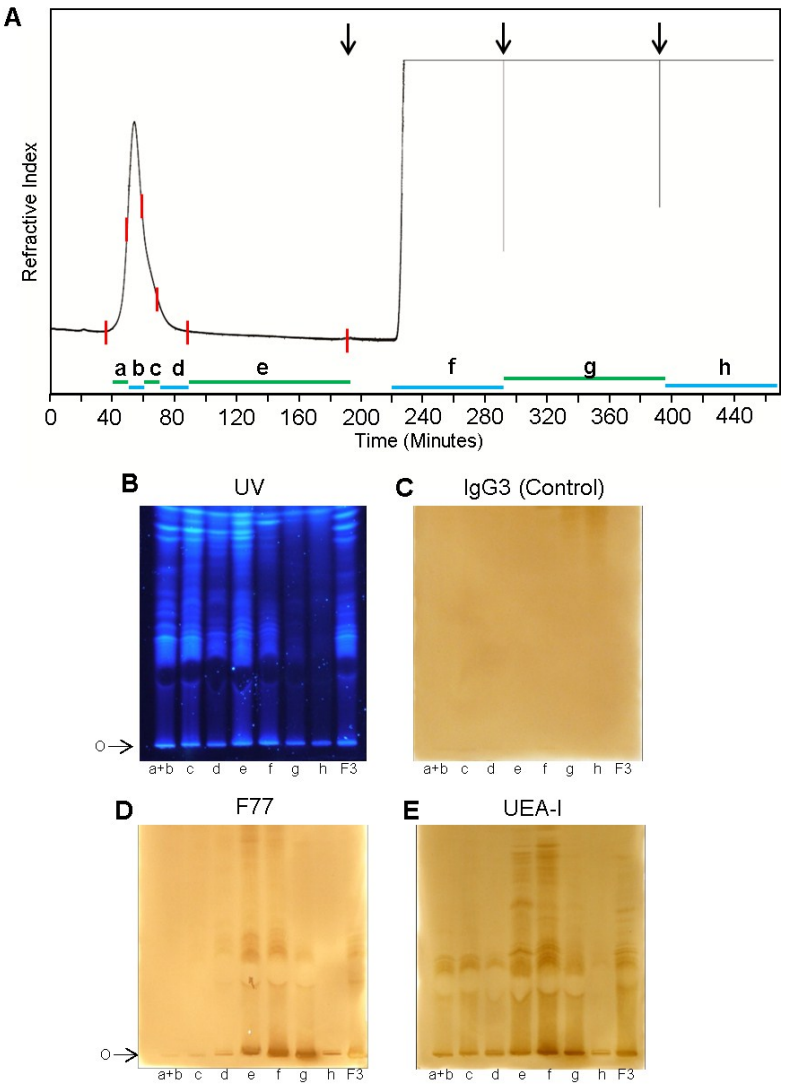


Figure 3.9 Affinity chromatography to enrich F77 antigen-positive *O*-glycan alditols from PSMF3 using UEA-I column. (**A**) Refractive index detection of the affinity chromatography using an UEA-I column (1.6×5cm). a to h are the fractions collected. Arrows indicate the time points changing the elution buffer. (**B**) TLC analysis of the NGLs derived from *O*-glycan alditols of the seven subfractions, a+b and c to h, revealed by UV light. (**C-E**) Chromatogram binding analysis of the IgG3 control, MG3-35 (**C**), mAb F77 (**D**), and UEA-I (**E**) on replicate TLC plates to panel (**B**). The fluorescent NGLs derived from the *O*-glycans in the seven subfractions of PSMF3 and the unfractionated F3 were chromatographed using $CHCl_3$/MeOH/H_2O, 60:35:8 (v/v), as solvent, visualized by UV light. Binding was detected as described in Figure 3.8. O indicates the origin.

Enrichment of F77 antigen-positive *O*-glycans was then attempted using the F77 affinity column. The procedures are described in Section 2.5.3 and the workflow is in Figure 3.10A. In total, five fractions were obtained and NGLs were generated (Figure 3.10B) with the *O*-glycans in these fractions. Surprisingly, except for the second eluent A2E, the fractions all contained F77 antigen-positive components (Figure 3.10C) and the binding patterns of these fractions were very similar to that observed with the starting material F3. These binding data indicated that the F77 affinity column had a very small capacity and could not be exploited for antigen enrichment.

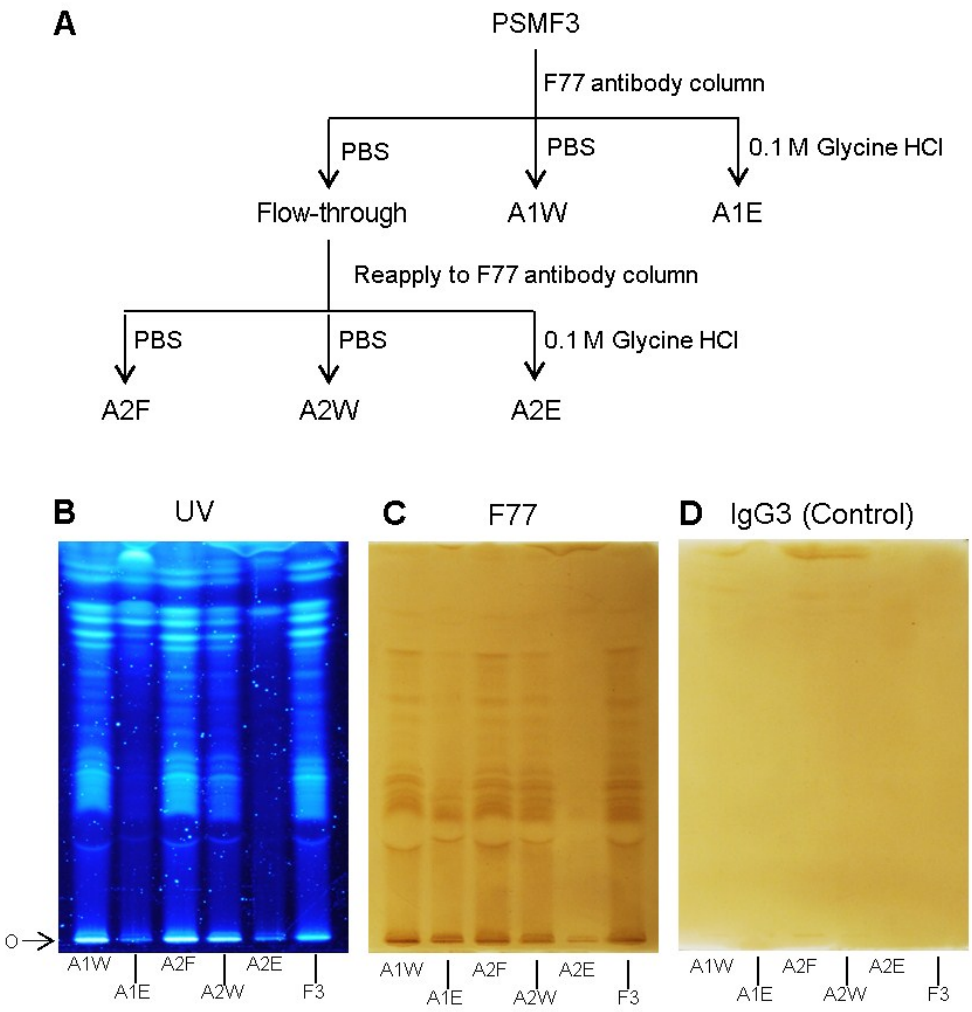


Figure 3.10 Affinity chromatography to enrich the F77 antigen-positive *O*-glycan alditols from PSMF3 using F77 antibody column. (**A**) Workflow of the F77 affinity chromatography. In total, five fractions were obtained, A1W, A1E, A2F, A2W and A2E. (**B**) TLC analysis of the NGLs derived from *O*-glycan alditols of the five subfractions in (**A**), revealed by UV light. (**C-D**) Chromatogram bindings of mAb F77 (**C**) and the IgG3 control (**D**) on replicate TLC plates to panel (**B**). The fluorescent NGLs derived from the *O*-glycan alditols in the five subfractions of F3 and the unfractionated F3 were chromatographed, visualized and detected as described in Figure 3.8. O indicates the origin.

In summary, although affinity chromatography of the *O*-glycans in fraction 3 with UEA-I or F77 showed fractionation to some extent, the limited capacities of the UEA-I and F77 columns precluded their application to the F77 antigen enrichment. Therefore other avenues were explored.

3.4.5 Separation of a F77 antigen-positive NGL by preparative TLC and HPLC

Separation of the F77 antigen-positive NGL component was then attempted by combined preparative TLC and HPLC (Section 2.7). For this, the NGLs in fraction 4 were resolved by semi-preparative TLC (Figure 3.11A) and the region designated 4M which includes the F77 antigen-positive component was harvested. The NGLs in 4M were resolved by HPLC (Figure 3.11B). Eight subfractions 4M-a to -h were obtained. F77 activity in each fraction was monitored using chromatogram-binding assays. One of the subfractions 4M-f was found to be F77-positive. The F77 antigen activity was detected in the top (4M-f1) of the triplex bands resolved by HPTLC (inset Figure 3.11B). The other HPLC fractions were F77 antigen-negative (not shown). Attempts to isolate the F77 antigen-positive component in subfraction 4M-f by a repeated HPLC were unsuccessful (data not shown). However, 4M-f could be well resolved into three bands by semi-preparative HPTLC (Figure 3.11C); and subfractions 4M-f1 to -f3 were harvested.

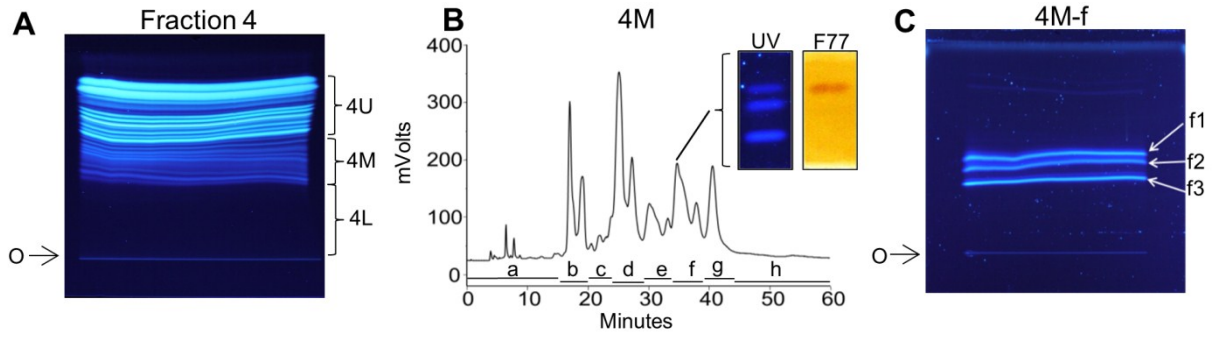


Figure 3.11 Isolation of a F77 antigen-positive component in fraction 4 of PSM *O*-glycans. (A). Chromatography of NGLs prepared from fraction 4 (Figure 3.8) on HPTLC plates. Three fractions (upper 4U, middle 4M and lower 4L) were harvested. *O* indicates origin. (**B**). Separation of fraction 4M into eight subfractions (a-h) by HPLC. Inset shows binding of mAb F77 to the upper band in subfraction 4M-f (designated 4M-f1). (**C**). Resolution of subfraction 4M-f by HPTLC. Three bands 4M-f1 to 4M-f3 were harvested. O indicates origin. All the HPTLC plates were chromatographed, visualized and detected as described in Figure 3.8.

3.4.6 Assignment of the sequence of the F77 antigen-positive NGL

NGL subfractions 4M-f1 to -f3 were analysed by negative-ion MALDI-MS (Figure 3.12). Subfraction 4M-f1 gave a $[M-H]^-$ at *m/z* 2033, corresponding to the NGL of a 6-linked branch with a composition $dHex_1Hex_2HexNAc_3$-OY (Figure 3.12A). Subfraction

4M-f2 contained two components, which gave $[M-H]^-$ at *m/z* 2240 and *m/z* 2297, corresponding to NGLs of 3-linked branches with compositions $dHex_2Hex_3HexNAc_2$-OX and $dHex_1Hex_3HexNAc_3$-OX, respectively (Figure 3.12B). The components in subfraction 4M-f3 gave $[M-H]^-$ ions at *m/z* 2094 and 2297 corresponding to $dHex_1Hex_3HexNAc_2$-OX and $dHex_1Hex_3HexNAc_3$-OX, respectively (Figure 3.12C).

In order to assign the oligosaccharide sequence of the F77 antigen-positive 4M-f1, MALDI-CID-MS/MS was performed (Section 2.9). The fragmentation pattern is shown in Figure 3.13. The oligosaccharide sequence was deduced based on the fragmentation pattern. Neutral loss of 146 Da from $[M-H]^-$ (*m/z* 2033) corresponded to the loss of dHex (-dHex). Similarly, loss of 162 Da from the fragment *m/z* 1887 and 203 from *m/z* 1725 correlated with the loss of Hex (-Hex) and HexNAc (-HexNAc), respectively. The large gap (365 Da) between *m/z* 1522 and 1157 corresponded to the loss of Hex.HexNAc, indicating the branching point as shown in the structure. After further loss of HexNAc (-HexNAc from *m/z* 1157), the fragment ion at *m/z* 954 was assigned to 6-linked fragment linked to ADHP (-OY). From information above, a dHex-Hex-HexNAc-Hex-HexNAc-OY sequence can be deduced. The fragment at *m/z* 1830 with a neutral loss of 203 Da from $[M-H]^-$ indicated the presence of a HexNAc branch. Taken together, the deduced sequence is clearly identified as an NGL derived from the 6-linked branch of the GalNAcol core with the hexasaccharide sequence shown in Figure 3.13.

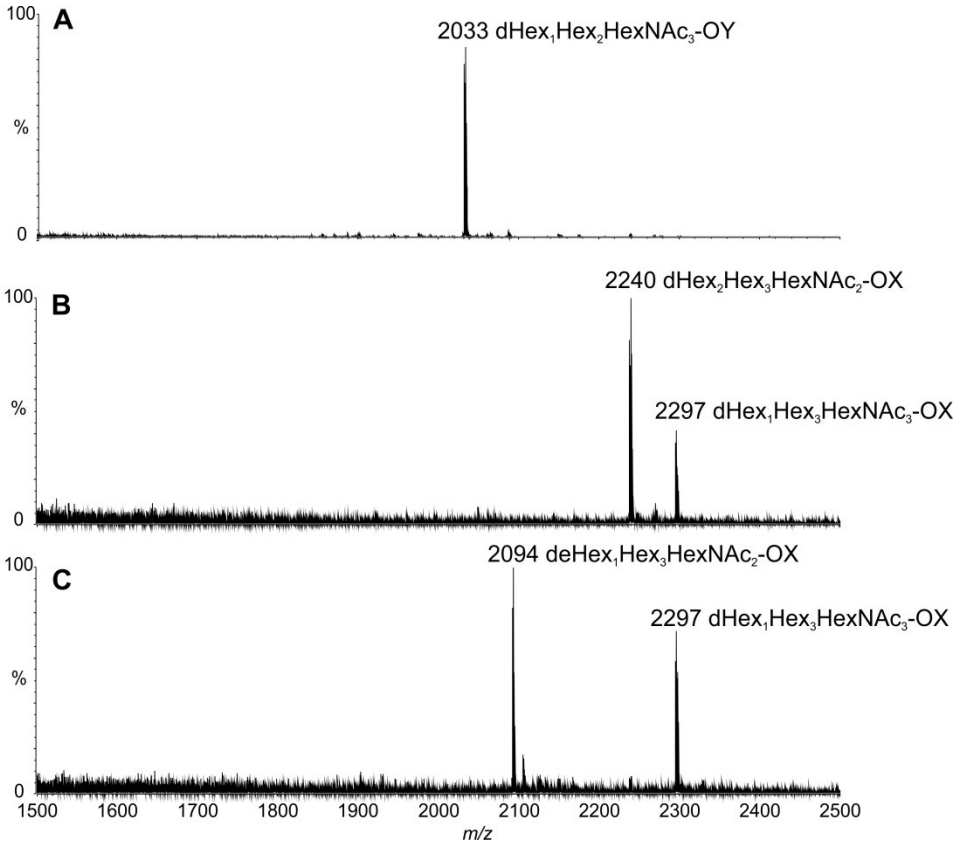


Figure 3.12 Negative-ion MALDI-MS analysis of the fraction 4M-f1 (A), 4M-f2 (B) and 4M-f3 (C). The deduced monosaccharide compositions were shown in each spectrum. dHex, deoxyhexose; Hex, hexose; HexNAc, N-acetetylhexosamine; OY and OX, 6- and 3-linked fragment of core GalNAc after periodate oxidation as described in Figure 3.8. OY: -OCH_2-CH_2-ADHP. OX: -OCH_2-CH(NHAc)-CH_2OH-CH_2-ADHP (304).

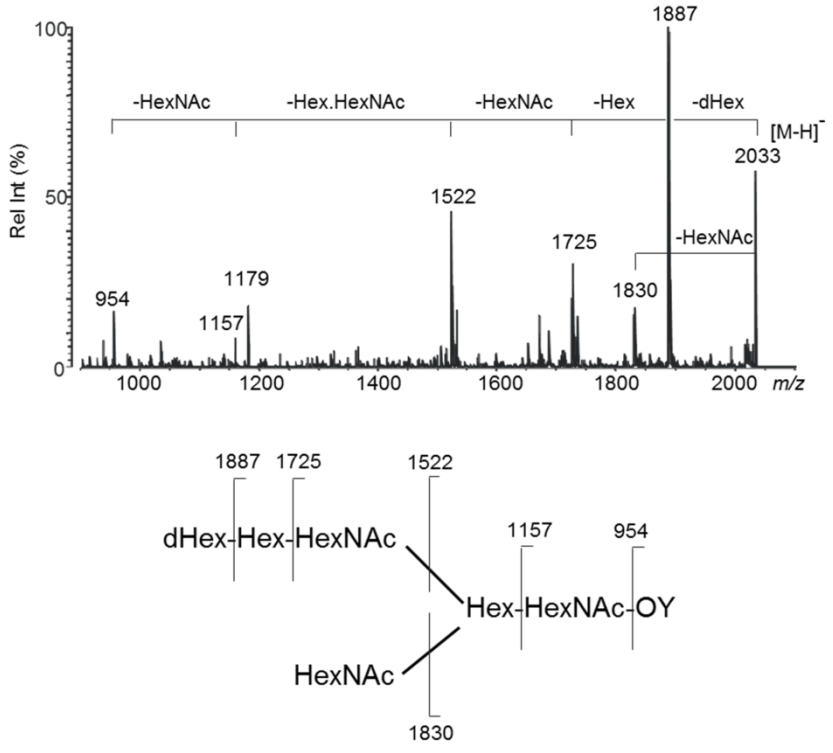


Figure 3.13 MALDI-CID-MS/MS analysis and the proposed sequence of the F77-positive 4M-f1. Abbreviations are as in legend to Figure 3.12.

Considering the origin of 4M-f1 is blood group H and A-active PSM, it is reasonable to predict that the component is a branched blood group H related structure. Indeed, when 4M-f1 was treated with α1-2 fucosidase as shown in Section 2.10, the $[M+H]^+$ shifted from *m/z* at 2035 to 1889 (Figure 3.14). The 146 Da loss from the parent NGL 4M-f1 confirms the presence of a α1,2-linked, blood group fucose.

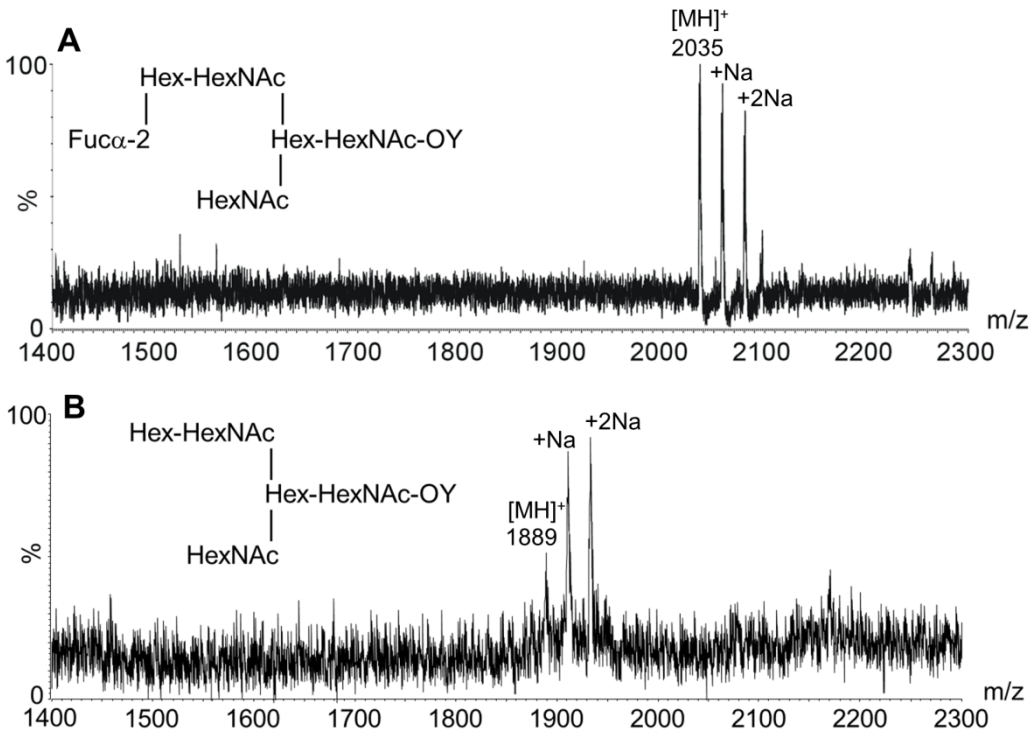
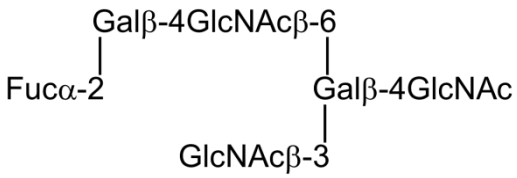
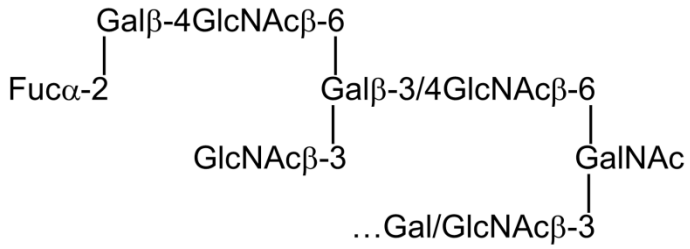


Figure 3.14 MALDI-MS analysis of NGL 4M-f1 before (A) and after (B) treatment with α1-2 fucosidase. The change in molecular ion $[MH]^+$ from m/z 2035 (A) to 1889 (B) indicated the capping sequence Fucα1-2Gal in 4M-f1. Abbreviations are as in legend to Figure 3.12.

In separate experiments (microarray analyses) described in Section 3.6, 4M-f1 was shown to be bound by UEA-I, but not anti-H type 1. Taking into account the F77 antigen activity of the branched blood group B glycolipid (BIII), the proposed oligosaccharide sequence of 4M-f1 is that of a blood group H on a branched, poly-LacNAc backbone (289), originating from the 6-branch of the core GalNAcol as depicted below.



Thus we can deduce that the parent *O*-glycan alditol before periodate oxidation is at least the octasaccharide as shown below.



3.5 Investigation of F77 antigen expression on blood group H-, A- and B-active sequences

Having identified the red cell-derived branched blood group B glycolipids BIII and BIV, and the mucin-derived branched blood group H-related *O*-glycan sequence (Section 3.4.6) as being bound by mAb F77, the question arose as to whether a branched blood group A analogue would also be bound by the antibody. If they were, it would be desirable to compare the antigenic activities of blood group H, A and B sequences.

To address this, I performed antigenic analyses with some linear and the branched blood group A and H glycolipids, also inhibition assays using the free oligosaccharides.

3.5.1 Chromatogram-binding analyses with linear and branched blood group H and A glycolipids

As the branched blood group H and A glycolipids were not present in the initial screening array (Appendix S1), we requested some preparations of the desired glycolipids from Dr. Michiko Fukuda at Sanford-Burnham Medical Research Institute. HPTLC and primulin analysis showed that each of these preparations from Dr Fukuda's archival collection contained multiple components (data not shown). To isolate the targeted glycolipids, I performed extensive purifications by HPTLC with monitoring by help of MALDI-MS analysis. Finally four human red cell-derived glycolipids were isolate: linear and branched blood group H, designated H_2 and H_3, respectively and linear and branched blood group A, designated Ab and Ad, respectively (Table 3.5).

The four glycolipid preparations Ab, Ad, H_2 and H_3 were resolved on an HPTLC plate and visualized by primulin (Figure 3.15A). Duplicate plates were probed for binding by mAb F77 (Figure 3.15B) and anti-H type 2 (Figure 3.15C). The branched H_3 and Ad but not the linear H_2 or Ab were bound by mAb F77. The anti-H type 2 antibody bound to both H_2 and H_3 but not to Ab and Ad, thus ruling out the presence of minor

H contaminants in the preparations of Ab and Ad. Collectively the results above showed that the mAb F77 recognizes the blood group H sequence on a 6-branched backbone but can also accommodate an additional α1,3-linked GalNAc or Gal, which constitute the blood group A or B analogues, respectively. These results, together with those obtained from the initial microarray analyses, were summarized in Table 3.5.

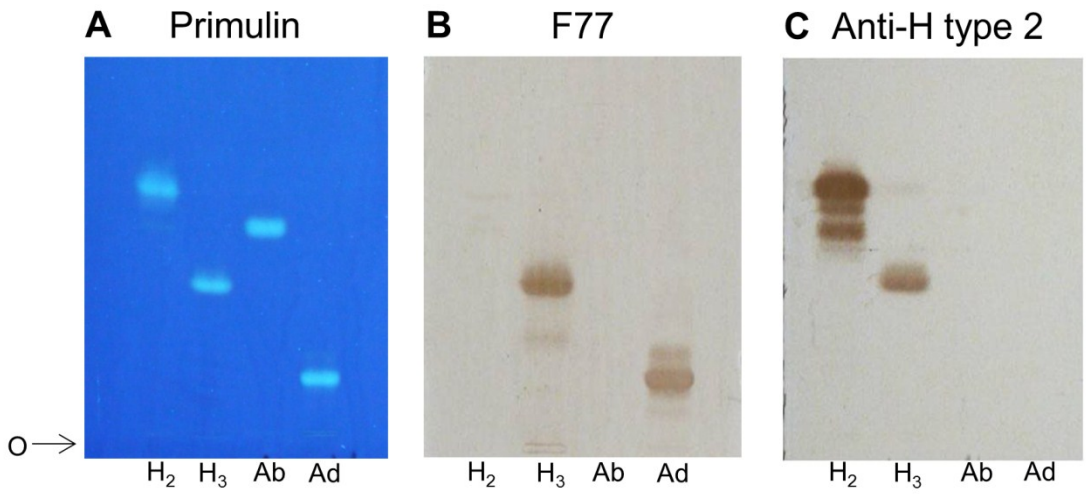
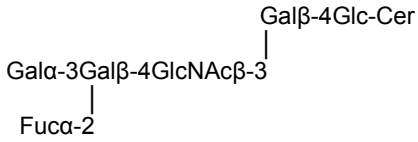
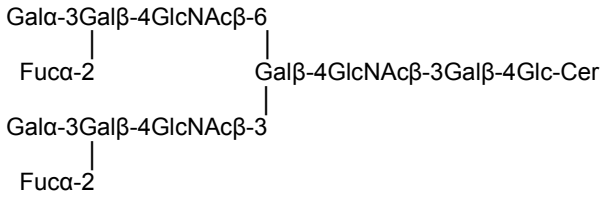
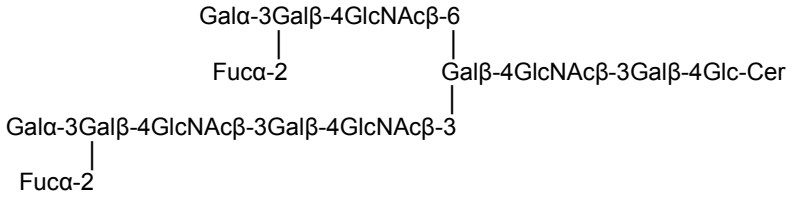
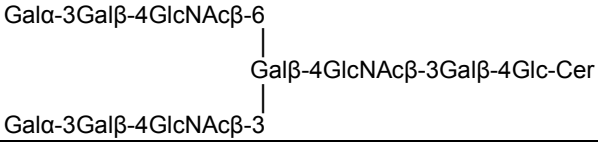


Figure 3.15 Chromatogram binding with mAbs F77 and anti-H type 2 to the four human blood group H- and A-derived glycolipids H_2, H_3, Ab, and Ad. (**A**). The four blood group H and A glycolipids were resolved by HPTLC and revealed by primulin staining for lipid. (**B**). Binding analysis of mAb F77 on a replicate TLC plate to (A). (**C**). Binding analysis of mAb anti-H type 2 on a replicate TLC plate to A. Solvent used for development was $CHCl_3$/MeOH/H_2O, 60:35:8 (v/v). *O* indicates the origin. Binding was detected as described in the legends to Figure 3.3.

Table 3.5 Blood group H-, A- and B-active and B-like glycosylceramides and their binding activities with mAb F77.

Designation	Carbohydrate sequence	mAb F77 binding
H_2*	Galβ-4GlcNAcβ-3Galβ-4Glc-Cer Galβ-4GlcNAcβ-3 Fucα-2	–
H_3‡	Galβ-4GlcNAcβ-6 Fucα-2 Galβ-4GlcNAcβ-3Galβ-4Glc-Cer Galβ-4GlcNAcβ-3 Fucα-2	+
Ab*	Galβ-4GlcNAcβ-3Galβ-4Glc-Cer GalNAcα-3Galβ-4GlcNAcβ-3 Fucα-2	–

Ad		+
B-hexa-		–§
BIII		+§
BIV		+§
B-like deca-		–§

*The asterisked glycolipid preparations contain a minor component with an additional fucose residue detected by MALDI-MS; most likely part of di-fucosylated Le^y sequence.
‡ The glycolipid H_3 contains a minor component lacking one fucose residue detected by MALDI-MS.
§These results were from the screening array analysis in Figure 3.2.

3.5.2 Inhibition assays with synthetic linear blood group A, B and O (H) oligosaccharides

It is desirable to corroborate the mAb F77 binding by other methods, e.g. inhibition assays. Unfortunately, only short and unbranched blood group-containing analogues were available in sufficient amounts. These were type 2-based blood group H, A, B, Le^x and Le^y-containing tetra- or pentasaccharides (Table 3.6). Although in direct binding assays, such as microarray analysis, the NGL derivatives of these short and unbranched oligosaccharides gave no binding signals (Appendix S1), it was considered possible that they could inhibit mAb F77 binding at high concentrations. Their inhibitory activities were tested in binding assays using PC3 cells and PSM as the reference immobilized substrates (Section 2.11.2).

When a monolayer of PC3 cells was immobilized on a microwell plate as substrate, mAb F77 showed good binding signals at concentrations between 3×10^{-3} and 3 µg/ml (Figure 3.16A). A concentration of 0.03 µg/ml of the antibody was selected for inhibition assays. At millimolar concentrations of oligosaccharide inhibitors, partial inhibitory activity (~50% inhibition) was recorded with blood group H sequence, H-tetra-T2, but not with the blood group A, B, Le^x and Le^y analogues (Figure 3.16B).

Table 3.6 Oligosaccharides included in the inhibition assays.

Designation	Carbohydrate sequence
H-tetra-T2	Galβ-4GlcNAcβ-3Gal | Fucα-2
A-penta-T2	GalNAcα-3Galβ-4GlcNAcβ-3Gal | Fucα-2
B-penta-T2	Galα-3Galβ-4GlcNAcβ-3Gal | Fucα-2
Le^X-tetra	Galβ-4GlcNAcβ-3Gal | Fucα-3
Le^Y-penta	Galβ-4GlcNAcβ-3Gal | | Fucα-2 Fucα-3

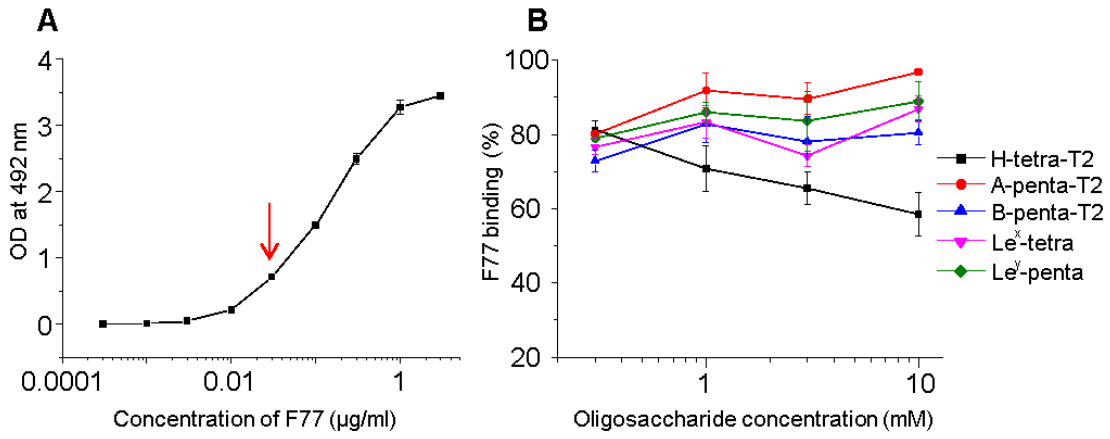


Figure 3.16 ELISA binding assays of mAb F77 to PC3 cells and inhibition of binding by short unbranched blood group oligosaccharides. (A). ELISA binding analysis of mAb F77 to PC3 cells. Arrow indicates the concentration of antibody used for inhibition assays. (**B**). Inhibition of mAb F77 binding to PC3 cells by the five oligosaccharides H-tetra-T2, A-penta-T2, B-penta-T2, Le^x-tetra and Le^y-penta. PC3 cells were grown in 96-well plates and reacted with mAb F77, with/without the presence of oligosaccharides between 3-10 mM, followed by incubation of biotinylated anti-mIgG and streptavidin-peroxidase polymer. Binding was detected using *FAST OPD* substrate.

When the A+H-active PSM was used as the immobilized substrate, mAb F77 binding was detected from 1×10^{-3} to 0.1 µg/ml (Figure 3.17A). A concentration of 0.01 µg/ml

was selected for inhibition assays. H, A and B oligosaccharides were almost equal potent in inhibiting binding of mAb F77 at high millimolar levels of inhibitors (Figure 3.17B).

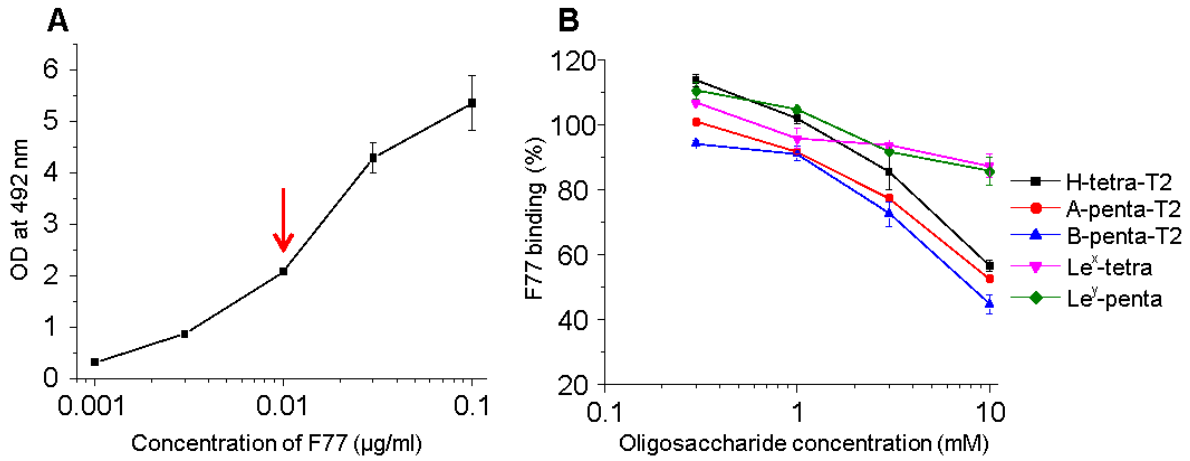


Figure 3.17 ELISA binding assays of mAb F77 to A+H antigen-positive PSM and inhibition of binding by short unbranched blood group oligosaccharides. (A). ELISA binding analysis of mAb F77 to PSM. Arrow indicates the concentration of antibody used for inhibition assays. (**B**). Inhibition of mAb F77 binding to PSM by the five oligosaccharides H-tetra-T2, A-penta-T2, B-penta-T2, Le^x-tetra and Le^y-penta. PSM was added in 96-well plates and reacted with mAb F77, with/without the presence of oligosaccharides between 3-10 mM. Binding was detected as described in the legend to Figure 3.16.

Thus when A+H active PSM was immobilized, H, A and B oligosaccharides were equally active as inhibitors, whereas when PC3 cells were used, the inhibitory activity of H oligosaccharide was stronger than the A and B analogues. In subsequent ELISA binding analysis, we found that the PC3 cells only express H antigen but lack of A and B antigens (Figure 3.18). It is likely that the abundant H antigen expression on PC3 cells made the H sequence a better inhibitor than the A and B analogues. Collectively, these results corroborate the ability of mAb F77 to accommodate the three blood group antigens but with a preference for the H analogue.

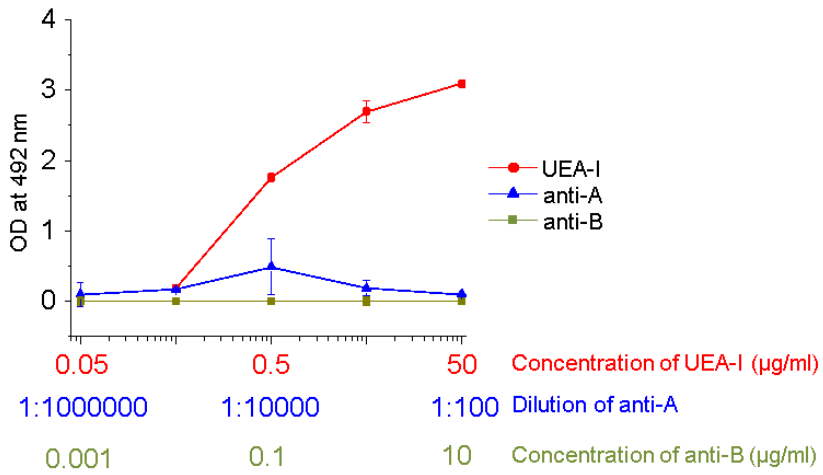


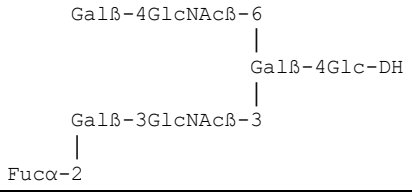
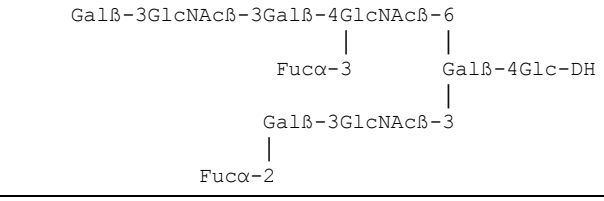
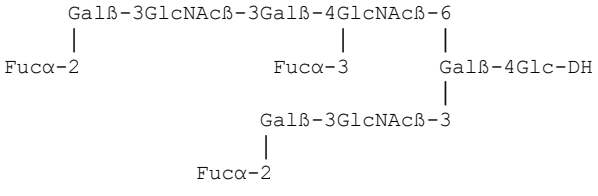
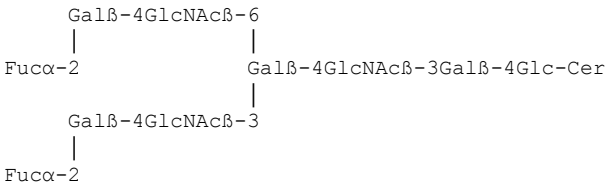
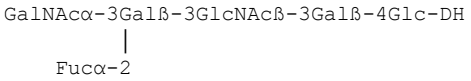
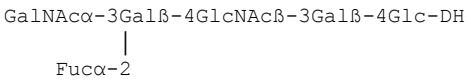
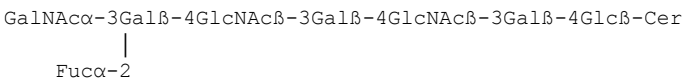
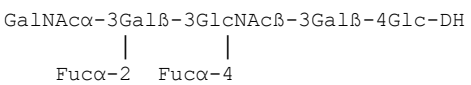
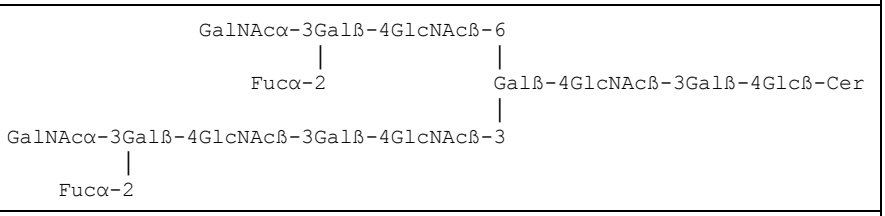
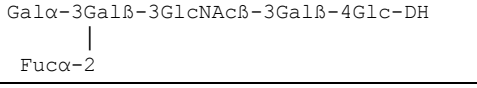
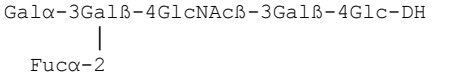
Figure 3.18 ELISA binding assays of UEA-I, anti-A and anti-B to PC3 cells. The PC3 cells were incubated with mAbs anti-A (dilutions between 1:10^6 and 1:100) or anti-B (10^{-3}-10 µg/ml), or biotinylated UEA-I (0.05-50 µg/ml), followed biotinylated secondary antibody and streptavidin-peroxidase polymer (for mAbs), or streptavidin-peroxidase polymer alone (for UEA-I). Binding was detected using *FAST OPD* substrate.

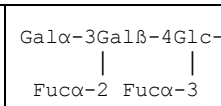
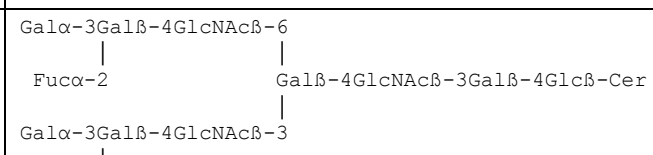
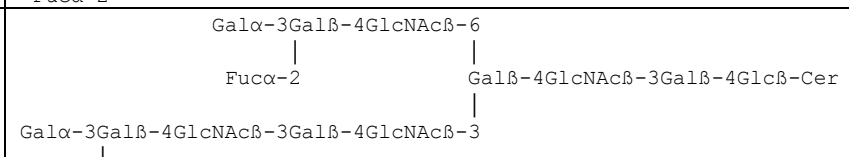
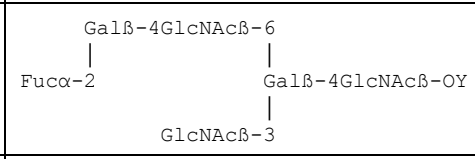
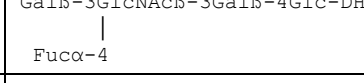
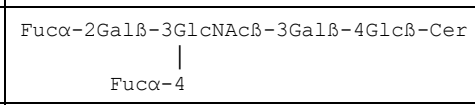
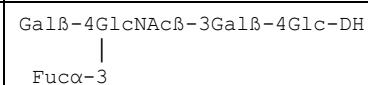
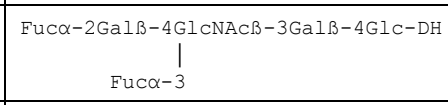
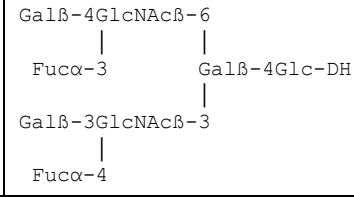
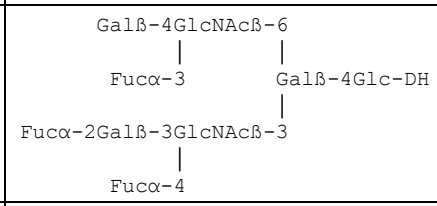
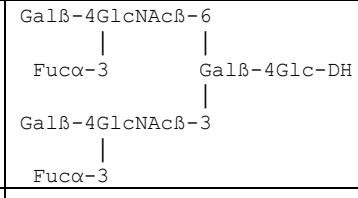
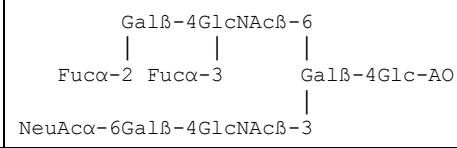
3.6 Further investigation of mAb F77 specificity using a focused glycan array

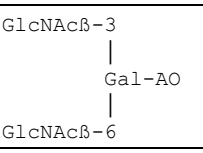
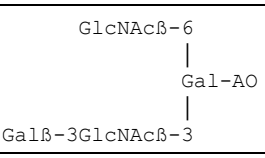
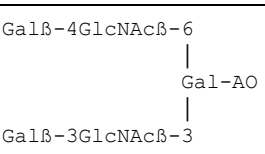
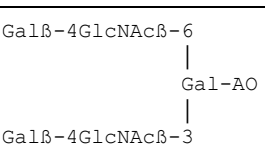
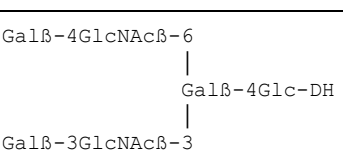
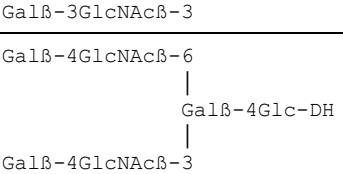
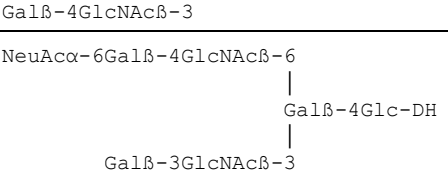
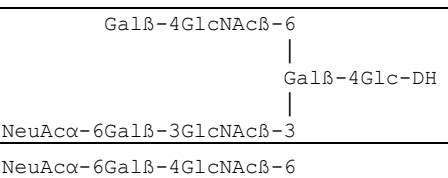
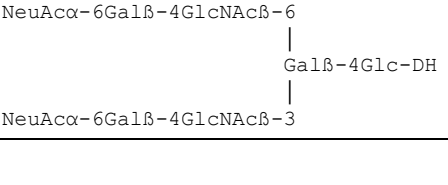
In order to complement the initial screening microarray analyses, a focused microarray (F77/Ii focused array) was assembled (Table 3.7). This included the aforementioned glycan probes with blood group A, B and H activities based on linear and branched poly-LacNAc sequences: H_2, H_3, Ab, Ad, BIII and BIV, and PSM-4M-f1 (probes 3, 7, 10, 12, 16, 17 and 18). The microarray also included twelve additional probes of the blood group H, A, B and Le^y series with type 1 and/or type 2 backbones (probes 1, 2, 4-6, 8-9, 11, 13-15 and 26). Other probes including selected Lewis antigen-containing sequences (probes 19-25), and linear and branched type 1 and/or type 2 backbone sequences (probes 27-54) were arrayed as well to further investigate the F77 binding specificity.

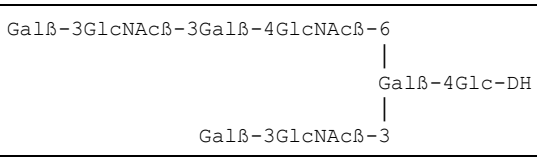
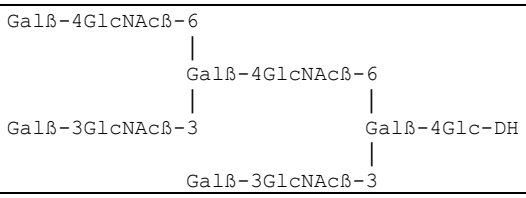
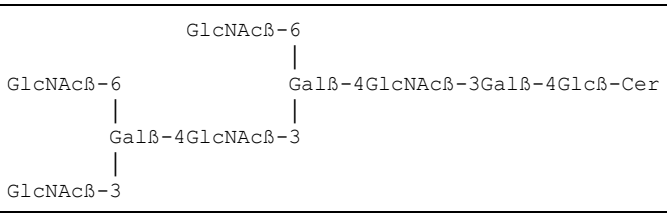
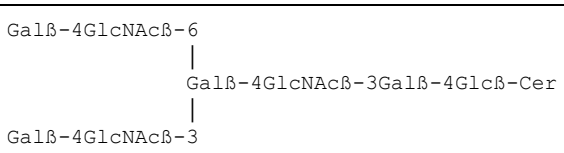
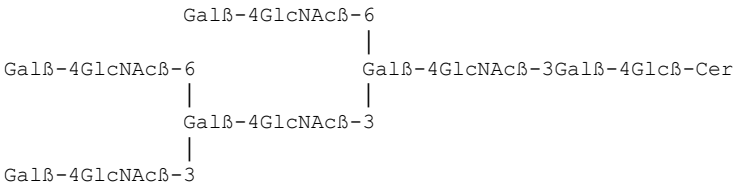
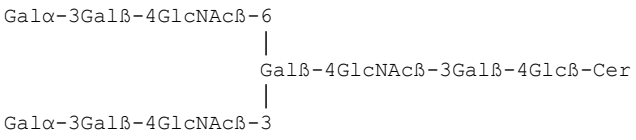
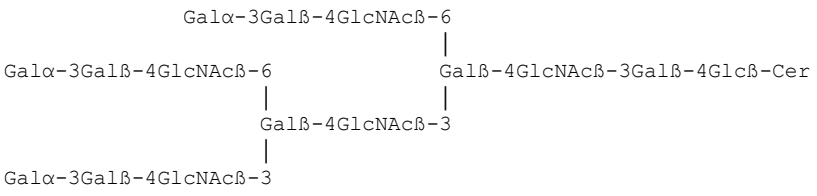
The mAb F77, together with UEA-I and mAbs anti-A, anti-B and anti-H type 1 were used to probe with the focused microarray (Figure 3.19). F77 binding was clearly restricted to the blood group H-, A- and B-active sequences with branched type 2 backbones. These included H_3 (probe 7), Ad (probe 12), BIII and BIV (probes 16 and 17) and the *O*-glycan-derived NGL PSM-4M-f1 (probe 18). Signals elicited by BIII and BIV were lower than with the two blood group H sequences H_3 and PSM-4M-f1, and the signal with Ad was even lower. The unbranched blood group H (probes 1-3), A (probes 8-10) and B (probes 13-14), A-Le^a (probe 11) and B-Le^y (probe 15) sequences were not bound. There was also no binding to probes with type 1 blood group H (probes 4, 5 and 6). Binding was also not detected to the Lewis antigen-containing sequences (probes 19-26), and to the non-fucosylated LacNAc and poly-LacNAc backbone sequences (probes 27-54). The binding of UEA-I, anti-A, anti-B and anti-H type 1 were in accord with their known specificities to blood group H type 2, A, B and H type 1 sequences, respectively. In particular, the binding of UEA-I to the purified PSM-4M-f1 (probe 18) and the lack of binding of anti-H type 1 corroborated the assignment of a type 2 H sequence.

Table 3.7 Oligosaccharide probes included in the F77/li focused array.

Pos[a]	Probe[b]	Sequence
1	LNFP-I	Fucα-2Galß-3GlcNAcß-3Galß-4Glc-DH
2	LnNFPI	Fucα-2Galß-4GlcNAcß-3Galß-4Glc-DH
3	H2 (& H2+Fuc)	Fucα-2Galß-4GlcNAcß-3Galß-4GlcNAcß-3Galß-4GlcNAcß-Cer
4	MFLNH-I	
5	DFiLNO(1-2,-,-3)	
6	TFiLNO(1-2,-2,-3)	
7	H3 (& H3-Fuc)	
8	A-Hexa-T1	
9	A-Hexa-T2	
10	Ab (& Ab+Fuc)	
11	A-Hepta	
12	Ad (& Ad+Fuc)	
13	B-Hexa-T1	
14	B-Hexa-T2	

15	B penta-AO	
16	B-III dodeca	
17	B-IV tetradeca	
18	PSM-4M-f1	
19	LNFP-II	
20	Leb-hexaosylceramide	
21	LNFP-III	
22	LNnDFH-I	
23	DFLNH(b)	
24	TFLNH	
25	DFLNnH	
26	MSDFLNnH	
27	Orsay-1	Galß-4GlcNAcß-6Gal-AO
28	Orsay-2	Galß-4GlcNAcß-3Gal-AO

29	Orsay-3	Galß-3GlcNAcß-6Gal-AO
30	Orsay-4	Galß-3GlcNAcß-3Gal-AO
31	LNT	Galß-3GlcNAcß-3Galß-4Glc-DH
32	LNnT	Galß-4GlcNAcß-3Galß-4Glc-DH
33	LSTa	NeuAcα-3Galß-3GlcNAcß-3Galß-4Glc-DH
34	LSTc	NeuAcα-6Galß-4GlcNAcβ-3Galβ-4Glc-DH
35	LSTd	NeuAcα-3Galß-4GlcNAcß-3Galß-4Glc-DH
36	pLNH	Galß-3GlcNAcß-3Galß-4GlcNAcß-3Galß-4Glc-DH
37	pLNnH	Galß-4GlcNAcß-3Galß-4GlcNAcß-3Galß-4Glc-DH
38	LNnO	Galß-4GlcNAcß-3Galß-4GlcNAcß-3Galß-4GlcNAcß-3Galß-4Glc-DH
39	O1.AO	
40	Orsay-5	
41	Orsay-6	
42	Orsay-7	
43	LNH	
44	LNnH	
45	MSLNH	
46	MSLNnH-I	
47	DSLNnH	

48	iLNO	
49	LND	
50	Nonaosylceramide	
51	I-octaosylceramide	
52	I-dodecaosylceramide	
53	B-like decaosylceramide	
54	B-like pentadecaosylceramide	

[a]Pos, Probe position in the screening microarray.
[b]The oligosaccharide probes are all lipid-linked, neoglycolipids (NGLs) or glycosylceramides. Unless otherwise specified the NGLs are prepared from reducing oligosaccharides by reductive amination with the amino lipid, 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE) (353); AO, NGL prepared from reducing oligosaccharides by oxime ligation with an aminooxy (AO) functionalized DHPE (310); Cer, natural glycolipids with various ceramide moieties; –OY is the 6-linked fragment of core GalNAc after periodate oxidation as further explained in Figure 3.7.

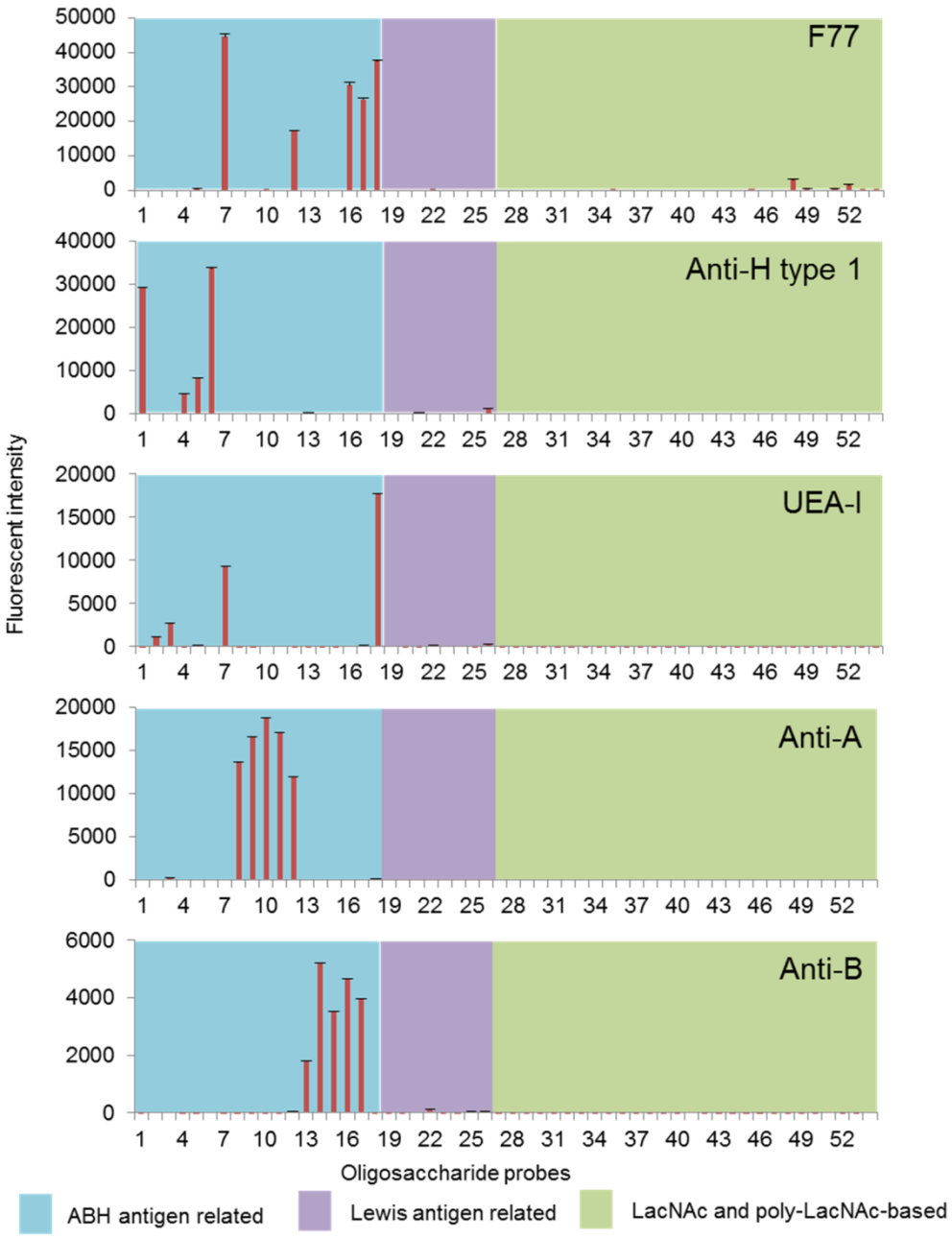


Figure 3.19 Microarray analysis of mAbs F77, anti-A, anti-B and anti-H type 1, and lectin UEA-I with oligosaccharide probes in F77/Ii focused array. The results are the means of fluorescence intensities of duplicate spots, printed at 5 fmol with error bars representing half of the difference between the two values. These 54 lipid linked probes (F77/Ii focused array) are arranged according to their epitopes and backbone sequences as annotated in the coloured panels below the figure. The probe sequences are in Table 3.7.

3.7 Haemagglutination properties and temperature-dependent binding of mAb F77

The results above have shown that mAb F77 binds specifically to blood group H, A and B sequences on branched poly-LacNAc backbones. The linear and branched poly-LacNAc-based blood group sequences are highly expressed on human red cells, with the linear (i-antigen type) being predominantly on cord cells and the branched (I-antigen type) on adult cells, respectively (30). We therefore tested if mAb F77 agglutinates human red cells (Table 3.8). The haemagglutination assays were done by our collaborator Dr. Don Siegel at Univerisity of Pennsylvania. Indeed, at ambient temperature, when dilutions of mAb F77 were tested with blood group O (H) adult cells and cord blood cells, there was, as predicted, agglutination of both cell types, but to a higher titre with the adult cells: haemagglutination of the adult and cord cells giving 4+ were at 625 and 5,000 ng/ml, respectively. Haemagglutination activity of mAb F77 at 37 ˚C was lower than at ambient temperature; the 4+ score with group O adult red cells was 1,250 ng/ml. Thus mAb F77 had features resembling those of anti-I cold-agglutinins (30).

Blood group B and A adult red cells were also agglutinated by mAb F77, but to lower titres with 4+ score at concentration of 1,250 ng/ml at ambient temperature. There is a resemblance here also to the so-called anti-HI in normal human sera that have been detected serologically in blood transfusion laboratories; these behave as low titre cold-agglutinins, with the hierarchy of titres: adult group O > adult groups A , B and AB, and >> cord blood (31).

Table 3.8 Haemagglutination assay of mAb F77 with adult and cord red blood cells at 20 and 37 ˚C. Data from Don Peehl.

Blood Group/Temp	mAb F77 concentrations (ng/ml)							
	5000	2500	1250	625	313	156	78	39
Adult O/20 ˚C	4+	4+	4+	4+	3+	3+	2+	0
Adult A/20 ˚C	4+	4+	4+	3+	2+	1+	0	0
Adult B/20 ˚C	4+	4+	4+	3+	3+	2+	1+	0
Cord O/20 ˚C	4+	3+	3+	2+	0	0	0	0

Adult O/37 ˚C	4+	4+	4+	3+	3+	2+	1+	0

To further investigate the temperature-dependent property of mAb F77, I performed microarray analysis of mAb F77 at three temperatures, 4, 20 and 37 ˚C, together with a typical anti-I cold-agglutinin, anti-I Ma. This latter antibody recognizes the β1-6-linked backbone branch Galβ-4GlcNAcβ-6Gal (19) which is present in the probes designated Orsay-1, -6, -7 and B-like deca (Table 3.7). The binding intensities of mAb F77 were indeed the highest at 4 ˚C and lower at 20 ˚C, and even lower at 37 ˚C (Figure 3.20A and B). The temperature effect was most prominent at 0.1 µg/ml (Figure 3.20B). The temperature-dependence of ligand binding was very similar to that of anti-I Ma (Figure 3.20C), whose binding was abolished at 37 ˚C.

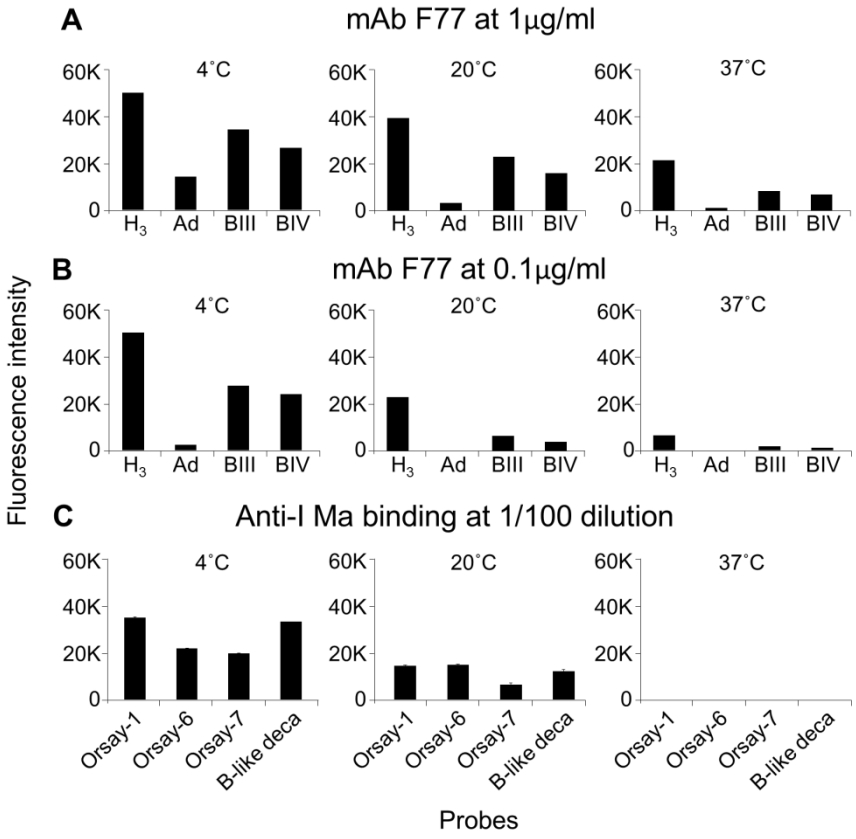


Figure 3.20 Temperature dependence of the binding intensities of mAb F77 and anti-I Ma. (**A** and **B**). Binding intensities elicited in microarray analyses of mAb F77, 1.0 (panel **A**) and 0.1 µg/ml (panel **B**), at 4 ˚C, 20 ˚C and 37 ˚C with the four branched human blood group H, A and B glycolipids, H_3, Ad, BIII and BIV. (**C**). Binding intensities elicited in microarray analyses of anti-I Ma (1:100) at 4 ˚C, 20 ˚C and 37 ˚C with NGLs of the chemically synthesized oligosaccharides designated Orsay-1, -6, -7 and the B-like decaosylglyceramide. The experiments were performed using the F77/Ii focused array and only probes relevant were shown. The carbohydrate sequences of the probes are listed in Table 3.7.

3.8 Corroboration of the specificity of mAb F77 with a chemically synthesized glycan

The assignment of the F77 antigen described in Section 3.4.5 was made using naturally occurring glycolipids and *O*-glycan. It was desirable to corroborate this finding with a chemically synthesized glycan and to gain insights into the size of the F77 antigen.

A nonasaccharide GSC-915 was synthesized in Laboratory of Makoto Kiso of Gifu University (Table 3.9). I performed treatments with specific glycosidases (Section 2.10) to sequentially remove Gal and GlcNAc on the 3-linked branch, to obtain oligosaccharides designated GSC-915-2 and GSC-915-3, respectively (Table 3.9). The Fuc and Gal on the 6-linked branch were sequentially removed from GSC-915-3 to obtain GSC-915-4 and GSC-915-5, respectively (Table 3.9). The oligosaccharide products were purified by HPLC and analysed by ES-CID-MS and ES-CID-MS/MS to corroborate their sequences (not shown).

Table 3.9 Sequences of the chemically synthesized nonasaccharide and its four enzymatic treatment products

Designation	Sequences
GSC-915	Galβ-4GlcNAcβ-6 | Fucα-2 Galβ-4GlcNAcβ-3Galβ-4Glc | Galβ-4GlcNAcβ-3
GSC-915-2	Galβ-4GlcNAcβ-6 | Fucα-2 Galβ-4GlcNAcβ-3Galβ-4Glc | GlcNAcβ-3
GSC-915-3	Galβ-4GlcNAcβ-6 | Fucα-2 Galβ-4GlcNAcβ-3Galβ-4Glc
GSC-915-4	Galβ-4GlcNAcβ-6Galβ-4GlcNAcβ-3Galβ-4Glc
GSC-915-5	GlcNAcβ-6Galβ-4GlcNAcβ-3Galβ-4Glc

NGLs were generated from these five oligosaccharides. MALDI-MS (Figure 3.21) analysis confirmed the high purity of each NGL product. $[M-H]^-$ of each NGL was consistent with the predicted molecular mass of each NGL product. The mass

differences between the products and the starting oligosaccharide, GSC-915, clearly indicated the successful removal of Gal, GlcNAc, Fuc and Gal, respectively.

Antigenic analyses were performed using NGLs of GSC-915 and the glycosidase digestion products (Figure 3.22). Clearly, F77 antigen-activity was retained after removal of the Gal and GlcNAc from the 3-linked branch. Defucosylation of the 6-linked branch markedly reduced mAb F77 binding but it was not until the 6-linked Gal was removed that the F77 antigen activity was abolished. The complete defucosylation of GSC-915-3 was confirmed by the lack of binding of GSC-915-4 by UEA-I and AAL. These results highlighted the importance of LacNAc on the 6-linked branch in the F77 antigen. The minimal sequence that elicited the strongest F77 binding signal was the unbranched blood group H sequence with the β1,6-linked LacNAc backbone. This contrasts with the lack of mAb F77 binding to the unbranched H with the β1,3-linked LacNAc backbone discussed in Section 3.6.

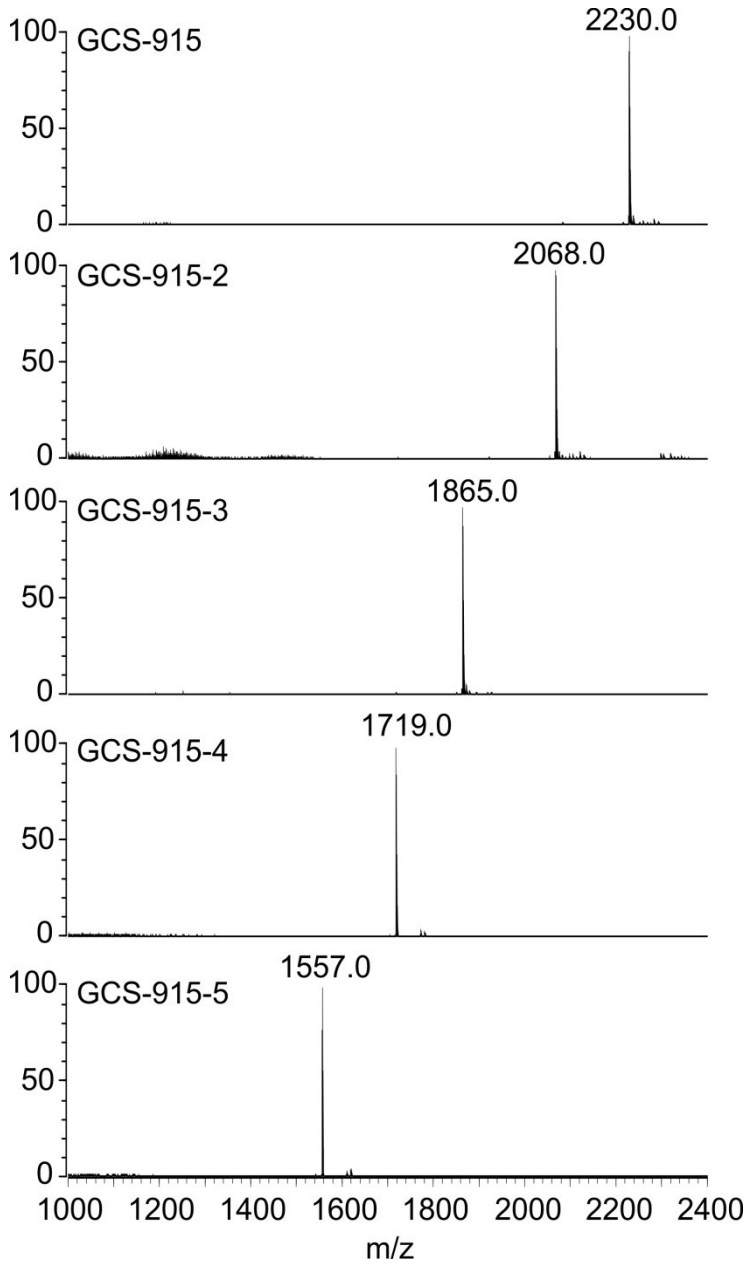


Figure 3.21 Negative-ion MALDI-MS analysis of the NGLs derived from the synthetic nonasaccharide, GSC-915, and its four glycosidase treated products GSC-915-2, -3, -4 and -5.

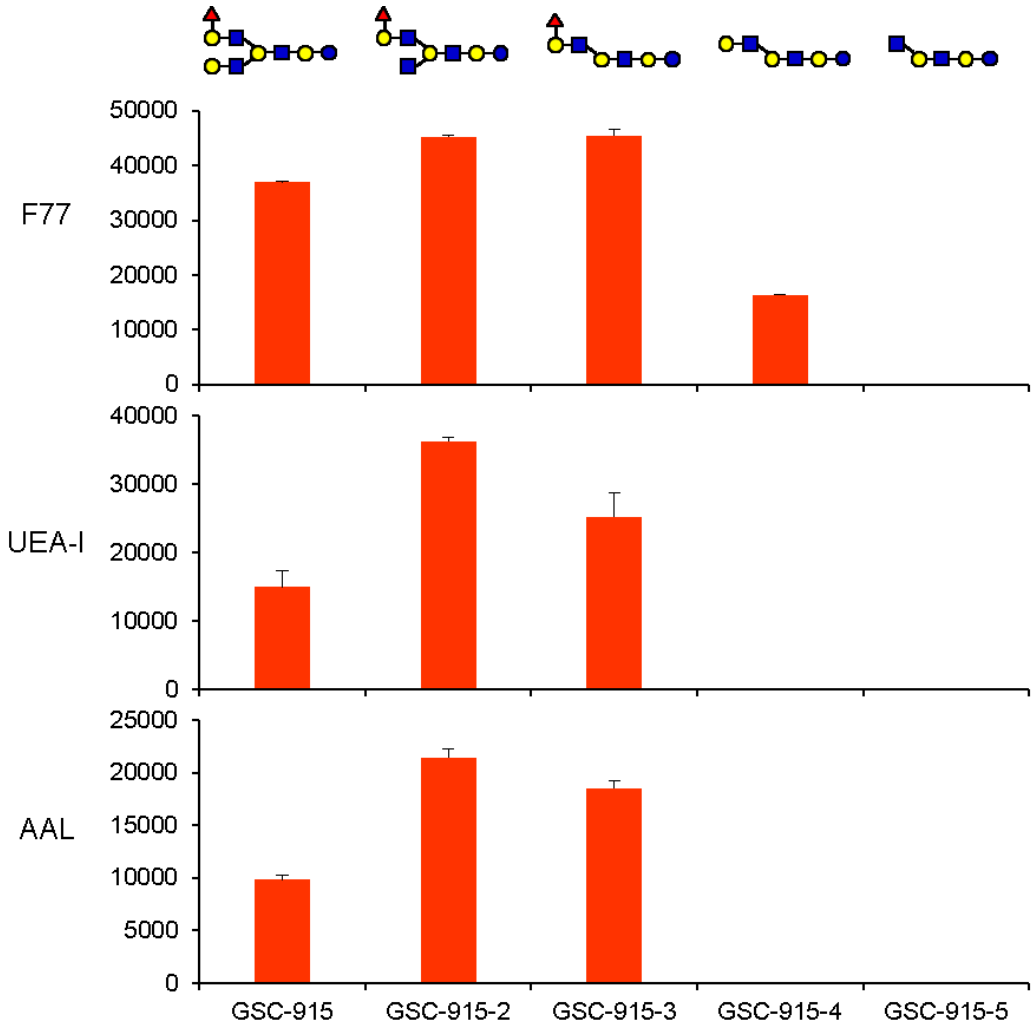


Figure 3.22 Microarray analysis of mAb F77, UEA-I and AAL with NGLs derived from the synthetic oligosaccharide GSC-915 and its four glycosidase treated products GSC-915-2, -3, -4 and -5. The results are the means of fluorescence intensities of duplicate spots, printed at 5 fmol with error bars representing half of the difference between the two values. The glycan sequences of the five NGLs are depicted on the top of the figure and listed in Table 3.9.

3.9 Discussion

This study illustrates the value of microarrays of sequence-defined glycans in conjunction with glycome-scale designer arrays for the elucidation of novel carbohydrate antigenic determinants. The initial microarray analysis of mAb F77 with existing glycan probes clearly pointed to the presence of the F77 antigen on the two blood group B sequences (BIII and BIV) based on branched poly-*N*-acetyllactosamine backbones. This was quite unpredicted as F77 antigen is known to be widely expressed in prostate cancer tissues (341) whereas the prevalence of blood group B in Caucasians is only 9% (364). The branched blood group H and A equivalents of BIII and BIV glycolipids were not available in the microarray initially. However, the lack of F77 antigen activity on the unbranched analogue, the blood group B hexaglycosylceramide, and on an analogue of the branched blood group B glycolipids lacking the terminal α1,2-linked blood group H fucose (so called B-like), was an important clue indicating the presence of the F77 antigen on the branched blood group H, and this was indeed corroborated by the studies that followed.

The F77-active glycolipids of PC3 cells were found to be very minor components within an extremely heterogeneous population. Therefore, the discovery of F77 antigen on an abundantly available mucin, PSM, opened up an alternative route to isolating the antigen, namely, by generating designer arrays from the PSM *O*-glycome. Antigenic analyses of the *O*-glycan population released from PSM and arrayed as NGLs led to the isolation and characterization of a F77 antigen-positive component, 4M-f1, as being a blood group H sequence on a 6-linked branch of a di-lactosamine backbone. This assignment is supported by the lack of F77 antigen activity of the two unbranched H-active sequences LnNFPI and H_2 (probes 2 and 3 in Figure 3.19).

It was essential thereafter to evaluate directly the F77 antigen expression on a branched blood group A analogue. This was achieved by analysis of the glycolipid Ad, the blood group A analogue of BIV, isolated from a glycolipid extract of human red cells. Ad was included in conventional chromatogram-binding analyses and compared with the red cell-derived branched blood group H glycolipid H_3. In addition

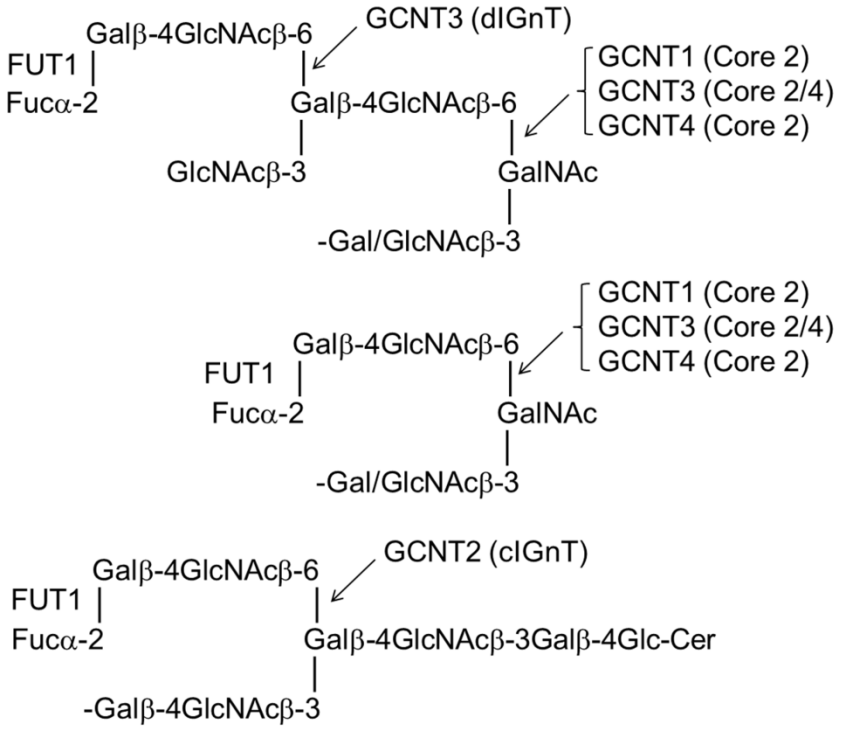
microarray analyses were performed using a newly generated set of probes among which Ad and H_3 glycolipids were included, also their respective analogues BIV and BIII, their unbranched analogues, the Lewis antigen-related probes and the linear and branched nonfucosylated backbones. It was thus shown that mAb F77 binds to the branched rather than the linear blood group A in addition to the branched blood group B and H on poly-*N*-acetyllactosamine backbones. The agglutination of red cells of A, B and O types, and more strongly those of adults than of cord blood, were also in accord with the conclusions above that mAb F77 can bind each of the branched A, B and H antigens. The specificity was clinched using a synthetic oligosaccharide GSC-915 and the products obtained by serial enzymatic treatments. The minimum sequence that mAb F77 recognizes was shown to be a type 2 blood group H (Fucα-2Galβ-4GlcNAc) linked to poly-LacNAc backbone through β-6 linkage; and the 3-linked branch was not required.

The approach of generating designer probes (NGLs) from *O*-glycans, following alkaline reductive release from PSM and mild periodate-oxidation, has three advantages: first, there is a higher yield of glycans than by non-reductive release (308); second, there are diagnostic MS features with respect to their origins from 3-linked (OX) or 6-linked (OY) branches at the core GalNAcol residues (363); and third, the cleavage at the core GalNAcol residues gives rise to a population of NGLs with reduced heterogeneity as there arise branches of the same size and sequence from different cores. Thus the antigen-positive components among the NGLs were resolved into discrete bands on HPTLC (Figure 3.8) unlike those derived from non-reductively released glycans which were not only larger but also more heterogeneous (Figure 3.5), and would be difficult to purify in sufficient amounts for detailed analyses. The patterns of binding of F77 to the NGLs from the reductively released *O*-glycans together with the fluorescence labelling of the NGLs facilitated both the visualization and isolation of the main antigen-positive component, 4M-f1.

In the previous study (341), there was a lack of a perceptible effect on F77 antigen expression on PC3 and DU 145 cells after treatment with the *O*-glycosylation inhibitor benzyl-α-GalNAc, contrasting with diminished antigen expression after treatment with the glycolipid synthase inhibitor 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol. The authors concluded that the F77 antigen was expressed on glycolipids rather than

O-glycosylated proteins. However, in our study, we identified PSM [which has *O*-glycan sequences that resemble those of humans (365)] to express strongly the F77 antigen and we isolated an antigen-positive *O*-glycan fragment. Thus our studies established the F77 antigen can be expressed not only on glycolipids, but also on *O*-glycans of glycoproteins. This conclusion is supported by the finding in our collaborator, Minoru Fukuda's lab that benzyl-α-GalNAc treatment abolished F77 antigen activity in another cell line human prostate epithelial 267B1 that had been co-transfected with FUT1 and GCNT3 (366). Collectively these results indicate that there is a predominance of glycolipids with F77 antigen activity in PC3 and DU 145 cells and a low content of *O*-glycosylated proteins relative to those on the transfected 267B1 cells such that the inhibition of *O*-glycan biosynthesis would not have a perceptible effect on expression of the F77 antigen. Recent more sensitive analyses using immunoprecipitation followed by Western blotting have revealed a glycoprotein that is bound by mAb F77 (Mark Greene lab, unpublished). The entity of the carrier protein is under investigation.

The marked differences in carbohydrate antigen expression on cancer cells compared with normal counterparts have been discussed and reviewed in Section 1.2. These changes reflect the altered activities of the corresponding glycosyltransferases. Highly relevant to F77 antigen expression are α1,2-fucosyltransferase encoded by FUT1, which adds α1,2-linked blood group H fucose (367) and, β1,6-*N*-acetylglucosaminyltransferase branching enzymes encoded by *GCNTs 1-4*, which synthesize the 6-linked GlcNAc for cores 2, core 4 and I-antigen type backbones (289,368), as depicted in Scheme 3.1.



Scheme 3.1 Key glycosyltransferases responsible for the biosynthesis of F77 antigen

With the exception of *GCNT3*, the expression of which was found to be down-regulated in colorectal cancer (369), the above-mentioned branching enzyme genes have been observed to be increased in epithelial cancers. Notably, over-expression of *GCNT1* has been reported to be associated with progression of prostate cancer (370,371). Indeed upregulation of *GCNT1* has been reported to be associated with invasiveness and high metastasis in many cancer types, including endometrial carcinoma (372), breast cancer (373), bladder cancer (374), testicular germ cell tumour (375), colorectal cancer and pulmonary carcinoma (376,377). Recently, *GCNT2* has been reported to be involved in metastasis of breast cancer through TGF-β signalling, and blocking the expression of *GCNT2* was shown to abrogate the tumour cell migration and invasion (378).

The question then arises as to why F77 antigen that includes a branched backbone sequence is strongly expressed in prostate cancer rather than in other cancers. We propose that the answers lie in the finding that the type 2 blood group H antigen, which is expressed in the normal prostate irrespective of the ABO blood group status, is increased in prostate cancer tissues (379-382). This contrasts with many other cancers in which the branching enzyme genes are overexpressed, but the type 2 H

antigen expression is decreased due to α1,3-fucosylation and the formation of Le^y antigen sequence, which we have shown not to be bound by mAb F77 (probe 26 in Figure 3.19). Alternatively, there may occur competition by sialylation on the terminal galactose instead of α1,2-fucosylation (156). Results of glycosyltransferase gene transfections in the Fukuda lab (366) are in complete accord both with our findings and our hypothesis. These include first, expression of F77 antigen was induced in 267B1 cells lacking the antigen by co-transfection of the gene for α1,2-fucosyltransferase and those for the branching enzymes GCNT1, GCNT2 or GCNT3; second, the antigen activity persisted when A or B enzyme was transferred into the cells but binding was less strong; third, RT-PCR analysis revealed the expression of the genes for FUT1 and GCNT2, and those for FUT1 and GCNT3 in F77 antigen-positive cells PC3 and LNCaP, respectively; and fourth, siRNA targeting these enzyme genes in antigen-positive cells resulted in significantly reduced levels of the antigen.

With knowledge of the structure, prevalence and potential biological function of F77 antigen in prostate cancer, the way is open to explore rationally its applications as a biomarker. Whereas direct use of mAb F77 in cancer sero-diagnosis and tumour imaging would be precluded because of the expression of the antigen on red cells. One possibility under current investigation is the detection of the antigen on circulating tumour cells. We are also exploring F77-antigen-positive prostatic glycoproteins as potential serum biomarkers of prostate cancer. Possible new approaches to immunotherapy of prostate cancer using the single chain variable region (scFv) of F77 antibody in are discussed in Chapter 5.

Chapter 4

STUDIES OF AN EPITHELIAL CANCER-ASSOCIATED CARBOHYDRATE ANTIGEN AE3

4.1 Introduction

Monoclonal antibody AE3 (IgM) was raised against sialidase treated (asialo) murine epiglycanin (383), which is a large (~500 kDa) sialylated mucin produced by murine mammary adenocarcinoma TA3-Ha cells (384). The mAb bound to high molecular weight (>1,000 kDa) human cancer-associated epithelial glycoproteins that are collectively designated HCA (human carcinoma antigen) (385). Among more than 40 mAbs raised against epiglycanin, mAb AE3 was considered the 'most carcinoma-specific' (386). Using mAb AE3, the HCA could be immunochemically detected in ascites fluid of patients with ovarian cancer (385) and in sera of patients with breast cancer (386). The AE3 antibody strongly stained human cancer tissues such as those of breast (386), prostate (387), bladder (388) and oesophagus (389).

The molecular identities of the murine epiglycanin and HCA have been investigated. By differential analyses of the murine mammary carcinoma cell variants that express or lack epiglycanin (designated TA3-Ha and TA3-St, respectively), a mouse epiglycanin cDNA fragment was first identified. Using homology searching, the human orthologue was cloned, expressed and designated MUC21 (390). This was followed by the clone of the mouse complete epiglycanin gene which was designated as Muc21 (391). The two human and murine orthogogues have been shown to be highly glycosylated transmembrane mucins with 28 and 98 tandem repeat domains, respectively, rich in serine and threonine residues (390,391).

In earlier studies, the binding of epiglycanin by most of the 40 mAbs was reduced after incubation of the glycoprotein with trypsin or pronase, suggesting a high molecular weight dependency for binding by the antibodies (392). The binding of these antibodies to epiglycanin was also attenuated by periodate treatment, and inhibited by peanut agglutinin (PNA) or high concentrations of the T disaccharide, Galβ-3GalNAc. Another plant lectin, *Ricinus communis* agglutinin I (RCA_{120}), could also inhibit the binding of one of these 40 antibodies (392). Treatment of the AE3 antigen-positive cells with endo-α-*N*-acetyl-D-galactosaminidase abolished binding of mAb AE3 (383). This enzyme removes the T disaccharide from the reducing terminal

on *O*-glycans. Collectively, these results suggested the involvement of carbohydrate, and specifically the Galβ-3GalNAc in the antigenic determinant.

The authors investigated the specificity of mAb AE3 by performing inhibition assays (inhibition of binding to epiglycanin) using T disaccharide and synthetic glycopeptides carrying this disaccharide sequence (393). The results are summarized in Table 4.1. Binding of mAb AE3 to epiglycanin was inhibited by the glycoproteins epiglycanin (number 1) or asialoglycophorin (number 2) at very low concentration (~0.7 μg/ml). Seven glycopeptides with various combinations of T disaccharide and Tn (GalNAc) monosaccharide (numbers 3 to 9, listed in Table 4.1) also inhibited AE3 binding, but at a higher concentration with IC_{50} between 150 and 5,000 μg/ml. The glycopeptides with triple Tn (number 10) did not show inhibitory activity. The free T disaccharide (number 11) could inhibit mAb AE3 binding although the inhibitory activity was relatively low (IC_{50} at 5000 μg/ml). These results suggested that mAb AE3 resembles PNA which recognizes the T antigen disaccharide Galβ-3GlcNAc. However, mAb AE3 differed from PNA in that the concentration of the T disaccharide required for inhibition was 10^4 times greater than for PNA, suggesting that the T disaccharide sequence forms part of a larger antigenic determinant recognized by mAb AE3 (393). In addition, among the glycopeptides, the one that had the strongest inhibitory activity was a glycopeptide with one Tn monosaccharide (GalNAc) on the first threonine residue and two T disaccharides on the second and third threonine residues (number 3). This was followed by the glycopeptide with one T on the middle threonine (number 4) and by the one with trivalent T on all three threonines (number 5). This was suggestive of the requirement for clustered presentation of T disaccharide, but not conclusive. The structure of AE3 antigen has remained an open question over the ensuing years. The molecular basis of ordering of the clustered mucin-type *O*-glycosylation has been investigated in detail. Recent results have shown that this is dependent on the specificities and catalytic activities of the UDP-GalNAc:polypeptide α-*N*-acetylgalactosaminyltransferases (ppGalNAc Ts), which initiate *O*-glycosylation, these, are influenced by the amino acid sequence and pre-existing GalNAc on the substrate (394,395).

Palma et al in the Glycosciences Laboratory (319) carried out microarray analysis of mAb AE3 using almost 500 glycan probes (Glycosciences Lab Array Sets 32-39).

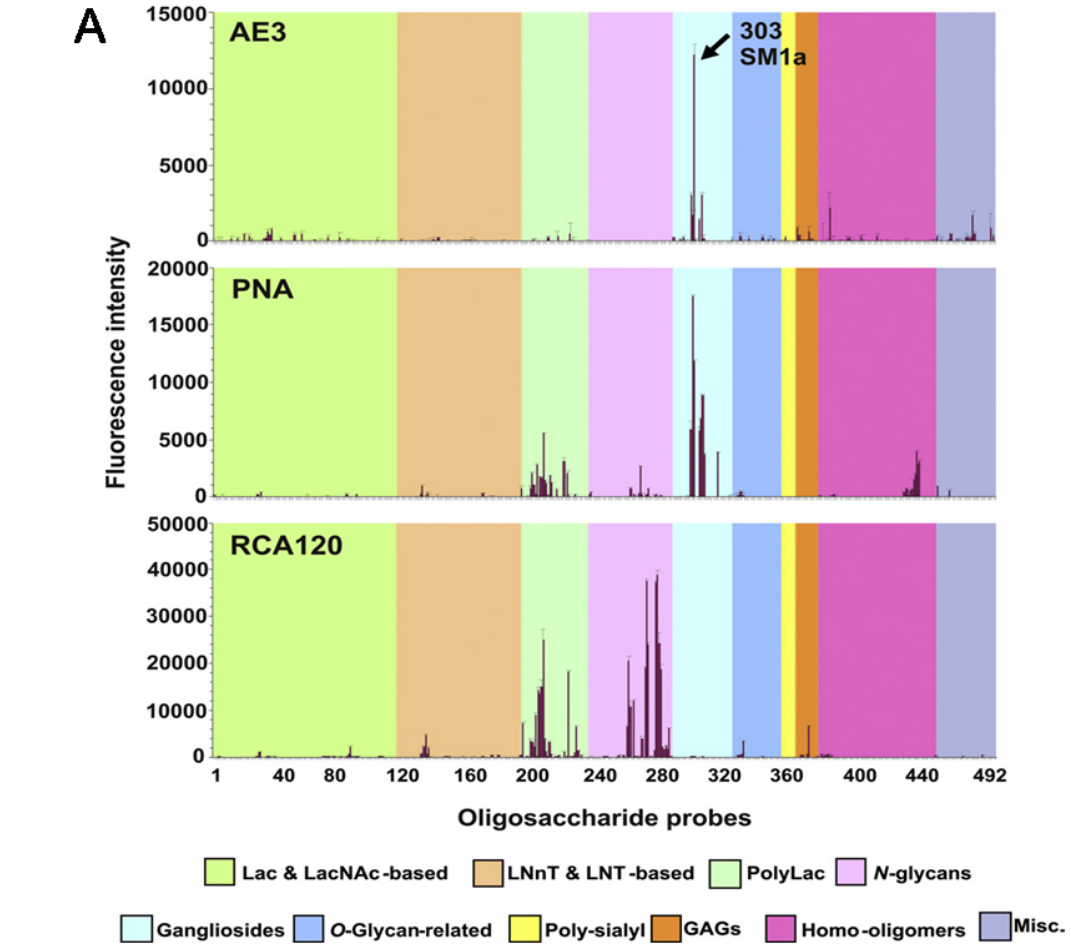
The binding profile for mAb AE3 partially overlapped with that of PNA but was clearly distinct from that of RCA_{120} (Figure 4.1A). There was an unpredicted finding that the strongest binding of mAb AE3 was to a sulphoglycolipid SM1a, an analogue of the sialoglycolipid GM1 (Figure 4.1B). The AE3 antibody bound relatively weakly to the three structurally-related sequences asialo-GM1, GM1 and GM1 (Gc) (probes 301, 306 and 308, respectively in Figure 4.1A and B). Additional sulphation on the terminal Gal impeded the AE3 binding as evidenced by the lack of binding to SB1a (probe 304, Figure 4.1A and B). This was the first report of a glycolipid carrier of human HCA.

Table 4.1 Inhibition of mAb AE3 binding to epiglycanin by glycoproteins, synthetic glycopeptides and T disaccharide. Data were taken from reference (393).

Number	Inhibitor	IC_{50} (μg/ml)
	Glycoprotein	
1	Asialoglycophorin	~0.7
2	Epiglycanin	~0.7
	Synthetic glycopeptides	
3	Tn T T Ac---P----T----T----T----P----I----S----T---NH_2	~150
4	Tn T Tn Ac---P----T----T----T----P----I----S----T---NH_2	~300
5	T T T Ac---P----T----T----T----P----I----S----T---NH_2	~1200
6	T Tn T Ac---P----T----T----T----P----I----S----T---NH_2	~2100
7	T T Tn Ac---P----T----T----T----P----I----S----T---NH_2	~2100
8	T Tn Tn Ac---P----T----T----T----P----I----S----T---NH_2	~3000
9	Tn Tn T Ac---P----T----T----T----P----I----S----T---NH_2	>5000
10	Tn Tn Tn Ac---P----T----T----T----P----I----S----T---NH_2	not inhibited
	T disaccharide	
11	Galβ1-3GalNAc	~5000

The carbohydrate sequence of SM1a has not been reported on mucin-type glycoproteins so far. Sequences related to SM1a have been described (348) among *O*-glycans of glycoproteins in normal descending colon (Figure 4.1C): These are structures **a**, with sulphate on Gal but lacking terminal Galβ1-3GalNAc and structure **b**, a Sd^a/Cad sequence (Figure 1.6 and Section 1.1.2.2) lacking a terminal Gal but with sialic acid instead of sulphate on Gal. Taken together, these findings led to a proposal that an *O*-glycan analogue of SM1a with a non-reducing terminal Galβ1-

3GalNAc, a sulphate on an inner galactose and the core 3 sequence at the reducing end (Figure 4.1C) may be present on mucin-type glycoproteins bound by mAb AE3.



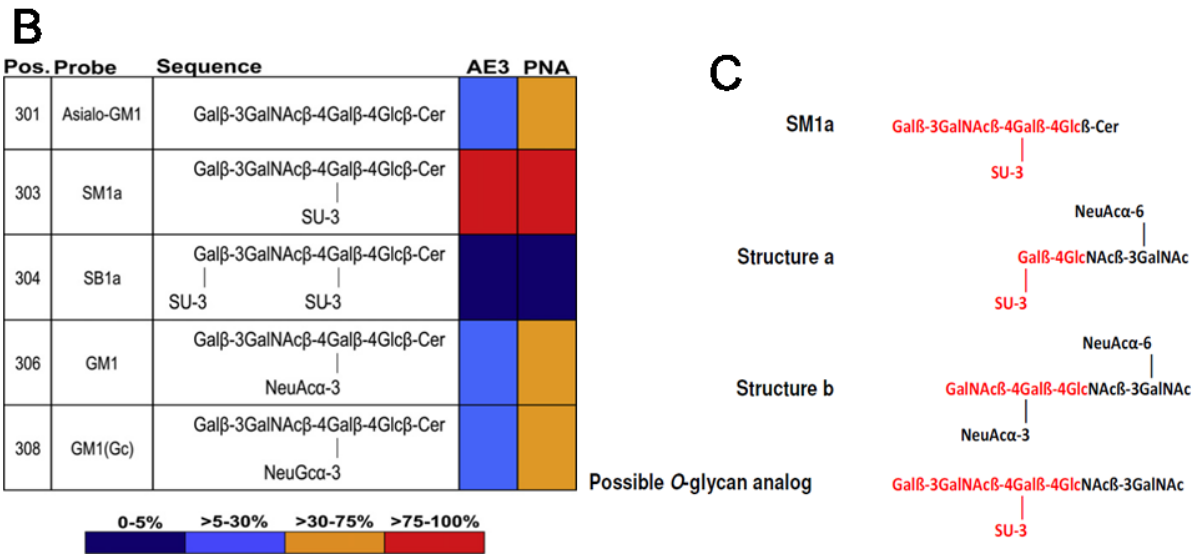


Figure 4.1 Microarray screening analyses of mAb AE3 and the plant lectins peanut agglutinin (PNA) and Ricinus communis agglutinin I (RCA_{120}). (**A**) The results with 492 lipid-linked probes (>370 mammalian type) printed at 5 fmol in the arrays (means of fluorescence intensities of duplicate spots with error bars). The probes are grouped according to their backbone sequences as annotated by the coloured panels. (**B**) Relative intensities of binding of AE3 and PNA to five glycolipids sharing the asialo-GM1 backbone sequence in the microarray

screening analyses. (**C**) Schematic presentation of oligosaccharide sequences, structures a and b, related to that of SM1a and based on the *O*-glycan core 3 that have been described among O-glycans of glycoproteins in descending colon; also shown is a possible *O*-glycan analogue of SM1a. Taken from (319) with permission.

This chapter has been to investigate whether the AE3 determinant among *O*-glycans indeed occurs as an SM1a analogue or if it is a sequence with which SM1a cross reacts. It is important to determine which glycan sequence constitutes the cancer-associated antigen and thereby to have an indication of which glycosyltransferases are involved.

4.2 Antigenic analysis of an AE3 antigen-positive glycolipid SM1a

The sulphoglycolipid SM1a that was unexpectedly bound by mAb AE3 in the microarray screening analysis was from a natural source. It could not be ruled out that a minor component existed in the preparation and could not be detected by MS. In order to corroborate the mAb AE3 binding to SM1a, two glycolipids, monosulphated SM1a and disulphated SB1a, were chemically synthesized in the Laboratory of Makato Kiso of Gifu University for this project.

Purity of the two synthetic glycolipids was corroborated by HPTLC and MS analyses. They were then arrayed for probing with mAb AE3 (Figure 4.2). The result showed that the synthetic and the naturally occurring SM1a were equally well bound by mAb AE3, whereas the disulphated glycolipid SB1a was completely AE3 antigen-negative. This corroborated the finding in the screening array analysis that AE3 binding was restricted to the monosulphated glycolipid SM1a. Sulphation on the terminal Gal masked the AE3 binding epitope.

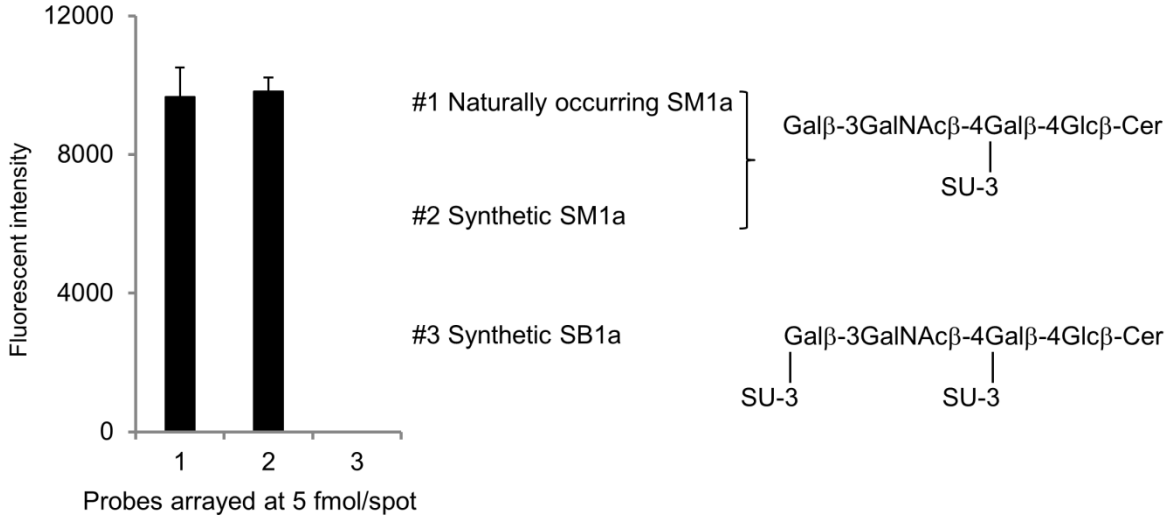


Figure 4.2 Microarray analyses of mAb AE3 with naturally occurring and chemically synthesized SM1a and synthetic SB1a. The results shown are binding intensities with the three glycolipids printed at 5 fmol in the arrays (means of fluorescence intensities of duplicate spots with error bars).

4.3 Identification of an AE3 antigen-positive ovarian cyst mucin, Cyst 756

In the hope of finding a source of the proposed *O*-glycan analogue of SM1a (Figure 4.1C), microarray analysis of mAb AE3 was performed using an array of 46 preparations of mucin-type glycoproteins (Mucin Array Set 2, Table 4.2). These included most of the 30 glycoproteins in Mucin Array Set 1 used in the F77 study (Table 3.1) and some additional glycoproteins: 11 enriched cystadenoma glycoprotein preparations being pepsin treated, 4 glycoprotein preparations of human milk and sheep stomach and 2 glycoproteins transferrin and invertase. A preparation of HCA served as the positive control. Two lectins PNA and RCA_{120} were tested in parallel to compare their binding profiles with that of mAb AE3.

Twenty eight of the mucin preparations gave binding signals with mAb AE3 (Figure 4.3). Glycoproteins giving moderate or high binding signals were those purified from cystadenomas (probes 29 to 32, Figure 4.3), preparations from meconia (probes 34 to 37), transferrin (probe 45) and the positive control, HCA (probe 42). The AE3 antibody bound only moderately or weakly to the enriched (but not purified) cystadenoma glycoproteins (probes 1, 5 to 12, 15, 16 and 23 to 26). Among them, three preparations of cystadenoma 756 (Cyst 756), probes 23 to 25, elicited relatively strong mAb AE3 binding. These samples had been enriched for high molecular mass glycoproteins (mucins) by trypsin, pronase or pepsin treatment, respectively, followed by ethanol precipitation (Section 2.2.3). The plant lectins PNA and RCA_{120} showed similar binding profiles which also overlapped with that of mAb AE3. In this sense, mAb AE3 indeed resembled the two plant lectins, albeit binding with a lower intensity. These results were in accord with the inhibition of binding of mAb AE3 and related mAbs raised against epiglycanin in the presence of RCA_{120} and PNA (392). However, mAb AE3 differed from the two plant lectins clearly in its lack of bindings to the two sheep stomach glycoproteins (probes 40 and 41). These findings suggested that the epitope of this antibody may partially overlap with those of the lectins but it is clearly not identical as indicated in the glycan array data in Figure 4.1A.

One of the samples most strongly bound by AE3, samples 29 to 32 and 35 to 36, would have been desirable sources of antigen-positive *O*-glycans for investigation. But these glycoproteins are available only in limited amounts. Thus an abundantly available ovarian cystadenoma glycoprotein preparation (Cyst 756) was selected for the studies described in this Chapter.

Table 4.2 Mucin-type epithelial glycoproteins arrayed as Mucin Array Set 2 for screening for mAb AE3 binding.

Position	Designation	Donor red cell blood-group	Secretor status
	Enriched cystadenoma glycoproteins		
1	Cys 301 (Pro)*		
2	Cys 301 (Pep)		
3	Cys 379 (Pro)	A1	Non-secretor
4	Cys 379 (Pep)		
5	Cys 444 (Trp)	Unknown	Non-secretor
6	Cys 444 (Pro)	Unknown	Non-secretor
7	Cys 444 (Pep)	Unknown	Non-secretor
8	Cys 446 (Pro)	B	Non-secretor
9	Cys 446 (Pep)	B	Non-secretor
10	Cys 461 (Try)	Unknown	Non-secretor
11	Cys 461 (Pro)	Unknown	Non-secretor
12	Cys 461 (Pep)	Unknown	Non-secretor
13	Cys 654 (Pro)	B	Non-secretor
14	Cys 654 (Pep)	B	Non-secretor
15	Cys 705 (Pro)	Unknown	Non-secretor
16	Cys 717 (Pro)	A1	Non-secretor
17	Cys 717 (Pep)	A1	Non-secretor
18	Cys 733 (Pro)	A1	Secretor
19	Cys 745 (Pro)	A	Non-secretor
20	Cys 745 (Pep)	A	Non-secretor
21	Cys 754 (Pro)	Unknown	Non-secretor
22	Cys 754 (Pep)	Unknown	Non-secretor
23	Cys 756 (Trp)	A	Non-secretor
24	Cys 756 (Pro)	A	Non-secretor
25	Cys 756 (Pep)	A	Non-secretor
26	Cys 762 (Pro)	O	Non-secretor
27	Cys 762 (Pep)	O	Non-secretor
28	Cys 765 (Pro)	O	Non-secretor
	Purified cystadenoma glycoproteins		
29	Og 10% from 20% (Pep)	Unknown	Non-secretor
30	Og 10% 2x (Pep)	Unknown	Non-secretor
31	N1 20% (Pep)	Unknown	Non-secretor
32	Tij 10% 2x (Pep)	B	Secretor
33	OC2	Unknown	Unknown
	Meconia		
34	Meconium B (Pro)	B	Unknown
35	Meconium Mo (Pro)	Unknown	Unknown
36	Meconium Wo (Pro)	Unknown	Unknown
37	Meconium He (Pro)	Unknown	Unknown

	Milk glycoproteins		
38	Milk batch #3	Unknown	Unknown
39	Milk batch #6	Unknown	Unknown
	Sheep stomach glycoproteins		
40	Sheep (S1+S10) (pep)		
41	New sheep pool (pep)		
	Other glycoproteins		
42	HCA	Unknown	Unknown
43	Porcine gastric mucin	A+H	(Sigma)
44	Bovine submaxillary mucin	Unknown	(Sigma)
45	Transferrin	Unknown	(Sigma)
46	Invertase	Unknown	(Sigma)

Details for the enrichment procedures for the mucin preparations 1-37, 40-41 and of 42 are given in Section 2.2.3. Samples 43 to 46 were from commercial sources.
*Trp, Pro and Pep refer to proteases, pronase, trypsin or pepsin, used in the process of solubilizing the mucins.

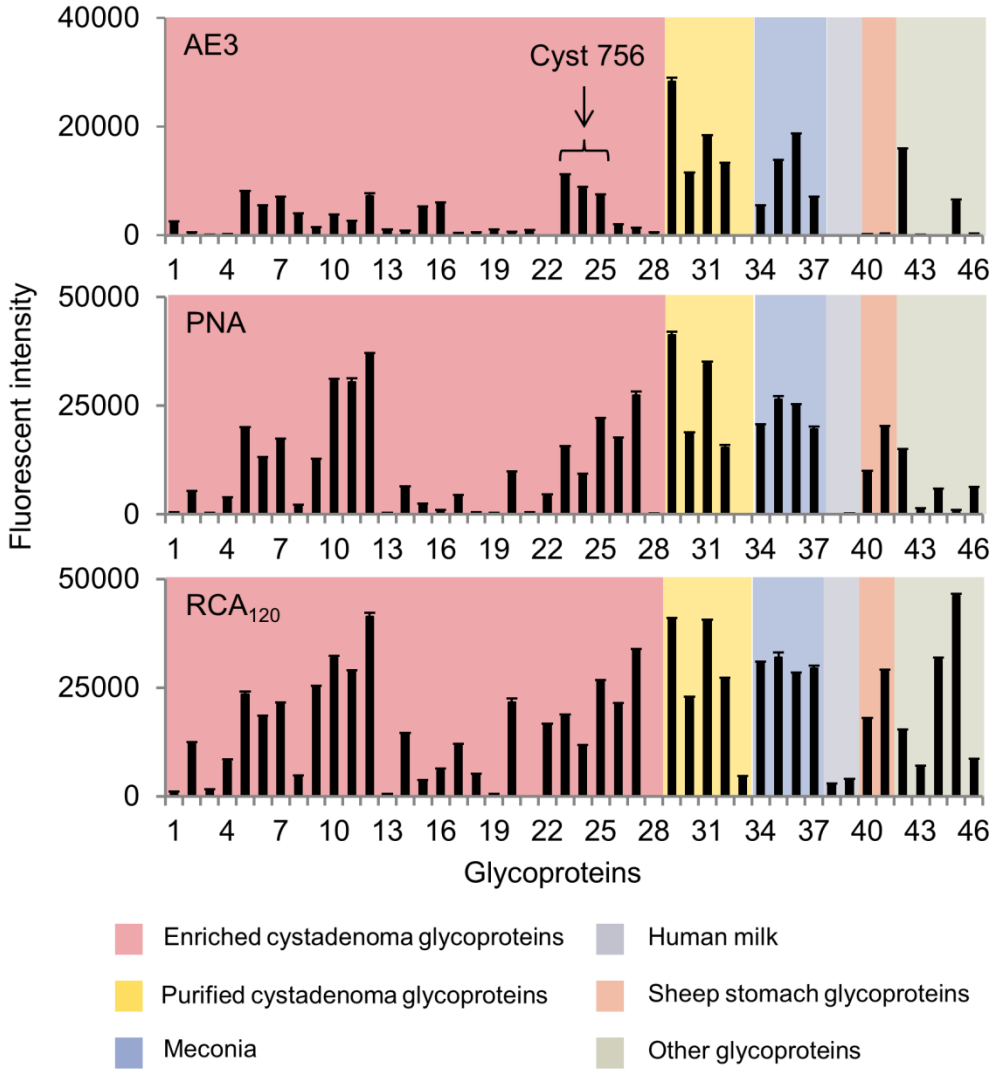
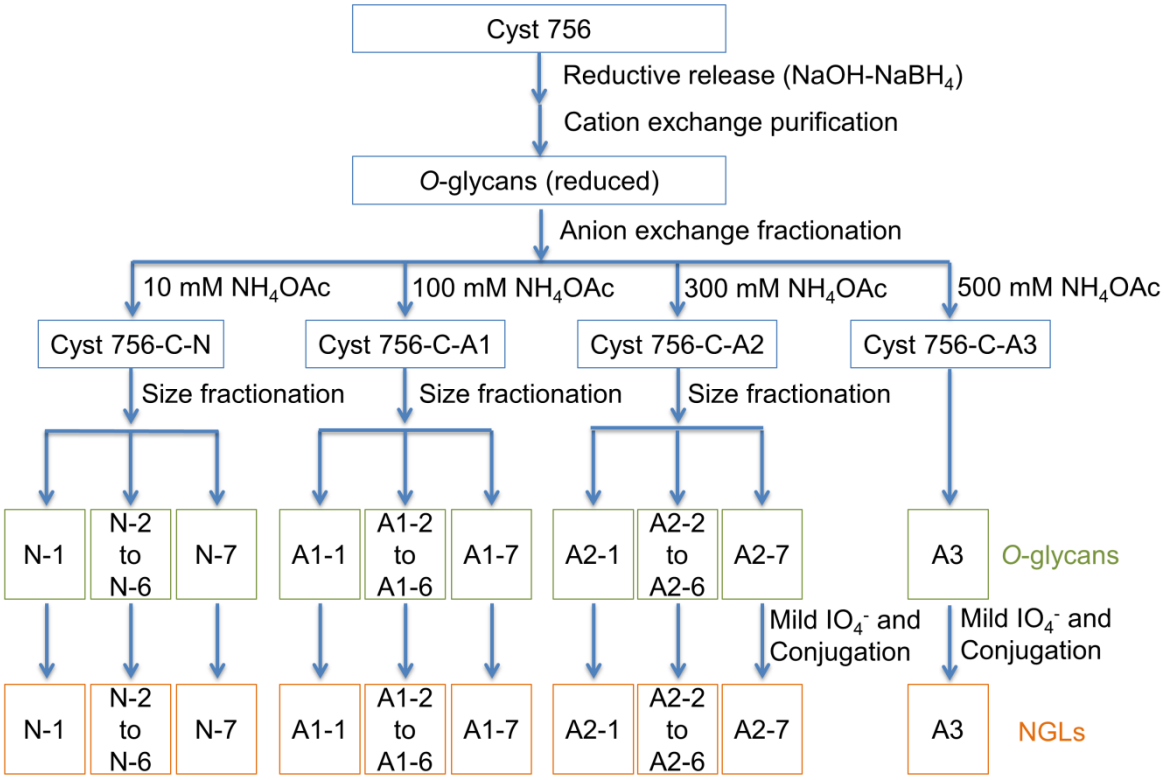


Figure 4.3 Microarray analyses of mAb AE3-containing culture supernatant, PNA and RCA_{120} with mucin-type glycoproteins. The mucin-type epithelial glycoproteins are from enriched and purified ovarian cystadenomas, meconia, human milk, sheep stomach and commercial glycoproteins such as porcine gastric mucin, bovine submaxillary mucin, transferrin and invertase. They are arranged according to their sources as

annotated in the *coloured panels* below the figure. Results are the means of fluorescence intensities of duplicate spots printed at 150 pg glycoprotein per spot. The error bars represent half of the difference between the duplicate values. In brackets are the three preparations of cystadenoma 756 selected for further investigation of the AE3 antigen.

4.4 Antigenic analyses of *O*-glycans reductively released from Cyst 756

Following the success in the F77 study, reductive release was initially attempted. The workflow is summarized in Scheme 4.1. Five hundred milligrams of the crude Cyst 756 lyophilised powder was used to release the *O*-glycans by reductive alkaline borohydride degradation. Total hexose content of the products was 12.4 mg determined by dot-orcinol assay. The products were initially fractionated by charge using DEAE-Sephadex A25 column (Section 2.5.1). Four fractions were successively eluted using 10, 100, 300 and 500 mM NH_4OAc buffer, and they were designated Cyst 756-C-N, Cyst 756-C-A1, Cyst 756-C-A2 and Cyst 756-C-A3, respectively (Scheme 4.1). The four subfractions were analysed by ES-MS and the monosaccharide compositions of each component in these fractions were deduced and listed in Table 4.3. One molecular ion may correspond to multiple possible monosaccharide compositions. Sometimes both with and without sialic acid (SA) or sulphate (SU) is possible. There were in total 72 molecular species based on the *m/z* values observed as singly, doubly and triply charged ions, corresponding to oligosaccharides ranging from disaccharide to hexadecasaccharide. The major components in Cyst 756-C-N were neutral oligosaccharides, ranging from disaccharide to heptasaccharide, whereas in Cyst 756-C-A1, Cyst 756-C-A2 and Cyst 756-C-A3 the major components were acidic and ranged from disaccharide to hexadecasaccharide. The ion corresponding to the predicted AE3 antigen-positive *O*-glycan (Figure 4.1C), SU1.Hex2.HexNAc2.HexNAcol, was not observed, however, suggesting that the sought-after antigen may be a minor component as was the case with F77 antigen in PSM or that it was absent.



Scheme 4.1. Workflow of the reductive *O*-glycan release, fractionation and conjugation to lipid to generate NGLs.

Table 4.3 Compositions of the reductively released *O*-glycans in fractions Cyst 756-C-N (N), Cyst 756-C-A1 (A1), Cyst 756-C-A2 (A2) and Cyst 756-C-A3 (A3) deduced from ES-MS analysis (tick indicates the corresponding ion is found in this fraction).

m/z			Monosaccharide composition	Fractions			
$[M-H]^-$	$[M-2H]^{2-}$	$[M-3H]^{3-}$		N	A1	A2	A3
384.1			Hex1, HexNAcol1	√			
	478.6		SA1, SU1-Hex1, HexNAc1, HexNAcol1				√
	482.6		SA2-Hex1, HexNAcol1			√	√
513.2			SA1-HexNAcol1		√	√	
530.2			dHex1, Hex1, HexNAcol1				√
	553.3		SU1-dHex2, Hex1			√	
	559.6		SA1, SU1-Hex2, HexNAc1, HexNAcol1			√	√
	584.2		SA2-Hex1, HexNAc1, HexNAcol1			√	
587.2			Hex1, HexNAc1, HexNAcol1	√			
623.2			SU3-Hex1, HexNAcol1				√
	632.7		SA1, SU1-dHex1, Hex2, HexNAc1, HexNAcol1				√
		640.2	SA1, SU1-dHex3, Hex3, HexNAc2, HexNAcol1 SU2-Hex7, HexNAc2, HexNAcol1 SA2, SU1-dHex1, Hex3, HexNAc2, HexNAcol1				√
	657.2		SU2-Hex2, HexNAc3, HexNAcol1 SU1-Hex5, HexNAc1, HexNAcol1 SA2-dHex1, Hex1, HexNAc1, HexNAcol1			√	
	665.2		SA2-Hex2, HexNAc1, HexNAcol1			√	
667.2			SU1-Hex1, HexNAc1, HexNAcol1			√	√

675.2			SA1-Hex1, HexNAcol1	√	√	√	√
	702.2		SA1-Hex3, HexNAc2, HexNAcol1	√			
733.2			dHex1, Hex1, HexNAc1, HexNAcol1	√			
	734.2		SA1, SU1-dHex1, Hex2, HexNAc2, HexNAcol1 SA1-dHex1, Hex5, HexNAcol1			√	
	742.2		SA1SU1-Hex3, HexNAc2, HexNAcol1 SA1-Hex6, HexNAcol1 SU1-dHex2, Hex3, HexNAc2, HexNAcol1			√	√
749.3			Hex2, HexNAc1, HexNAcol1	√			
755.2			SA1, SU1-Hex1, HexNAcol1				√
785.2			SU3-Hex2, HexNAcol1				√
793.2			SU3-dHex3, Hex3, HexNAc1, HexNAcol1		√		
812.3			SU1-dHex1, Hex1, HexNAc1, HexNAcol1	√			
	815.2		SU1-dHex3, Hex3, HexNAc2, HexNAcol1 SA1, SU1-dHex1, Hex3, HexNAc2, HexNAcol1 SA1-dHex1, Hex6, HexNAcol1 dHex3, Hex6, HexNAcol1			√	√
829.2			SU1-Hex2, HexNAc1, HexNAcol1			√	√
878.3			SA1-Hex1, HexNAc1, HexNAcol1	√	√	√	√
	887.7		SA1, SU1-dHex2, Hex3, HexNAc2, HexNAcol1				√
	892.2		SU2-dHex1, Hex4, HexNAc3, HexNAcol1 SA2-dHex2, Hex3, HexNAc1, HexNAcol1 SA3-Hex3, HexNAc1, HexNAcol1				√
	898.7		SA2, SU2-dHex1, Hex3, HexNAc1, HexNAcol1				√
895.3			dHex1, Hex2, HexNAc1, HexNAcol1	√			
931.3			SU3-dHex1, Hex2, HexNAcol1				√
	920.8		SA1-dHex3, Hex3, HexNAc2, HexNAcol1 SA2-dHex1, Hex3, HexNAc2, HexNAcol1 SU2-Hex4, HexNAc4, HexNAcol1 SA1, SU1-dHex3, Hex5, HexNAcol1 SU1-Hex7, HexNAc2, HexNAcol1			√	
952.3			Hex2, HexNAc2, HexNAcol1	√			
958.2			SA1, SU1-Hex1, HexNAc1, HexNAcol1				√
		958.0	SA2, SU1-dHex2, Hex5, HexNAc4, HexNAcol1 dHex4, Hex4, HexNAc7, HexNAcol1 SA1-dHex2, Hex4, HexNAc7, HexNAcol1			√	
	960.8		SA1, SU1-dHex3, Hex3, HexNAc2, HexNAcol1 SU2-Hex7, HexNAc2, HexNAcol1 SA2, SU1-dHex1, Hex3, HexNAc2, HexNAcol1				√
966.3			SA1-dHex2, Hex1, HexNAcol1 SA2-Hex1, HexNAcol1			√	√
	971.8		SA2, SU2-dHex2, Hex3, HexNAc1, HexNAcol1 SU1-dHex2, Hex6, HexNAc1, HexNAcol1				√
980.2			SA1, SU2-dHex1, Hex1, HexNAcol1				√
988.3			SU3-Hex2, HexNAc1, HexNAcol1				√
	997.8		SA1, SU1-dHex1, Hex4, HexNAc3, HexNAcol1 SU1-dHex3, Hex4, HexNAc3, HexNAcol1 SA1-dHex1, Hex7, HexNAc1, HexNAcol1			√	

		1008.0	SA1, SU1-dHex5, Hex8, HexNAc2, HexNAcol1 SA1, SU2-dHex5, Hex5, HexNAc4, HexNAcol1 SA1, SU2-Hex7, HexNAc6, HexNAcol1 SU2-dHex2, Hex7, HexNAc6, HexNAcol1 SA2-dHex3, Hex6, HexNAc4, HexNAcol1			√	
1024.3			SA1-dHex1, Hex1, HexNAc1, HexNAcol1	√	√		
1040.4			SA1-Hex2, HexNAc1, HexNAcol1	√	√	√	√
	1070.8		SA1, SU1-dHex2, Hex4, HexNAc3, HexNAcol1 SA1-dHex2, Hex7, HexNAc1, HexNAcol1 SA2, SU1-Hex4, HexNAc3, HexNAcol1 SU2-dHex4, Hex6, HexNAc1, HexNAcol1 SU1-dHex4, Hex4, HexNAc3, HexNAcol1 dHex4, Hex7, HexNAc1, HexNAcol1			√	
	1110.9		SA1, SU1-dHex2, Hex7, HexNAc1, HexNAcol1 SU1-dHex4, Hex7, HexNAc1, HexNAcol1	√			
	1111.3		SU2-dHex4, Hex4, HexNAc3, HexNAcol1 SU1-dHex4, Hex7, HexNAc1, HexNAcol1 SA2-Hex5, HexNAc3, HexNAcol1 SA2-dHex5, Hex3, HexNAc1, HexNAcol1 SA1, SU1-dHex2, Hex7, HexNAc1, HexNAcol1		√	√	
1114.4			Hex3, HexNAc2, HexNAcol1	√			
1120.3			SA1, SU1-Hex2, HexNAc1, HexNAcol1				√
1142.3			SA1, SU2-dHex1, Hex2, HexNAcol1				√
1160.4			SU2-dHex2, Hex3, HexNAcol1 SA1, SU1-Hex1, HexNAc2, HexNAcol1	√			
1178.3			SA1-dHex1, Hex2, HexNAc2, HexNAcol1		√		
		1178.4	SA2, SU2-dHex4, Hex6, HexNAc5, HexNAcol1 SA1, SU2-dHex1, Hex8, HexNAc7, HexNAcol1 SA1, SU2-dHex6, Hex6, HexNAc5, HexNAcol1			√	
1186.4			SA1-dHex1, Hex2, HexNAc1, HexNAcol1	√	√		
1194.4			SU1-Hex3, HexNAc2, HexNAcol1 Hex6, HexNAcol1			√	
	1216.4		SA2, SU1-dHex2, Hex4, HexNAc3, HexNAcol1 SA2-dHex2, Hex7, HexNAc1, HexNAcol1			√	
		1226.7	SA2, SU2-Hex8, HexNAc7, HexNAcol1 SA2, SU2-dHex5, Hex6, HexNAc5, HexNAcol1 SA1, SU2-dHex2, Hex8, HexNAc7, HexNAcol1			√	
1243.4			SA1-Hex2, HexNAc2, HexNAcol1		√		
	1253.4		SA1, SU1-dHex2, Hex5, HexNAc4, HexNAcol1			√	
1260.4			dHex1, Hex3, HexNAc2, HexNAcol1	√			
1296.4			SU3-dHex1, Hex3, HexNAc1, HexNAcol1				√
1306.4			SU2-dHex3, Hex3, HexNAcol1	√			
	1326.4		SA1, SU1-dHex3, Hex5, HexNAc4, HexNAcol1			√	
1340.4			SU1-dHex1, Hex3, HexNAc2, HexNAcol1		√	√	
1405.4			SA1-Hex3, HexNAc2, HexNAcol1	√	√		
	1439.4		SA2, SU1-dHex2, Hex8, HexNAc2, HexNAcol1 SA1, SU2-dHex4, Hex5, HexNAc4, HexNAcol1 SA1, SU1-dHex4, Hex8, HexNAc2, HexNAcol1			√	

	1450.4		SA2, SU2-dHex3, Hex8, HexNAc1, HexNAcol1			√	
1485.4			SA1, SU1-Hex3, HexNAc2, HexNAcol1				√
1551.5			SA1-dHex1, Hex3, HexNAc2, HexNAcol1	√	√		
1587.4			SU3-dHex3, Hex3, HexNAc1, HexNAcol1		√		

Abbreviations: SA, NeuAc; SU, sulphate; dHex, deoxyhexose; Hex, hexose; HexNAc, *N*-acetylhexosamine; HexNAcol, *N*-acetylhexosaminitol.

The fractions Cyst 756-C-N, Cyst 756-C-A1 and Cyst 756-C-A2 were further fractionated by size using Bio-Gel P4 and the elution profiles are in Figure 4.4. Seven pooled subfractions were obtained in each of the fractions. These were N-1 to N-7 in Figure 4.4A, A1-1 to A1-7 in Figure 4.4B and A2-1 to A2-7 in Figure 4.4 C. As the material in Cyst 756-C-A3 was very limited, it was not further fractionated. Twenty-one subfractions of Cyst756-C-N, -A1 and -A2, together with the unfractionated Cyst756-C-A3, were quantified by hexose assay (Table 4.4). The subfractions N-1 to N-7 were the most abundant, with hexose content ranging from 300-2,300 µg. The hexose content was less in subfractions in Cyst 756-C-A1 and -A2 (10 to 340 µg and 7 to 260 µg, respectively) and even less (140 µg) in Cyst756-C-A3.

Table 4.4 Quantitation of hexose in the subfractions N-1 to -7, A1-1 to -7 and A2-1 to -7 obtained from size fractionation and A3.

Fractions	Total Hex (µg)	Fractions	Total Hex (µg)	Fractions	Total Hex (µg)	Fractions	Total Hex (µg)
N-1	2314.6	A1-1	337.8	A2-1	258.5	A3	140.0
N-2	1867.9	A1-2	233.9	A2-2	99.2		
N-3	1835.8	A1-3	212.1	A2-3	75.5		
N-4	1351.3	A1-4	323.4	A2-4	24.9		
N-5	1147.5	A1-5	112.3	A2-5	82.3		
N-6	1280.9	A1-6	34.7	A2-6	35.6		
N-7	300.0	A1-7	10.8	A2-7	7.3		
Total	10098.0	Total	1265.1	Total	583.2	Total	140.0

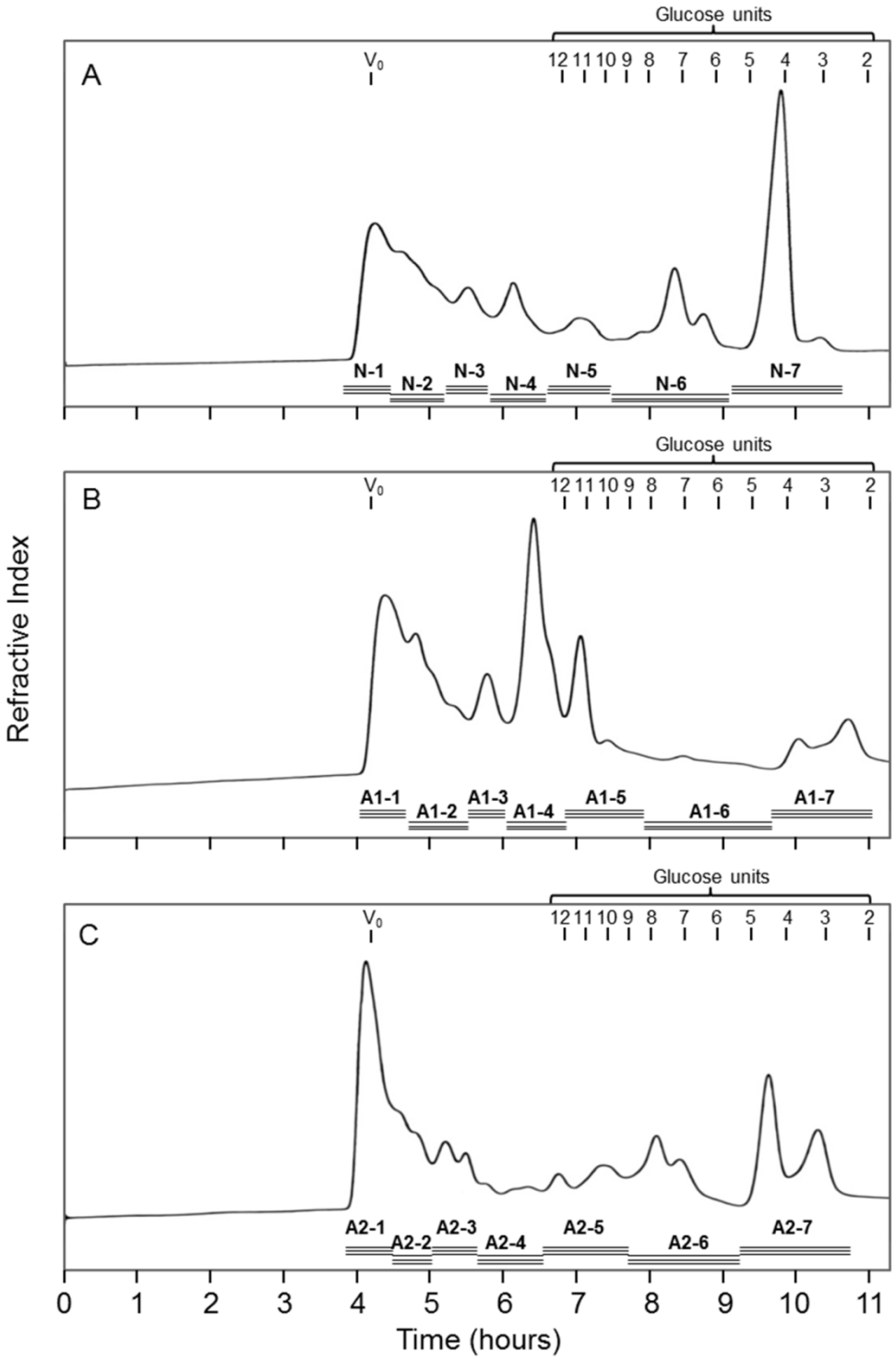


Figure 4.4 Size fractionation of the three subfractions, Cyst756-C-N (A), Cyst756-C-A1 (B) and Cyst756-C-A2 (C). The chromatography was on a Bio-Gel P4 column (1.6×90cm) eluted with 0.05 M NH_4OAc. V_0 is the void volume of the column; numbers 2-12 indicate the positions of elution of oligosaccharides with degrees of polymerization 2-12 in an acid hydrolysate of dextran glucose units. N-1 to N-7, A1-1 to A1-7 and A2-1 to A2-7 designate the pooled fractions in each preparation.

Fluorescent NGLs of these 22 subfractions were prepared using ADHP after mild periodate oxidation. The reaction mixture in each fraction was resolved by HPTLC together with the positive control SM1a (Figure 4.5). Numerous fluorescent bands

were detected in each fraction under UV light. The same plates were then probed for binding with mAb AE3 (Figure 4.5). The SM1a control was clearly bound by mAb AE3 on each of the plates. Immunostained bands were detected in subfractions 5 and 6 of Cyst 756-C-N, subfractions 2 to 5 of Cyst 756-C-A1, subfractions 2 to 5 of Cyst 756-C-A2, and Cyst 756-A3 (Figure 4.5). However the intensities of these immunostained bands seemed to correlate with the amounts of NGLs visualized by fluorescence. This raised the possibility that the immunostained bands were non-specific.

The reaction mixtures of the two fractions Cyst 756-C-A1-3 and Cyst 756-C-A2-2 (squared in Figure 4.5) were then selected for further fractionation on a silica column (Section 2.7) followed by binding analysis (Figure 4.6). The reaction mixture A1-3 yielded four subfractions **a** to **d** (Figure 4.6A); the other, A2-2, yielded five subfractions **a** to **e** (Figure 4.6B). In both cases, subfraction **a** contained mainly excess lipid (not shown) and was not studied further. The remaining subfractions were chromatographed on two HPTLC plates with lactose AD-NGL (Lac.AD) and SM1a in the control lane. The plates were visualized by UV light and probed for binding by mAb AE3 (panel C and D, Figure 4.6). Clearly, numerous fluorescent bands with differing mobilities were observed in subfractions **b** to **d** in A1-3 and **b** to **e** in A2-2. In the control lane, only the fluorescent Lac.AD was visualized by UV. The position of the non-fluorescent glycolipid SM1a was marked. Immunostaining was observed only with SM1a by mAb AE3 on these two plates (panel C and D, Figure 4.6).

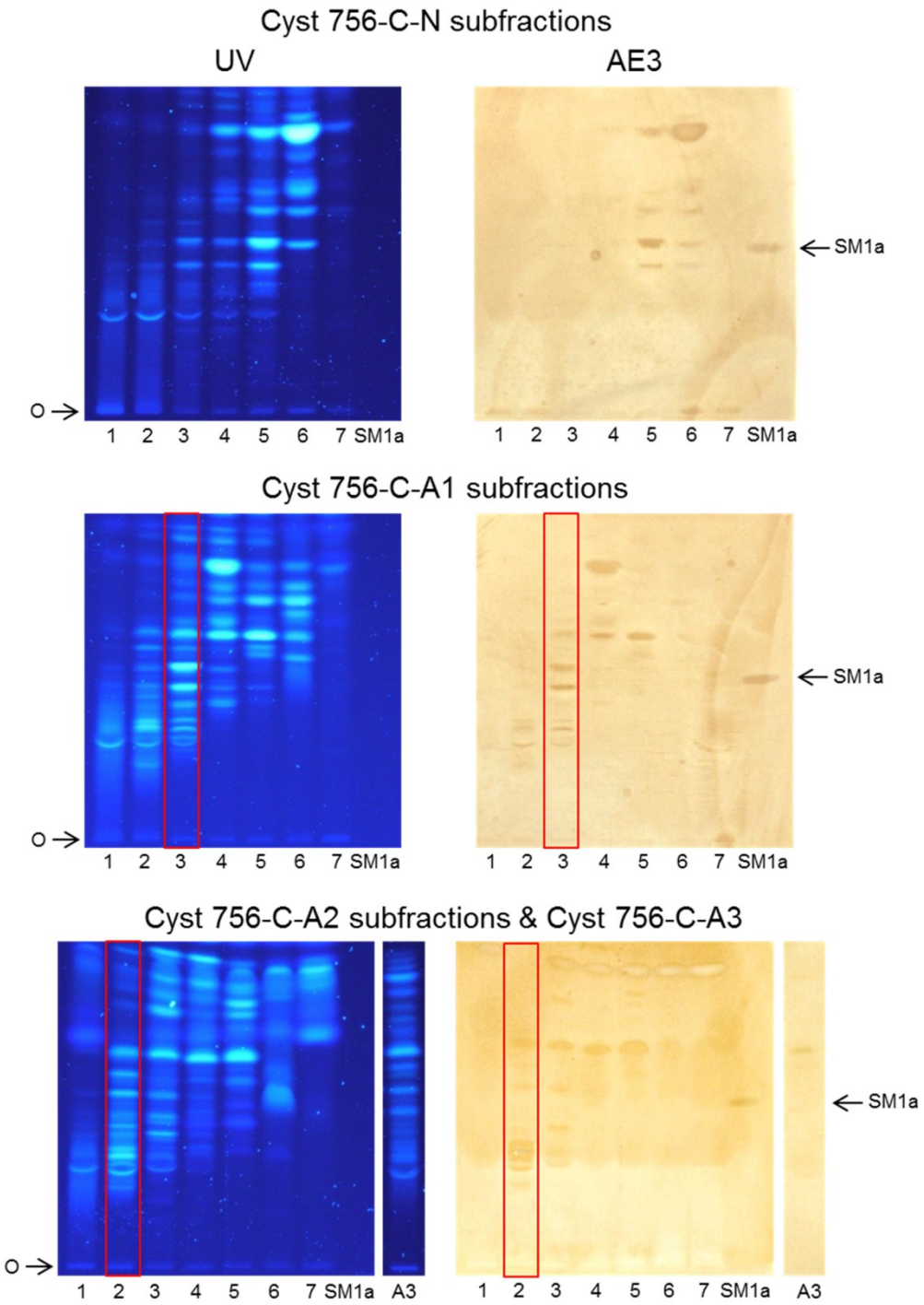


Figure 4.5 Chromatogram binding analysis of mAb AE3 to NGLs in subfractions of Cyst 756-C-N, Cyst 756-C-A1 and Cyst 756-C-A2, and of fraction Cyst 756-C-A3. The NGLs in the fractions were chromatographed together with the glycolipid SM1a on HPTLC plates, using $CHCl_3$/MeOH/H_2O, 60:35:8 (v/v), as solvent. The fluorescent NGLs were visualized under UV light (left panel). The same plate was incubated with mAb AE3 (right panel), followed by biotinylated anti-mouse IgM (μ-chain specific). Binding was visualized by colour development using the streptavidin-peroxidase followed by DAB liquid substrate system. *O* indicates the origin. Arrow indicates the position of SM1a, which was revealed by immunostaining with mAb AE3. Squares highlight the two subfractions Cyst 756-C-A1-3 and Cyst 756-C-A2-2 which were selected for further fractionation.

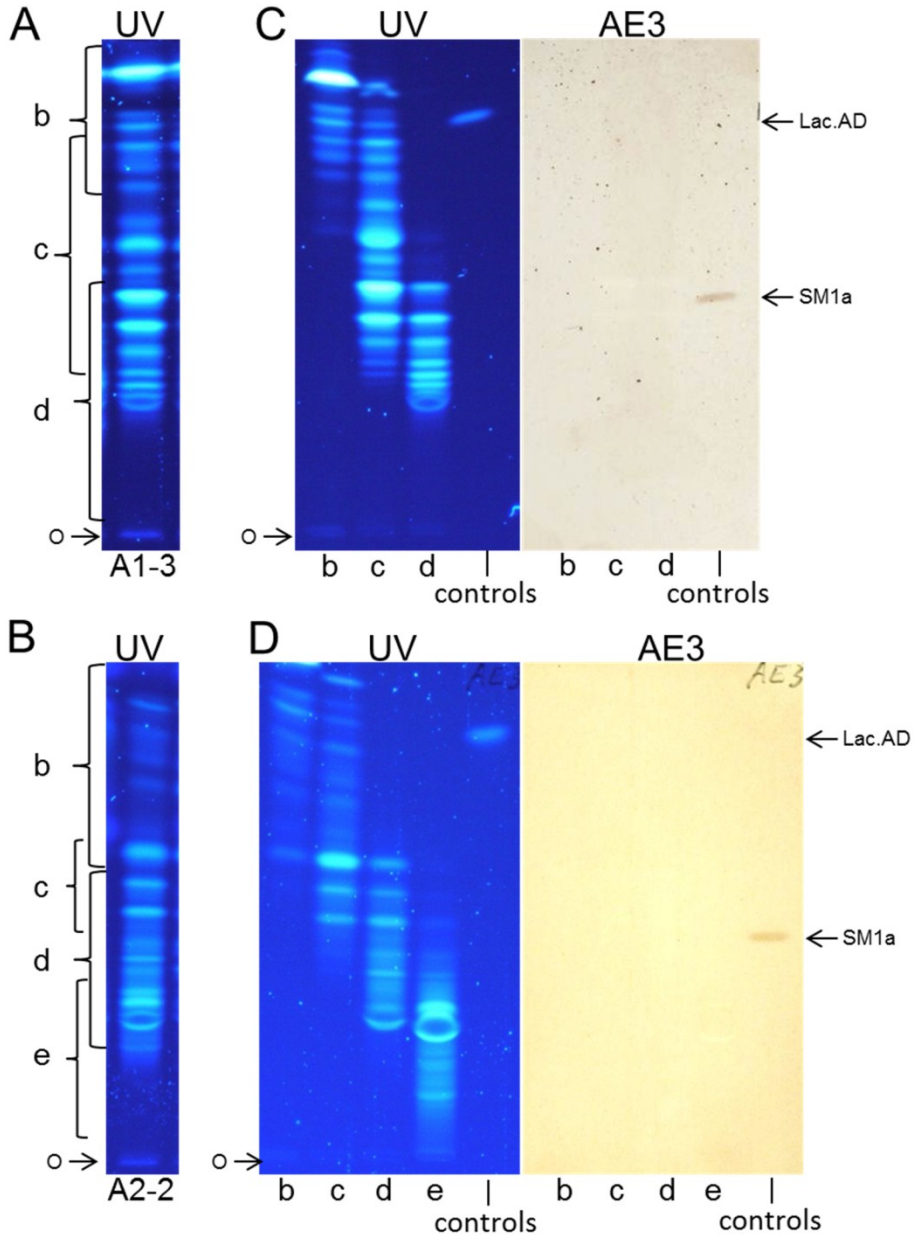


Figure 4.6 TLC analysis and mAb AE3 binding analyses of the NGLs in fractions Cyst 756-C-A1-3 and Cyst 756-C-A2-2. (**A**). TLC analysis of NGLs in Cyst 756-C-A1-3 (A1-3). (**B**). TLC analysis of NGLs in Cyst 756-C-A2-2 (A2-2). b to e indicate subfractions obtained in in the prep-TLC of A1-3 and A2-2. The subfractions a which contained excess lipid in the two preparations was not shown. (**C**). Binding analysis of mAb AE3 using the NGLs in subfractions b to d of A1-3. (**D**). Binding analysis of mAb AE3 using the NGLs in subfractions b to e of A2-2. The NGLs were chromatographed together with the glycolipid SM1a and Lac.AD (Lane controls) on HPTLC plates, using $CHCl_3$/MeOH/H_2O, 60:35:8 (v/v), as solvent. The fluorescent NGLs were visualized under UV light (left panel). The same plate was incubated with mAb AE3 (right panel), followed by biotinylated anti-mouse IgM (μ-chain specific). Binding was visualized as in the legend to Figure 4.5. Arrows indicate the positions of Lac.AD and SM1a. *O* indicates the origin.

The NGLs in subfractions N-1 to N-7, A1-1 to A1-7, and A2-1 to A2-7 as well as fraction A3 (Scheme 4.1) were analysed by MALDI-MS; the results are listed in three tables. Table 4.5 contained MS data of the seven subfractions in Cyst 756-C-N, Table 4.6 for the nine subfractions in Cyst 756-C-A1 and Table 4.7 for the ten subfractions in Cyst 756-C-A2 and the unfractionated Cyst 756-C-A3. In total, thirty-

six molecular species were identified in NGL subfractions of N-1 to N-7, fifty in A1-1 to A1-7 and fifty-one in A2-1 to A2-7. There were fourteen molecular species detected in the unfractionated A3 NGL fraction. Note that only major components are observed in the MALDI-MS spectra, very minor components cannot be seen. The same molecular ion may arise from different NGL components. In the absence of additional data (such as MS/MS), it is difficult to assign unambiguously the compositions of each component and to draw conclusions as to the number of sialylated or sulphated species in each subfraction. Therefore only the possible monosaccharide compositions corresponding to individual ions are listed in the three tables. The major components deduced to be present in N-1 to N-7 ranged from monosaccharide to nonasaccharide and most of these were neutral (Table 4.5). The deduced major components in subfractions A1-1 to A1-7 contained acidic moieties such as sialic acid and sulphate. In addition, there were neutral components as in N-1 to N-7. Among subfractions of Cyst 756-C-A2 components with multiple negatively charged moieties, sulphate and sialic acid were deduced to be present. However, none of the molecular ions deduced corresponded to that of the putative AE3 antigen-positive *O*-glycan (Figure 4.1C), SU1.Hex2.HexNAc2-OX.

Thus from the results of antigenic analysis and MS we concluded that *O*-glycans reductively released from Cyst 756 contained little if any of the proposed AE3 antigen-positive *O*-glycan.

Table 4.5 Compositions of the NGLs in the seven subfractions of Cyst 756-C-N (tick indicates the corresponding ion is found in this fraction).

m/z	Monosaccharide composition	Subfractions of Cyst 756-C-N						
		1	2	3	4	5	6	7
954.3	-OY				√	√		√
1055.3	-OX				√	√	√	√
1157.3	HexNAc1-OY				√	√	√	
1217.3	Hex1-OX	√	√	√	√	√	√	√
1258.4	HexNAc1-OX						√	√
1319.3	Hex1, HexNAc1-OY	√	√	√	√	√	√	√
1363.4	dHex1, Hex1-OX							√
1379.3	Hex2-OX							√
1420.3	Hex1, HexNAc1-OX	√	√	√	√	√	√	√
1465.0	dHex1, Hex1, HexNAc1-OY				√	√	√	
1522.3	Hex1, HexNAc2-OY			√	√	√	√	
1525.4	dHex1, Hex2-OX							√
1538.3	SU2-Hex2-OX					√	√	

1566.4	dHex1, Hex1, HexNAc1-OX				√	√	√	√
1582.3	Hex2, HexNAc1-OX		√	√	√	√	√	√
1640.3	SU2-Hex2, HexNAc1-OY			√	√			
1684.3	Hex2, HexNAc2-OY			√	√	√		√
1728.3	dHex1, Hex2, HexNAc1-OX		√	√	√	√	√	
1785.3	Hex2, HexNAc2-OX				√	√		
1830.3	dHex1, Hex2, HexNAc2-OY		√	√	√	√		
1887.3	Hex2, HexNAc3-OY SU2-dHex1, Hex2, HexNAc1-OX				√			
1932.2	dHex1, Hex2, HexNAc2-OX			√	√	√		
1947.2	Hex3, HexNAc2-OX SU1-HexNAc4-OX				√	√		
1976.2	dHex2, Hex2, HexNAc2-OY SA1, SU2-dHex1, Hex2-OX			√	√			
2033.2	dHex1, Hex2, HexNAc3-OY			√	√			
2049.2	Hex3, HexNAc3-OY			√	√			
2077.3	dHex2, Hex2, HexNAc2-OX			√		√		
2093.4	dHex1, Hex3, HexNAc2-OX			√	√	√		
2151.1	Hex3, HexNAc3-OX			√	√			
2195.2	dHex1, Hex3, HexNAc3-OY			√	√			
2239.2	dHex2, Hex3, HexNAc2-OX			√	√	√		
2297.1	dHex1, Hex3, HexNAc3-OX			√	√			
2341.2	dHex2, Hex3, HexNAc3-OY SA1, SU2-dHex1, Hex3, HexNAc1-OX		√	√	√			
2444.1	dHex2, Hex3, HexNAc3-OX			√				
2458.3	dHex1, Hex4, HexNAc3-OX SA2, SU2-dHex3, Hex2-OY SU1-dHex1, Hex1, HexNAc5-OX				√			
2604.1	SA2, SU2-dHex4, Hex2-OY dHex2, Hex4, HexNAc3-OX SU1-dHex2, Hex1, HexNAc5-OX		√	√	√			

Abbreviations: –OX and –OY are the 3- and 6- linked fragments of core GalNAc after periodate oxidation; -OX: -OCH_2-CH(NHAc)-CH_2OH-CH_2-ADHP; OY: -OCH_2-CH_2-ADHP; the rest abbreviations are in legends to Table 4.3.

Table 4.6 Compositions of the NGLs in the nine subfractions of Cyst 756-C-A1 (tick indicates the corresponding ion is found in this fraction).

m/z	Monosaccharide composition	Subfractions of Cyst 756-C-A1								
		1	2	3b	3c	3d	4	5	6	7
954.3	-OY			√	√	√	√	√	√	
1055.3	-OX			√	√		√	√	√	
1157.3	HexNAc1-OY				√	√	√			
1217.3	Hex1-OX	√	√	√		√	√	√	√	√
1319.3	Hex1, HexNAc1-OY	√	√	√	√	√	√	√	√	
1420.3	Hex1, HexHexNAc1-OX	√	√	√	√		√	√	√	√
1465.0	dHex1, Hex1, HexNAc1-OY				√	√	√	√	√	
1508.3	SA1-Hex1-OX				√			√		
1522.3	Hex1, HexNAc2-OY				√				√	
1564.4	SU2-dHex1, HexNAc1-OX							√		
1566.4	dHex1, Hex1, HexNAc1-OX				√		√		√	
1582.3	Hex2, HexNAc1-OX	√	√	√	√		√		√	

1640.3	SU2-Hex2, HexNAc1-OY				√	√			√	
1654.9	SA1-dHex1, Hex1-OX					√				
1668.3	SU2-dHex2, Hex1-OX				√					
1684.3	Hex2, HexNAc2-OY		√		√		√	√	√	
1698.4	Hex1, HexNAc2-OY				√					
1728.3	dHex1, Hex2, HexNAc1-OX	√	√		√	√	√	√	√	
1749.5	SA1, SU1-Hex2-OX							√		
1785.3	Hex2, HexNAc2-OX				√		√	√		
1830.3	dHex1, Hex2, HexNAc2-OY	√	√		√	√	√			
1844.3	SU2-Hex2, HexNAc2-OY SA2-dHex1, Hex1-OY				√	√				
1854.0	SA1-HexNAc3-OY		√							
1873.0	SA1-Hex2, HexNAc1-OX		√							
1887.3	Hex2, HexNAc3-OY				√		√	√	√	
1932.2	dHex1, Hex2, HexNAc2-OX		√		√	√	√			
1947.3	Hex3, HexNAc2-OX SU1-HexNAc4-OX				√					
1976.4	dHex2, Hex2, HexNAc2-OY SA1, SU2-dHex1, Hex2-OX	√			√	√				
2005.0	SA2, SU2-dHex1, Hex1-OY					√				
2033.2	dHex1, Hex2, HexNAc3-OY				√	√	√			
2049.3	Hex3, HexNAc3-OY				√	√				
2077.3	dHex2, Hex2, HexNAc2-OX				√					
2093.4	dHex1, Hex3, HexNAc2-OX	√	√		√	√				
2142.0	SU1-dHex3, Hex1, HexNAc2-OX		√							
2151.1	Hex3, HexNAc3-OX					√				
2195.2	dHex1, Hex3, HexNAc3-OY	√	√		√	√				
2204.5	SA1, SU2-dHex2, HexNAc2-OX SA1, SU1-dHex2, Hex3-OX			√						
2239.2	dHex2, Hex3, HexNAc2-OX	√	√			√				
2297.1	dHex1, Hex3, HexNAc3-OX					√				
2341.2	dHex2, Hex3, HexNAc3-OY SA1, SU2-dHex1, Hex3, HexNAc1-OX	√	√	√		√				
2376.3	SU1-dHex1, Hex3, HexNAc3-OX			√						
2378.5	dHex1, Hex1, HexNAc5-OX SA1, SU1-Hex4, HexNAc2-OY SA1, SU2-Hex1, HexNAc4-OY			√						
2394.3	SU2-dHex1, Hex7-OY SA2, SU1-dHex2, Hex3-OY SA2, SU2-dHex2, HexNAc2-OY dHex2, HexNAc5-OX			√						
2444.1	dHex2, Hex3, HexNAc3-OX		√			√				
2458.3	dHex1, Hex4, HexNAc3-OX SA2, SU2-dHex3, Hex2-OY SU1-dHex1, Hex1, HexNAc5-OX					√				
2549.2	SU2-dHex4, Hex4, HexNAc1-OY SA2, SU1-Hex2, HexNAc3-OY SA2-Hex5, HexNAc1-OY	√								

2560.1	dHex1, Hex4, HexNAc4-OY		√							
2604.1	dHex2, Hex4, HexNAc3-OX SU1-dHex2, Hex1, HexNAc5-OX	√	√			√				
2706.0	dHex2, Hex4, HexNAc4-OY		√							
2809.0	SA1, SU2-dHex1, Hex4, HexNAc3-OY SU2-dHex3, Hex4, HexNAc3-OY SA2-dHex4, Hex3, HexNAc1-OY		√							

Abbreviations are in the legends to Table 4.5.

Table 4.7 Compositions of the NGLs in the ten subfractions of Cyst 756-C-A2 and the unfractionated A3 (tick indicates the corresponding ion is found in this fraction).

m/z	Monosaccharide composition	Subfractions of Cyst 756-C-A2										A3
		1	2b	2c	2d	2e	3	4	5	6	7	
954.3	-OY			√					√			√
1055.3	-OX			√					√			√
1157.3	HexNAc1-OY		√	√	√							√
1217.3	Hex1-OX	√	√	√	√		√	√	√			√
1319.3	Hex1, HexNAc1-OY		√	√	√	√	√	√	√			√
1409.0	dHex2, Hex1-OY						√					
1420.3	Hex1, HexNAc1-OX		√	√	√		√	√	√	√	√	√
1464.9	dHex1, Hex1, HexNAc1-OY			√	√	√	√	√	√			√
1504.4	SU1-dHex1, Hex2-OY							√				
1508.3	SA1-Hex1-OX			√								
1522.3	Hex1, HexNAc2-OY							√	√			
1564.4	SU2-dHex1, HexNAc1-OX						√	√				
1566.4	dHex1, Hex1, HexNAc1-OX								√			√
1582.3	Hex2, HexNAc1-OX		√	√	√	√	√	√	√			√
1625.2	SU2-dHex1, Hex1, HexNAc1-OY											√
1640.3	SU2-Hex2, HexNAc1-OY			√	√			√	√			√
1654.9	SA1-dHex1, Hex1-OX dHex3, Hex1-OX			√	√	√						
1668.3	dHex1, Hex1, HexNAc2-OY			√	√							
1684.3	Hex2, HexNAc2-OY			√	√	√	√	√				√
1694.4	SA1-dHex1, HexNAc1-OX							√				
1705.9	SU3-dHex1, Hex1, HexNAc1-OY					√						
1721.9	Su3-Hex2, HexNAc1-OY					√						
1728.3	dHex1, Hex2, HexNAc1-OX	√		√	√		√	√				√
1749.5	SA1, SU1-Hex2-OX						√					
1785.3	Hex2, HexNAc2-OX				√							
1794.4	SA1, SU1-dHex1, Hex2-OY							√				
1800.9	dHex2, Hex1, HexNAc1-OX				√	√						
1830.3	dHex1, Hex2, HexNAc2-OY			√	√	√	√					√
1844.3	SU2-Hex2, HexNAc2-OY SA2-dHex1, Hex1-OY				√							
1851.9	SA1, SU1-Hex2, HexNAc1-OY					√						
1887.2	Hex2, HexNAc3-OY SU2-dHex1, Hex2, HexNAc1-OX				√							

1932.2	dHex1, Hex2, HexNAc2-OX SA1, SU2-Hex2, HexNAc1-OY			√	√	√	√					
1947.2	Hex3, HexNAc2-OX SU1-HexNAc4-OX				√							
1977.2	dHex2, Hex2, HexNAc2-OY SA1, SU1-dHex1, HexNAc2-OX SA1-dHex1, Hex3-OX				√							
1995.1	SU1-dHex2, Hex1, HexNAc2-OX dHex2, Hex4-OX						√					
2005.0	SA2, SU2-dHex1, Hex1-OY						√					
2033.2	dHex1, Hex2, HexNAc3-OY				√							
2050.2	Hex3, HexNAc3-OY SU2-dHex1, Hex3, HexNAc1-OX				√	√						
2094.2	dHex1, Hex3, HexNAc2-OX SA1, SU2-Hex3, HexNAc1-OY				√	√	√					
2151.1	Hex3, HexNAc3-OX SA3-Hex2-OY				√		√					
2195.2	dHex1, Hex3, HexNAc3-OY SU1-dHex1, HexNAc5-OY SA1, SU2-Hex3, HexNAc1-OX				√	√						
2275.1	SU1-dHex1, Hex3, HexNAc3-OY					√						
2297.1	dHex1, Hex3, HexNAc3-OX				√	√						
2342.2	dHex2, Hex3, HexNAc3-OY SU2-dHex3, Hex3, HexNAc1-OX SA1, SU2-dHex1, Hex3, HexNAc1-OX				√	√						
2376.3	SU1-dHex1, Hex3, HexNAc3-OX		√									
2394.3	SU2-dHex1, Hex7-OY SA2, SU1-dHex2, Hex3-OY SA2, SU2-dHex2, HexNAc2-OY dHex2, HexNAc5-OX		√									
2421.1	SU1-dHex2, Hex3, HexNAc3-OY					√						
2443.1	SA1, SU2-dHex1, Hex3, HexNAc2-OY dHex2, Hex3, HexNAc3-OX				√	√						
2560.1	dHex1, Hex4, HexNAc4-OY SA1-dHex4,Hex2, HexNAc2-OY SA1, SU3-Hex1, HexNAc4-OX SA1, SU2-Hex4, HexNAc2-OX					√						
2706.0	dHex2, Hex4, HexNAc4-OY					√						
2787.0	SA1-dHex2, HexNAc6-OY SA2-dHex3, HexNAc4-OY dHex2, Hex7, HexNAc2-OY SU1-dHex2, Hex4, HexNAc4-OY SU3-dHex3, Hex4, HexNAc2-OX SA1, SU3-dHex1, Hex4, HexNAc2-OX					√						

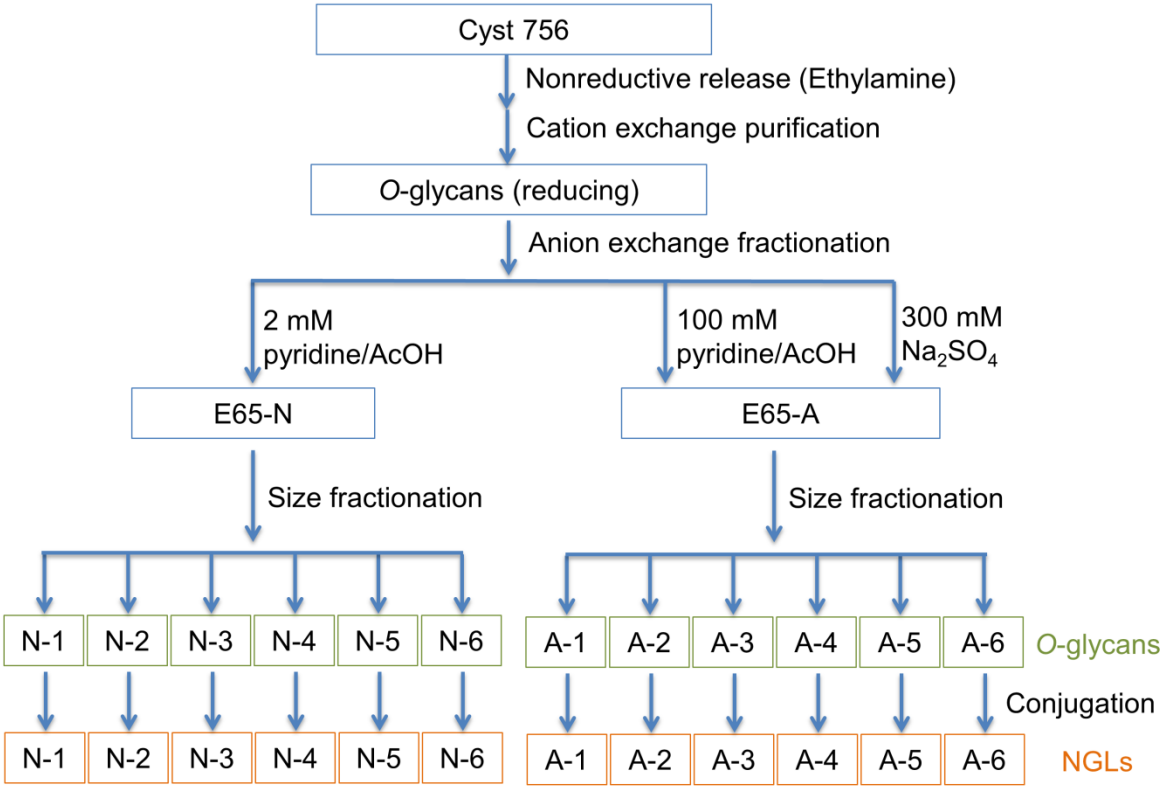
Abbreviations are in the legends to Table 4.5.

4.5 Antigenic analyses of *O*-glycans non-reductively released from Cyst 756

As discussed in Section 3.4.3, reductively released *O*-glycans need to be oxidized by periodate before conjugation to obtain reactive aldehydes at their reducing ends. The periodate oxidation causes cleavage of the core GalNAcol. However, as the core GalNAc may be a part of the AE3 antigenic determinant on the possible *O*-glycan structure (Figure 4.1C), non-reductive release of *O*-glycans was carried out. The resulting reducing oligosaccharides would be conjugated without periodate oxidation.

Cyst 756 was subjected to preparative-scale (500 mg) non-reductive degradation using ethylamine at 22 °C or 65 °C as described in Section 2.4. This procedure lends itself better to large-scale degradation in a biochemical laboratory, rather than the use of hydrazine which is highly flammable and toxic and unsuitable for large scale use in a basic biosafety level 2 (risk group 2) laboratory environment. The two ethylamine conditions were referred to as E22 or E65. Hexose content of the products, as determined by dot-orcinol assay, was 3.2 for E22 and 6.6 mg for E65. It is well known that the yield of non-reductive was less than reductive release. The products from E22 were not investigated further.

The workflow of non-reductive release under condition of E65 is summarized in Scheme 4.2. The products of E65 were fractionated by anion exchange chromatography using AG1-X8 as described in Section 2.5.1. Three fractions were successively eluted using 2 mM and 100 mM pyridine/AcOH (pH 5.0), and 300 mM Na_2SO_4. The first fraction was designated E65-N and the latter two were combined and designated E65-A (Scheme 4.2). Size fractionation of the E65-N and the E65-A fractions was carried out using a Bio-Gel-P4 (Figure 4.7). Twelve subfractions were obtained in the E65-N and the E65-A fractions (N-1 to N-6, and A-1 to A-6) based on the individual elution profile. These twelve subfractions were quantified by hexose assay (Table 4.8). Hexose content of the subfractions of N-1 to N-6 and A-1 to A-6 ranged from 380-2,000 µg and 100-520 µg, respectively.



Scheme 4.2 Workflow of the non-reductive *O*-glycan release, fractionation and conjugation.

Table 4.8 Quantitation of hexose in the subfractions N-1 to -6, and A-1 to -6 obtained from size fractionation.

Fractions	Total Hex (µg)	Fractions	Total Hex (µg)
N-1	2020.0	**A-1**	538.8
N-2	1159.2	**A-2**	264.0
N-3	528.4	**A-3**	146.4
N-4	372.4	**A-4**	184.8
N-5	591.8	**A-5**	376.8
N-6	386.2	**A-6**	105.6
Total	5058.0	**Total**	1616.4

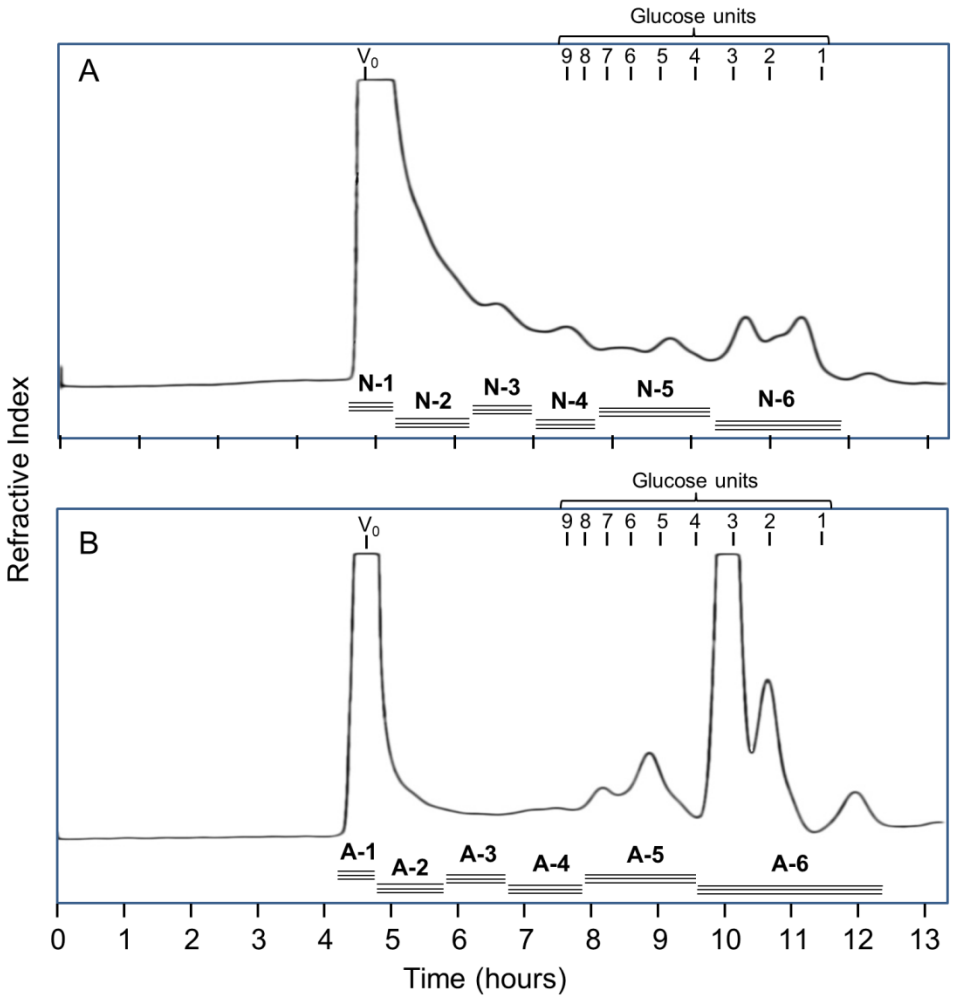


Figure 4.7 Size fractionation of the two subfractions, E65-N (A) and E65-A (B). The chromatography was on a Bio-Gel P4 column (1.6×90cm) eluted with 0.05 M NH_4OAc. V_0 is the void volume of the column; numbers 1 to 9 indicate the positions of elution of oligosaccharides with degrees of polymerization 1 to 9 in an acid hydrolysate of dextran glucose units. N-1 to N-6, and A-1 to A1-6 designate the pooled fractions in each preparation.

Fluorescent NGLs of these 12 subfractions were prepared using ADHP. The reaction mixture in each fraction was resolved by HPTLC together with the positive and negative controls, SM1a and Lactose-AD (Figure 4.8). Numerous fluorescent bands were detected in the NGL fractions N-1 to N-6 and A-1 to A-6 by UV light. The mobilities of the NGLs generated gradually increased from N-1 to N-6 and from A-1 to A-6. This was in accord with the decreased size of the *O*-glycans in the corresponding fractions. Faint or no fluorescent bands were detected in N-1 and A-1, suggesting the limited amounts of NGLs in these two fractions. When the same plates were probed for binding with mAb AE3, only the positive control SM1a glycolipid gave immunostaining. No binding was detected to the NGLs in the twelve subfractions of E65-N and E65-A (Figure 4.8).

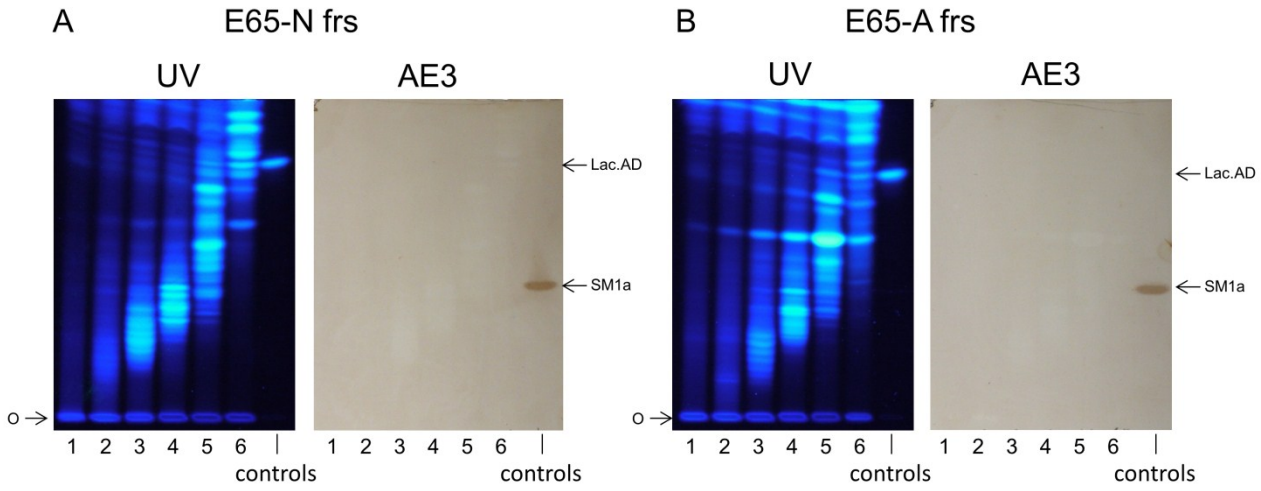


Figure 4.8 Chromatogram binding analysis with mAb AE3 of NGLs in subfractions of Cyst 756-E65-N (A), and Cyst 756-E65-A (B). The NGLs in the fractions were chromatographed together with the glycolipid SM1a and Lac-AD (lane controls) on HPTLC plates, using $CHCl_3$/MeOH/H_2O, 60:35:8 (v/v), as solvent. The fluorescent NGLs were visualized under UV light (left panel in **A** and **B**). The same plate was incubated with mAb AE3 (right panel), followed by biotinylated anti-mouse IgM (μ-chain specific). Binding was detected as in the legend to Figure 4.5. *O* indicates the origin.

Thus, although innumerable fluorescent NGLs were obtained from *O*-glycans non-reductively released from Cyst 756, no AE3 antigen-positive bands were detected among them. This approach was not pursued further. Rather, we plan to chemoenzymatically synthesize the proposed core-3-based *O*-glycan sequence (below) and examine its AE3 antigen activity. This will be discussed in Section 4.7.

Galβ-3GalNAcβ-4Galβ-4GlcNAcβ-3GalNAc
|
SU-3

4.6 AE3 binding to a T antigen-containing glyco-amino acid

I next revisited the T disaccharide sequence, Galβ-3GalNAcα-, as a recognition motif for mAb AE3 using a novel approach. The starting material was a commercially available glyco-amino acid Galβ-3GalNAcα-O-Ser, in which the core GalNAc is intact and is linked to the hydroxyl group of the Ser in α-configuration as in mucin-type glycoproteins.

The glyco-amino acid was converted into an NGL by using an aldehyde-functionalized DHPE developed by Dr Wengang Chai (to be described elsewhere). This lipid designated DHPA can be conjugated to the amine-terminating Ser by a peptide-type bond. In this NGL (Galβ-3GalNAcα-O-Ser-DHPA), designated T-Ser-DHPA (Figure 4.9A), the ring-closed α form of the GalNAc is preserved as the conjugation reaction involves the amino group of the amino acid moiety. The AE3 antigenicity of the T-Ser-DHPA was compared with that of two conventional NGLs. The first is T-DHPE (Galβ-3GalNAc-DHPE) designated T-DH generated by reductive amination and the second is T-AOPE (Galβ-3GalNAc-AOPE) designated T-AO generated by oxime ligation. In T-DH, the core GalNAc is in ring-open form. In T-AO, a proportion of GalNAc is in ring-closed form, but in β-configuration (310) (Figure 4.9A).

These three NGLs, together with the SM1a as the positive control and Lac.AD as a negative control were chromatographed on a HPTLC plate. The plate was visualized by primulin staining (Figure 4.9B). Clearly the glyco-amino acid derivative T-Ser-DHPA had different mobility from the two glycan derivatives, T-DH and T-AO. Two replicate plates were probed for binding by mAb AE3 and the plant lectin PNA (Figure 4.9C and D). The T-Ser-DHPA and SM1a were bound by mAb AE3. The glycan NGL derivatives, T-DH and T-AO, and the negative control Lac.AD gave no detectable binding. This binding pattern was very similar to that of PNA.

These results show that the T disaccharide-containing glyco-amino acid with intact GalNAcα- core is recognized by mAb AE3, whereas the ring-opening and the β-

configuration of the core GalNAc in T-DH and T-AO, respectively abolish binding by mAb AE3.

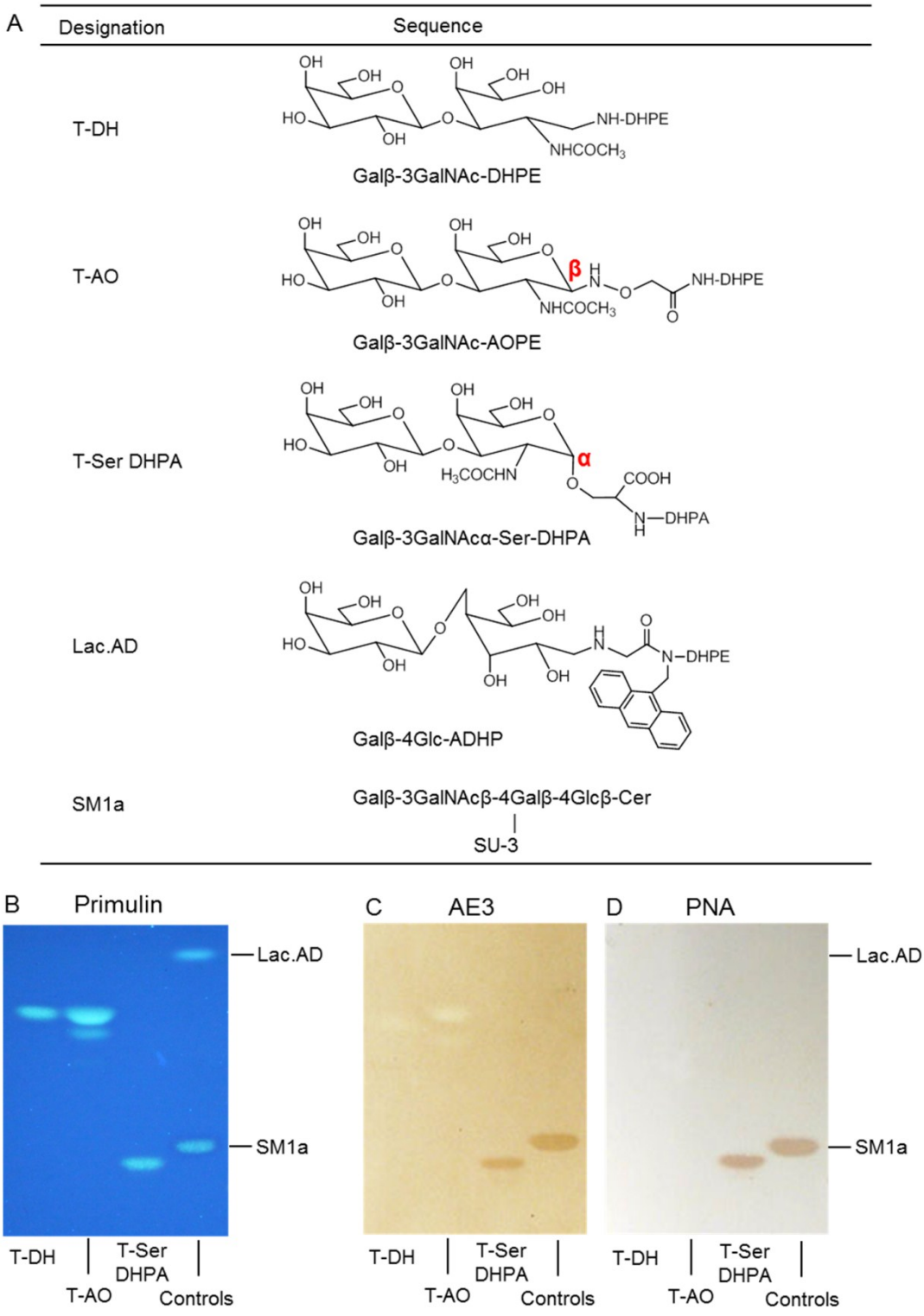


Figure 4.9 Chromatogram binding analysis of mAb AE3 and PNA with two T disaccharide-related NGLs. (**A**) Sequences of the T glycan-derived NGLs T-DH and T-AO, a glyco-amino acid derived T-Ser-DHPA and controls Lac.AD and SM1a. The anomeric configurations of T-AO and T-Ser-DHPA were shown in red. (**B**) HPTLC analyses of the NGLs and glycolipid visualized by primulin lipid staining. (**C** and **D**) Binding analysis of mAb AE3 and PNA on replicate HPTLC plates to A. The plates were developed by $CHCl_3$/MeOH/H_2O, 60:35:8

(v/v) as solvent. Then the plates were incubated with mAb AE3 followed by biotinylated anti-mIgM or biotinylated PNA directly. Detection of binding was in the legend to Figure 4.5.

4.7 Discussion

Early work on mAb AE3 in the 1990s had indeed suggested that the T disaccharide (Galβ-3GalNAc) is involved in mAb AE3 binding but it was concluded that it might be only a part of a larger antigenic determinant (393). Our new results show that the core GalNAc must be intact and in α-configuration for mAb AE3 recognition. In the initial large screening microarray analysis (Section 4.1), the T-disaccharide was represented, but as DH- and AO-NGLs. These two probes are in ring-open and ring-closed β-configuration, respectively. Thus our new results account for the lack of mAb AE3 binding to these.

In the course of investigating the binding specificity of mAb AE3 in relation to the *O*-glycan core region, we collaborated with other labs using recombinant or synthetic glycoproteins carrying different glycoforms including T disaccharide. As discussed below, their results corroborate our new findings.

Professor Joy Burchell at King's College London has shown that mAb AE3 could bind to the T disaccharide α-linked to polyacrylamide [T-PAA, (396)], and to recombinant MUC1 expressing T-disaccharide (designated MUC1-T) (Figure 4.10, Burchell and Taylor-Papadimitriou unpublished). A glycosylation variant, MUC1-Tn with only GalNAc-linked MUC1, was not bound, nor were MUC1-sialylated T (MUC1-ST) with Neu5Acα-3Galβ-3GalNAc or the non-glycosylated MUC1. The Laboratory of Professor Ola Blixt in University of Copenhagen performed microarray analysis of mAb AE3 using a series of synthetic MUC5Ac-based glycopeptides, including non-glycosylated and various glycosylation variants of the glycopeptide, Tn-, T-, STn-(Neu5Acα-6GalNAc-), 3ST- (Neu5Acα-3Galβ-3GalNAc-), 6ST- [Galβ-3(Neu5Acα-6)GalNAc-] and diST- [Neu5Acα-3Galβ-3(Neu5Acα-6)GalNAc-]. The binding of mAb AE3 was restricted to the MUC5Ac-T (Figure 4.11, Blixt unpublished).

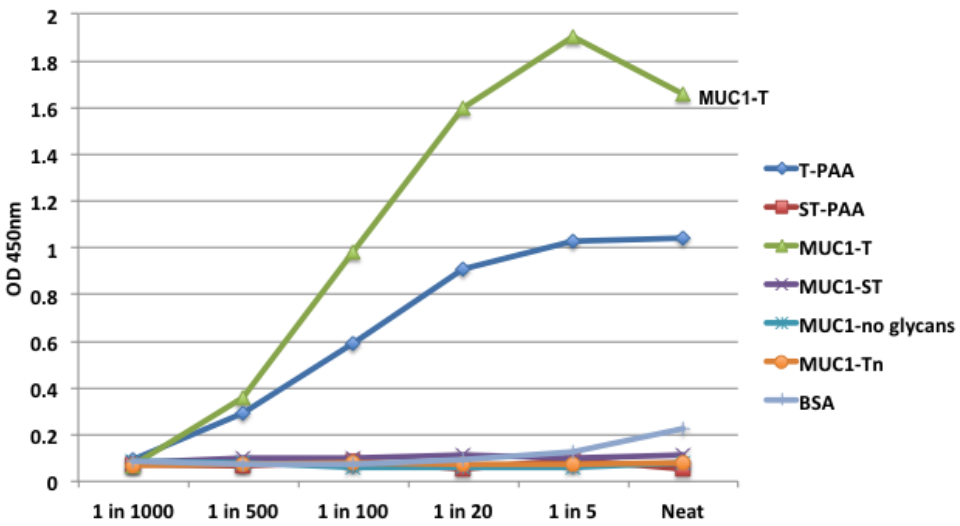


Figure 4.10 ELISA binding assay of mAb AE3 to different glycoforms (T-, ST-, Tn) of MUC1, naked MUC1 and polyacrylamide (PAA) conjugated T and ST. Results obtained from Joy Burchell (unpublished).

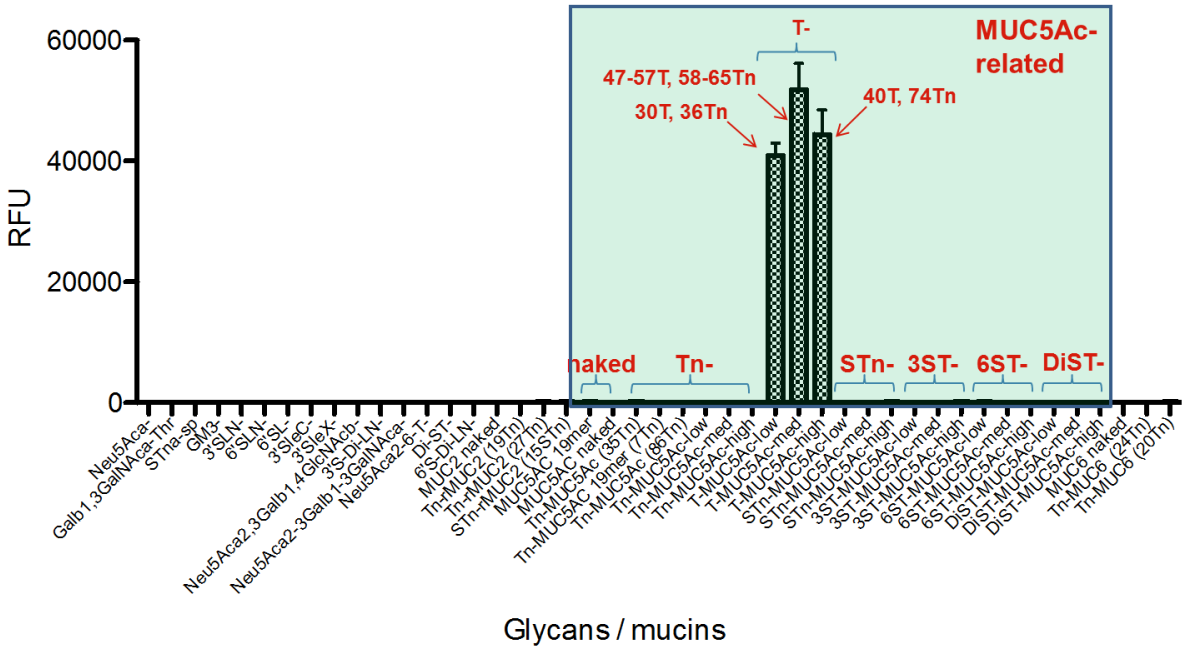


Figure 4.11 Microarray analysis of mAb AE3 with 15 glycans and 30 mucin-type glycoproteins. The glycoproteins are based on MUC2, MUC5 or MUC6, with different combinations of glycoforms. Results obtained by curtsey of Ola Blixt (unpublished).

The naturally occurring glyco-amino acids, as part of *O*-glycopeptides, all have α-linkage between the core GalNAc and the polypeptide backbone. The T-PAA also has an α-linkage in the core region. In order to confirm that the configuration of the core GalNAc is important for mAb AE3 binding, Blixt lab performed microarray analysis using more than 310 sequence-defined synthetic glycan probes which encompassed a variety of mammalian type sequences, representative of complex-

type *N*-glycans, core and peripheral regions of *O*-glycans, blood group antigen-related sequences and gangliosides. The α- and the β- isomers of T disaccharide were printed on the microarray with a propyl spacer (designated Sp8, which is -CH_2-CH_2-CH_2-NH-). The α-isomer, Galβ-3GalNAcα-O-Sp8, was the only probe that gave positive binding with mAb AE3. There was no detectable binding to the β-analogue (Figure 4.12, Blixt unpublished). This result corroborates our finding that the anomeric configuration of the core GalNAc is important in the recognition by mAb AE3.

Taken together, the results above and the loss of AE3 antigen after treatment of cells with endo-α-*N*-acetyl-D-galactosaminidase observed earlier (383) show that the α-linked T disaccharide Galβ-3GalNAcα-O- is indeed a moiety recognized by mAb AE3. Modification to either of the two monosaccharide residues including sialylation of the galactose, ring-opening, or splitting of the core GaNAc obviates the mAb AE3 recognition.

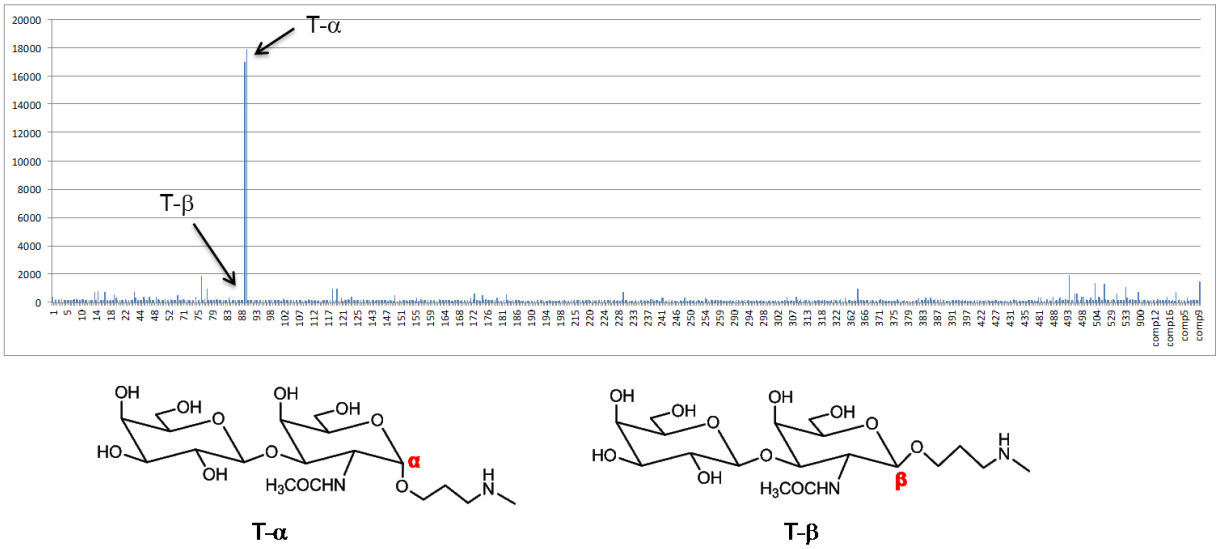
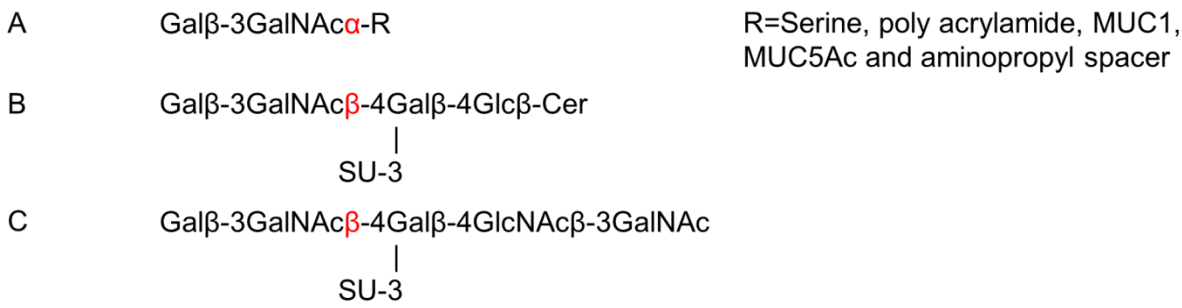


Figure 4.12 Microarray analysis of mAb AE3 with more than 310 sequence-defined glycans. These glycans were covalently immobilized on solid membranes. Two sequences below the chart are the AE3 antigen-positive probe T-α and a close comparison, T-β, which lacks AE3 binding activity. The microarray contains a variety of mammalian type glycan sequences, representative of complex-type *N*-glycans, core and peripheral regions of *O*-glycans, blood group antigen-related sequences and gangliosides. Results obtained by curtsey of Ola Blixt (unpublished).

The results above do, however, need to be reconciled with our previous data that show both the natural and synthetic forms of the sulphated glycolipid SM1a to be bound by mAb AE3. Therefore, there are two structurally and biosynthetically distinct

carbohydrate sequences bound by mAb AE3 (Structures A and B in Scheme 4.3). One is the T-disaccharide with GalNAc in α-configuration (Structure A in Scheme 4.3) and the other is a sulpho-oligosaccharide sequence with GalNAc in β-configutaion (Structure B in Scheme 4.3). This was the impetus for me to attempt to isolate an *O*-glycan (Scheme 4.3C) containing such a sequence. I performed microarray analyses with mucin type glycoproteins, identified an abundantly available AE3 antigen-positive glycoprotein preparation, Cyst 756, and carried out antigenic analyses of the reductively and non-reductively released *O*-glycans therefrom. We reasoned that although the conjugation of both the reduced and the reducing *O*-glycans to ADHP by reductive amination would sacrifice the core GalNAc, the remaining backbone sequences would be available for binding with mAb AE3. However, neither immunoreactivity nor MS of the NGLs were suggestive of these sequences. These findings suggest that such *O*-glycans, if present, must be extremely minor components on the mucin preparation investigated, which in fact was not among the most antigenically active substances in the glycoprotein array.



Scheme 4.3 Two glycan sequences (A and B) recognized by mAb AE3 and the proposed *O*-glycan sequence recognized by AE3 (C).

Evidence for the existence of two kinds of AE3 active antigens has been obtained from immunohistochemical analyses in Joy Burchell Laboratory (Figure 4.13, Burchell and Taylor-Papadimitriou unpublished) using two MUC1-reactive antibodies in addition to mAb AE3. HMFG2, an antibody reacting with a wide range of MUC1 glycoforms, showed strong and homogeneous binding to the membrane. The mAb AE3 showed not only strong immunoreactivity in areas bound by HMFG2, but also to cytoplasmic components. Another antibody, 1B9, which is known to bind to T-MUC1, gave more heterogeneous membrane-associated staining. The results suggest that

mAb AE3 binds to two types of components in breast cancer tissue; these may be T antigen-carrying MUC1 and glycolipids. This will require investigation.

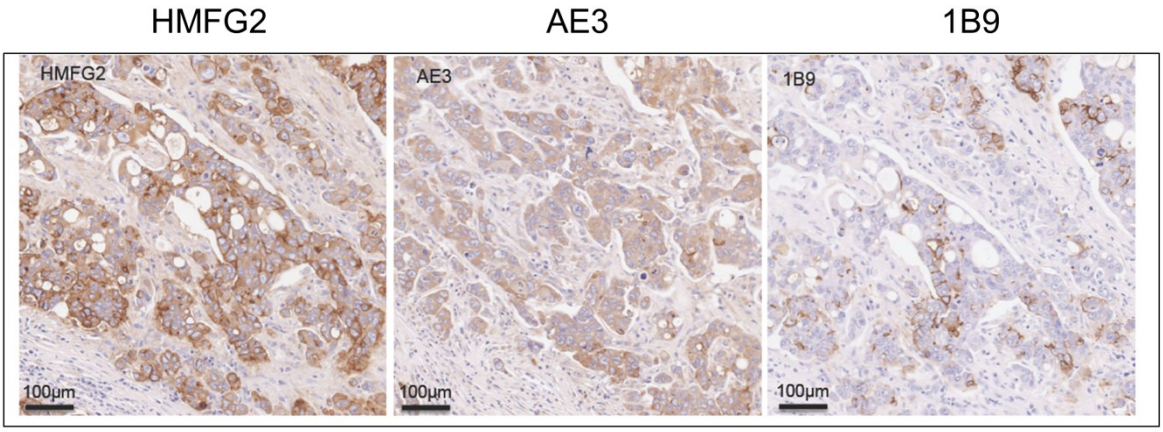


Figure 4.13 Immunostaining of mAb AE3 and two other T-antigen targeting antibodies HMFG2 and 1B9 to primary breast cancers. Formalin fixed paraffin embedded sections of primary breast cancers were used. Results obtained by curtsey of Joy Burchell (unpublished).

Two avenues are being considered to prove the existence of the two types of AE3 antigen. One is to extract these AE3 antigen-positive components from breast tumour tissues and determine their glycan sequences. The other is to synthesize the proposed core-3-based *O*-glycan sequence (Scheme 4.3C) by a chemoenzymatic approach and examine its AE3 antigen activity. Assignment of the sequences of the two AE3 antigens would help to reveal the key glycosyltransferases required for their synthesis and will lead to genetic studies of the corresponding genes that are altered in epithelial oncogenesis.

Another study under current investigation in our collaborator Professor Mark Greene's Lab in University of Pennsylvania is the crystallographic studies of mAb AE3 complexed with the two different glycan sequences. It is hoped that a comparison of the complexes generated, will elucidate the structural basis of the apparent dual specificity of mAb AE3.

Chapter 5
DISCUSSION

Two cancer-associated carbohydrate antigens F77 and AE3 were studied in this thesis. Their higher levels of expression in cancer cells and tissues than in normal raise the possibility of their involvement in cancer biology through interaction with endogenous binding receptors. The detailed understanding of the two antigens, the key glycosyltransferases and the two antibodies also gives rise to opportunities for exploring clinical applications, as cancer biomarkers and therapeutic agents. This chapter is concerned with these aspects.

5.1 Possible biological relevance of antigens of F77 and AE3

In the pages that follow, I discuss the possible involvement of the two antigens, F77 and AE3 and their corresponding glycosyltransferases in the pathobiology of cancer cells.

5.1.1 F77 antigen and related carbohydrate sequences

The F77 antigen encompasses the type 2 blood group H sequence β1,6-linked to a poly-LacNAc backbone. As reviewed in Chapter 3, there is elevated expression of branching *N*-acetylglycosaminyltransferases, GCNTs, and persistent expression of both type 2 blood group H antigen and corresponding α1-2 fucosyltransferase FUT1 in prostatic cancer tissues and cell lines compared to normal prostate tissues and cells. In a recent study, using the cancer progression model of low (androgen-dependent) and high passage (androgen-independent) LNCaP cells, Bennun *et al* showed that the major glycosylation difference between the two states was the increased expression of type 2 blood group H and Le^y antigens. There was elevated FUT1 activity in the more aggressive high passage cells (397). Similarly, real time PCR showed FUT1 expression was elevated in prostate cancer tissue relative to normal tissue (398). The expression of blood group H may also arise from loss of expression of blood group A and B antigens, which unmasks blood group H antigens, among these is F77 antigen.

The biological relevance of the blood group H expression on cancer cells has not been well understood. In different biological contexts, some correlations have been described with respect to apoptotic potential of cancer cells and expression of blood group H antigen. It has been reported that mAb F77 can induce modest apoptosis of prostate cancer cells (341). However, in a colon carcinoma model, poorly tumorigenic REG cells transfected with human (FUT1) or rat α1,2-fucosyltransferases (FTA and FTB) were more resistant to apoptosis; knockdown of these enzymes in the spontaneously tumorigenic PRO cells increased sensitivity to apoptosis induced by serum deprivation (399). The anti-apoptotic effect of the α1,2-fucosylated antigens was suggested to be associated with the evasion of CD4 lymphocyte-mediated immune surveillance (399).

The F77 antigen may be involved in the modulation of the growth of prostatic gland. It was found that in serum-free culture medium, the addition of the type 2 blood group H specific lectin, UEA-I, significantly reduced testosterone-stimulated growth and branching morphogenesis of rat ventral prostates compared with the addition of other lectins (400). This suggested that blood group H antigen and the corresponding fucosyltransferases may somehow be involved in normal prostatic development. It is tempting to predict that the type 2 blood group H may be also involved in the growth of prostate cancer given that blocking the F77 antigen by mAb F77 has been noted to inhibit tumour growth (341).

It is also possible that F77 antigen is involved in prostate tumour metastasis. As reviewed in Section 1.2.3, the decreased expression of blood group A and B is associated with increased cellular motility (165,166). In particular, loss of capping of the H antigen by the blood group A and B monosaccharides in prostate cancer has been reported (380,381,401). It is thus possible that F77 antigen facilitates tumour metastasis by promoting prostate cancer cell motility.

The proteins carrying the F77 antigen are not known. One of the carrier proteins has been detected from the extracts of prostate cancer cells (Hongtao Zhang in Mark Greene's laboratory, unpublished), as mentioned in Chapter 3. But the identity of it is not clear. Some glycoproteins have been reported to carry blood group antigens, such as a splice variant of CD44 and integrin. These glycoproteins are involved in

cell-cell and cell-matrix adhesions. Alteration of glycosylation of these molecules can potentially influence their biological functions such as in tumour cell invasion and metastasis. In a highly tumourigenic clone of rat colon carcinoma, blood group H antigen was found to be largely carried by a splice variant of CD44 (402). It was found that the tumorigenicity could be influenced by blood group H antigen modification of CD44 (402). Integrins were found to be a major carrier of the type 2 blood group A and B antigens in some human colon cancer cell lines (403). Expression of blood group A or B antigen on integrins α3, α6 or β1 was suggested to affect the interactions between the α and β subunits, thereby changing the matrigel-dependent haptotactic motility (which is a measure of invasive activity of tumour cells) of colonic tumour cells (165,166). The glycosylation of integrins was also found important in the interaction between integrins and laminin in the extracellular matrix (404). Furthermore, integrin-mediated adhesion and growth can be promoted by expression of hyaluronic acid (Figure 1.1) and large tumour-associated glycoproteins such as mucins . Evidence has been presented that these large molecules can mechanically push matrix-bound integrins to enter a focal adhesion where integrins are clustered and integrin-dependent growth factor signalling is elicited. This was believed to be independent of the biochemical interactions of the glycans with their binding proteins (405).

Although from the discussion above it can be inferred that F77 antigen participates in a number of ways in prostate cancer progression, the biological receptor of F77 antigen has not been described. Here I propose that the biological functions of F77 antigen and its poly-LacNAc backbone sequences are mediated, at least in part by galectins. Proteins of the galectin family has been reported to be variously involved in cancer pathology, including cell proliferation, apoptosis, angiogenesis, and metastasis. Particularly, it has been known that many prostate cancer cell lines, including PC3, express galectin-3 (406). The galectin-3 has also been reported to be expressed in human prostate cancer tissues (406,407) but some results using various polyclonal or monoclonal antibodies have been contradictory (408). In a recent study, galectin-3 was uniformly detected in the basal cell layer of prostate cancer tissues (409). Basal cells are suggested to be cells of origin of prostate cancers (410). Indeed, in prostate cancer cells, knockdown of galectin-3 was associated with reduced cell proliferation, migration and invasion *in vitro*, and with

decreased tumour formation and increased apoptosis *in vivo* (409,411). Galectin-1, which was reported to be the most abundant galectin in prostate cancers, was also positively correlated with the disease progression (412). Immunostaining of galectin-1 on capillary endothelial cells was increased in prostate tumour tissues compared to the non-tumoural stroma area (413). Expression of this endogenous lectin was highly associated with angiogenesis. Knockdown of galectin-1 reduced the formation of new vasculature in prostate cancer (412). Recently it was found that binding of galectin-1 to β1,6-linked poly-LacNAc on VEGFR2 could initiate VEGF-like signalling, and could lead to the tumour neovascularization (152).

There are many reports on galectins in cancer events mediated by interaction with poly-LacNAcs on *N*-glycans as reviewed in Section 1.2.2. Relatively few reports have focused on interactions of galectins with poly-LacNAcs on *O*-glycans. In bladder tumour cells which were shown to highly express the branching enzyme GCNT1, poly-LacNAc containing *O*-glycans were detected on MHC class I-related chain A (MICA). Galectin-3 binding to core 2 *O*-glycans on MICA reduced the affinity of MICA to its biological receptor NKG2D (natural killer group 2 member D) on NK cells (374). In addition, MUC1 was found to be expressed on both bladder and prostate tumour cells. On these tumour cells expressing GCNT1, MUC1 was found highly *O*-glycosylated, carrying poly-LacNAc chains. Engagement of galectin-3 with its ligands on MUC1 attenuated the interaction of tumour cells with NK cells, leading to the reduced granzyme B secretion by NK cells. The interaction of galectin-3 with MUC1 also interfered with the access of tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) to the tumour cell surface. Thus poly-LacNAc on *O*-glycans could mediate the evasion of tumour cells from the NK cell-mediated killing, facilitating tumour dissemination through the circulation (414,415).

Although multiple galectins are implicated in the pathobiology of prostate cancer, there is currently a lack of evidence of galectin interactions with the branched blood group H sequence, although it is well-known that most galectins bind well to poly-LacNAc sequences. Prediction of the interaction between F77 antigen and galectins is based on two pieces of evidence. First, earlier studies of Ten Feizi's lab showed that blood group H antigen on a linear backbone was clearly bound by galectin-1; the branched poly-LacNAc sequences could also be bound (18). Second, Cummings'

group corroborated those findings by microarray analyses of galectins 1, 2 and 3 with sequence-defined glycans. It was found that these galectins, at different concentrations, could bind to blood group H on linear poly-LacNAc backbones (17). It is therefore reasonable to predict that the F77 antigen which combines blood group H and the branched poly-LacNAc would also interact with galectins. We will shortly investigate this.

5.1.2 AE3 antigen and related carbohydrate sequences

One of the sequences recognized by mAb AE3 is T disaccharide α-linked to serine or threonine (Galβ-3GalNAcα-Ser/Thr). It is a well-known pan-carcinoma antigen, variously designated as Thomsen-Friedenreich antigen, TF antigen, T antigen or core 1. This antigen is found on the surface of about 90% of all human carcinomas including colon, breast, bladder, prostate, liver, ovary and stomach (416), but is lacking on normal adult tissues (417). The expression pattern of T antigen was found to overlap considerably with AE3 antigen on many epithelial cancers (385-389), implying a relationship between the T disaccharide and the antigens that mAb AE3 recognizes. The mechanism for the increased expression of T antigen in cancers is not fully understood. It is likely that the expression of such a structure is the consequence of the alterations of several glycosyltransferases in conjunction with other mechanisms such as the increased availability of substrate UDP-Gal (416). This needs investigation.

In spite of the high prevalence of T antigen in tumour cells, little is known about the carrier proteins. A recent study using an enzymatic approach to label the T antigen on human breast adenocarcinoma MCF-7 cells identified more than 700 putative T antigen-carrying glycoproteins (418). Among these glycoproteins, MUC1 has been extensively investigated. Immunohistochemical studies revealed that MUC1 was the predominant carrier of T antigen in gastric and colorectal adenocarcinomas (419). In one study it was observed that galectin-3 in the circulation promoted adhesion of human breast or colon cancer cells to human umbilical vein endothelial cells through an interaction with MUC1 carrying T antigen (420). The interaction was suggested to change the distribution of MUC1 on tumour cell surface, exposing cell adhesion

molecules, which would otherwise be “shielded”, and thus allowing epithelium to endothelium interaction (420).

Given its broad distribution in cancers and lack of expression in normal tissues, the T antigen has been suggested to be associated with tumour formation. It has been implicated in cancer cell proliferation, metastasis and angiogenesis (416). Interestingly, investigations of these associations of T antigen in cancer cell biology so far have also focused on galectin-mediated interactions, especially galectin-3. Expression of galectin-3 could be induced on endothelial cells by T antigen-bearing glycoproteins or cancer cells. Galectin-3 could then mediate docking of T antigen-expressing tumour cells onto endothelium (421). Galectin-3 was also found to be expressed on breast and prostate cancer cells. It could help the homotypic aggregation of tumour cells in the circulatory system (422,423). In addition to tumour cell-endothelium interactions, galectin-3 has been suggested to facilitate the evasion of tumour cells from immune surveillance by inducing apoptosis of T cells (424,425).

However, there has been evidence contradicting the results above. A detailed study of galectins 1, 2 and 3 using glycan microarray analysis indicated that galectin-1 and -3 did not bind significantly to T antigen and suggested T antigen may not be the glycan that was responsible for the effects reported for the galectins (17). This needs to be further investigated as the highly clustered presentation of T antigen on the cancer cell surface may enhance the avidity for the galectins. A T antigen-carrying glycopeptide was recently isolated from Pacific cod and was shown to bind to galectin-3 with picomolar affinity. The glycopeptide was able to inhibit galectin-3-mediated angiogenesis, tumour-endothelial cell interaction, metastasis and T cell apoptosis (426). Earlier studies reported that galectin-3 interacted with human serum albumin carrying T antigen; this interaction could be inhibited by either a T antigen-targeting peptide (designated P30) or a T antigen-mimicking glyco-amino acid lactulosyl-L-leucine (421,422). Further studies demonstrated that T antigen-mediated tumour cell adhesion and metastasis could be inhibited by antibodies (such as JAA-F11) against T antigen (427) or galectin-3 (428). It would be interesting to test if mAb AE3 shows inhibitory effects similar to those of T antigen-targeting antibodies.

The other carbohydrate sequence that mAb AE3 recognizes is the sulphoglycolipid SM1a. So far this glycolipid has only been described as a minor component in rat kidney (429) and in the monkey kidney cell line Verots S3 (349). SM1a was postulated to be a precursor of the di-sulphated glycolipid analogue SB1a (349), which was shown to be abundantly expressed on the surface of human hepatocellular carcinoma cell line PLC/PRF/15 but not detectable in glycolipid extracts from cirrhotic liver or normal liver using a mAb against SB1a (430). SB1a glycolipid was shown to mediate binding of human colon carcinoma CCK-81 cells to galectin-4 coated plates. This binding of the cancer cells was inhibited by antibodies against SB1a or galectin-4 (431). The sulphated *O*-glycan analogue of SM1a on mucin type glycoprotein has not been described to our knowledge. The involvement of SM1a and the proposed *O*-glycan analogue based on core 3 in cancer pathology is yet to be investigated.

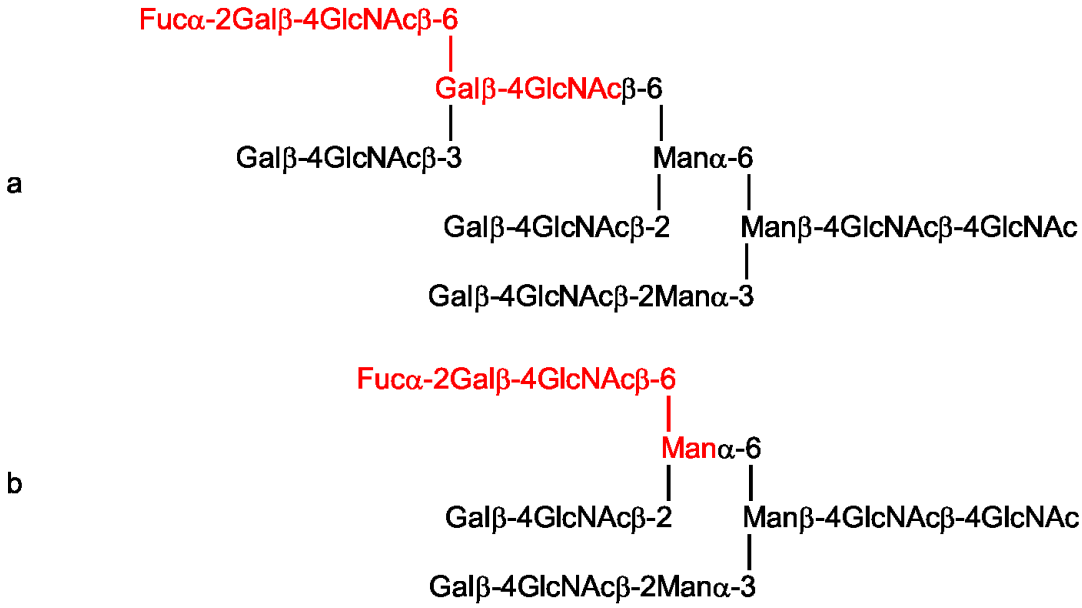
In sum, although the sequences of the two carbohydrate antigens F77 and AE3 are distinct, one thing in common is both of them can be potentially recognized by galectins. This will be investigated in the follow-up studies.

5.2 Potential clinical applications of the antigens and antibodies of F77 and AE3

In the pages that follow, I discuss possible uses of the F77 and AE3 systems as biomarkers or in cancer therapy.

5.2.1 Diagnostic and prognostic biomarkers

There are limitations to the direct use of F77 antigen as a serum biomarker because of the presence of the antigen on red blood cells. As referred to in Chapter 3, there is potential, however, to explore the F77 antigen expressing glycoproteins as biomarkers, given that F77 has now been detected on glycoproteins of prostate cancer cells. Although it is still not known whether F77 antigen is present on *N*-glycans, it is conceivable that mAb F77 would bind to the *N*-glycan sequences **a** and **b** depicted below. Highlighted in red are the residues potentially involved in the recognition of mAb F77.



Both **a** and **b** are type 2 blood group H-based sequences. Structure **a** is a type 2 blood group H on poly-LacNAc β-6 linked to *N*-glycan backbone on the 6-linked Man branch. It is the *N*-glycan equivalent of the F77 antigen sequence characterized on

O-glycans and glycolipids. In addition, the type 2 blood group H sequence directly linked to the 6-branch Man expressed on *N*-glycan as depicted in structure **b** may also be bound.

The question then arises as to whether such *N*-glycans and *O*-glycans of the type described in Chapter 3 can occur on glycoproteins, such as the prostate cancer-associated glycoproteins PSA, prostate-specific membrane antigen (PSMA), and prostate stem cell antigen (PSCA). If they indeed contain F77 antigen-positive sequences, targeting the F77 glycoforms of the glycoproteins could potentially improve the sensitivity and specificity of the current assays. In the past, evidence has been presented for the elevated expression of blood group H (Fucα1,2-Gal-) antigen on PSA in sera of prostate cancer patients, relative to that of patients with benign prostatic hyperplasia (398).

As mentioned in Section 5.1.2, the T antigen recognized by mAb AE3 is broadly expressed in many cancers but rarely in normal tissues. Thus it has been extensively investigated as to whether this carbohydrate motif can be used as a diagnostic or prognostic biomarker (432). Historically, the detection of T antigen has relied on lectins such as *Amaranthus caudatus* lectin, *Artocarpus integrifolia*/jacalin and PNA, and mAbs such as A78-G/A7 and HH8. However, most of these reagents are not purely specific to Galβ-3GalNAcα- sequence. Lectins of *A. caudatus* and *A. integrifolia/jacalin* can bind to GalNAcα-, as in Tn antigen, and α2,3-linked sialyl-T antigen NeuAcα-3Galβ-3GalNAc (433,434). PNA and antibody A78-G/A7 are able to bind to Galβ-3GalNAcβ- such as asialo-GM1 (433,435). The antibody HH8 could accommodate C6 substituted GalNAc (core 2) (436). The differing binding specificities of these reagents may have led to contradictory results on the value of T antigen in prognosis and metastasis. Having said that, generally higher levels of T antigen expression have been associated with poorer prognosis in many types of cancers (437). In this regard, although mAb AE3 is not purely specific to the α-linked T disaccharide (Galβ-3GalNAcα-) either, it is clear from our investigation that asialo-GM1 or C6 substituted GalNAc are not bound by mAb AE3. It would be interesting therefore to test whether mAb AE3 is able to better detect α-linked T antigen than the above-mentioned antibodies or lectins.

An alternative to detecting glycans or glycoproteins directly is to analyse the antigen expression on circulating tumour cells (CTCs). This can be applied to both F77 and AE3 antigens. Our collaborators are now investigating if there occur CTCs containing F77 antigen in patients with prostate cancer. If CTCs can be detected, it will be desirable to evaluate whether their presence correlates with prostate tumour progression.

Glycosyltransferases are another type of good candidate for biomarkers. As suggested by the glycosyltransferase transfection study (366) the branching glycosyltransferases GCNTs and the fucosyltransferase FUT1 play a key role in the biosynthetic pathway of the F77 antigen. As these glycosyltransferases are essential for the synthesis of F77 antigen, their levels of expression in the tumour cells may be correlated with prostate cancer progression and thus be of prognostic value.

Another strategy is to look for autoantibodies, which have been considered as prime candidates for biomarkers in early stage cancer (242). The presence of autoantibodies against F77 antigen in sera of prostate cancer patients requires investigation. In order to perform this investigation, the blood samples should be collected at 37 °C to avoid absorption of the potential autoantibodies to red cells. Clearly, patients carrying these autoantibodies are likely to be only mild cases, otherwise large amounts of autoantibodies would cause anaemia given their ability to agglutinate red cells and would have been detected. The autoantibodies against the AE3 antigen, T disaccharide, may be used as diagnostic biomarkers. It was reported that all humans have naturally occurring antibodies against T antigen (438). Early studies suggested that these antibodies were probably elicited by our own intestinal microbiome, which contain strains of bacteria that express T antigen (439). Level of autoantibodies against T antigen can be used as an indication of the stage of cancer. Higher levels of autoantibodies in sera of patients with stomach and breast cancer were found to correlate with better survival rates (440,441). Perhaps this is a reflection of lower levels of the circulating T antigen. In addition, glycosylation pattern of the IgG isotype of autoantibodies have also been suggested to have diagnostic and prognostic value in gastric cancer (442,443). This needs systematic investigation.

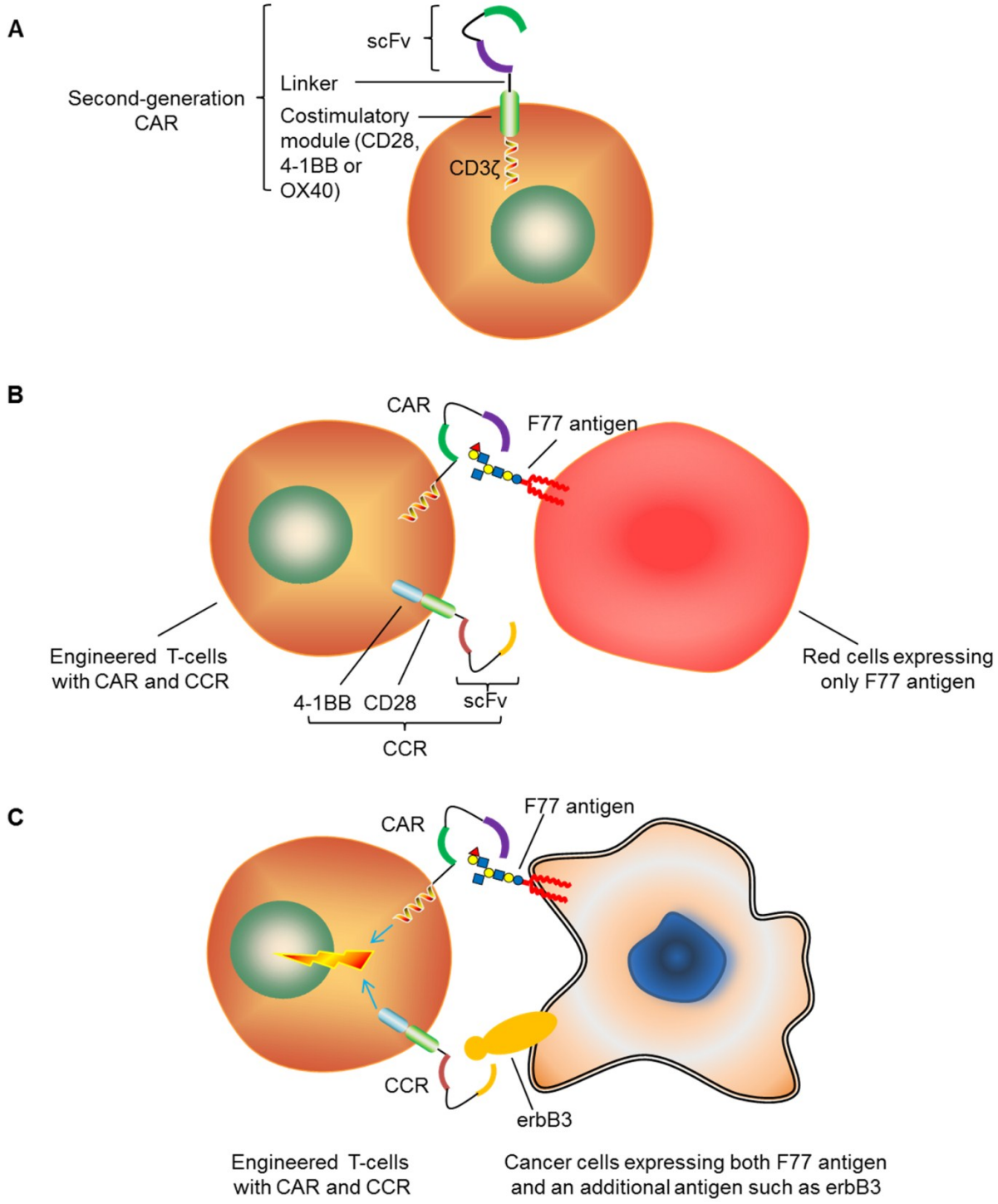
5.2.2 Therapy

The two antibodies F77 and AE3 do offer therapeutic possibilities. Antibody AE3 with specificity to T antigen deserves investigation in tumour therapy and imaging. Recently an antibody JAA-F11 which is highly specific to α-linked Galβ-3GalNAc has shown promising results in preclinical settings (444,445). It has strong anti-metastasis activity *in vivo* and *in vitro* (427,445,446). JAA-F11 stained 32 of 41 human breast cancer cell lines, irrespective of their estrogen, progesterone or HER2 receptor status (447). This antibody was also capable of imaging human triple-negative breast tumours in mice (447). Therefore as an antibody targeting the same antigen, it would be desirable to compare the capability of mAb AE3 in the same settings as JAA-F11.

Although direct applications of mAb F77 in tumour imaging and tumour therapy are precluded on account of binding of this IgG3 antibody to red cells, engineering of the antibody for cancer therapy is a possible strategy. Efforts are being made to generating single chain variable region (scFv) and humanized forms of mAb F77 by our collaborators. There is considerable potential to capitalize on the cold reactive ('cold agglutinin') property of the native antibody, namely the diminished antigen binding at temperatures above 20 ˚C. A monomeric scFv F77 antibody will further reduce the binding affinity. Therefore at body temperature, it is possible that the low affinity scFv would not agglutinate human red cells. If this is indeed the case, creating a hybrid molecule fusing the F77 scFv and a second prostate-specific scFv would target different antigenic determinants on prostate tumour cells. This hybrid antibody could also be used for immunodetection of human prostate cancer.

Another avenue has been to engineer the T lymphocytes and other immune cells to direct them to cancer cells using chimeric antigen receptors (CARs). These receptors are engineered recombinant transmembrane molecules that often consist of scFv of an antibody and a transmembrane- and cytoplasmic domain such as CD3-ζ, to transduce signals to activate the T cells. As CARs can target not only processed antigens but also native cell surface antigens such as glycans and glycolipid structures, T cells grafted with CARs open immunotherapy possibilities of directing at

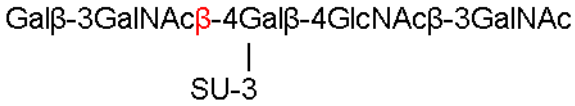
cancer-associated carbohydrate antigens. Examples of CARs targeting glycans currently under investigation include those targeting GD2, GD3 and Le^y (448). In order to confer stronger signalling properties and persistence to the T cells, a second generation of CARs has been designed by fusing the antigen-binding site with the activating receptor and the cytoplasmic domain of a costimulatory receptor such as CD28, 4-1BB and OX40 (Scheme 5.1A). Recently, engineered T cells containing scFv of an anti-GD3 mAb (MB3.6) in frame with ζ-chain and CD28 dual signalling showed great growth inhibitory of subcutaneously transplanted melanoma cells in the presence of IL-2 (449). Many of these second-generation CARs are in clinical trials (448). One of the big challenges to overcome is the paucity of truly tumour-restricted antigens. Recently a new strategy has been reported by splitting the activating and costimulatory signals, and installing two different receptors, a CAR and a chimeric costimulatory receptor (CCR) to a T cell. The two receptors target two different antigens. Although neither antigen is strictly tumour-specific, the co-expression of the two tumour-associated antigens can efficiently differentiate the tumour cells from normal cells. Using this strategy, Sadelain group produced T cells carrying CAR and CCR against PSCA and PSMA, respectively (450). They selected a low-affinity scFv targeting PSCA to generate a CAR with diminished activities such that T cells could not be activated without the costimulatory signal from CCR. The engineered T cells specifically targeted double positive prostate cancer cells and subsequently killed these, leaving tumour cells expressing only one antigen intact (450). We propose the potential application for mAb F77 is to create engineered T-cells transduced with both a CAR containing scFv of F77 and a CCR that recognizes a second prostate cancer-associated antigen, such as erbB3. By critical adjustment of activating signals, a F77 CAR could be generated with low binding activity to F77 antigen. Therefore normal cells such as red cells expressing F77 antigen alone would not activate T cells (Scheme 5.1B). The hypothesis is that when prostate cancer tumour cells expressing both F77 antigen and erbB3 are recognized, the costimulatory signal from the recognition by CCR, in conjunction with the signal from CAR, would activate T cells, leading to the specific attack of tumour cells (Scheme 5.1C).



Scheme 5.1 Scheme of the engineered T cell with second-generation CAR and the strategy for designing T cells with two receptors: CAR and CCR to spare normal cells and selectively attack tumour cells. (**A**) A second-generation CAR is usually composed of an scFv, a CD3ζ activating subunit and a costimulatory motif which can be CD28, 4-1BB or OX40. A strategy is proposed to specifically target tumour cells by generating T cells transduced with a CAR that recognizes F77 antigen with low affinity and a CCR (with two costimulatory motifs CD28 and 4-1BB) that binds with high affinity to another antigen (such as erbB3). When these T-cells encounter normal cells, such as red cells expressing F77 antigen as in (**B**), they cannot be activated because the signal from the low-affinity CAR alone is not strong enough. When the engineered T-cells encounter tumour cells expressing both antigens as in (**C**), they receive both CAR and CCR signals and become activated.

5.3 Future prospects

The studies in this thesis have fully characterized the prostate cancer-associated antigen F77, as well as partially characterized the broadly distributed epithelial cancer-associated antigen AE3. As mAb AE3 has been found to recognize two distinct glycan sequences (structures a and b in Scheme 4.3), and the likely *O*-glycan analogue of structure b has proven difficult to isolate, future work will be focused on the chemo-enzymatic synthesis and antigenic analysis of the proposed AE3-active *O*-glycan:



One of the future studies planned, which I am interested to pursue, is to examine whether the F77 and AE3 antigens are ligands for galectins, and whether mAbs F77 and AE3 can inhibit recognition by galectins. It is hoped this follow-up study will contribute to a better understanding of these antigens in epithelial cancer progression.

With knowledge of the structure of the two antigens F77 and AE3, the way is open to explore rationally their applications as biomarkers. The two well-characterized mAbs F77 and AE3 can be used as tools, to detect CTCs and specific glycoforms of glycoproteins; and these in turn would serve as biomarkers in monitoring cancer progression during treatment. The two antigens and the related glycan sequences generated in this thesis project are valuable reference probes for glycan arrays. These can be used in serological studies in the search for tumour-associated autoantibodies. There are good prospects for such autoantibodies as biomarkers in cancer diagnosis.

With the advent of technologies for engineered T cells, especially with the two-receptor strategy, there is considerable potential for exploiting scFvs of antibodies F77 and AE3 in cancer therapy.

In sum, the two antibodies and the corresponding antigens have tremendous biological and functional implications. Further research should be carried out to

understand how the aberrant glycosylations occur during cancer progression, and to elucidate details of the contributions of these glycan sequences to the neoplastic processes, and to determine whether therapies targeting these modifications can lead to beneficial clinical outcomes.

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APPENDICES

Appendix S1 Oligosaccharide probes included in the screening array (Glycosciences Array Set 32-39) and the binding signals (means of the fluorescence intensity) elicited with mAb F77 and 89-F anti-B at 5 fmol probe per spot.

Appendix S2 Summary of permission for third party copyright works