Glycoconjugate Journal 17, 553–565, 2000

Progress in deciphering the information content of the 'glycome' — a crescendo in the closing years of the millennium*

Ten Feizi

The Glycosciences Laboratory, Imperial College School of Medicine, Northwick Park Campus, Harrow, HA1 3UJ, United Kingdom

The closing years of the second millennium have been uplifting for carbohydrate biology. Optimism that oligosaccharide sequences are bearers of crucial biological information has been borne out by the constellation of efforts of carbohydrate chemists, biochemists, immunochemists, and cell- and molecular biologists. The direct involvement of specific oligosaccharide sequences in protein targeting and folding, and in mechanisms of infection, inflammation and immunity is now unquestioned. With the emergence of families of proteins with carbohydrate-binding activities, assignments of information content for defined oligosaccharide sequences will become more common, but the pinpointing and elucidation of the bioactive domains on oligosaccharides will continue to pose challenges even to the most experienced carbohydrate biologists. The neoglycolipid technology incorporates some of the key requirements for this challenge: namely the resolution of complex glycan mixtures, and ligand binding coupled with sequence determination by mass spectrometry.

Keywords: **blood group antigens, carbohydrate ligands, differentiation antigens, embryonic development, galectins, inflammation, leukocyte adhesion, mass spectrometry, monoclonal antibodies, neoglycolipids, oligosaccharide ligands, oligosaccharide probes, selectins**

Abbreviations: **DHPE, L-1,2-dihexadecyl-*sn*-glyccro-3-phosphoethanolamine; ADHP, *N*-aminoacetyl-*N*-(9-anthracenyl-methyl)-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine.**

Introduction

Oligosaccharides are co- or post-translational modifications of proteins and they also occur in lipid-linked form or as free molecules in secretions. Oligosaccharides are formed by the sequential actions of cellular glycosyltransferases and other enzymes. There are core, backbone and peripheral domains on oligosaccharides that are common to many glycoproteins, although the prevalence and proportions differ in various cell types and in the various proteins that they contain. Other oligosaccharides are genetically determined in individuals, as illustrated *par excellence* by the major blood group antigens A, B, H, Le^a and Le^b. The natural immune responses to the A and B antigens in those who lack them, and the resulting clinically serious sequelae of mismatched blood transfusions served to focus research into the chemical structures and the genetic basis of the blood group antigens. The pioneering biochemical and immunochemical work in the groups of Morgan and Watkins in the UK and of Kabat in the USA [1–3] not only led to the elucidation of the oligosaccharide sequences that constitute the major blood group antigens, but also laid the foundations for the recent research into roles of oligosaccharides related to these antigens as ligands for effector proteins that have key roles in the trafficking of leukocytes in inflammation [3a].

I entered the field of carbohydrate biology in the course of studies on an autoimmune haemolytic disorder, which commonly occurs in patients with *Mycoplasma pneumoniae* infection [4–6]. I became intrigued by this condition which is characterized by transiently elevated titres of serum antibodies (cold agglutinins) directed to the blood group I antigen, and regarded it as an excellent model of autoimmunity. There is little if any I antigen on the mycoplasma [7]. This infective agent has the property of adhering to human red cells. Having established that the mycoplasma, when bound to I-positive red

*This article is dedicated to the late Elvin A. Kabat my mentor who, instead of offering to work up the first preparation of I-antigen-active glycoprotein that I made in 1969, invited me to bring it to his laboratory where I would be trained in carbohydrate studies.

Tel.: + 44(0)20 8869 3460; Fax: + 44(0)20 8869 3455;
E-mail: t.feizi@ic.ac.uk

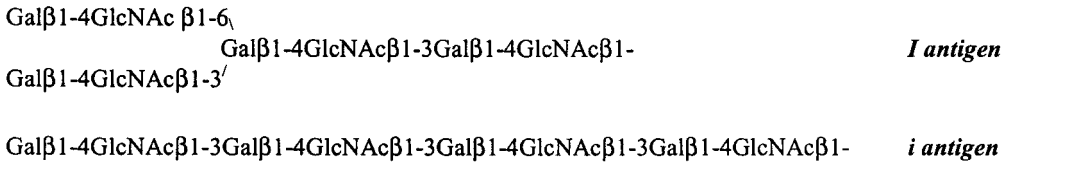

Figure 1. Poly-N-acetyllactosamine sequences of I antigen type (branched) and i antigen type (linear). Individual monoclonal autoantibodies recognize different domains on these sequences.

cells, could trigger the transient production of auto-anti-I in an experimental model [8], the way forward seemed to be, to elucidate the nature of the I antigen, and the biochemical basis of the microbe–host cell interaction. The transiently occurring anti-I antibodies are mono- or oligoclonal [9]; thus they resemble the persistent monoclonal autoantibodies with the same specificity which occur in the monoclonal gammopathy known as chronic cold agglutinin disease. A collection of sera assembled from patients with chronic cold agglutinin disease [10], containing monoclonal antibodies to the I antigen or to the related i antigen, turned out to be powerful reagents in the studies that followed. In this article I highlight the way this research program took me deep into carbohydrate biology, a field that has burgeoned in the last decade of the millennium.

The era of monoclonal antibodies, and clues to bioactivities of blood group – related oligosaccharides

In collaboration with Elvin Kabat, we showed the I and i antigens are expressed on precursor sequences (backbones) of the major blood group antigens, and we characterized the trisaccharide sequence (a branched domain Galβ1-4GlcNAcβ1-6-, on poly-*N*-acetyllactosamine chains, see Figure 1) recognized by one of the antibodies, anti-I Ma [11,12]. Later, with Sen Hakomori, we characterized the various domains on branched poly-*N*-acetyllactosamine sequences that are recognized by other anti-I, and on linear sequences recognized by anti-i [13,14].

Oligosaccharides of poly-*N*-acetyllactosamine type are minor components among the sugar chains of glycoproteins and glycolipids, and because they are a family of isomeric sequences, they are difficult to separate from one another. After characterizing the different tri- to octasaccharide sequences of poly-*N*-acetyllactosamine type recognized by the individual monoclonal autoantibodies, I realized the power of these antibodies as biochemical tools [15]. Cancer-associated changes could be detected in the expression of these antigens among gastrointestinal glycoproteins [16–18].

It seemed likely that the poly-*N*-acetyllactosamine chains at the cell surface would dwarf the relatively short oligosaccharide chains, and that they could form a carbohydrate layer with special function [15]. From their very structure and because of the different carbohydrate epitopes that they display, the Ii antigens seemed good candidates for bioactive components on cell membranes [15]. Some five years after elucidating the Ii antigens, my colleagues and I showed that the sialylated form of the I antigen is the host-cell attachment site for *M. pneumonie* [19,20].

One approach to investigating the possible roles of the Ii type sequences as ligands for endogenous receptors was to observe the effects of cross-linking and redistributing the Ii antigens of human peripheral blood lymphocytes by incubating them at 4°C with the anti-I or -i (NB these antibodies bind only at low temperatures) followed by fluorescent second antibodies, and then warming to 37°C. It was observed that the resulting patches at the lymphocyte surface were identical to the binding sites for the mitogen, ConA [21], indicating that Ii antigen-bearing molecules of lymphocyte membranes are structurally associated with counter-receptors for mitogens. In separate experiments [22–24], oligosaccharide sequences of poly-*N*-acetyllactosamine type, I- and i-active, were detected on multiple glycoproteins of human lymphocytes, the *O*-glycans of leukocyte common antigen (CD45) were among these. We had observed that an animal lectin, 14 kDa β-galactoside binding protein (now termed galectin 1), binds strongly to glycoproteins with Ii activities rather than to those with ABH activities, and we isolated a Ii-active substance from calf heart that was strongly bound by the 14 kDa calf heart lectin [25]. We proposed that the Ii antigens might be natural ligands for such an endogenous lectin. There have been some interesting developments in this context recently. These are discussed below under the heading of animal lectins.

It had been shown by blood bank serology that the expression of the I and i antigens on human red cells changes during the first year of life. There is a predominance of i antigen and a lack of the I at birth, and an increase in the I coincident with diminution of the i in the course of the first year of life [26]. Prompted by this knowledge and the observations of Takashi Muramatsu that early embryonic tissues are rich in oligosaccharide chains of poly-*N*-acetyllactosamine type [27,28], we used the human monoclonal anti-I and anti-i as reagents in studies of early embryonic development in the mouse, and observed marked changes in expression of the branched and linear sequences they recognize, during successive stages of differentiation (see Figure 2) [29]. This lead to the suggestion that oligosaccharides of this family may be involved in the developmental processes [29,30]. This view was markedly boosted by our demonstration that the hybridoma-defined, murine stage-specific embryonic antigen, SSEA-l, is expressed on αl-3 fucosylated lactosamine sequence (Figures 2 and 3), the isomer of blood group Lc^a, termed Le^x [31]. SSEA-1 appears on the 8-cell embryo coincident with the onset of compaction,

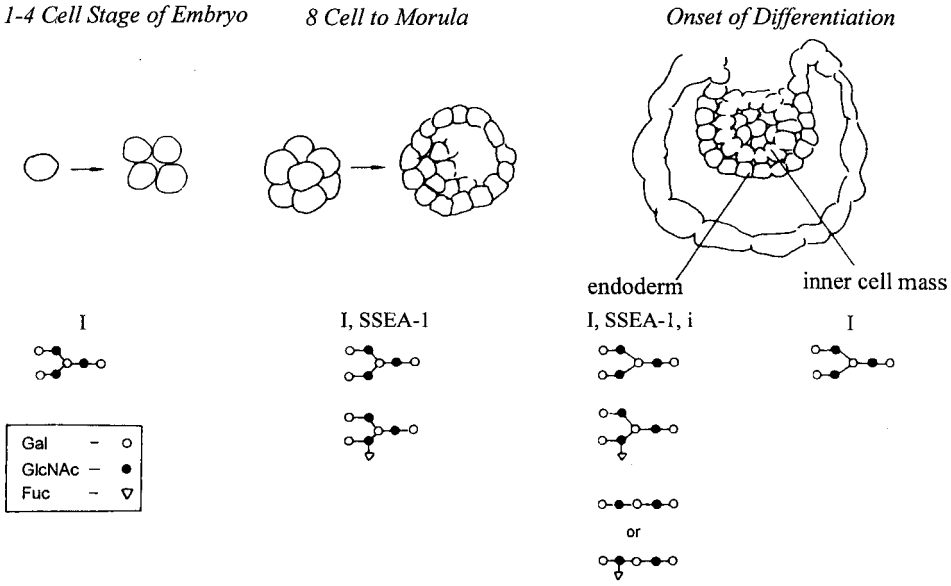

Figure 2. Sequential changes in antigens expressed on poly-N-acetyllactosamine chains during early embryogenesis in the mouse (taken from ref 45 with permission).

the first cell–cell adhesion event in the developing embryo [32]. We suggested [31] that there may exist a carbohydrate-binding protein which mediates this adhesive event which is crucial for the further development of the embryo. Such an embryonic protein has not yet been discovered, although as discussed below, Le^x-related sequences are ligands for cell adhesion proteins that have important functions later in life. The precise role of the Le^x sequence in the developing embryo is at present unknown. There have been two reports that Le^x-containing compounds can inhibit the embryonic compaction [33,34]. Attempts in my laboratory to inhibit the compaction process with oligosaccharides and with mucins that express the Le^x sequence were unsuccessful, although it was possible to markedly delay the compaction of recently compacting 8–16 cell embryos by decompacting them by calcium chelation and treating them with endo-β-galactosidase [35]. This enzyme cleaves linear oligosaccharide sequences of poly-*N*-acetyllactosamine type [36]. Hopefully, work with the recently described fucosyltransferase, Fuc-T9, which is thought to be the enzyme that generates the SSEA-1 capping structure on oligosaccharides of poly-*N*-acetyllactosamine type [37], will provide new insights into the role of SSEA-1 in the complex molecular interactions that lead to embryonic compaction.

Further interest in carbohydrate biology was stimulated by the demonstration that numerous hybridoma antibodies raised to *neo*-antigens of tumour cells recognize specific carbohydrate sequences, often blood group-related [38,39]. Initial hope that such antibodies would serve as immuno-therapeutic tools in cancer were tempered by the finding that the tumour-associated carbohydrate antigens are for the most part expressed normally in unrelated tissues [40]. There is nevertheless a limited number of carbohydrate antigens that are sufficiently restricted in their expression to warrant pursuance for human tumour therapy [41]. In my opinion, a major message from the demonstration that carbohydrate structures of glycoproteins and glycolipids are 'oncodevelopmental' antigens was that the various carbohydrate sequences may have biological functions as area codes, for example, that determine pathways of cell migration [42], or that they may serve as ligands for regulators of cell growth and differentiation [15,40,43,44].

Support for the concept of the oligosaccharides as area codes has come from leukocyte biology. When lectin domains were discovered on the selectins, two of which, the E-and P-selectins, bind to myeloid cells, there was intense interest in the Le^x, sialyl-Le^x and related sequences which are distinctive markers of these blood cells [45–50]. As further discussed in sections that follow, roles have now been established for these sequences [51], and their sulphated analogues [52] as ligands for the selectins which mediate interactions crucial for initiating leukocyte recruitment to sites of inflammation. These developments have not only served to dispel some of the skepticism prevailing among biologists about the biological functions of the sugar chains of glycoproteins, but have opened new avenues for designs of carbohydrate-based inhibitors for treating disorders of inflammation.

Families of animal lectins – the emergence of much awaited 'missing links' in carbohydrate biology

A key requirement for elucidating the roles of particular oligosaccharide domains is to identify receptors that specifically recognize them in appropriate body compartments. Two striking examples have been revealed in the course of biochemical and cell biological studies of important household functions in cells, namely, the folding and the routing of newly

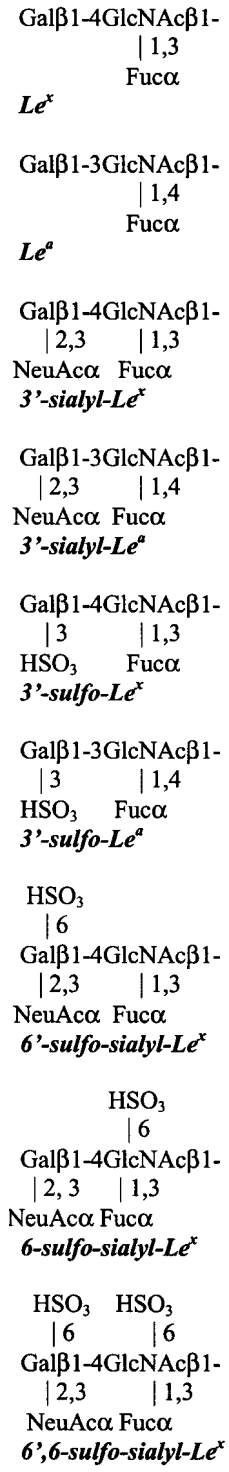

Figure 3. Capping sequences on oligosaccharides of the Le^x and Le^a types referred to in this article.

synthesized proteins. The chaperone proteins calnexin and calreticulin have been shown to operate through transient associations with the partially processed, glucosylated *N*-glycans of cellular and viral glycoproteins [53–55]. The delivery to lysosomes of newly synthesized hydrolytic enzymes is through recognition of the mannose-6-phosphate modification of their high-mannose *N*-glycans. Two mannose-6-phosphate-specific receptors are involved in this routing [56–58].

As with plant lectins (these are not within the scope of this review), it has not been straightforward to elucidate the precise functions of mammalian proteins that were first discovered by virtue of a carbohydrate-binding property. A prime example is the large family of *β*-galactoside-binding proteins, now termed galectins [59]. For example, the first described member of this family, the widely distributed 14 kDa, *β*-galactoside-binding protein (galectin 1), with its preference for poly-*N*-acetyllactosamine (Ii antigen-active) glycoconjugates [25] seemed a promising candidate for a protein with a role in development or cell growth regulation [15,43,60]. The developmentally regulated changes in galectin levels [59], and the detection of increased levels of multiple proteins antigenically related to the lectin in transformed and mitogen-stimulated lymphocytes [61] are consistent with such roles. Disappointingly though, we could not detect changes in the growth of freshly isolated human and bovine mononuclear cells in the presence of the lectin isolated from bovine heart muscle (Yoko Katagiri and Ten Feizi, 1980, unpublished). However, following the report of Linda Baum and colleagues that recombinant, bacterially-expressed galectin 1 induces apoptosis when added to immature thymocytes and activated thymocytes [62], there have been intriguing reports on signalling activities of this protein [63,64]. Among these is the phosphorylation of the T cell receptor *ζ*-chain, and antagonism of antigen induced signals in T cells which have been incubated with recombinant galectin 1 [65]. Carbohydrate-mediated galectin 1 binding has been demonstrated, *in vitro*, to defined lymphocyte membrane glycoproteins including CD45 ref [62], CD2 and CD3 ref [66], and CD7 ref [67]. The mechanisms of T cell apoptosis induced by the exogenously added recombinant galectin is not yet clear, but there is a correlation with the presence of poly-*N*-acetyllactosamine sequences in susceptible cell types [68]. Other studies [68a] have implicated immunoregulatory lattice formations between galectin 3 and glycoproteins with *N*-glycans of poly-*N*-acetyllactosamine type [cf refs 43 and 60].

An important development for the field of carbohydrate biology was Gilbert Ashwell's discovery of the hepatic receptors for clearance of glycoproteins [69]. This lead to the cloning, sequencing and description by Kurt Drickamer and others of the structural features of a protein module that is characteristic of a family of calcium-dependent (C-type) animal lectins [70–73]. These include several proteins of the innate immune system: the collectins (serum mannan-binding proteins, pulmonary surfactant proteins, and conglutinin), leukocyte-endothelium adhesion molecules (E-, L- and P-selectins), and the macrophage endocytosis receptor. A special feature of the scrum mannan-binding proteins, on which detailed structural information is available, is that the ligation of carbohydrate occurs via co-ordination bonds with a calcium

ion which is also bound to amino acids in the lectin protein. A considerable number of proteins with similar lectin-type modules have been reported. However many of these lack the amino acids predicted to ligate a calcium ion that could mediate the carbohydrate binding. These have been referred to as C-type lectin-like proteins that may not bind carbohydrates [72,74]. One member of this 'atypical' group of proteins does nevertheless bind to a decasaccharide of hyaluronan [75]. Others bind to glycoproteins in non-glycosylated recombinant form, although it is considered possible that the carbohydrate moieties on the natural proteins contribute to binding strength [76,77]. Binding to sulphated polysaccharides has been reported for at least three of these proteins [76,78]. There is indeed a precedent for dual recognition of carbohydrate and protein among the C-type lectins. As discussed below, both a sialyl-Lc^x glycan and sulphated tyrosine residues contribute to the strength of binding of P-selectin to the glycoprotein counter-receptor, PSGL-1 [79]. Further work is required to identify the ligands of the 'atypical' lectin type domains [80,81].

One of the lectins of the immune system, the macrophage endocytosis receptor, contains in addition to multiple C-type lectin modules, a cysteine-rich module, which is a lectin in its own right. As first shown by Jacques Baenziger, this domain of the macrophage receptor binds the pituitary hormones, luteinizing and thyroid stimulating hormones, via their sulphated carbohydrate chains (4′-sulphated at terminal *N*-acetylgalactosamine), and is thought to serve as a clearance receptor for these glycoproteins [82]. In collaboration with Michel Nussenzweig, we have identified three additional classes of sulphated oligosaccharides for this domain. These are chondroitin sulphates A and B (which contain terminal 4′-sulphated *N*-acetylgalactosamine), sulphated blood group chains (3′-sulphated at terminal galactose) [83], and sulphated glycolipids (these are also 3′-sulphated at terminal galactose) [84]. The binding is independent of calcium ions. The crystal structure of the cysteine-rich domain complexed with 4′-sulphated *N*-acetylgalactosamine, and molecular modelling in Pamela Bjorkman's group [85] have rationalized the way that the receptor can accommodate the four classes of oligosaccharide ligands which are biosynthetically distinct. The domain, which folds into an approximately three-fold symmetric β-trefoil shape resembling fibroblast growth factor, binds the sulphate in a neutral pocket. The lack of discrimination between sulphated galactose and sulphated *N*-acetylgalactosamine is explained by the absence of contacts between the *N*-acetyl group and the protein. In the model, the 3′-sulphated galactose can fit into the binding site without hindrance. It is predicted moreover that the sugar ring adjoining the terminal sulphated sugar would not contact the protein thus explaining the observation that the identity of the sugar linked to the terminal carbohydrate did not affect the ability to bind. This is clearly a multifunctional protein domain which was shown previously to bind to distinct areas of lymphoid tissues [86]. This binding is via the site for sulphated carbohydrate [83]. The way is now open to investigate whether this carbohydrate-binding module is an effector in antigen capture. Among the questions that I would raise are (a) a possible role in innate immunity, e.g. endocytosis and clearance of tumour cells that bear the sulphated carbohydrate antigens; (b) a possible role in adaptive immunity, e.g. capture for presentation of sulphated glycoprotein and glycolipid antigens; and (c) possible involvement in autoimmunity e.g. in inflammatory joint disease with cartilage damage, and in demyelinating diseases.

Another family of animal lectins which is the subject of active research is the siglec family which form a distinct group within the immunoglobulin superfamily, and bind oligosaccharides with 3′ or 6′-sialyl termini [87–91]. The first described member is sialoadhesin of macrophages. Other members include CD22 of lymphocytes, and myelin associated protein of oligodendrocytes.

Principles established in the quest for the ligands of the selectins

Arguably the most dramatic development for carbohydrate biology has been the finding that the three selectins are members of the C-type lectin family, with the features characteristic of calcium ligation [51,92,93]. This development, and the knowledge that these adhesion molecules have critical roles at the initial stages of leukocyte extravasation in inflammation, served to stimulate intense research into their carbohydrate ligands and counter-receptors [94]. The field became of biotechnological interest as it is clear to vascular biologists that ligand analogues which can inhibit the adhesion mediated by these molecules could be the basis of anti-inflammatory treatments (for some comments see ref [95]).

Initial developments in identifying oligosaccharide ligands for the selectins occurred rapidly, and by multiple approaches from different groups, Knowledge that human E- and P-selectins bind granulocytes and monocytes, served to focus research on 3′-fucosyl-*N*-acetyllactosamine (Le^x), and sialyl-Le^x sequences, which had been shown to be differentiation antigens of myeloid cells [40,50]. The 3′-sialyl-Le^x and the isomeric sequence 3′-sialyl-Le^a were readily shown to be recognized by all three selectins [51,93,96]. A picture has emerged, however, of differences in the binding specificities of the selectins such that variant carbohydrate sequences related to sialyl-$Le^{a/x}$, and additional elements on the counter-reccptors, elicit preferential binding by one or other of these receptors [97–100]. Carbohydrate ligands identified for E-selectin, apart from the above-mentioned sialyl sequences on leukocytes, include the 3′-sulpho-Le^a and 3′sulpho-Le^x sequences (Figure 3) as found on epithelial mucins [52]. The sulpho-Le^a and -Le^x are also bound by the L- and P-selectins [101]. However, other sulphate-containing motifs have been described on glycoprotein counter-receptors for L- and P-selectins. These have been shown to be critical elements for high avidity binding of P-selectin to its major counter-receptor

PSGL-1, and of L-selectin to the endothelium glycoprotein GlyCAM-1, sulphate being carried on protein and on carbohydrate respectively [98,102]. I discuss below, the salient conclusions thus far concerning the recognition elements for the three selectins which must be regarded as an interim report, as there remains much more to be learned about the full repertoire.

Direct binding experiments [103] with structurally defined oligosaccharide sequences have revealed that the sialyl-Le^x sequence that is modified by 6-*O*-sulfation at *N*-acetylglucosamine, as found on the G1yCAM-1 [104], rather than 6-*O*-sulfation at the outer galactose (see Figure 3 for capping sequences), is the most potent ligand thus far for L-selectin. The potency is enhanced if the sialic acid is non-acetylated [103,105,106]. On PSGL-l, the presence of sulphated tyrosine residues, in addition to a sialyl-Le^x glycan, assembled at the amino-terminal tip of the counter-receptor, is required for high avidity binding by P-selectin [107–112]. PSGL-1 is also bound by L- and E-selectins; for L-selectin binding, but not for E-selectin, again there appears to be a requirement for both the sialyl-Le^x and the sulphotyrosine [98,111]. Thus, the P- and L-selectins differ from E-selectin in their recognition of an amino-acid-associated determinant as well as carbohydrate.

Interactions of L-selectin with several other acidic compounds have been described when these are presented in the clustered state. These include sulphated galactosyl-ceramides (sulphatides), sulphated ganglio-series glycolipids, and neoglycolipids derived from glycosaminoglycan disaccharides reviewed in refs [101,113]. P-selectin also binds to these compounds ([114] and R. A. Childs, unpublished observations). Here, the position of the sulphate on the sulphatides [115] and the glycosaminoglycan disaccharides [116] is not critical. This is in contrast to the requirement for 3-*O*-sulphation at terminal galactose of thc $Le^{a/x}$ [117] and the 6-*O*-sulphation of the *N*-acetylglucosamine of the sialyl-Le^x sequence.

The binding of all three selectins to the acidic fucooligosaccharides is calcium-dependent, whereas the L- and P-selectin binding to the sulfatides and glycosaminoglycans is less sensitive to calcium chelation [114,116]. P-selectin binding to the *N*-terminal sulpho-glycopeptide domain of PSGL-1, bearing sulphate at tyrosines 46, 48, and 51, is also calcium-dependent as shown by studies with synthetic tyrosine-sulphated analogues of this domain [112]. In recent experiments, we have obtained evidence that there are two functionally distinct binding sites on P-and L-selectins: one for the fuco-oligosaccharides, and another for sulpho-tyrosine and other short sulpho-oligosaccharides (C. Galustian, R. A. Childs M. Stoll, H. Ishida, M. Kiso and T. Feizi, submitted for publication). This is in accord with the recent crystal structure [117a] of a P-selectin PSGL-1 complex. The possibility of there being two binding sites on a lectin domain should be kept in mind for other lectin-type proteins, which clearly bind to particular non-glycosylated proteins but appear also to have a saccharide-binding property.

Challenges in the quest for bioactive oligosaccharides

The pinpointing of oligosaccharides with bioactivities, identifying the bioactive domains on them, and determining their chemical structures is a most challenging area of cell biology. As exemplified by antibodies and the selectins the recognition sequences may be tri-, tetra-, and larger oligosaccharide sequences; these may be modified, for example by sulphate in specific linkages. Once an oligosaccharide ligand is detected, there are the 'downstream' demands of analyses of monosaccharide composition, sequence, linkage and anomeric configurations by enzymatic procedures in combination with mass spectrometry. When released from protein, oligosaccharides have low affinities for the recognition proteins, and by conventional methods, it is exceptional to have sufficient purified material for bioactivity and concomitant structure determination. In the classical studies [2,3], the determination of the oligosaccharide sequences that constitute the major blood group antigens could be achieved with what was to become a 'trick of the trade', namely the use of glycoproteins other than those of blood cells which are rich sources of the antigens in question, and also the use of free oligosaccharides of human milk. The glycoproteins (mucins) were derived from the secreted fluids in ovarian cystadenomas of patients of differing blood group status. Before the days of early diagnosis, these cysts sometimes grew to very large sizes. Ovarian cyst mucins were invaluable also in elucidation of the I and i antigens [11,12], SSEA-1 [31], the carbohydrate differentiation antigen of human granulocytes, VEP8/9 (this was identified as Le^x, now termed CD 15) [45,46], and other onco-developmental antigens [40,118]. The work-up of the oligosaccharides released from the mucins was extremely labour intensive. Moreover, the amounts of oligosaccharides required for the assignments were often prohibitive. Large amounts of oligosaccharides, milligram amounts, would need to be committed for inhibition of binding studies without a guarantee of success. This is in contrast to the relatively small amounts of glycolipids required for inhibition studies as they can be presented in the clustered state on liposomes [14].

Thus, it was clear that improved technologies were needed to facilitate and decrease the amounts of glycoprotein glycans required for assays of their bioactivities, and in particular to be able to pinpoint bioactive oligosaccharides within the highly heterogeneous populations typically released from glycoproteins. In discussion with Yuan-Chan (Ed) Lee, my colleagues and I introduced the neoglycolipid (NGL) technology [119] which was further developed with my colleague Mark Stoll, and a guest investigator Tsuguo Mizuochi [120], incorporating also the chromatogram-binding technique [121] that had been formulated by John Magnani for binding experiments with natural glycolipids resolved on thin layer chromatograms. NGLs are probes derived by chemical conjugation of oligosaccharides to lipid [119]. Much of our work has been carried out with the aminolipid 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE) to which oligosaccharides are

conjugated by reductive amination. The oligosaccharides may be *N*- or *O*-glycans released from glycoproteins or released from glycolipids, or synthesized chemically [122–124]. As with natural glycolipids, the hydrophobic lipid enables the oligosaccharides to be coated onto matrices for solid-phase binding experiments. Through clustering of the lipid moieties, the oligosaccharides are presented in an oligomeric state, which generates the avidities required for readily detectable binding. NGLs have the advantage that they contain a single lipid moiety, contrasting with the heterogeneous lipids of natural glycolipids. Following conjugation, each oligosaccharide remains a discrete entity, rather than a population of oligosaccharides conjugated to a macromolecular carrier. Thus, mixtures of NGLs are amenable to resolution by TLC for binding experiments on chromatograms. NGLs have excellent ionization property which my colleagues, Alex Lawson and Wengang Chai, have exploited, and developed a powerful mass spectrometric strategy, whereby components resolved on chromatograms and bound components can be sensitively analyzed, *in situ*, by liquid secondary ion mass spectrometry [125,126]. By this means, molecular mass, monosaccharide sequence, branching pattern and the presence of other groups such as sulphate or phosphate can be detected on bioactive oligosaccharide components within mixtures released from glycoproteins [52,122].

The key feature of the NGL technology is that it enables the pinpointing of the oligosaccharides containing the recognition motifs within highly heterogeneous mixtures derived from natural glycoconjugates. Aliquots of total oligosaccharide populations (or of sub-populations fractionated on the basis of charge or size) derived from desired glycoproteins, glycolipids or whole cells are converted into NGLs, resolved by TLC, and ligand-bearing oligosaccharides detected by chromatogram-binding experiments. Thus work can be focussed on oligosaccharide fractions containing the desired ligand-bearing components. These are subjected to successive liquid chromatographies, with monitoring of the bioactivities within aliquots converted into NGLs, until the desired components are isolated. The technology has been powerful in the elucidation of oligosaccharide-binding specificities of antibodies and endogenous carbohydrate-binding proteins, and in studies of thc roles of specific oligosaccharides in cell signaling and microbial adhesion studies and as substrates for glycosyltransferases, as reviewed in refs. [123,127–129]. Notable examples have included the discovery of a unique oligosaccharide antigen on the adhesive proteoglycan of the sponge, *Microciona prolifera* [130], a family of linear and branched oligosaccharides of the lacto- and neolacto-series capped with sulphated-Le^a and sulphated-Le^x sequences which are potent ligands the selectins [52,131], and assignments of the sequences of novel *O*-mannosyl glycans on mammalian brain glycoproteins [132,133].

A limitation of DHPE, shared with the naturally occurring ceramides, is that it does not contain a chromophore, and the derived NGLs have only the UV absorbing property of the parent oligosaccharide for detection by HPLC. Detection and quantitation of NGLs has depended on staining for lipid, hexose or sialic acid residues using primulin, orcinol or resorcinol, respectively [122]. HPLC of NGLs with UV detection has not been convenient because of the UV-absorbing property of the solvents required for separation. There was a need, therefore, for a new lipid reagent, which contains a UV-absorbent or fluorescent chromophore. This has now been addressed, and we have introduced a second generation of NGLs which are fluorescent [134]. For this purpose, DHPE has been modified to incorporate a fluorescent label, anthracene. This new lipid reagent, *N*-aminoacetyl-*N*-(9-anthracenylmethyl)-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (ADHP), synthesized from anthracenaldehyde and DHPE, gives an intense fluorescence under UV light. Fluorescent NGLs derived from a variety of neutral and acidic oligosaccharides by conjugation to ADHP, by reductive amination, can be detected and quantified by spectrophotometry and scanning densitometry, and resolved by TLC and HPLC with sub-picomol detection. Antigenicities of the ADHP-NGLs are well retained, and picomol levels can be detected using monoclonal carbohydrate sequence-specific antibodies. Among *O*-glycans from an ovarian cystadenoma mucin, isomeric sialyl-Le^a- and sialyl-Le^x-active sequences, could be resolved by HPLC as fluorescent NGLs, and sequenced by liquid secondary ion mass spectrometry [134]. Thus the NGL technology now uniquely combines high sensitivity of immuno-detection with a comparable sensitivity of chemical detection. Principles are thus established for a streamlined technology whereby an oligosaccharide population is carried through ligand detection and ligand isolation steps, and sequence determination by mass spectrometry, enzymatic sequencing and other state-of-the-art carbohydrate analyses. Our recent studies of an epitope on heparan sulphate recognized by a monoclonal antibody, which binds to the earliest lesions in prion disease, indicate that the applications of the fluorescent NGL technology extend to discoveries of bioactive determinants also on glycosamino-glycans [134a].

Powerful though the NGL technology is for establishing the carbohydrate-binding properties of antibodies and receptors, and for pinpointing and sequence determination of the oligosaccharides bound, this does not necessarily mean that the oligosaccharides concerned are available for binding on the natural glycoproteins. A striking example is the influence of carrier protein on *N*-glycan recognition by the collectins, conglutinin and mannan-binding protein, a topic that we have been pursuing in detail with Dolores Solis and colleagues. Although the two lectins show qualitatively similar binding specificities toward free high mannose *N*-glycans and the NGLs derived from them [135,136], only conglutinin binds to the Man_{8-9} *N*-glycan (at Asn_{917}) on glycoprotein C3b which is a proteolytically pruned form of the complement glycoprotein C3 [137]. There is no binding by either lectin to the parent glycoprotein C3 or to C3c which is a further proteolyzed fragment of C3 containing the same *N*-glycan. We have

observed a similar phenomenon with the glycoprotein RNAse B, which contains at a single glycosylation site (Asn_{34}), one or other of five high mannose oligosaccharides (Man_{59}) [137]. On the native glycoprotein, the oligosaccharide is not bound by the either lectin, whereas binding occurs when the protein is reduced and denatured. Thus the protein moieties of these glycoproteins exert pronounced effects on the presentation of the oligosaccharides thereby modulating recognition by the lectins. We have pursued the model system afforded by the isolated RNAase B Man_8 glycoform [138,139]. NMR analyses reveal that the three-dimensional structure of the protein moiety is essentially identical to that of non-glycosylated RNAse (RNAase A). Thus there are no perceptible differences between the RNAse protein forms that could account for the differential availability of the *N*-glycan for conglutinin binding. After reduction and denaturation, the NMR spectrum becomes typical of a non-structured polypeptide, although the conformational preferences of the N-glycosidic linkage are unchanged, and the oligosaccharide retains the average conformational behavior of the free oligosaccharide irrespective of the protein fold. This conformational freedom is clearly not translated into full availability of the oligosaccharide for the carbohydrate recognition protein. We propose therefore that the differing availability of the glycan is a reflection of the existence of different geometries of presentation of the carbohydrate determinant within the glycan:protein ensemble [138].

Concluding remarks

The field of carbohydrate biology, now widely referred to as glycobiology, is well launched, and is all the better for having the close involvement of molecular biologists and genome scientists. Discoveries of novel families of animal lectins can be anticipated, and continued advances in the frontiers of knowledge in the biosynthesis of oligosaccharides that they recognize [140]. An increased momentum is anticipated in knowledge of the bioactivities of glycosaminoglycan sequences as modulators of the activities of cytokines, chemokines, growth factors, and of their receptors and as sensors in developmental processes [141,142]. The involvement of oligosaccharides, including the glycosaminoglycans in microbe–host interactions [141,143], and the increasing knowledge on microbe-specific pathways of surface carbohydrate synthesis and microbial lectins that mediate host-cell attachment, raise interesting possibilities of carbohydrate-based anti-microbial therapeutics [144].

The molecular biology of glycosyltransferases and other carbohydrate modifying enzymes is providing important information on substituents that are of key importance in oligosaccharide recognition [145,146a]. Nevertheless, ligand discovery for known and emerging families of carbohydrate-recognizing receptors is likely to remain a challenging exercise in the post-genome era, largely because oligosaccharides cannot be cloned! I believe that there remains a need for further developments of technologies for generating oligosaccharide probes that can be readily resolved, probed and sequenced. Ideally, the core monosaccharide ring should be preserved in the new probes, and glycopeptide probes should be developed that incorporate the amino acids flanking the oligosaccharides on glycoproteins. Synthetic carbohydrate chemistry will, as ever, remain a staunch ally of carbohydrate biology, providing large quantities of defined oligosaccharide sequences essential for corroborating specificity [147,148], and even providing clues to hitherto unsuspected, natural modifications of saccharide ligands that occur in the course of cell regulation [105].

Acknowledgement

I acknowledge with deep gratitude the late W. Lawrence Marsh who drew my attention to the occurrence of I antigen in human milk, and to Richard M. Krause who had instructed me in polysaccharide extraction methods, which I applied in preparing I antigen. The cited work from my laboratory has been supported by the Medical Research Council, the Arthritis Foundation, the Arthritis Research Council, the Cancer Research Campaign, the European Union, the Human Frontiers Science Program and the Leukaemia Research Fund.

References

1 Morgan WTJ, Some immunological aspects of the products of the human blood group genes. In *Ciba Foundation Symposium* pp. 194–216 (1959).
2 Watkins WM, Biochemistry and genetics of the ABO, Lewis and P blood group systems, *Advances in Human Genetics*, **10**, 1–136, 379–85 (1980).
3 Kabat EA, Contributions of quantitative Immunochemistry to knowledge of blood group a, B, H, Le, I and i antigens, *Clin Pathol* **78**, 28l–92 (1982).
3a Feizi T, Lloyd KO, An appreciation of Elvin A. Kabat (1914–2000): scientist, educator and a founder of modern carbohydrate biology, *Glycoconj J* **17**, 439–42 (2000).
4 Feizi T, Maclean H, Sommerville RG, Selwyn JG, The role of mycoplasmas in human disease, *Proc Roy Soc Med* **59**, 1109–12 (1966).
5 Feizi T, Cold agglutinins, the direct Coombs' test and serum immunoglobulins in Mycoplasma pneumoniae infection. *Ann NY Acad Sci* **143**, 801–12 (1967).
6 Feizi T, Carbohydrate differentiation antigens Ii, SSEA-l (Le^x) and related structures. In *New comprehensive biochemistry-Glycoproteins II*, edited by Montreuil J, Vliegenthart JFG, Schachter H, (Elsevier Science, Amsterdam, 1997), pp. 571–86.
7 Feizi T, Taylor-Robinson D, Cold agglutinins anti-I and *Mycoplasma pneumoniae Immunology*, **13**, 405–9 (1967).
8 Feizi T, Taylor-Robinson D, Shields MD, Carter RA, Production of cold agglutinins in rabbits immunized with human erythrocytes treated with *Mycoplasma pneumoniae*, *Nature*, **222**, 1253–6 (1969).
9 Feizi T, Schumacher M, Light chain homogeneity of post-infective cold agglutinins, *Clin Exp Immunol* **3**, 923–9 (1968).

10 Feizi T, Lamda chains in cold agglutinins, *Science* **156**, 1111–2 (1967).
11 Feizi T, Kabat EA, Vicari G, Anderson B, Marsh WL, Immunochemical studies on blood groups XLVII. The I antigen complex-Precursors in the A, B, H, Le^a and Le^b blood group system-Hemagglutination inhibition studies, *J Exp Med* **133**, 39–52 (1971).
12 Feizi T, Kabat EA, Vicari G, Anderson B, Marsh WL, Immunochemical studies on blood groups XLIX. The I antigen complex: Specificity differences among anti-I sera revealed by quantitative precipitin studies; partial structure of the I determinant specific for one anti-I serum, *J Immunol* **106**, 1578–92 (1971).
13 Niemann H, Watanabe K, Hakornori S, Chiids RA, Feizi T, Blood group i and I activities of Lacto-N-*nor*hexaosyl ceramide and its analogues: the structural requirements for i-specificities, *Biochem Biophys Res Commun* **81**, 1286–93 (1978).
14 Feizi T, Childs RA, Watanabe K, Hakomori SI, Three types of blood group I specificity among monoclonal anti-I autoantibodies revealed by analogues of a branched erythrocyte glycolipid, *J Exp Med* **149**, 975–80 (1979).
15 Feizi T, Structural and biological aspects of blood group I and i antigens on glycolipids and glycoproteins, *Blood Trans Immunohaematol* **23**, 563–77 (1980).
16 Feizi T, Turberviile C, Westwood JH, Blood-group precursors and cancer-related antigens, *Lancet* **ii**, 391–3 (1975).
17 Picard JK, Waldron Edward D, Feizi T, Changes in the expression of the blood group A, B, H, Le^a and Le^b antigens and the blood group precursor associated I (Ma) antigen in glycoprotein-rich extracts of gastric carcinoma, *J Clin Lab Immunol* **1**, 119–28 (1978).
18 Feizi T, The blood group Ii system: a carbohydrate antigen system defined by naturally monoclonal or oligoclonal autoantibodies of man, *Immunol Commun* **10**, 127–56 (1981).
19 Loomes LM, Uemura K-I, Childs RA, Paulson JC, Rogers GN, Scudder PR, Michalski JC, Hounsell EF, Taylor-Robinson D, Feizi T, Erythrocyte receptors for Mycoplasma pneumoniae are sialylated oligosaccharides of Ii antigen type, *Nature* **307**, 560–63 (1984).
20 Loomes LM, Uemura K, Feizi T, Interaction of Mycoplasma pneumoniae with erythrocyte glycolipids of I and i antigen types, *Infect Immun* **47**, 15–20 (1985).
21 Feizi T, Kapadia A, Yount WJ, I and i antigens of human peripheral blood lymphocytes cocap with receptors for concanavalin, *A Proc Natl Acad Sci USA* **77**, 376–80 (1980).
22 Childs RA, Kapadia A, Feizi T, Expression of blood group I and i active carbohydrate sequences on cultured human and animal cell lines assessed by radioimmunoassays with monoclonal cold agglutinins, *Eur J Immunol* **10**,79–84 (1980).
23 Childs RA, Feizi T, Differences in carbohydrate moieties of high molecular weight glycoproteins of human lymphocytes of T and B origins revealed by monoclonal autoantibodies with anti-I and anti-i specificities, *Biochem Biophys Res Commun* **102**, 1158–64 (1981).
24 Childs RA, Dalchau R, Scudder P, Hounsell EF, Fabre JW, Feizi T, Evidence for the occurrence of O-glycosidically linked oligosaccharides of poly-N-acetyllactosamine type on the human leucocyte common antigen, *Biochem Biophys Res Commun* **110**, 424–31 (1983).
25 Childs RA, Feizi T, Calf heart lectin reacts with blood group Ii antigens and other precursor chains of the major blood group antigens, *FEBS Lett* **99**, 175–9 (1979).
26 Marsh WL, Anti-i; a cold antibody defining the Ii relationship in human red cells, *Brit J Haematol* **7**, 200–9 (1961).
27 Muramatsu T, Gachelin G, Nicolas JF, Condamine H, Jakob H, Jacob F, Carbohydrate structure and cell differentitation: unique properties of fucosyl-glycopeptides isolated from embryonal carcinoma cells, *Proc Natl Acad Sci USA* **75**, 2315–9 (1978).
28 Muramatsu T, Gachelin G, Damonneville M, Delarbre C, Jacob F, Cell surface carbohydrates of embryonal carcinoma cells: polysaccharidic side chains of F9 antigens and of receptors to two lectins, FBP and PNA, *Cell* **18**, 183–91 (1979).
29 Kapadia A, Feizi T, Evans MJ, Changes in the expression and polarization of blood group I and i antigens in post-implantation embryos and teratocarcinomas of mouse associated with cell differentiation, *Exp Cell Res* **131**, 185–95 (1981).
30 Feizi T, Kapadia A, Gooi HC, Evans MJ, Human monoclonal autoantibodies detect changes in expression and polarization of the Ii antigens during cell differentiation in early mouse embryos and teratocarcinomas. In *Teratocarcinoma and embryonic cell interactions*, edited by Muramatsu T, Gachelin G, Moscona A, Ikawa Y, (Japan Scientific Societies Press & Academic Press, Tokyo, 1982), pp. 201–15.
31 Gooi HC, Feizi T, Kapadia A, Knowles BB, Solter D, Evans MJ, Stage specific embryonic antigen SSEA-1 involves α1–3 fucosylated type 2 blood group chains, *Nature*, **292**, 156–58 (1981).
32 Solter D, Knowles BB, Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1), *Proc Natl Acad Sci USA* **75**, 5565–9 (1978).
33 Bird JM, Kimber SJ, Oligosaccharides containing fucose linked α(1–3) and α(1–4) to N-acetylglucosamine cause decompaction of mouse morulae, *Developmental Biology* **104**, 449–60 (1984).
34 Fenderson BA, Zehavi U, Hakomori S, A multivalent lacto-N-fucopentaose III-lysyllysine conjugate decompacts preimplantation-stage mouse embryos while the free oligosaccharide is ineffective, *J Exp Med* **160**, 1591–6 (1984).
35 Rastan S, Thorpe SJ, Scudder P, Brown S, Gooi HC, Feizi T, Cell interactions in pre-implantation embryos: evidence for involvement of saccharides of the poly-N-acetyllactosamine series, *J Embryol Exp Morph* **87**, 115–28 (1985).
36 Scudder P, Hanfland P, Uemura K, Feizi T, Endo-*β*-galactosidases of *Bacteroides fragilis* and *Escherichia freundii* hydrolyse linear but not branched oligosaccharide domains of glycolipids of the neolacto series, *J Biol Chem* **259**, 6586–92 (1984).
37 Kaneko M, Kudo T, Iwasaki H, Ikehara Y, Nishihara S, Nakagawa S, Sasaki K, Shiina T, Inoko H, Saitou N, Narimatsu H, Alpha1,3-fucosyltransferase IX (Fuc-TIX) is very highly conserved between human and mouse; molecular cloning, characterization and tissue distribution of human FucTIX, *FEBS Lett* **452**, 237–42 (1999).
38 Hakomori S, Aberrant glycosylation in cancer cell membranes as focused on glycolipids: Overview and perspectives, *Cancer Res* **45**, 2405–14 (1985).
39 Feizi T, Carbohydrate antigens in human cancer, *Cancer Surveys* **4**, 245–69 (1985).

40 Feizi T, Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are oncodevelopmental antigens, *Nature* **314**, 53–7 (1985).

41 Lloyd KO, The chemistry and immunochemistry of blood group A, B, H, and Lewis antigens: past, present and future, *Glycoconj J* **17**, 531–41 (2000).

42 Feizi T, Carbohydrate differentiation antigens, *Trends Biochem Sci* **6**, 333–5 (1981).

43 Feizi T, Childs RA, Carbohydratcs as antigenic determinants of glycoproteins, *Biochem J* **245**, 1–11 (1987).

44 Feizi T, Childs RA, Growth regulating network? Nature, **329**, 678 (1987).

45 Feizi T, Carbohydrate differentiation antigens. In *Fetal antigens and cancer. Ciba Foundation Symposium 96 (1992)*, edited by Everett D, Whelan J (Pitman, 1983), pp. 216–21.

46 Gooi HC, Thorpe SJ, Hounsell EF, Rumpold H, Kraft D, Forster O, Feizi T, Marker of peripheral blood granulocytes and monocytes of man recognized by two monoclonal antibodies VEP8 and VEP9 involves the trisaccharide 3-fucosyl-N-acetyllactosamine, *Eur J Immunol* **13**, 306–12 (1983).

47 Thorpe SJ, Feizi T, Species differences in the expression of carbohydrate differentiation antigens on mammalian blood cells revealed by immunofluorescence with monoclonal antibodies, *Biosci Reps* **4**, 673–85 (1984).

48 Fukuda, MN, Dell, A, Tiller PR, Varki A, Klock JC, Fukuda M, Structure of a novel sialylated fucosyl lacto-*N*-nor-hexaosylceramide isolated from chronic myelogenous leukemia cells, *J Biol Chem* **261**, 2376–82 (1986).

49 Macher BA, Buehler J, Scudder P, Knapp W, Feizi T, A novel carbohydrate differentiation antigen on fucogangliosides of human myeloid cells recognized by monoclonal antibody VIM-2, *J Biol Chem* **263**, 10186–91 (1988).

50 Fukuda M, Hiraoka N, Yeh JC, C-type lectins and sialyl Lewis X oligosaccharides. Versatile roles in cell–cell interaction, *J Cell Biol* **147**, 467–70 (1999).

51 Bevilacqua MP, Nelson RM, Selectins, *J Clin Invest* **91**, 379–87 (1993).

52 Yuen C-T, Lawson AM, Chai W, Larkin M, Stoll MS, Stuart AC, Sullivan FX, Ahern TJ, Feizi T, Novel sulfated ligands for the cell adhesion molecule E-selectin revealed by the neoglycolipid technology among O-linked oligosaccharides on an ovarian cystadenorna glycoprotein, *Biochemistry* **31**, 9126–31 (1992).

53 Helenius A, How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum, *Mol Biol Cell* **5**, 253–65 (1994).

54 Bergeron JJ, Brenner MB, Thomas DY, Williams DB, Calnexin: a membrane-bound chaperone of the endoplasmic reticulum, *Trends Biochem Sci* **19**, 124–8 (1994).

55 Molinari M, Helenius A, Chaperone selection during glycoprotein translocation into the endoplasmic reticulum, *Science* **288**, 331–3 (2000).

56 Sly WS, Fischer HD, The phosphomannosyl recognition system for intracellular and intercellular transport of lysosomal enzymes, *J Cell Biochem* **18**, 67–85 (1982).

57 von Figura K, Hasilik A, Lysosomal enzymes and their receptors, *Ann Rev Biochem* **55**, 167–93 (1986).

58 Kornfeld S, Structure and function of the mannose 6-phosphate/insulin-like growth factor -II receptors, *Annu Rev Biochem* **61**, 307–30 (1992).

59 Barondes SH, Castronovo V, Cooper DNW, Cummings RD, Drickamer K, Feizi T, Gitt MA, Hirabayashi J, Hughes C, Kasai K, Leffler H, Liu F.-T, Lotan R, Mercurio AM, Monsigny M, Pillai S, Poirier F, Raz A, Rigby PWJ, Rini JM, Wang JL, Galectins: A family of animal β-galactoside-binding lectins. [Letter to the Editor], *Cell* **76**, 597 (1994).

60 Feizi T, Childs RA, Carbohydrate structures of glycoproteins and glycolipids as differentiation antigens, tumour-associated antigens and components of receptor systems, *Trends Biochem Sci* **10**, 24–9 (1985).

61 Carding SR, Thorpe SJ, Thorpe R, Feizi T, Transformation and growth related changes in levels of nuclear and cytoplasmic proteins antigenically related to mammalian β-galactoside-binding lectin, *Biochem Biophys Res Commun* **127**, 680–6 (1985).

62 Perillo NL, Pace KE, Seilhamer JJ, Baum LG, Apoptosis of T cells mediated by galectin-1, *Nature* **378**, 736–9 (1995).

63 Rabinovich GA, Alonso CR, Sotomayor CE, Durand S, Bocco JL, Ricra CM, Molecular mechanisms implicated in galectin-1-induced apoptosis: activation of the AP-1 transcription factor and downregulation of bcl-2, *Cell Death Differ* **7**, 747–53 (2000).

64 Salvatore P, Benvenuto G, Pero R, Lembo F, Bruni CB, Chiariotti L, Galectin-1 gene expression and methylation state in human T leukemia cell lines, *Int Oncol* **17**, 1015–18 (2000).

65 Chung CD, Patel VP, Moran M, Lewis LA, Carrie MM, Galectin-1 induces partial TCR zeta-chain phosphorylation and antagonizes processive TCR signal transduction, *J immunol* **165**, 3722–9 (2000).

66 Walzel H, Blach M, Hirabayashi J, Kasai KI, Brock J, Involvement of CD2 and CD3 in galectin-1 induced signaling in human Jurkat T-cells, *Glycobiology* **10**, 131–40 (2000).

67 Pace KE, Hahn HP, Pang M, Nguyen JT, Baum LG, CD7 delivers a pro-apoptotic signal during galectin-1-induced T cell death, *J Immunol* **165**, 2331–4 (2000).

68 Galvan M, Tsuboi S, Fukuda M, Baum LG, Expression of a specific glycosyltransferase enzyme regulates T cell death mediated by galectin-1, *J Biol Chem* **275**, 16730–7 (2000).

68a Dimitriou M, Granovski M, Quaggin S, Dennis JW, Negative regulation of T-cell activation by *Mgat5 N*-glycosylation, *Nature* **409**, 733–9 (2001).

69 Ashwell G, Harford J, Carbohydrate-specific receptors of the liver, *Ann Rev Biochem* **51**, 531–54 (1982).

70 Drickamer K, Two distinct classes of carbohydrate-recognition domains in animal lectins, *J Biol Chem* **263**, 9557–60 (1988).

71 Weis WI, Drickamer K, Structural basis of lectin-carbohydrate recognition, *Ann Rev Biochem* **65**, 441–73 (1996).

72 Weis WI, Taylor ME, Drickamer K, The C-type lectin superfamily in the immune system, *Immunol Rev* **163**, 19–34 (1998).

73 Hakansson K, Lim NK, Hoppe HJ, Reid KBM, Crystal structure of the trimeric alpha-helical coiled-coil and the three lectin domains of human lung surfactant protein D, *Structure* **7**, 255–64 (1999).

74 Drickamer K, Dodd RB, C-Type lectin-like domains in Caenorhabditis elegans: Predictions from the complete genome sequence 1, *Glycobiology* **9**, 1357–69 (1999).

75 Day AJ, The C-type carbohydrate recognition domain (CRD) superfamily, *Biochem Soc Trans* **22**, 83–8 (1994).

76 Parham P, NK cell receptors: of missing sugar and missing self, *Curr Biol* **10**, R195–R197 (2000).

77 Colonna M, Moretta A, Vely F, Vivier E, A high-resolution view of NK-cell receptors: Structure and function 1, *Immunol Today* **21**, 428–31 (2000).

78 Childs RA, Galustian C, Lawson AM, Dougan G, Benwell K, Frankel G, Feizi T, Recombinant soluble human CD69 dimer produced in *Escherichia coli*: Reevaluation of saccharide binding, *Biochem Biophys Res Commun* **266**, 19–23 (2000).

79 McEver RP, Selectin-carbohydrate interactions during inflammation and metastasis, *Glycoconj J* **14**, 585–91 (1997).

80 Kogelberg H, Montero E, Bay S, Lawson AM, Feizi T, Reevaluation of monosaccharide binding property of recombinant soluble carbohydrate recognition domain of the natural killer cell receptor NKR-P1A, *J Biol Chem* **274**, 30335–6 (1999).

81 Kogelberg H, Lawson AM, Muskett FW, Carruthers RA, Feizi T, Expression in Escherichia coli, folding *in vitro*, and characterization of the carbohydrate recognition domain of the natural killer cell receptor NKR-P1A, *Protein Expression and Purification* **20**, 10–20 (2000).

82 Fiete D, Beranek MC, Baenziger JU, A cysteine-rich domain of the "mannose" receptor mediates GalNAc-4-$S0_4$ binding, *Proc Natl Acad Sci USA* **95**, 2089–93 (1998).

83 Leteux C, Chai W, Loveless RW, Yuen CT, Uhlin-Ilansen L, Combarnous Y, Jankovic M, Maric SC, Misulovin Z, Nussenzweig MC, Feizi T, The cysteine-rich domain of the macrophage mannose receptor is a multispecific lectin that recognizes chondroitin sulfates A and B and sulfated oligosaccharides of blood group Lewis^a and Lewis^x types in addition to the sulfated N-glycans of lutropin, *J Exp Med* **191**, 1117–26 (2000).

84 Leteux C, Nussenzweig MC, Ishizuka I, Feizi T, The cysteine-rich domain of the macrophage endocytosis receptor recognizes multiple classes of sulphated oligosaccharides. Proceedings Abstract: 20th International Carbohdyrate symposium August 27–September 1, 2000, Hamburg, Germany.

85 Liu Y, Chirino AJ, Misulovin Z, Leteux C, Feizi T, Nussenzweig MC, Bjorkman PJ, Crystal structure of the cysteine-rich domain of mannose receptor complexed with a sulfated carbohydrate ligand, *J Exp Med* **191**, 1105–16 (2000).

86 Martinez-Pomares L, Kosco VM, Darley E, Tree P, Herren S, Bonnefoy JY, Gordon S, Fc chimeric protein containing thc cysteine-rich domain of the murine mannose receptor binds to macrophages from splenic marginal zone and lymph node subcapsular sinus and to germinal centers, *J Exp Med* **184**, 1927–37 (1996).

87 Nath D, Anton van der Merwe P, Kelm S, Bradfield P, Crocker PR, The amino-terminal immunoglobulin-like domain of sialoadhesin contains the sialic acid binding site, *J Biol Chem* **270**, 26184–91 (1995).

88 Law CL, Aruffo A, Chandran KA, Doty RT, Clark EA, Ig domains 1 and 2 of murine CD22 constitute the ligand-binding domain and bind multiple sialylated ligands expressed on B and T cells, *J Immunol* **155**, 3368–76 (1995).

89 Tomschy A, Fauser C, Landwehr R, Engel J, Homophilic adhesion of E-cadherin occurs by a co-operative two- step interaction of N-terminal domains, *EMBO J* **15**, 3507–14 (1996).

90 Crocker PR, Kelm S, Hartnell A, Freeman S, Nath D, Vinson M, Mucklow S, Sialoadhesin and related cellular recognition molecules of the immunoglobulin superfamily, *Biochem Soc Trans* **24**, 150–6 (1996).

91 Angata T, Varki A, Siglec-7: A sialic acid-binding lectin of the immunoglobulin superfamily, *Glycobiology* **10**, 431–8 (2000).

92 Harlan JM, Liu DY, *Adhesion: Its role in Inflammatory Disease*, (W.H. Freeman & Co., New York).

93 Brandley BK, Swiedler SJ, Robbins PW, Carbohydrate ligands of the LEC cell adhesion molecules, *Cell* **63**, 861–3 (1990).

94 Ley K, Functions of selectins. In *Mammalian carbohydrate binding proteins*, edited by Crocker PR, (Springer-Verlag, Berlin Heidelberg New York, 2000), pp. 175–98.

95 Feizi T, Bundle D, Carbohydrates and glycoconjugates, Editorial Overview, *Curr Opin Struct Biol* **4**, 673–6 (1994).

96 Feizi T, Cell-cell adhesion and membrane glycosylation, *Curr Opin Struct Biol* **1**, 766–70 (1991).

97 McEver RP, Moore KL, Cummings RD, Leukocyte trafficking mediated by selectin-carbohydrate interactions, *J Biol Chem* **270**, 11025–8 (1995).

98 Rosen SD, Bertozzi CR, Leukocyte adhesion: Two selectins converge on sulphate, *Curr Biol* **6**, 261–4 (1996).

99 Feizi T, Galustian C, Novel oligosaccharide ligands and ligand-processing pathways for the selectins, *Trends Biochem Sci* **24**, 369–72 (1999).

100 Feizi T, Carbohydrate ligands for the leukocyte-endothelium adhesion molecules, selectins. In *Mammalian carbohydrate binding proteins*, edited by Crocker PR, (Springer-Verlag, Berlin Heidelberg New York, 2000), pp. 199–221.

101 Crocker PR, Feizi T, Carbohydrate recognition systems: functional triads in cell–cell interactions, *Curr Opin Struct Biol* **6**, 679–91 (1996).

102 McEver RP, Moore KL, Cummings RD, Leukocyte trafficking mediated by selectin–carbohydrate interactions, *J Biol Chem* **270**, 11025–8 (1995).

103 Galustian C, Lawson AM, Komba S, Ishida H, Kiso M, Feizi T, Sialyl-Lewis^x sequence 6-*O*-sulfated at *N*-acetylglucosamine rather than at galactose is the preferred ligand for L-selectin and de-*N*-acetylation of the sialic acid enhances the binding strength, *Biochem Biophys Res Commun* **240**, 748–51 (1997).

104 Hemmerich S, Leffler H, Rosen SD, Structure of the *O*-glycans in GlyCAM-1, an endothelial-derived ligand for L-selectin, *J Biol Chem* **270**, 12035–47 (1995).

105 Komba S, Galustian C, Ishida H, Feizi T, Kannagi R, Kiso M, The first total synthesis of 6-sulfo-de-*N*-acetylsialyl Lewis^x ganglioside: A superior ligand for human L-selectin, *Angew Chem Int Ed* **38**, 1131–3 (1999).

106 Mitsuoka C, Ohmori K, Kimura N, Kanamori A, Komba S, Ishida H, Kiso M, Kannagi R, Regulation of selectin binding activity by cyclization of sialic acid moiety of carbohydrate ligands on human leukocytes, *Proc Natl Acad Sci USA* **96**, 1597–602 (1999).

107 Sako D, Comess KM, Barone KM, Camphausen RT, Cumming DA, Shaw GD, A sulfated peptide segment at the amino terminus of PSGL-1 is critical for P-selectin binding, *Cell* **83**, 323–31 (1995).

108 Pouyani T, Seed B, PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus, *Cell* **83**, 333–43 (1995).

109 Li F, Erickson HP, James JA, Moore KL, Cummings RD, McEver RP, Visualization of P-selectin glycoprotein ligand-1 as a highly extended molecule and mapping of protein epitopes for monoclonal antibodies, *J Biol Chem* **271**, 6342–8 (1996).

110 Liu W, Ramachandran V, Kang J, Kishimoto TK, Cummings RD, McEver RP, Identification of N-terminal residues on P-selectin glycoprotein ligand-1 required for binding to P-selectin, *J Biol Chem* **273**, 7078–87 (1998).

111 McEver RP, Cummings RD, Role of PSGL-1 binding to selectins in leukocyte recruitment, *J Clin Invest* **100**, S97–103 (1997).

112 Lcppanen A, White SP, Helin J, McEver RP, Cummings RD, Binding of glycosulfopeptides to P-selectin requires sterospecific contributions of individual tyrosine sulfate and sugar residues, *J Biol Chem* **275**, 39569–78 (2000).

113 Feizi T, Oligosaccharides that mediate mammalian cell–cell adhesion, *Curr Opin Struct Biol* **3**, 701–10 (1993).

114 Needham LK, Schnaar RL, The HNK-1 reactivity Sulfoglucuronyl Glycolipids are ligands for L-selectin and P-selectin but not E-selectin, *Proc Natl Acad Sci USA* **90**, 1359–63 (1993).

115 Suzuki Y, Toda Y, Tamatani T, Watanabe T, Suzuki T, Nakao T, Murase K, Kiso M, Hasegawa A, Tanado-Aritomi K, Ishizuka I, Miyasaka M, Glycolipids are ligands for a lymphocyte homing receptor, L-selectin (LECAM1), binding epitope in sulfated sugar chain, *Biochem Biophys Res Commun* **190**, 426–34 (1993).

116 Green PJ, Yuen C-T, Childs RA, Chai W, Miyasaka M, Lcmoine R, Lubineau A, Smith B, Ueno H, Nicolaou KC, Feizi T, Further studies of the binding specificity of the leukocyte adhesion molecule, L-selectin, towards sulphated oligosaccharides—Suggestion of a link between the selectin and the integrin-mediated lymphocyte adhesion systems, *Glycobiology* **5**, 29–38 (1995).

117 Galustian C, Childs RA, Yuen C-T, Hasegawa A, Kiso M, Lubineau A, Shaw G, Feizi T, Valency dependent patterns of reactivity of human L-selectin towards sialyl and sulfated oligosaccharides of Le^a and Le^x types: Relevance to anti-adhesion therapeutics, *Biochemistry* **36**, 5260–6 (1997).

117a Somers WS, Tang J, Shaw GD, Camphausen RT, Insights into molecular basis of leukocyte tethering and rolling revealed by structures of P- and e-selectin bound to Sle(X) and PSGL-1, *Cell* **103**, 467–79 (2000).

118 Feizi T, Antigenicities of mucins–their relevance to tumour associated and stage specific embryonic antigens. In *Mucus in health and disease–II. Advances in experimental medicine and biology*, edited by Chantler EN, Elder JB, Elstein M, (Plenum Press, New York, 1982), pp. 29–37.

119 Tang PW, Gooi HC, Hardy M, Lee YC, Feizi T, Novel approach to the study of the antigenicities and receptor functions of carbohydrate chains of glycoproteins, *Biochem Biophys Res Commun* **132**, 474–80 (1985).

120 Stoll MS, Mizuochi T, Childs RA, Feizi T, Improved procedure for the construction of neoglycolipids having antigenic and lectin-binding activities from reducing oligosaccharides, *Biochem J* **256**, 661–4 (1988).

121 Magnani JL, Spitalnik SL, Ginsburg V, Antibodies against surface carbohydrates: determination of structure, *Methods in Enzymol* **138**, 195–8 (1987).

122 Feizi T, Stoll MS, Yuen C-T, Chai W, Lawson AM, Neoglycolipids: probes of oligosaccharide structure, antigenicity and function, *Methods Enzymol* **230**, 484–519 (1994).

123 Feizi T, Childs RA, Neoglycolipids: probes in structure/function assignments to oligosaccharides, *Methods Enzymol* **242**, 205–17 (1994).

124 Osanai T, Feizi T, Chai W, Lawson AM, Gustavsson ML, Sudo K, Araki M, Araki K, Yuen C-T, Two families of murine carbohydrate ligands for E-selectin, *Biochem Biophys Res Commun* **218**, 610–5 (1996).

125 Lawson AM, Chai W, Cashmore GC, Stoll MS, Hounsell EF, Feizi T, High-sensitivity structural analyses of oligosaccharide probes (neoglycolipids) by liquid-secondary-ion mass spectrometry, *Carbohydr Res* **200**, 47–57 (1990).

126 Chai W, Cashmore GC, Carruthers RA, Stoll MS, Lawson AM, Optimal procedure for combined high-performance thin-layer chromatography/high-sensitivity liquid secondary ion mass spectrometry, *Biol Mass Spectrom* **20**, 169–78 (1991).

127 Loveless RW, Holmskov U, Feizi T, Collectin-43 is a serum lectin with a distinct pattern of carbohydrate recognition, *Immunology* **85**, 651–9 (1995).

128 Feizi T, Carbohydrate-mediated recognition systems in innate immunity, *Immunol Rev* **173**, 79–88 (2000).

129 Feizi T, 'Glyco-epitope' assignments for the selectins: advances enabled by the neoglycolipid (NGL) technology in conjunction with synthetic carbohydrate chemistry, *Adv Exp Med Biol*, **491**, 65–78 (2001).

130 Spillmann D, Hard K, Thomas OJ, Vliegenthart JFG, Misevic G, Burger MM, Finne J, Characterization of a novel pyruvylated carbohydrate unit implicated in the cell aggregation of the marine sponge Microciona prolifera, *J Biol Chem* **268**, 13378–87 (1993).

131 Chai W, Feizi T, Yuen C-T, Lawson AM, Nonreductive release of *O*-linked oligosaccharides from mucin glycoproteins for structure/function assignments as neoglycolipids: Application in the detection of novel ligands for E-selectin, *Glycobiology* **7**, 861–72 (1997).

132 Yuen C-T, Chai W, Loveless RW, Lawson AM, Margolis RU, Feizi T, Brain contains HNK-1 immunoreactive *O*-glycans of the sulfoglucuronyl lactosamine series that terminate in 2-linked or 2,6-linked hexose (mannose), *J Biol Chem* **272**, 8924–31 (1997).

133 Chai W, Yuen CT, Kogelberg H, Carruthers RA, Margolis RU, Feizi T, Lawson AM, High prevalence of 2-mono- and 2,6-di-substituted Manolterminating sequences among O-glycans released from brain glycopeptides by reductive alkaline hydrolysis, *Eur J Biochem* **263**, 879–88 (1999).

134 Stoll MS, Feizi T, Loveless RW, Chai W, Lawson AM, Yuen C-T, Fluorescent neoglycolipids: improved probes for oligosaccharide ligand discovery, *Eur J Biochem* **267**, 1795–804 (2000).

134a Leteux C, Chai W, Nagai K, Lawson AM, Feizi T, 10E4 antigen of scrapie lesions contains an unusual non-sulphated heparan motif, *J Biol Chem* in press.

135 Mizuochi T, Loveless RW, Lawson AM, Chai W, Lachmann PJ, Childs RA, Thiel S, Feizi T, A library of oligosaccharide probes (neoglycolipids) from N-glycosylated proteins reveals that conglutinin binds to certain complex type as well as high-mannose type oligosaccharide chains, *J Biol Chem* **264**, 13834–9 (1989).

136 Childs RA, Drickamer K, Kawasaki T, Thiel S, Mizuochi T, Feizi T, Neoglycolipids as probes of oligosaccharide recognition by recombinant and natural mannose-binding proteins of the rat and man, *Biochem J* **262**, 131–8 (1989).

137 Solis D, Feizi T, Yuen CT, Lawson AM, Harrison RA, Loveless RW, Differential recognition by conglutinin and mannan-binding protein of *N*-glycans presented on neoglycolipids and glycoproteins with special reference to complement glycoprotein C3 and ribonuclease B, *J Biol Chem* **269**, 11555–62 (1994).

138 Solis D, Bruix M, Gonzalcz L, Diaz-Maurino T, Rico M, Jimenez-Barbero J, Feizi T, Carrier protein-modulated presentation and recognition of an N-glycan. Observations on the interactions of Man8 glycoform of ribonuclease B with conglutinin, *Glycobiology* **11**, 31–6 (2001).

139 Gonzalcz L, Bruix M, Diaz-Maurino T, Feizi T, Rico M, Solis D, Jimenez-Barbero J, Conformational studies of the Man8 oligosaccharide on native ribonuclease B and on the reduced and denatured protein, *Arch Biochem Biophys* **383**, 17–27 (2000).

140 Schachter H, The joys of HexNAc. The synthesis and function of N- and O- glycan branches, *Glycoconj J* **17**, 465–83 (2000).

141 Lindahl U, "Heparin"—from anticoagulant drug into new biology, *Glycoconj J* **17**, 597–605 (2000).

142 Hascall VC, Hyaluronan: A common thread, *Glycoconj J* **17**, 607–16 (2000).

143 Beeson JG, Chai W, Rogerson SJ, Lawson AM, Brown GV, Inhibition of binding of malaria-infected erythrocytes by a tetradecasaccharide fraction from chondroitin sulfate A, *Infect Immun* **66**, 3397–402 (1998).

144 Sharon N, Ofek I, Safe as mother's milk: Carbohydrates as future antiadhesion drugs for bacterial disease, *Glyconjugate J* **17**, 659–64 (2000).

145 Maly P, Thall AD, Petryniak B, Rogers CE, Smith PL, Marks RM, Kelly RJ, Gersten KM, Cheng G, Saunders TL, Camper SA, Camphausen RT, Sullivan FX, Isogai Y, Hindsgaul O, von Andrian UH, Lowe JB, The alpha(1,3)fucosyltransferase Fuc-TVII controls leukocyte trafficking through an essential role in L-, E-, and P-selectin ligand biosynthesis, *Cell* **86**, 643–53 (1996).

146 Hemmerich S, Rosen SD, Carbohydrate sulfotransferases in lymphocyte homing, *Glycobiology* **10**, 849–856 (2000).

146a Muramatsu T, Protein-bound carbohydrates on cell-surface as targets of recognition: an odyssey in understanding them, *Glycoconj J* **17**, 577–95, (2000).

147 Augé C, Dagron F, Lemoine R, Le Narvor C, Lubineau A, Syntheses of sulfated derivatives as sialyl Lewis^a and sialyl Lewis^x analogues, *Carbohydrate mimics: concepts and methods. Verlag Chemie Weinheim, RFA* 365–83 (1997).

148 Sears P, Wong CH, Carbohydrate Mimetics: A New Strategy for Tackling the Problem of Carbohydrate-Mediated Biological Recognition, *Angew Chem Int Ed Engl* **38**, 2300–24 (1999).