BLOOD GLYCOCONJUGATE RECOGNITION BY TISSUE CARBOHYDRATE BINDING PROTEINS: GLYCOPROTEIN RECOGNITION BY HUMAN HEART GALECTIN-1

A thesis submitted to

The Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram in partial fulfilment of the requirements of the degree of Doctor of Philosophy in the subject of Biochemistry

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JULY 2005

CERTIFICATE

I, SANGEETHA.S.R hereby certify that I had personally carried out the work described in the thesis entitled "BLOOD GLYCOCONJUGATE RECOGNITION BY TISSUE CARBOHYDRATE BINDING PROTEINS: GLYCOPROTEIN RECOGNITION BY HUMAN HEART GALECTIN-1"

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DECLARATION

This is to certify that Ms. Sangeetha.S.R of the department of Biochemistry of this institute has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph.D degree of the Sree Chitra Tirunal Institute for medical Sciences and Technology, Thiruvananthapuram. The work relating to the thesis entitled "BLOOD GLYCOCONJUGATE RECOGNITION BY TISSUE CARBOHYDRATE BINDING PROTEINS: GLYCOPROTEIN RECOGNITION BY HUMAN HEART GALECTIN-1" was carried out under my direct supervision.

20. July. 2005

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The thesis entitled

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ABBREVIATIONS

AHIg	:	anti human immunoglobulin
Anti -α-Gal	:	anti -α-galactoside antibody
BSA	:	bovine serum albumin
CLGG	:	cross-linked guar gum
Con A	:	concanavalin A
CRD	:	carbohydrate recognition domain
DIg	:	dextran-binding immunoglobulin
DJPGP	:	desialylated jacalin binding-plasma glycoproteins
EDTA	:	ethylene diamine tetra acetic acid
Gal	:	galactose
GalNAc	:	N-acetyl galactosamine
Glc	:	glucose
GlcNAc	:	N-acetyl glucosamine
GP IIbα	:	glycoprotein IIbα
GP IIb/IIIa	:	glycoprotein IIb/IIIa
HHL	:	human heart 14 kDa galactose-binding lectin
HHL-G	:	human heart 14 kDa galactose-binding lectin along with endogenous glycoproteins
HRP	:	horse radish peroxidase
IgA1	:	immunoglobulin A1
IgA1P	:	IgA1 purified using polystyrene-immobilized jacalin
IgA1S	:	IgA1 purified using Sepharose-immobilized jacalin
IgA2	:	immunoglobulin A2
IgG	:	immunoglobulin G
IgM	:	immunoglobulin M

IL-1β	:	interleukin 1, β
JPGP	:	jacalin-binding plasma glycoproteins
JRBCGP	:	jacalin-binding erythrocyte membrane glycoproteins.
LacNAc	:	N-acetyl lactosamine
Man	:	mannose
Man-6-P	:	mannose -6-phosphate
2-ME	:	2-mercaptoethanol
1-O-methyl α-Gal	:	1-O- methyl- α-D galactopyranoside
1-methyl -β-Gal	:	1-O-methyl-β-D-galactopyranoside
NAc	:	N- acetyl moiety
NANA	:	N-acetyl neuraminic acid
NeuNAc	:	N-acetyl neuraminic acid
OPD	:	orthophenylene diamine.
PAGE	:	polyacrylamide gel electrophoresis
PBS	:	20 mM potassium phosphate buffer pH 7.4 containing 150 mM NaCl
PMSF	:	phenyl methyl sulphonyl fluoride
PNA	:	peanut agglutinin
PNP-α-Gal	:	Para- nitro phenyl-α-D-galactopyranoside
PNP-β-Gal	:	Para-nitro phenyl-β-D galactopyranoside
PVDF	:	polyvinylidene difluoride
RCA1	:	*Ricinus communis* agglutinin
SDS	:	sodium dodecyl sulphate
T antigen	:	Thomsen-Friedenreich antigen
TAG	:	terminal α-linked galactose moiety
TEMED	:	N, N, N', N'-Tetra methyl ethylenediamine

CHAPTER I
INTRODUCTION

INTRODUCTION

Complex carbohydrates are widely distributed in animal tissues. There is extensive glycosylation of proteins in the serum and extracellular matrix and of proteins and lipids at the cell surface [Sharon and Lis, 1982; Hakomori, 1981]. Role for complex carbohydrates has been implicated in recognition processes including cell-cell and cell-extracellular matrix adhesion and cell surface receptor recognition by ligand-bearing biomolecules [Yamada, 1983; Edelman, 1985; Hook et al., 1984; Florman and Wassarman, 1985]. In addition, carbohydrates are recognized as differentiation markers and antigenic determinants [Feizi, 1981; Feizi and Childs, 1987]. They have an enormous potential for encoding biological information. In peptides and oligonucleotides, the information content dictated by the degree of structural variation is based only on the number of monomeric units and their sequence, whereas in carbohydrates information is also encoded in the position and anomeric configuration of the glycosidic units and in the occurrence of branch points. Alternative isomeric linkages between sugars and the formation of branched structures can generate an enormous number of structures from even a small number of saccharide units [Hughes, 1983]. Animal lectins, which by definition have the potential to decode the information found in carbohydrate sequences, are complementary to carbohydrates in the biological recognition discussed above and have been widely investigated in the recent decades.

Lectins are proteins, capable of specific recognition of and reversible binding to carbohydrate moieties of complex glycoconjugates without altering the covalent structure of any of the recognized glycosyl ligands. They constitute a super family of ubiquitously distributed proteins that exhibit a specific carbohydrate binding activity. They combine reversibly and non-covalently with mono- or oligosaccharides, both simple and complex whether free in solution or on cell surfaces. Sugar binding of lectins may involve several forces, mostly hydrophobic and hydrogen bonds; only rarely electrostatic forces are involved since most carbohydrates recognized on glycoconjugates are devoid of prominent electrical charge. Lectins are known to recognize not only terminal sugar moieties, but inner ones as well of the oligosaccharide sequence [Sharon and Lis, 1989]. Lectin carbohydrate interactions satisfy additional requirements expected of a cellular recognition system, such as speed and reversibility.

Lectins were first described in 1888 by Stillmark working with castor bean extracts. Many members of the lectin family agglutinate red blood cells as a result of three-dimensional cross-linking of the cells. Some lectins can bind to cells and not cause agglutination, as exemplified by ricin from castor beans, which possesses only one sugar binding site per molecule and by lectins chemically modified to reduce their binding sites to one [Mandal and Brewer, 1993]. Since agglutination of cells is the assay

most generally employed to measure the activity of lectins, many non-agglutinating lectins may have escaped detection. Multivalency may not be an absolute requirement for biological activity as seen in the case of ricin, even though it is still an important factor for most lectins. Although lectins are similar to carbohydrate-binding antibodies in their ability to bind reversibly to glycoconjugates, they differ from immunoglobulins in being non- immune in origin and diverse in structure [Barondes, 1981; Mirelman and Ofek, 1986; Sharon, 1977]. The specificity of a lectin is defined in terms of the monosaccharide(s) or simple oligosaccharide, which inhibits the lectin-induced cell agglutination or precipitation reaction. Such specific inhibitors are commonly effective at concentrations in the millimolar range or lower. However sometimes a specific oligosaccharide structure involved in the binding of a lectin to a biological glycoconjugate might be quite different from its best monosaccharide inhibitor [Molin et al., 1986]. The specificity is not absolute, so that each lectin has a spectrum of sugars or sugar sequences which it can recognize. The differential binding of sugars to lectins depends on subtle changes in disposition of amino acid side chains near the sugar binding sites. Lectins have been purified from extracts of many tissues and cells by affinity chromatography on immobilized monosaccharides, oligosaccharides or glycoproteins [Harrison and Chesterton, 1980; Barondes 1981,1984; Ashwell and Harford, 1982].

Lectins generally have no apparent catalytic activity like enzymes, but a significant number of exceptions are evident now. Ricin, the oldest known lectin, is the enzyme RNA-N-glycosidase [Endo et al., 1987; 1988; Endo and Tsurugi, 1987] and galectin-10 is lysophospholipase [Leffler, 1997].

Many lectins have been isolated and characterized from plants and animals. In general, animal lectins are more labile than plant lectins. Based on structural information and requirements for ligand binding, animal lectins are divided into five groups [Drickamer, 1988]: (i) The C-type lectins (including the selectins) containing a carbohydrate recognition domain with 18 conserved amino acid residues and exhibiting Ca^{2+} dependent ligand binding, (ii) P-type lectins, which derive their name from their ability to bind phosphorylated mannose residues, (iii) I type lectins including siglecs and other immunoglobulin- like sugar binding proteins, (iv) L-type lectins related in sequence to the leguminous plant lectins and (v) The galectins or the S-type lectins.

Galectins are a family of related carbohydrate binding proteins found in all metazoans, including sponges, invertebrates, fungi and mammals [Cooper, 2002; Leffler, 2001]. This wide distribution among species coupled with a highly evolutionary conserved sequence within the carbohydrate recognition domain, suggests that galectins are involved in biologically crucial and therefore evolutionarily conserved biological processes [Barondes et al., 1994a,b; Kasai and Hirabayashi, 1996;

Kilpatrick, 2002]. However, the in vivo biological functions of galectins in mammals remain enigmatic and the function of a given galectin can vary from site to site depending on the nature of available ligands. Although there have been many studies on bovine galectin-1, there have only been a few studies to date on the carbohydrate-binding specificity of human galectin-1 [Hirabayashi et al., 2002; Kopitz et al., 1998; Lee et al., 1990]. There is also uncertainty surrounding the carbohydrate-binding requirements of the galectins, particularly of galectin-1, the first galectin family member identified. Moreover the number of endogenous human galectin-1- binding proteins characterized so far is very limited. Elucidation of the physiological function of the galectin-1 would require identification and characterization of its endogenous glycoconjugate receptors.

Blood cells and plasma proteins being in circulation have the maximum opportunity for encounter with tissue galectins. In addition, most blood cell surface and plasma glycoproteins are heavily glycosylated, with few notable exceptions like hemoglobin and albumin. Therefore in the search for galectin-1-interacting glycoproteins and the specific sugar group involved in this interaction, the present study focused attention on blood cell surface and plasma glycoproteins. Another reason for concentrating on blood component glycoproteins was that a balanced distribution of N- and O-glycosylations had been noted on them unlike on other tissue glycosylated proteins such as mucins or proteoglycans.

OBJECTIVES OF THE STUDY

1. Purification of human heart galectin-1 and characterization of sugar-specifically bound endogenous glycoproteins that co-purify with the lectin during affinity chromatography.

2. Demonstration of T antigen (Gal β1→3 Gal NAc) specificity of human heart galectin-1.

3. Study of interaction of human galectin-1 with erythrocyte and erythrocyte membrane glycoproteins.

4. Preparation of human erythrocyte membrane glycopeptides and demonstration of their galectin-1 inhibition.

5. Identification of galectin-1-interacting glycoprotein subunits on human platelet and lymphocyte membranes.

REVIEW OF LITERATURE

GALECTINS

Galectins, formerly known as S-type lectins or galaptins, are a family of β-galactosyl-binding lectins characterized by a typical motif of conserved amino acids in their carbohydrate recognition domain(s) (CRDs) [Hirabayashi and Kasai, 1993; Barondes et al., 1994b; Hirabayashi, 1996; Leffler, 1997]. Most galectins require a reducing environment but not divalent cations for their binding activity. This reducing environment is to prevent oxidation of a particular –SH group that is crucial for their sugar-binding activity. Although initially described in vertebrate taxa, their presence has since been documented in prochordates, invertebrates, and fungi [Hirabayashi et al. 1992a, 1992b, 1996; Pfeifer. et al., 1993; Klion and Donelson, 1994; Cooper et al., 1997; Newton et al .,1997] suggesting that they are widely distributed in nature. Almost all galectins discovered so far are small (14-36 kDa) soluble proteins with one or two CRDs. The first galectins were discovered as part of a quest to find proteins recognizing cell surface carbohydrates thought to be involved in cell adhesion [Ginsburg and Neufeld, 1969; Barondes, 1970; Roseman, 1970]. Tissue or cell extracts which tested positive for agglutinating activities were subjected to affinity chromatography on immobilized β-galactosides and bound proteins eluted

with lactose, a β-galactoside that resembled known structures on cell surface [Barondes, 1984; Barondes, 1997]. In this way a 14 kDa protein, now known as galectin-1 was first isolated from electric eel by Teichberg et.al in 1975 [Teichberg, 1975]. After this investigation a number of β-galactoside binding lectins were identified in tissues from rat, chick, bovine, human, etc and their biochemical properties and histological distributions were studied in relation to development and differentiation.

Galectins are predominantly, cytoplasmic proteins and lack a trans- membrane segment [Ohyama et al., 1986; Cleves et al., 1996]. They also hold many features of cytoplasmic proteins ie, have no disulphide bridges, no sugar chains, no signal sequences, biosynthesis occurs on free ribosomes and in most cases their N-terminal amino acids are acetylated. However, their histological localization is diverse and they occur not only in cytoplasm but also in nuclei, on cell surfaces and in extra cellular spaces depending on galectin species. The occurrence of vertebrate lectins both in the cytoplasm and on the surface of various cells [Barondes, 1984; Zanetta et al., 1984; Zanetta et al., 1985] suggests their role in cell-cell recognition, cellular adhesion and cell migration [Cooper et al., 1991; Skrincosky et al., 1993; Hughes, 1992] which are important during immune defense, microbial infection, tumorigenesis and metastasis. Cell-cell adhesion mediated by binding of added soluble ligand glycoprotein to galectins at the cell surface has also been proposed.

In 1994, it was proposed that mammalian galectins be numbered in the order of discovery [Barondes, 1994a]. This numbering system cannot be applied directly to non-mammalian galectins because it is difficult to correspond non-mammalian galectins to mammalian ones and vice versa. Fourteen galectins have been identified in mammals and some organisms such as *Caenorhabditis elegans* may have many more [Cooper and Barondes, 1999; Rabinovich et al., 2002a]. The evolutionary conservation of galectins likely reflects the essential roles of galectins in development and function of multicellular organisms including cell adhesion, migration, differentiation and death [Perillo et al., 1998; Leffler, 2001; Goldring et al., 2002]. The most common galectin having a single CRD per subunit is galectin-1, which is suggested to have growth regulatory and immunomodulatory activities [Wells and Mallucci, 1991; Lutomski et al., 1995]. The principal physiological roles of this protein in humans remain unknown. It has been suggested that they differ according to whether galectin-1 is predominantly monomeric or dimeric [Perillo et al., 1995].

Galectin-1 behaves as a stable dimer. However, hamster galectin-1 was found to dissociate into monomers with a Kd of about 7μM [Cho and Cummings, 1995]. Other species of galectin-1 may also dissociate into monomers but perhaps at lower concentrations. Galectin-1 may, hence be dimeric and cross-link ligands if present at a high enough concentration,

which is well within the range found in nature. But it may also act as a monomer at lower concentrations as suggested [Giudicelli et al., 1997; Blaser et al., 1998]. Brewer et.al have demonstrated that galectin-1, like some plant lectins can form ordered arrays with glycoproteins and based on this sorting out of one glycoprotein from another can occur [Gupta et al., 1996]. Galectin-3 is isolated as a monomer but undergoes multimerization on binding to surfaces that contain glycoconjugate ligands and the N-terminal half of the protein is required for this property [Massa et al., 1993].

Many vertebrate tissues contain β-galactoside binding lectins, which are extractable with lactose and are specifically inhibited by β-galactoside containing saccharides. Although all galectins bind lactose, a considerable diversity in their carbohydrate-binding specificities has been recognized [Leffler and Barondes, 1986; Oda et al., 1993; Ahmed and Vasta, 1994]. It has been proposed that these subtle differences in carbohydrate-binding patterns may reflect the different endogenous ligands recognized by various galectin types and ultimately their distinct biological roles. For example laminin is the most likely endogenous ligand for galectin-1, a lectin proposed to mediate muscle development [Cooper et al., 1991] and induce apoptosis of activated T cells by binding with CD45 glycoprotein [Perillo et al., 1995]. In contrast, mucin, IgE, fibronectin, cytokeratin and

laminin have been shown to interact in vitro with galectin-3 [Sato and Hughes, 1992; Bresalier et al., 1996] a lectin proposed to inhibit apoptosis [Yang et al., 1996].

The galectins are characterized by their hemagglutinating activity and the carbohydrate binding activity of galectins is essential for many of the family's functions. These lectins usually require a thiol-reducing reagent such as 2- mercaptoethanol or dithiothreitol for maintenance of their hemagglutinating activity [Barondes, 1984,1986], so that they were designated as S-type lectins [Drickamer, 1988]. Though many scientists adopted this terminology, it was not always relevant. First, galectin-3 never requires a thiol-reducing reagent for the maintenance of activity [Frigeri et al., 1990]. Second, Whitney et al. [1986] reported that galectin-1 became resistant to oxidative inactivation when it was treated with a cysteine–modifying reagent, monoiodoacetamide . Finally, Hirabayashi and Kasai, [1991] proved no cysteine residue is required for the sugar binding function by means of site directed mutagenesis, substituting one of the 6 Cys with Ser with no change in sugar binding activity. According to the present knowledge a few reactive cysteine residues are critical for oxidative inactivation; these residues form abnormal disulphide bridge(s) to cause drastic change in the three dimensional structure leading to inactivation. Though the physiological significance of this phenomenon is not known, it

is possible that the lectin is regulated by the oxidative state of its environment [Hirabayashi et al., 1987]. An attractive speculation is that change in conformation may switch on another function of the protein [Hirabayashi and Kasai, 1993]. Levi and Teichberg [1981] reported that electric eel lectin does not contain any cysteine residue, although it still requires a thiol-protecting reagent for maintenance of hemagglutinating activity. They suggested that a single tryptophan residue is implicated in the binding. Inactivation of electrolectin was linked to oxidation of tryptophan side chain to form an oxindole derivative. Oxidation is prevented by lactose and the fluorescence data and pH dependence of lactose binding suggest the tryptophan residue interacts directly with the saccharide [Abbott and Feizi, 1991; Levi and Teichberg, 1981]

Immunochemical studies with conventional antisera and aminoacid analysis have suggested that the β-galactoside binding lectins among phylogenetically related species are antigenically and structurally related [Childs and Feizi, 1979; Levi and Teichberg, 1982; Roff and Wang 1983]. The molecular properties of vertebrate galactose-binding lectins are strikingly similar from chicken to cow to man [Barondes, 1981]. The amino acid identity in the carbohydrate–binding domains among different known galectins from one mammalian species ranges from about 20 to 40% [Oda et al, 1993]. The aminoacid sequence homology of the same galectin

from different mammalian species is 80-90%. Among the amino acid residues that are substantially conserved among various galectins, His^{44}, Asn^{46}, Arg^{48}, Asn^{61}, Trp^{68}, Glu^{71}, and Arg^{73} (residue numbers are those of bovine spleen) [Liao et al., 1994] are recognized as critical for sugar binding [Lobsanov et al., 1993; Bourne et al., 1994], whereas Ser^{29}, Phe^{30}, Asn^{33}, His^{44}, Asn^{46}, Arg^{48}, Asn^{61} and Arg^{111} interact with each other to provide the architecture of the CRD.

Fourteen kDa lectin is abundantly expressed in a variety of vertebrate organs including rat and human brain. Its distribution in the central nervous system is developmentally regulated [Joubert et al., 1987,1988, 1989; Kuchler et al., 1989]. In rat brain, this lectin is predominantly expressed inside the neuron cytosol and in neuritic processes. It is also transiently secreted and found on the surface of neuronal cells. The 14 kDa lectin presumably participates in intracellular traffic of glycosylated molecules in nerves. It is possible that cytosolic lectin also plays a role in the construction of the cytoskeleton since the lectin was shown to bind sugar independently to actin molecules [Joubert, 1992]. Reversible association of human brain galectin-1 (HBL) with both actin and glycoconjugates was proposed as a mechanism to regulate actin metabolism in brain though further work on this hypothesis has not been reported. It has been suggested that galectin-1 participates in neurite

fasciculation, synaptogenesis [Joubert et al 1989], construction of the cytoskeleton [Joubert et al., 1992] and interactions with the extracellular matrix [Avellana-Adalid et al., 1994]. In the peripheral nervous system only the oxidized form of galectin-1 promoted axonal regeneration [Horie et al., 1999] and the carbohydrate binding activity of galectin-1 was not necessary for axonal regeneration. But the reduced form did not show such activity. On the contrary the carbohydrate binding activity of galectin-1 was essential for astrocyte differentiation and production of brain-derived neurotrophic factor (BDNF) [Tasuku Sasaki et al., 2004], a neuro protective polypeptide, which appears to play an important role in the survival, differentiation and synaptic plasticity of neurons [Barde, 1989; Ming et al 2002; Thoenen, 1995]. These results indicate that galectin-1 is a bifunctional protein and plays different roles depending on whether it is in the oxidized or reduced form.

So far, the secretory pathway of galectins remains unknown. One of the reasons is that all known galectins lack signal peptides necessary for their insertion into the endoplasmic reticulum membrane and its subsequent secretion via the classical pathway [Cleves et al., 1996]. An alternative pathway for secretion being considered is membrane blebbing [Cooper and Barondes, 1990; Mehul and Hughes, 1997]. When galectin-1 secretion starts during muscle cell development, the galectin is first accumulated underneath

the plasma membrane, then in small membrane evaginations, the blebs, which are about 2 μm in diameter. These are subsequently released from the cell and their membrane disintegrates, releasing the lectin. Nothing is known about what triggers this process, its mechanisms or how the protein to be secreted gets specifically targeted to the blebs. Recently a sequence in the N-terminal domain of galectin-3 has been proposed as a determinant for its secretion [Menon and Hughes, 1999]. However the presence of such secretion signal sequence in other galectins and the precise mechanism of galectin-3 secretion remain unknown.

The reason galectins are secreted by non-classical pathways is not known. One possibility is to segregate them from complementary glycoconjugate ligands so that galectins and the ligands interact only after externalization. Another possibility is that, in contrast with the single classical secretion pathway, there may be multiple non-classical secretory mechanisms so that different galectins in a cell might be selectively secreted in response to specific signals, as suggested for other proteins [Rubartelli et al., 1992.]. It is well known that tumor necrosis factor (TNF) α and IL-1β also lack signal peptide like galectins. Non-classical secretion of galectin-1 has been studied in skeletal muscle, where the protein moves from a diffusely intracellular to an extracellular location during in vivo development. In cultured myoblasts, galectin-1 remains in the cytosol until

it is externalized during differentiation, apparently by membrane evagination [Cooper and Barondes, 1990; Harrison and Wilson, 1992]. Extracellular galectins may modulate cell adhesion and induce intracellular signals by cross-linking cell-surface and extracellular glycoproteins, possibly forming supramolecular ordered arrays [Sacchettini et al., 2001].

On the basis of structural architecture, β-galactoside binding lectins are classified into three; proto, chimera and tandem repeat types [Hirabayashi and Kasai, 1993]. The proto type, which is the best-studied type and is often called the 14 kDa type because all members so far studied have mobilities corresponding to 14 kDa on sodium dodecyl sulphate polyacrylamide gel electrophoresis. Lectins belonging to this type have a single CRD and are presumed to form non-covalent dimers under physiological conditions. The chimera type lectins are multidomain proteins consisting of a lectin domain and another non-lectin domain(s). Mammalian lectins having a 29-35 kDa subunit size are chimeras consisting of a C-terminal lectin domain and an N-terminal domain homologous to components of heterogeneous nuclear ribonucleoprotein complex (hnRNP) and the second exon of L-myc gene product. Galectin-3 is the only chimeric galectin identified in mammals. The tandem-repeat type was found for the first time in *C.elegans*. The nematode 32 kDa lectin is composed of two tandemly repeated lectin domains, which show similar extents of homology

to vertebrate lectins (25-30% identities). The linker regions connecting the two domains are likely to be susceptible to proteases and to generate truncated proto-type products. Tandem repeats should be the result of gene duplication and the two domains would subsequently have diverged.

X-ray crystallography of several galectins has shown that their carbohydrate recognition domains (CRD) consist of about 134 amino acids arranged in a tightly folded conserved β-sandwich structure formed by a six strand sheet (S1–S6) and a five strand sheet (F1–F5), with conserved carbohydrate-binding amino acids in strands S4–S6 [Rini and Lobsanov, 1999; Loris, 2002]. Most galectins have a core-binding site for lactose and related disaccharides. The galactose residue is most tightly bound and interacts with the protein along one side via hydrogen bonds involving OH on C4 and C6, the ring O, and van der Waals interaction between the hydrophobic patch formed by CH 3-5 and a Trp or Tyr in the protein.The glucose residue also interacts, mainly via OH3 and for most galectins lactose binds with about 100 fold higher affinity (Kd 0.5 mM) than galactose alone. However there is some room for variation of the Glc residue. If the C-2 hydroxyl of the latter is replaced by acetamido group to form GlcNAc, the affinity goes up by a factor of about ten for some galectins but not others, due to interactions involving the NAc group. Gal bound β1→3 to GlcNAc also binds well because here OH4 of GlcNAc takes the steric place of OH3 of GlcNAc in LacNAc (Gal β1→4 GlcNAc).

The carbohydrate binding specificities of galectin-1 and -3 are similar in that both of them bind to LacNAc and lactose residues [Barondes et al., 1994b; Gabius,1997; Kasai and Hirabayashi, 1996]. However differences exist, including the observation that galectin-3 binds to internal and terminal LacNAc residues in polylactosamine chains, whereas galectin-1 prefers non- reducing terminal LacNAc residues [Ahmad et al., 2002]. Galectin-1 did not bind to polylactosamine sequences terminating in either a β1-3-linked GlcNAc residue or a β1-4-linked GalNAc residue indicating the requirement for a terminal non-reducing β1-4-linked Galactose residue. Galectin-1 could bind to polylactosamines, provided the chains have a length of at least 3 repeating lactosamine units [Zhou and Cummings, 1993]. Polylactosamine sequences are selectively expressed on glycoproteins such as laminin and lysosome-associated membrane proteins. The terminal sequences of many complex type N-glycans in most mammalian glycoproteins contain the non-reducing LacNAc motif, which in turn is further modified by sialylation, fucosylation, galactosylation and sulphation [Kornfeld and Kornfeld, 1985; Spiro, 2002]. Such modifications of the LacNAc unit may be important in regulating the affinity and thereby the degree of biological activity of galectin-1 towards these LacNAc containing ligands. Chondroitin sulphate B that lacks any LacNAc motif was recently identified as a potential human galectin-1 ligand [Moiseeva et al., 2003].

The galectin composition varies between different cell types, but all cells appear to express at least one galectin. Galectin-1 is abundant in

adult muscle and other cells of mesodermal origin; galectin-3 is abundant in various epithelial cells and in macrophages; galectin-4 is confined to epithelial cells of the alimentary tract and galectin-7 is found in epidermis [Colnot et al., 1996;Gitt et al., 1998a; Cooper and Barondes, 1999; Timmons et al., 1999]. Much less is known about the distribution of galectin-2, which is expressed in hepatomas. Galectin-3 surface expression has been shown in normal human monocytes and its level increases as monocytes differentiate into macrophages [Liu et al., 1995]. High expression is seen in a few selected cell types and stages for each galectin during development. Galectin expression can also be induced by various stimuli [Chiariotti et al., 1999]. For example, inflammatory mediators modulate galectin-3 [Cherayil et al., 1989; Liu, 1993; Liu et al., 1995] and galectin-9 that acts as a potent chemoattractant selectively for eosinophils is induced by allergic stimulation in monocytes [Matsumoto et al., 1998].

The general designation of the genes encoding galectins is LGALS (lectin, galactoside-binding, soluble) and gene numbering is being kept, consistent with the numbering of the proteins so that LGALS1 encodes galectin-1, etc. There is likely to be only one gene for the soluble 14 kDa galactoside binding lectin in each species [Abbott and Feizi, 1989]. In humans LGALS1 and LGALS2 have been mapped to the q12-q13 region of chromosome 22 [Mehrabian et al., 1993], and LGALS3 has been mapped

to chromosome 1p13 [Raz et al., 1991]. In the mammalian galectin genes published so far, the CRDs are encoded by three consecutive exons [Gitt and Barondes, 1991; Barondes et al., 1994b; Gitt et al., 1998b]. The middle exon encodes almost all the conserved residues that make up the galectin signature and are known to interact with bound carbohydrates [Lobsanov et al., 1993; Barondes et al., 1994b].

Existence of metal-independent β-galactoside binding lectins in invertebrate [Hirabayashi, et al., 1992a, 1992b] widened the horizon of research on this lectin family, implying that this family existed at a very early stage in the evolution of animal species and consequently that it may have a fundamental role (s) in almost all animals. Based on deletion mutagenesis analysis of the bovine 14 kDa lectin, Abbott and Feizi concluded that almost all of the polypeptide chain is necessary for the integrity of the CRD [Abbott and Feizi, 1991]. In this context the central region is the most important, but may not be sufficient to express full activity.

Programmed cell death or apoptosis is indispensable for proper development of multicellular organisms. To date, only two families of proteins have been described as death-inducing ligands: the tumor necrosis factor (TNF) family of proteins and the galectin family [Zimmermann and Green, 2001; Rabinovich et al., 2002b]. TNF ligands bind to cognate TNF

receptor polypeptides to initiate cell death. In contrast proapoptotic galectins bind to specific saccharide ligands on cell surface glycoproteins and or glycolipids to initiate cell death. Inside the cell, additional families of proteins promote or prevent death initiated by extracellular death ligands and receptors. Similar to the Bcl family of proteins, which is the best-characterized family of intracellular death regulators, galectins also function intracellularly to promote cell survival or cell death [Yang et al., 1996; Kuwabara et al., 2002]. Galectins are unique among molecules regulating cell viability because they act both outside the cell to initiate death signals and inside the cell to regulate susceptibility to death. The multivalent nature of the galectins facilitates the glycan cross linking believed to be essential in initiating cell signals, including those leading to death. Galectin-1, -7, -8, -9 and –12 are all proapoptotic. Galectin-3 is the only antiapoptotic family member. Recombinant human galectin-1 induced apoptosis of activated human T cells [Duvall and Wyllie ,1986]. Membrane blebbing, margination of chromatin and cell shrinkage were visible within 90 min after galectin–1-treatment of T cells and apoptotic bodies appeared within 5 h.

Cell death is a critical process regulating T cell development in the thymus and in controlling the immune response in the periphery. Over 90% of developing T cells (thymocytes) die in the thymus while learning to distinguish self from non-self. Both galectin-1 and galectin-9 are expressed by thymic epithelial cells in the cortex and kill thymocytes

[Baum et al., 1995a; Perillo et al., 1997;Wada et al., 1997]. Galectin-1 and galectin-9 may therefore participate in selection events critical for the development of a functional and self tolerant T cell repertoire and contribute to the elimination of antigen activated peripheral T cells because galectin-1 killed activated but not resting human T cells and galectin-9 killed activated T cells in a murine nephritis model [Perillo et al., 1995; Tsuchiyama et al., 2000]. CD45, a T cell glycoprotein appears to be essential for triggering galectin-1 induced apoptosis. Multiple isoforms of CD45 are created by alternative splicing of exons in the N-terminal domain of the molecule [Trowbridge and Thomas, 1994]. Expression of specific isoforms distinguishes thymocytes and T cells at different points in the maturation and activation pathway. Galectins may therefore regulate immune homeostasis both during development in the thymus and in the periphery. Both galectin-1 and galectin-3 trigger a calcium flux in T cells [Dong and Hughes, 1996; Pace et al., 2000; Walzel et al., 2000]. Galectin-1 binding to neutrophils results in increased production of reactive oxygen species [Timoshenko et al., 1997; Almkvist et al., 2002] and that to T cells results in increased AP-1 DNA binding activity [Rabinovich et al., 2000]. The rapid time course of galectin-1- induced death makes it unlikely that de novo transcription is required for the initiation of death, but transcription may be required for other galectin-1 effects [Pace et al., 1999].

Galectins have been used successfully as therapeutics in several T_H1 mediated autoimmune disease models. Before the effects of galectin-1 on T cells were known, galectin-1 administration was used to treat animal models of myasthenia gravis and encephalomyelitis [Levi et al., 1983; Offner et al., 1990]. Galectin-1 treatment resulted in decreased antigen-induced T cell proliferation. The antigen-specific T cell lines capable of transferring disease could not be selected from galectin-1-treated animals, suggesting that administration of galectin-1 led to the deletion or anergy of the autoreactive T cells [Levi et al., 1983; Offner et al., 1990]. In some animal models, galectin-1 treatment resulted in a decrease in T_H1 cytokines and an increase in T_H2 cytokines suggesting that galectin-1 may preferentially suppress or delete T_H1CD 4+ T cells. In vivo, galectin-1 therapy ameliorated disease in models of hepatitis, nephritis, arthritis, inflammatory bowel disease, and multiple sclerosis [Hernandez and Baum, 2002; Santucci et al., 2003].

In addition to the, the S-type lectins, which are ubiquitous and are characterized by distinct molecular weight and isoelectric point, additional lectins have been isolated from some tissues, such as lung. Six isoforms of bovine galectin-1 have been identified in the spleen, whereas in the heart only four isoforms can be detected based on their isoelectric point [Ahmed et al., 1996]. Human brain lectin and other 14 kDa lectins

are known to resolve into multiple band patterns upon isoelectric focusing [Clerch et al., 1988; Avellana-Adalid et al., 1990; Bladier et al., 1989; Lotan et al., 1989; Hirabayashi et al., 1987; Allen et al., 1987].

Many bacteria carry β-galactoside containing saccharides on their surface, making it likely that they interact with galectins. One group is the gram-negative non-enteric bacteria such as Haemophilus spp.and Neisseria spp. that, have, instead of LPS, lipooligosaccharides (LOS) with shorter outer carbohydrate chains that often contain β-galactosides. Galectin–3 has been found to bind via its CRD to some such saccharides [Mandrell et al., 1994; Mey et al., 1996; Gupta et al., 1997]. However, galectin-3 also binds the lipid-A moiety of LOS and LPS via its non-carbohydrate binding N-terminal [Mey et al., 1996]. *Entamoeba histolytica* adheres to human colonic mucus, colonic epithelial cells and other target cells via a galactose binding surface lectin [Petri, 1987].

Several lines of evidences strongly suggest that β-galactoside binding lectins in human and murine metastatic tumor cell surfaces may be involved in the formation of tumor emboli [Raz and Lotan, 1987; Lotan and Raz, 1988; Gabius, 1988]. Lectins may function by binding complementary glycoconjugates on the surface of other tumor cells to mediate homotypic aggregation, or on the surface of host cells to mediate

heterotypic aggregation or attachment to endothelial cells or extracellular matrix. It was proposed that increased amounts of surface-expressed galectin would increase cell adhesion in turn, perhaps resulting in increased metastasis. Secreted or cell surface galectin-1 could enhance tumors by suppressing anti tumor immunity via its apoptotic effect on antigen-activated T cells [Perillo et al., 1998]. Galectin-3 could be tumorigenic due to its intracellular growth-promoting anti-apoptotic effect on the tumor cells themselves [Yang et al., 1996]. Endogenous β-galactoside binding lectins have been localized at the surface of different viable cultured tumor cells and after fixation and permeabilization, in intracellular pools. The surface distribution was in the form of microclusters suggesting that the membrane-associated lectin molecules were laterally mobile and subject to rearrangement apparently mediated by endogenous ligands. Recently evidences for specific induction of a galectin-8-like protein in prostate cancer [Su et al., 1996], of galectin-9 in Hodgkin's lymphoma [Tureci et al., 1997] and galectin-4 in liver cancer and breast cancer have been reported [Kondoh et al., 1999]. Galectin-7 was found to be over-expressed in chemically induced rat mammary tumors [Lu et al., 1997]. There is also evidence for altered subcellular distribution of galectins in tumor cells.

β–Galactoside binding lectins are considered to be involved in differentiation and developmental events, because they are often found in

embryonic tissues. It has been found that human placenta lectin stimulated the production by macrophages of a cytotoxin capable of destroying malignant cells suggesting the role of the lectin in immune system [Kajikawa et al., 1986]. Cooper et al suggested roles of galectin-1 in maturation and organization of muscle fibers [Cooper and Barondes, 1990; Cooper et al., 1991; Gu et al., 1994].

Galectin-1 and Galectin -3 null mice have been generated to investigate the roles of galectins in development and immune regulation [Poirier and Robertson, 1993, Colnot et al., 1998 a]. The galectin-1 null mutants have an altered guidance of neurons in the olfactory bulb [Puche et al., 1996]. The galectin-3 null mutants have an altered recruitment/ survival of neutrophils in inflammation and an altered terminal differentiation of chondrocytes in fetal bone growth plates [Colnot et al., 1998b].

The same galectin can apparently affect cells in a variety of ways depending on the cell type and circumstances. For instance, galectin-1 can stimulate or inhibit cell proliferation [Wells and Mallucci, 1991; Adams et al., 1996; Yamaoka et al., 1996] and can either stimulate or inhibit cell adhesion to extracellular matrix [Cooper et al., 1991; Van Den Brule et al., 1995]. There is also evidence that galectins can simultaneously

have distinct intracellular and extracellular functions. For instance both galectin-1 and galectin-3 have been implicated in pre-mRNA splicing [Dagher et al., 1995; Vyakarnam et al., 1997].

Recently, a protein GRIFIN (galectin-related interfiber protein) was identified in the eye lens and was shown to have high sequence similarity to galectin-1 but no lactose binding activity [Ogden et al., 1988]. Subunits of legume lectins [Sharon, 1993] and serum amyloid P component (a pentraxin with lectin activity) [Emsley et al., 1994] have the same topology and very similar tertiary structure as the galectin carbohydrate-binding domains have but show no significant sequence homology. This suggests that galectins are a subset of a larger group of proteins sharing conserved folding motifs.

A consideration of molecular evolution suggests that lectins belonging to this family probably existed in the Precambrian era. Ubiquitous occurrence of these homologous lectins with shared sugar specificity suggests that they are involved in "essential minimum" functions of multicellular animals possibly in cooperation with their cognate glycoconjugates.

GLYCOPROTEINS

Glycoproteins are proteins to which carbohydrates are covalently linked through glycosidic bonds [Spiro, 1973] and occur in all organisms.

Glycoproteins are found in soluble form in the extracellular fluids and in insoluble form in membrane and intercellular matrix components. This class of compounds includes enzymes, hormones, immunoglobulins, lectins, toxins, carriers and structural proteins. Although nearly 200 different monosaccharides are found in nature only 11 are known to occur in glycoproteins [Sharon and Lis, 1981].

The carbohydrate content of glycoproteins varies from less than 1% to over 90% of the total weight and the carbohydrate moieties vary in size from mono- or disaccharides to oligosaccharides and they are distributed unevenly along the polypeptide back bone. The numbers of oligosaccharide chains attached to the glycoprotein are also varied. Derivatization to sulphate or phosphate is also observed occasionally. Apparently most of the proteins in nature are glycoproteins. Proteoglycans are a diverse group of proteins containing a large number of glycosaminoglycan side chains and the distinction between glycoproteins and proteoglycans resides in the level and types of carbohydrate modifications.

The oligosaccharide chains of most glycoproteins are linked to the polypeptide backbone by either of two types of carbohydrate-peptide linkages: The N-glycosidic and O-glycosidic [Montreuil, 1980; Zinn et al.,

1977]; they differ markedly in their chemical properties, in particular their stability to acid and base. The N-glycosidic linkage is between the anomeric carbon atom of N-acetyl–D-glucosamine and the amide nitrogen of asparagine in the polypeptide chain [Kornfeld and Kornfeld, 1976]. The carbohydrate units linked to the asparagine contain a common pentasaccharide core: Man α-1→6 (Man α-1→3) Man β1→4 GlcNAc β 1→4GlcNAc [Montreuil, 1980]. Depending on the type of additional saccharide units attached to the pentasaccharide core the N-linked oligosaccharides are categorized as high mannose, complex and hybrid types. The high mannose oligosaccharides have additional mannose residues attached to the core. The total number of mannose residues in this type ranges from 6 to 12 and the chains are often branched. The complex type contains the disaccharide N-acetyl lactosamine (Galβ1→4GlcNAc) attached to the core. Sialic acid residues may or may not be linked to Gal. The most common trisaccharide sequence found attached to the pentasaccharide core in complex type chains is NeuAcα (2→3) Gal β(1→4) GlcNAc. The third class of N-linked oligosaccharides is the hybrid type, which contains the features of both high mannose and complex types [Tai et al., 1977; Yamashita et al., 1978]. Most hybrid molecules contain a "bisecting" N-acetyl glucosamine linked β1→4 to the β-linked mannose residue, although some exceptions exist [Hunt et al., 1983;Varki and Kornfeld, 1983; Yamashita et al., 1983].

In the polypeptide chains, the amino acid sequence containing the glycosylated asparagine is Asn-X-Ser/Thr where X can be any amino acid other than proline and aspartic acid [Marshall, 1972]. Only about one third of the potential Asn-X-Ser/Thr sites in proteins are actually glycosylated. It has been suggested that rapid folding of the nascent polypeptide is responsible for the lack of glycan chains at potential glycosylation sites. Since glycosylation occurs co-translationally once the protein has folded potential glycosylation sites are no longer accessible to the glycosyltransferases.

N-linked glycosylation commences with the enzymatic transfer enbloc of a pre-synthesized core oligosaccharide, $(GlcNAc)_2$ $(mannose)_9$, $(Glucose)_3$ borne by a dolichol lipid to potential Asn residues of the protein [Kornfeld and Kornfeld, 1985] . This oligosaccharide is then subjected to stepwise modification. Initial trimming of the core oligosaccharide is carried out by glucosidase and mannosidase enzymes in the endoplasmic reticulum. This results in all three glucose residues and one of the mannose residues being removed. Upon passage of the glycoprotein through the Golgi, five more mannoses are removed by mannosidase. This leaves a residual $(GlcNAc)_2$ $(mannose)_3$ structure, which acts a frame work for the glycosyltransferase-mediated addition of GlcNAc ,galactose, fucose and sialic acid.

The second major type of saccharide-peptide linkage occurring in glycoproteins is the O-glycosidic linkage, which is found in mucins, blood group active glycoproteins, certain plasma glycoproteins and membrane glycoproteins. In O-glycans, carbohydrate is attached to hydroxyl groups of amino acids, serine and threonine. O-glycans are divided into multiple subgroups depending on the nature of the amino acid residue and sugar group involved in the carbohydrate-protein linkage. The sugar linked to serine or threonine is N-acetylgalactosamine (GalNAc) in mucin-type O-glycosylated proteins, N-acetylglucosamine in intracellular glycoproteins and xylose in proteoglycans.

Mucin type O-glycosylation is initiated by enzymatic addition of GalNAc to serine or threonine by the UDP-GalNAc T: polypeptide N-acetyl-galactosaminyltransferase family of enzymes in the Golgi. Depending on which saccharide groups are subsequently attached to this first GalNAc residue, mucin O-glycans are divided into four major subtypes [Schachter and Brockhausen, 1992]. (i) The core 1 structure is formed by addition of galactose to form Galβ1-3 GalNAc- Ser/Thr. (ii) The core 2 structure requires the core 1 structure as substrate and is formed by addition of GlcNAc to form Galβ1-3 (GlcNAcβ1-6) GalNAc-Ser/Thr. (iii) The core 3 structure is formed by the addition of GlcNAc to form GlcNAc β1-3 GalNAc-Ser/Thr. (iv) The core 4 structure requires the core 3 structure as substrate and is formed by addition of GlcNAc to form GlcNAcβ1-3

(GlcNAcβ1-6) GalNAc-Ser/Thr. Other modifications to the core GalNAc structure have also been found but appear to be uncommon. Of the four main core O-glycan structures the core 1 and 2 structures are widely distributed while the core 3 and core 4 structures are less common and expression has been mostly associated with mucin producing tissue of the digestive tract. Commonly the core 2 and the core 4 branches are elongated with one or multiple lactosamine structures (Gal β1-4 GlcNAc) [Lowe, 2001; Daniels et al., 2002].

Core 1 O-glycan or T antigen, the immediate precursor of the human blood group MN antigens [Springer and Ansell, 1958] was discovered about 70 years ago by Thomsen and Friedenreich as a laboratory curiosity. The phenomenon of panagglutinability acquired by bacterially contaminated human red blood cells led to the discovery of T antigen [Thomsen, 1927]. The Tn immunodeterminant group is GalNAc linked α-to the hydroxyl group of Serine or Threonine in the amino terminal region of glycoproteins. T and Tn are usually covered by covalently linked carbohydrates [Lloyd and Kabat, 1968; Jirgensons and Springer, 1968], holding high negative charge density in the case of sialic acid substitution [Springer and Desai, 1982] or are physically separated from the immune system [Pierce-Cretel et al., 1981]. Unmasked T and Tn specific epitopes are unique carcinoma markers.

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O-glycan biosynthesis is simpler than asparagine (N)-linked oligosaccharide generation in that a lipid-linked oligosaccharide precursor for transfer to protein is not required. Also in contrast to N-glycosylation, a consensus sequence for GalNAc addition to polypeptides has not been found, although predictive algorithms do exist. However, O-glycans are less branched than most N-glycans and are commonly biantennary structures.

Glycosylation is one of the most frequent and important post-translational modifications. The glycosylation of a protein is not directly specified in the genome but is determined by levels of glycosyltransferase expression, accessibility of glycan attachment sites, concentrations of nucleotide sugar donors and other environmental factors. Though variable, the glycosylation of a protein is often characteristic of the cell type where it was synthesised. The glycosylation profile and the glycosyltransferase expression vary widely between different cell types and can change significantly during development or stimulation of a cell.

The biological roles of oligosaccharides appear to span the spectrum from those that are trivial, to those that are crucial for the development, growth, function or survival of an organism. Feasible approaches to explore the biological roles of sugar chains on glycoproteins include the enzymatic or chemical removal of completed sugar chains,

prevention of initial glycosylation, alteration of oligosaccharide processing, elimination of specific glycosylation sites, and the study of natural variants and genetic mutants in glycosylation.

The carbohydrates of glycoproteins modify the physicochemical properties of proteins by changing their hydrophobicity, electrical charge, mass and size. The attachment of sugars to proteins is known to increase the solubility of the latter. Sialic acids in salivary glycoproteins are responsible for the high viscosity of the mucous solutions [Gottschalk and Thomas, 1961]. Negatively charged acylneuraminic acid residues impart physical strength to cell membranes because of their mutual repulsion and influence the mutual adhesion of cells in organ structure. The antifreeze glycoprotein of antarctic fish depends on the integrity of the disaccharide (Gal β1-3 GalNAc) units for their activity. The ability of these glycoproteins to depress the freezing point of water is lost on removal or modification of the saccharide side chains [Vandenheede et al., 1972].

Glycoproteins rich in sugar are relatively resistant to proteolysis [Gottschalk et al 1960]. The finding that the enzymatic removal of terminal NeuAc from ovine submaxillary mucin enhanced its susceptibility to trypsin digestion supported this. The protection against proteolytic degradation is attributed to steric hindrance by the carbohydrate as well as the more stable

conformation adorned by the glycosylated protein. The sugar chains of glycoproteins are involved in the initiation of correct polypeptide folding in the rough endoplasmic reticulum (ER) and in the subsequent maintenance of protein solubility and conformation. Many proteins that are incorrectly glycosylated failed to fold properly and /or fail to exit the ER, and are consequently degraded. For some glycoproteins the addition of asparagine (Asn)-linked oligosaccharides are required for glycoprotein transport to the cell surface [Soderquist and Carpenter, 1984; Fliesler and Basinger, 1985; Gaun et al., 1985]. However glycosylation is not obligatory for protein export or translocation. For many proteins glycosylation is not a prerequisite for biological activity.

The carbohydrate units of membrane glycoproteins are always located on the outer surface of the cell and the coating of glycoconjugates covering a whole cell can present a 'glycocalyx'of substantial proportions. All most all cells carry carbohydrates on their surfaces in the form of glycoproteins, glycolipids and polysaccharides [Cook, 1986]. The greatest variation in glycosylation pattern tends to be found among the outermost (non-reducing terminal) regions of glycans on cell surfaces and extracellular molecules [Lis and Sharon, 1993; Tsuji, 1996]. These regions of sugar chains are best positioned to mediate recognition by carbohydrate binding proteins (lectins) [Baenziger, 1985]. Carbohydrates also serve as important

recognition markers. The classical work of Aswell and his coworkers had demonstrated that removal of sialic acid from circulating glycoproteins leads to a dramatic enhancement in the rate of glycoprotein clearance from the circulatory system [Ashwell and Morell, 1977]. Cell surface glycoproteins are the immunodeterminant structures of blood group A, B, H and M/N specificities. [Watkins and Morgan, 1952]. The Man-6-P recognition marker on the lysosomal enzymes function as an intracellular traffic signal to direct acid hydrolases to lysosomes [Sly and Stahl, 1978].

Certain oligosaccharides can act as highly specific receptors for a variety of viruses, bacteria and parasites. They are also receptors for many plant and bacterial toxins and serve as antigens for autoimmune and alloimmune reactions. Certain commensal gut bacteria in animals and some root–nodule-forming bacteria in plants appear to mediate their binding to host cell surfaces via specific sugar sequences. In these cases inter-species recognition via oligosaccharides serves a function useful to both organisms involved. The addition of specific monosaccharides or modifications masks the sugar sequences recognized by microorganisms, toxins or autoimmune antibodies. For example, the addition of a single O-acetyl ester to the 9-position of terminal sialic acid residues abrogates binding of the highly pathogenic influenza A viruses and extension of the oligosaccharide chain of GM1 would prevent binding of cholera toxin [Schauer, 1985]. Likewise,

the addition of galactose and sialic acid to the Tn antigen would abolish its autoimmune reactivity. Oligosaccharide sequences on soluble glycoconjugates such as mucins can also act as 'decoys' for microorganisms and parasites. Thus pathogenic organisms attempting to gain access to mucosal membranes might first encounter their cognate oligosaccharide ligands attached to soluble mucins. Upon binding to these sequences they could then be swept away by ciliary action, leaving the underlying mucosal cells untouched.

Many enzymes are glycosylated proteins; though deglycosylation in most cases does not lead to the loss of enzyme activity [Baw and Shah, 1977]. When the hormone human β chorionic gonadotropin (β-HCG) is deglycosylated, it still binds to its receptor with similar affinity, but fails to transmit a signal via stimulation of adenylate cyclase [Bahl et al., 1974; Moyle et al., 1975]. Thus an agonist is converted into an antagonist. O-glycans have been reported to function in sperm binding to the egg. The mammalian egg coat (the zona pellucida) contains a large number of O-glycans, as well as some N-glycans. Removal of egg N-glycans by glycosidase treatment does not destroy sperm binding, but loss of O-glycans following mild alkali treatment ablates sperm binding [Bleil and Wassarman, 1988; Florman and Wassarman, 1985].

The thymus tissue of all vertebrates studied from lizards to humans exhibits a change in peanut agglutinin lectin reactivity that marks the cortical-medullary boundary. During T-cell development in the thymus, loss of peanut agglutinin reactivity occurs as thymocytes mature into T cells and enter the medullary compartment. This is due to the induction of the sialyltransferase ST3Gal-I, which attaches a terminal sialic acid moiety that masks the peanut-agglutinin-binding epitope. By inactivating ST3Gal-I function, it has been discovered that this modification regulates T-cell viability.

Carbohydrates play important structural and functional role in various disease states. Changes in the sugar moieties on cell surface occur when normal cells are transformed into malignant ones. Such transformation also results in loss of contact inhibition suggesting the involvement of sugars in cellular recognition and intercellular communication. Cell surface glycoproteins may be regarded as principal candidates for involvement in tumor cell spread since they are generally oriented towards the exterior of the cells and thus ideally suited to mediate the interaction of metastatic cells with their environment. Alterations in cell surface glycoconjugates are considered to be relevant to the abnormal properties of cancer cells, such as uncontrolled cell growth, altered cell expression, avoidance of immunological destruction, invasiveness and metastatic spread [Bhavanandan, 1991].

Different molecules of the same glycoproteins may differ in size and composition of their carbohydrate units, a phenomenon known as micro heterogeneity, which is believed to be an outcome of the positional and temporal differences between protein and oligosaccharide biosyntheses, the latter being a post-translational event.

CHAPTER II
MATERIALS AND GENERAL METHODS

MATERIALS AND METHODS

MATERIALS

Fetuin, neuraminidase from *Clostridium perfringens,* human IgG, ortho phenylene diamine (OPD), concanavalin A (Con A), soluble guar gum, horse radish peroxidase, myosin, β-galactosidase, bovine serum albumin, ovalbumin, trypsin, Cytochrome C, amido black, Coomassie brilliant blue G, divinyl sulphone, cyanogen bromide, Tween 20, 4-chloro-1-naphthol, sodium dodecyl sulphate, acrylamide, N, N'- methylene bis acrylamide, TEMED, mannose, galactose, melibiose, 1-O-methyl α-D-galactoside, 1-O-methyl-β-D-galactoside, 1-O-methyl-α-D-glucoside, PNP-α-D-galactopyranoside, PNP-β-D- galactopyranoside, PNP-α-D-mannopyranoside, 2-mercaptoethanol ,Iodoacetamide, potassium thiocyanate, and antibodies to human IgA, IgG, IgM raised in goat , Jack bean- β-galactosidase , Ficoll, Sodium diatrizoate and lactose were purchased from Sigma Chemicals Company, U.S.A. Sephadex G-100, Sephadex G-200, Sepharose 4B, were the products of Pharmacia fine Chemicals, Uppsala, Sweden. Biogel P-4 was purchased from Bio- Rad laboratories, 32nd & Griffin Ave, Richmond, California. Nitrocellulose filters were from Millipore USA, PVDF and Coomassie brilliant blue R-250 were purchased from Pierce chemical company USA. Pronase was obtained from

Koch-light laboratories Ltd, England. Triton X-100, phenyl methyl sulphonyl fluoride, benzamidine hydrochloride and Potassium borohydride were obtained from Fluka, Buchs Switzerland. Other chemicals were of analytical grade and obtained from local sources. The seeds of *Artocarpus Mintegrifolia* and *Ricinus communis* were obtained locally.

METHODS

TISSUE COLLECTION

Human heart muscle was collected within 12 h after death due to accidents from Forensic Medicine Department, Medical College Hospital, Thiruvananthapuram. After removing fat deposits, heart muscle was washed in ice cold PBS 7.4 and kept frozen at –20° C before use. Human plasma and blood used were collected soon after expiry from blood bank, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram.

Protein estimation (Bradford's Method)

Reagent: Coomassie brilliant blue G-250 dye solution was prepared as a 0.06% solution in 3% perchloric acid. Sixty mg dye was dissolved in 100 ml distilled water containing 2.5 ml perchloric acid. The reagent was filtered through Whatman No: 1 filter paper before use. The reagent and protein solution were mixed in the ratio 1:1 and the absorbance at 620 nm measured immediately [Bradford, 1976].

Protein estimation (Lowry's method)

- Reagents.

  a. 2 % sodium potassium tartarate.

b. 1 % copper sulphate

c. 2 %sodium carbonate solution in 0.1 N sodium hydroxide.

d. Alkaline copper reagent: 1 ml of reagent 'a' and 'b' were mixed t the time of experiment and made up to 100 ml with reagent 'c'.

e 1 N Folin Ciocalteau reagent (Folin's reagent)

0.5 ml of protein solution was mixed with 2.5 ml of alkaline copper reagent and incubated at 25°C for 10 minutes. This was followed by the addition of 0.25 ml of 1 N Folin's reagent and incubation at 25°C for 30 minutes. Absorbance was measured at 660 nm. Ovalbumin was used as standard [Lowry et al., 1951].

Preparation of human heart galectin-1 (HHL-G)

All procedures were performed at 4°C. Thawed and minced human heart tissue (35 g) was homogenized in 250 ml PBS (20 mM potassium phosphate buffer pH 7.4 containing 150 mM NaCl) with 50 mM lactose, 0.2 mM phenyl methyl sulphonyl fluoride, 2 mM benzamidine hydrochloride, 2 mM EDTA and 5 mM 2-mercaptoethanol (2-ME) using POLYTRON homogenizer and stirred for 1 h at 4°C. After centrifugation at 16,000 g for 20 min the supernatant was subjected to 70 % ammonium sulfate precipitation. Proteins were precipitated by centrifugation at 16,000 g

for 20 min and the precipitated proteins were re-dissolved in 30 ml PBS containing 5 mM 2-mercaptoethanol and dialyzed against the same buffer to remove lactose. After another centrifugation as above, the supernatant was applied to lactose- Sepharose equilibrated in PBS-2-ME. After washing out unbound proteins with PBS-1M NaCl-2-ME and PBS-1M NaCl alone, PBS containing 150 mM lactose and 50 mM iodoacetamide was used to elute bound proteins. The sample (HHL-G) was dialysed against PBS and concentrated using AMICON PM10 ultra filtration membrane to 1-2 mg protein per ml.

Preparation of purified galectin-1 (HHL) from (HHL-G) using HPLC

HHL-G concentrated up to 1-2 mg/ml was used for the isolation of pure lectin HHL on a Pharmacia Biotech HPLC system using BIOSEP-SEC-S-2000 (300 x 7.8 mm) size-exclusion column. One hundred µl sample was injected at a time and the separation was achieved with a flow rate of 0.2 ml /min. Protein in column eluate was monitored using a UVCORD detector and 280 nm filter.

Hemagglutination assay

Agglutination titers of protein (galectin) samples were determined using U bottom 96-well polystyrene microtitre plates (Laxbro, New Delhi). Wells were first blocked by one-hour contact with PBS 7.4 containing 0.05%

Tween 20 (PBS-T). The protein samples were also brought to the same medium. In the drained wells 2-fold serial double dilutions of protein samples were made in a volume of 100 µl. Erythrocytes were then added (25 µl of 5% suspension in PBS 7.4) to each well and mixed. Agglutination titre was scored after 1 h with mixing. Settling of erythrocytes within 2 minutes after the final mixing was marked as positive agglutination.

Hemagglutination inhibition assay

Minimum hemagglutination quantity (MAQ) of human heart lectin using trypsinised rabbit / human RBC was determined by the method described above. Serial two fold dilutions of glycoproteins / sugars were incubated with twice the minimum agglutinating quantity of HHL for 1 h at 4^0 C. After the incubation erythrocytes were added (25 µl of 5% suspension) to each well and mixed. The agglutination titer was scored after 45 min.

Trypsinisation of human / rabbit erythrocytes

Blood was collected in trisodium citrate-dextrose solution (anticoagulant). The cells were washed using cold PBS and settled by centrifugation at 400 g for 10 min. This process was repeated twice. After the last washing step 100 µl of the packed cell was added to 1.9 ml of 0.1 % trypsin solution in PBS, mixed gently and incubated at 37^0 C for 1 h with

occasional mixing. After incubation the cells were washed three times using cold PBS with centrifugation. The final volume of the suspension was made up to 2 ml with PBS to make the suspension 5% v/v [Appukuttan, 2002].

Neuraminidase treatment of human erythrocytes

Discarded left over blood collected in presence of anticoagulant in our blood bank on the same day was used as the source of human erythrocytes. Approximately 1 ml blood was mixed gently with 15 ml of cold PBS and centrifuged at 400 g for 10 min to settle the erythrocytes. The supernatant was discarded and process was repeated twice. From the final undisturbed RBC pellet, 100 µl was made up to 1 ml with PBS containing neuraminidase solution (0.12 U/ml), the solution mixed gently and incubated at 37^{0}C for 1 h with occasional mixing. After incubation, the cells were washed with cold PBS three times and final volume of the suspension was made up to 2 ml to get a cell suspension of 5% v/v.

Lactose –Sepharose 4B matrix

Lactose was covalently attached to Sepharose using divinyl sulfone as the cross-linker. Lactose-divinyl sulfone Sepharose 4B was prepared as described by Dean et al. [1985]. Briefly, Sepharose 4B (20 g, moist weight) was washed in distilled water under suction and suspended in 20 ml 1M Na_2CO_3 pH 11. After adding 4 ml divinyl sulfone, the

suspension was stirred with a magnetic bar for 1h at room temperature. Activated gel thus obtained was washed thoroughly in distilled water and the wet cake of gel obtained by suction filtration over sintered glass funnel was added to 30 ml saturated solution of lactose in 1 M Na_2CO_3 and stirred over night at room temperature as above. The reacted beads were washed successively with the following.

1) 20 ml of 1 M Na_2CO_3 pH 11

2) 500 ml of 0.2 M glycine-HCl, pH 3,containing 1 M NaCl to block unreacted activated groups in the gel.

3) 500 ml of 1 M NaCl and

4) 500 ml of distilled water.

This lactosyl-Sepharose 4B matrix was equilibrated in PBS 7.4 and packed into a chromatographic column of required dimension.

Immobilization of lectins /glycoproteins to Sepharose 4B by CNBr activation method

Sepharose 4B (40 ml) (Pharmacia Biotech, Sweden) was activated using cyanogen bromide [Cuatrecasas and Anfinsen, 1971] in Na_2CO_3 (2 N) at 8^0C for 5 min and washed with 0.1 M $NaHCO_3$ buffer pH 8.5. The protein sample in 0.1 M $NaHCO_3$ was added to the activated gel

(2-4 mg/ml gel) and the mixture stirred gently overnight at 4^{0}C followed by incubating the gel in 0.1 M ethanolamine hydrochloride to block the unconjugated activated groups on the gel. The coupled gel was washed successively with 20 times the gel volume with the following

1) 0.1 M $NaHCO_3$
2) Distilled water
3) Acetate buffer 50 mM, pH 5 containing 1 M NaCl
4) Distilled water
5) PBS 7.4

The protein –Sepharose 4B affinity matrix can be stored at 4^{0}C with 0.02 % sodium azide or packed into a column of required size for chromatography.

Conjugation of Horse radish peroxidase (HRP) to lectins/antibodies

Lectin or antibody (1 mg) in 10 mM sodium bicarbonate buffer pH 9.5 (1 ml) was mixed with 0.67 mg periodate activated horse radish peroxidase in the same buffer and incubated at 25^0 C for 2 h in the dark. Then potassium borohydride solution in distilled water (1%) was added to a final concentration of 0.1%. After 30 min the mixture was dialysed against PBS with one change over night. The labeled lectin/antibody was stored in ice [Heyderman et al., 1989].

Carbohydrate estimation by phenol -sulphuric acid method

The total neutral sugar was estimated by phenol-sulphuric acid method of Dubois et al. [1956] in a total volume of 5.5 ml with galactose as standard.

❖ Reagents

a. Sulphuric acid.

b. 5% phenol (prepared by diluting distilled phenol 1:20 with water)

The sample was made up to 0.5 ml with water and mixed with 1 ml of 5% phenol. To this was added 4 ml of chilled sulphuric acid quickly and the mixture vortexed thoroughly. After 15 minutes incubation at room temperature, absorbance was measured at 485 nm.

Alkaline - PAGE

Alkaline-PAGE at pH 8.3 was done as described by Davis [1964].

❖ Reagents

A. One hundred ml Tris (1.5 M) containing 24 ml 1 N HCl and 0.12 ml TEMED pH 8.8

B. One hundred ml Tris (0.5 M) containing 48 ml 1 N HCl and 0.46 ml TEMED, pH 6.8.

C. 28 g acrylamide and 0.735 g bis acrylamide dissolved in 100 ml distilled water.

D. 20 g acrylamide and 5 g bis acrylamide dissolved in 100 ml distilled water.

E. 4 mg riboflavin dissolved in 100 ml distilled water.

F. 14 mg ammonium persulphate dissolved in 10 ml distilled H_2O.

Separating gel (Acrylamide 7%)

One part A was mixed with one part C .To this mixture, an equal volume of F was added and mixed.

Spacer gel

One part B, one part D, one part E and 5 parts distilled water were mixed.

Reservoir Buffer

Tris (0.05 M) / glycine (0.38 M), pH 8.3.

The gels were cast in 5 mm glass tubes (BROVIGA DISC electrophoresis apparatus) and electrophoresis run at 3 mA per tube till the bromophenol blue used as tracking dye had reached the bottom of the gel .The gels were fixed in 12.5 % trichloroacetic acid. Staining was done using Coomassie brilliant blue R-250 and destaining with methanol: acetic acid: water (1: 1.5: 17.5, v/v).

Isolation of IgA1P

IgA1 that binds to polystyrene-immobilized jacalin (IgA1P) was isolated from the naturally occurring dextran-binding human immunoglobulin (DIg) [Chacko and Appukuttan, 2003]. Wells of polystyrene 96 well U-bottom ELISA plates (Dynex, U.S.A) were coated with jacalin by incubating with 300 µl PBS solution of the lectin (50 µg / ml) at 4°C for 24h. Wells were blocked by 30 min. treatment with PBS containing 0.5% Tween 20 at 37° C and washed at 25°C with PBS containing 0.05% Tween 20 (PBS-T). Wells were then incubated with 50 µg DIg in 300 µl PBS-T for 2 h at 4°C. Unbound protein in supernatant (non-IgA1P DIg) were collected and concentrated by ultrafiltration. After washing wells with PBS alone, bound IgA1P was eluted using 50 mM 1-O-methyl-α-D galactoside in PBS, dialysed against the same buffer and concentrated by ultrafiltration.

Enzyme treatment of glycoproteins on dot blots and microwells.

Dot blots of 2 µg glycoprotein in 2 µl PBS on 5 mm x 5 mm polyvinyl difluoride membranes were allowed to dry in air for 20 min. and blocked with PBS containing 0.2% Tween 20 for 2 h at 25°C. For coating to polystyrene wells 200 µl PBS containing the specified amount of glycoprotein was added to a 96 well ELISA plate (4HBX; Dynex, USA)

and incubated at 37°C for 3 h. Wells were then washed with PBS-T, blocked with PBS containing 0.5% Tween 20 for 30 min. at 37°C and again washed with PBS-T. Enzyme treatment of the dot-blotted or polystyrene well coated glycoproteins were uniformly in 0.5% Tween 20 at 37°C under the following conditions: neuraminidase (0.12 U per ml) in PBS for 1h; α-mannosidase (1U per ml) in 100 mM citrate-phosphate buffer pH 4.5 containing 0.5 mM Zn^{2+} for 12 h.

β-Galactosidase treatment of glycoproteins

The glycoprotein coated on polystyrene micro wells were treated with neuraminidase (0.12 U/ml) for 1 h at 37°C after blocking with PBS containing 0.5 % Tween-20. After neuraminidase treatment the wells were washed with 0.05 % PBS-T three times and treated with Jack bean β-galactosidase. The glycoproteins were incubated in the enzyme solution (0.4 U/ml) in 0.2 M citrate-phosphate buffer, pH 4 for 3 h at 37° C and washed with 0.05 % PBS-T.

HRP-labeled lectin or antibody recognition of microplate-coated and dot-blotted glycoproteins.

Microwell-coated glycoproteins treated with respective enzyme if any as described above, were washed with PBS-T three times at 25°C. HRP conjugate of antibody or lectin (200 µl containing 7.5 µg lectin or

antibody per ml or as indicated) was added before incubation at 4°C for 2 h. After three washings with PBST at 4°C, the bound HRP activity was assayed by incubation at 25°C for 30 min. with 200 µl orthophenylene diamine (0.5 mg/ml) in 0.1 M citrate-phosphate buffer pH 5.0 containing 0.03% H_2O_2, followed by addition of 50 µl 12.5% H_2SO_4 and reading of absorption at 490 nm in an ELISA reader (BIOTECH, USA). To probe glycoproteins immobilized on polystyrene wells with Con A, to the blocked wells Con A solution (10 µg/ml) in TBS 7.4 was added and incubation done at 4^0 C for 2 h. Wells were then washed and incubated with HRP solution (5 µg/ml) in PBS for 2 h at 4^0 C. The bound HRP was measured using OPD.

Dot blots treated with or without enzyme as above were incubated with HRP conjugates (70 µg lectin or HRP per ml) in PBS containing 0.5% Tween 20 at 4°C for 2h. After three washings with PBS-T, bound conjugate was detected by dipping the dots for 5 min. in HRP substrate solution (1 ml 0.3% 4-chloronaphthol in anhydrous methanol mixed with 5 ml PBS and 3 µl 30% H_2O_2) at 25°C, followed by washing with PBS.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The molecular weight range of glycoproteins were determined by SDS-PAGE on 7.5.% slab gels according to the method of Laemmli [1970].

❖ Reagents

A. Gel Solution: 15 g of acrylamide and 0.4 g of bis acrylamide were dissolved in distilled water and the volume made up to 50 ml. The solution was filtered and stored at 4°C in amber colored bottle.

B. Buffer I: 0.614 M Tris/HCl pH 8.8. For 100 ml of buffer, 164 mg of SDS was dissolved.

C. Buffer II: 0.147 M Tris/HCl pH 6.8 .For 100 ml of the buffer, 108 mg of SDS was dissolved.

D. Chamber buffer: 0.25 M Tris/0.192 M glycine pH 8.3 containing 0.1% SDS.

E. Ammonium per sulfate (15 mg/ml) in distilled water.

The preparation of 7.5% separating gel, involved the mixing of 2.25 ml of "A" ,0.75 ml of glass distilled water, 4.25 ml of buffer I ,0.45 ml of ammonium per sulfate and 10 µl of TEMED at the time of polymerization .For spacer gel 0.5 ml of "A ",4.25 ml of buffer II, 0.25 ml of ammonium per sulfate and 5 µl of TEMED were mixed at the time of polymerization. Myosin (205 kDa), β-galactosidase (116 kDa), crystalline bovine serum albumin (67 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa) and cytochrome C (12.5 kDa) were used as molecular weight marker proteins. The mini slab gel (Hoefer Scientific 7.3x8.3 cm) was run at 15 mA till the

bromophenol blue dye had reached the bottom of the gel. The proteins in the gels were fixed using 50 % methanol, stained with Coomassie brilliant blue R-250 and destained with methanol: acetic acid: water (1: 1.5: 17.5,v/v).

Western Blot

SDS polyacrylamide slab gel electrophoresis (7.5%) as described by Laemmli [1970] was performed for the separation of glycoproteins (1mg/ml). Separated glycoproteins were transferred to polyvinyl difluoride (PVDF) membrane using transfer buffer consisting of 25 mM Tris, 192 mM glycine and 15 % methanol, pH 8.3 and applying a constant current of 0.8 mA/cm^2 membrane for 2 h at 25^{0}C as described by Towbin et al. [1979]. The strips 3-4 mm wide were cut out from the transferred membrane, blocked with PBS containing 0.2 % Tween 20 and probed with lectin conjugate with horse radish peroxidase (HRP) for 2 h at 4^0 C. The strips were washed twice with 0.05% PBS-T and once with PBS alone and stained using freshly prepared 4-chloronaphthol solution as described earlier. After staining for 2-5 minutes with 4-chloronaphthol solution, strips were washed again in PBS and photographed.

Isolation of glycoprotein-rich fraction from human serum

Human serum glycoprotein rich fraction was prepared from a

mixture of six consecutive samples from healthy donors by two successive ammonium sulphate precipitations. Human serum (pooled) was centrifuged at 10,000 g for 15 min and 50 ml of supernatant was mixed with 100 ml of cold PBS. Neutral, saturated ammonium sulphate solution was added drop wise to the diluted plasma at 25^{0}C with mixing until the solution was 45% saturated with ammonium sulphate. The mixing was continued for 1 h and the sample centrifuged at 10,000 g for 30 min. The supernatant was discarded and the precipitate was re-dissolved in 25 ml cold PBS .The solution was dialyzed twice against PBS 7.4 at 4^{0}C.

Preparation of non-O glycosylated serum proteins

Non-O-glycosylated serum glycoproteins were prepared from glycoprotein rich fraction from human serum by removing the O-glycosylated proteins by passing through two successive jacalin-Sepharose 4B columns (10 ml each) in PBS, pH 6.5 and collecting the unbound.

Preparation of N-linked oligosaccharides from glycoproteins

For preparation of peripheral N-linked oligosaccharides from IgA1S, fetuin or serum glycoprotein-rich fraction, solution of each (2 mg/ml) was treated with α-mannosidase (1U per ml) in 100 mM citrate phosphate buffer pH 4.5 containing 0.5 mM Zn^{2+} for 12 h. The released oligosaccharides were separated from proteins by ultrafiltration, desalted by gel filtration in Biogel P4 column in water and lyophilized.

Preparation of PNA-binding bovine/human tissue glycoproteins

To prepare soluble glycoproteins 30 g (bovine/human) tissue was homogenized in 200 ml PBS 6.5 containing 0.2 mM phenyl methyl sulphonyl fluoride, 2 mM benzamidine hydrochloride and 2 mM EDTA, homogenate stirred for 1 h and centrifuged at 10,000 g for 20 min. From the supernatant, proteins were precipitated at 70% ammonium sulphate saturation and sedimented by centrifugation at 10,000 g. The pellet was re-dissolved in and dialyzed against PBS 6.5 (2 changes). After a further centrifugation at 10,000 g the supernatant containing dissolved proteins was passed through unmodified Sepharose 4B and unbound protein from the column was passed through PNA-Sepharose. The column was washed until it was protein-free with PBS containing 1M NaCl and eluted with 150 mM lactose in PBS in 3 ml fractions. Eluted fractions containing proteins were pooled, dialyzed against PBS until free from lactose and concentrated by ultrafiltration using AMICON PM10 membrane.

Purification of dextran-binding immunoglobulin (DIg) from human plasma

DIg was purified from human plasma using cross-linked dextran (Sephadex G-100 from Pharmacia, Sweden) as affinity chromatography matrix. Plasma (70 ml) dialysed for two 6 h durations against PBS was centrifuged at 10,000 g for 15 min. The clear plasma supernatant was passed

through 40 ml Sephadex column at a flow rate of 30 ml/hour. The unbound and non-specifically retained proteins were removed from the column by washing with 5-6 bed volumes of PBS. The column was eluted with 500 mM 1-0 methyl α-D glucoside in PBS. The eluate was collected in 4 ml fractions in which protein was monitored by Bradford method. The protein rich fractions were pooled, the solution was concentrated using AMICON PM 30 ultra filtration membrane to 1-2 mg/ml, dialyzed and stored in ice [Chacko and Appukuttan, 2003]

Preparation of cross-linked guar gum (CLGG)

Soluble guar galactomannan was cross-linked to form an insoluble gel by a modification of the procedure described by Appukuttan et al. [1977]. 10 g guar gum powder was mixed thoroughly with a finely dispersed emulsion of 2 ml epichlorohydrin and 25 ml 3 N NaOH until the mixture became a solid cake. It was then left at 40^{0}C in a water bath for 24 h and then at 70^{0}C for 10 h. The resulting gel was soaked in distilled water and repeatedly washed with water until washings were neutral. The gel was then equilibrated with PBS and homogenized in a blender to obtain particles of about 300 μm size. Fine particles were discarded by repeated decantation.

Preparation of jacalin

Jacalin (Jack fruit seed agglutinin) was isolated from the seeds of *Artocarpus integrifolia* (jack fruit seed) by the procedure described by

Suresh kumar et al. [1982]. Thirty g of Jack fruit seeds were dehusked and soaked in PBS 6.5 for 12 h. The seeds were then cut into small pieces, homogenized in 300 ml PBS 6.5 and stirred 2 h at 4°C. The supernatant of homogenate obtained by centrifugation at 14,500 g for 20 min was subjected to 70 % ammonium sulphate saturation and stirred for 30 min at 4°C. The precipitated proteins recovered by a similar centrifugation were dissolved in PBS 6.5 and dialysed against the same buffer before loading on to cross-linked gaur galactomannan column. The column was washed with PBS 6.5 and eluted with 0.15 M galactose in PBS 6.5. Fractions containing proteins were pooled and dialysed against PBS 7.4.

Preparation of Anti -α-Gal

Isolation of anti-α-Gal was done by the method of Jaison and Appukuttan [1992]. All steps were carried out at 4°C. Out-dated human plasma was disinfected by incubation at 56°C for 30 minutes. It was then dialyzed exhaustively against PBS containing 5 mM EDTA and 35 ml was passed through a column of CLGG in the same buffer. The column was washed with PBS-EDTA until washings were protein free and eluted with 0.15 M galactose in the same buffer in 3 ml fractions. Protein- containing fractions were pooled, concentrated by ultrafiltration through an AMICON PM 30 membrane and dialysed against PBS to remove galactose. Protein was assayed by the method of Bradford. Activity of the purified anti-α-gal was determined by agglutinating assay using trypsinised rabbit RBC.

Preparation of jacalin-binding erythrocyte membrane glycoproteins (JRBCGP)

Out dated human blood (25-30 days old) collected from the hospital blood bank was washed three times using PBS 7.4 by centrifuging at 400 g for 10 minutes in HS-4 swinging bucket rotor of Sorvall RC5B centrifuge to get erythrocytes free from plasma proteins and other cell types. After the third washing 50 ml packed cells mixed with 450 ml lysis buffer (7.5 mM phosphate buffer, pH 7.3) was stirred well. Membrane ghosts were pelleted by centrifugation at 1,00,000 g for 1 h and settled ghosts were washed with the same buffer and again allowed to settle by centrifugation. The pellet obtained was resuspended in PBS 6.5. Triton X-100 was added to the membrane pellet with stirring to make it 1% with respect to the detergent. This yielded a completely solubilized membrane, which was dialysed against PBS 6.5 with one change overnight. Dialysed sample was centrifuged at 10,000 g for 30 min to remove any residual matter present. This sample was then loaded on to a jacalin-Sepharose column equilibrated with PBS 6.5. The column was washed with PBS 6.5 till the washings were protein free and the bound protein were eluted using 150 mM galactose in PBS 6.5 containing 3.5 M potassium thiocyanate. The protein rich fractions were pooled and dialysed against PBS 7.4 with two changes.

Preparation of jacalin-binding plasma glycoproteins (JPGP)

All procedures were at 4^{0}C. Human plasma (70 ml) was dialysed twice against cold PBS 6.5 using Serva dialysis membrane (molecular weight cut off 10,000 Da). After dialysis, possible cells and any suspended material were removed by centrifugation at 10,000 g for 15 minutes using Sorvall RC5B refrigerated centrifuge. The clear supernatant was filtered through a loose cotton plug to remove fine fatty layer on the surface. The filtered plasma was loaded on to a cross-linked guar gum (CLGG) column to remove anti-α-Gal antibody which otherwise binds to Sepharose. The unbound from the CLGG column was passed through a column of jacalin-Sepharose (40 ml) previously equilibrated with PBS, at a flow rate of 30 ml/hr. The column was washed with PBS 6.5 until washings were protein free and eluted in 3 ml fractions with 150 mM galactose and 3.5 M potassium thiocyanate. Protein rich fractions were pooled and dialysed against PBS 7.4 with two changes. The jacalin-binding plasma glycoproteins thus obtained were concentrated to approximately 10 ml and passed through activated Sepharose column to remove any contaminating Sepharose-binding protein. The unbound protein was concentrated using AMICON PM10 ultra filtration membrane to a protein concentration of 2 mg/ml.

Preparation of PNA

All preparations were done at 4^{0}C. Fifty gram dehusked peanut soaked for 24 h in 20 mM phosphate buffer, pH 6.5 containing 150 mM NaCl

was homogenized in the same buffer using a POLYTRON homogenizer and stirred for 1 h. After 15,000 g centrifugation the lipid slab on top was removed and proteins from supernatant, precipitated by 70% ammonium sulphate saturation, were dissolved in and dialyzed against PBS 6.5. Supernatant after 15,000 g centrifugation of the dialysate was passed through a 40 ml CLGG column equilibrated in the same buffer. After washing out unbound proteins using PBS 6.5, bound proteins were eluted using 0.15 M lactose in the same buffer and concentrated using AMICON PM10 ultrafiltration membrane.

Preparation of α-mannosidase

α-mannosidase from jack bean was prepared according to the method of Li and Li [1972]. All operations were carried out at 4°C. Fifty gram jack bean soaked for 2 days in 300 ml water, was homogenized in 20 mM TBS pH 7.4 using a POLYTRON homogenizer and stirred for 1 h. The suspension was strained through cheesecloth. To the filtrate solid ammonium sulphate was added to obtain 30 % saturation. After the mixture had stood for 2 hours, the precipitate was removed by centrifugation (10,000 g) and more ammonium sulphate was added to the supernatant to obtain 60 % saturation. After standing overnight followed by a similar centrifugation the precipitate was redissolved in 250 ml of 0.1 M sodium phosphate buffer pH 7. To the crude enzyme fraction thus obtained, 95 % ethanol was added

drop wise at room temperature with constant stirring to a concentration of 25 %. The mixture was cooled to -10^0C. The precipitate was collected by centrifugation at -10^0C, dissolved in 100 ml of 0.1 M sodium phosphate buffer pH 7. The enzyme rich fraction was applied to a Sephadex G-200 column , which had been equilibrated with 0.1 M sodium phosphate buffer pH 7. The column was eluted with the same buffer at a flow rate of 30 ml per hour. The fractions in the first protein peak containing α-mannosidase were pooled and dialysed overnight against saturated ammonium sulphate. The precipitated protein was collected by centrifugation, re- dissolved in 100 ml of 0.05 M sodium phosphate buffer pH 7. To remove any β-N acetyl hexosaminidase co-purified with the enzyme during gel filtration, five milliliters of α-mannosidase containing 500 units of enzyme were mixed with 0.5 ml of pyridine and incubated at room temperature for 1 h. The precipitated protein was removed by centrifugation and the clear supernatant which contained α-mannosidase was dialysed thoroughly against 0.05 M sodium phosphate buffer to remove pyridine. The purified enzyme thus obtained was concentrated by ultrafiltration.

Alpha-mannosidase assay

The standard assay system for α-mannosidase contained 200 μmoles of citrate-phosphate buffer pH 4.2, 0.5 μmole of p-nitrophenyl-α-D-mannopyranoside, 100 μg of bovine serum albumin, 1 μmole of $ZnSO_4$ and

suitable amount of enzyme in a total volume of 0.5 ml. The mixture was incubated at 37°C for 30 min. The reaction was terminated by heating at 100°C for 30 sec. The contents, after cooling were mixed with 2.5 ml of 0.4 M Glycine-NaOH buffer pH 10.5 and centrifuged at 2000 g for 5 min. The yellow colour formed due to the liberation of p-nitrophenol was measured at 405 nm.

Isolation of lymphocytes from whole blood

Lymphocytes were prepared from human blood by the procedure described by Boyum [1984].

Stock solution

Ficoll 9 % (w/v) in distilled water.

Sodium diatrizoate 33.9 % in distilled water.

For use 12 parts of Ficoll stock were mixed with 5 parts of sodium diatrizoate. (Density of this mixture = 1.077g/cc).

Fifty ml blood collected with anticoagulant citrate phosphate dextrose adenine (CPD-A) solution 7 ml.

CPD-A
- Citric acid 0.3 g
- Sodium citrate 2.63 g
- Mono basic Sodium phosphate (monohydrate) 0.22 g

- Dextrose (monohydrate) 3.19 g
- Adenine (anhydrous) 0.0275 g
- 100 ml water.

The anti coagulated blood was diluted (1:1) with PBS and 3 ml layered on to the Ficoll -sodium diatrizoate mixture (3 ml). The sample was centrifuged at 400 g for 30 min at room temperature in HS-4 swinging bucket rotor of Sorvall RC5B centrifuge. The supernatant was discarded and the lymphocytes present at the interface were transferred to a fresh tube. The lymphocytes thus obtained were washed twice by centrifugation with PBS at 400 g for 30 min. The lymphocyte pellet obtained was finally resuspended in cold hypotonic buffer (7.5 mM phosphate buffer). The lysate was centrifuged at 1, 000,00 g for 1 hour at 4^0C to get plasma membrane enriched fraction of lymphocytes as sediment.

Isolation of *Ricinus communis* agglutinin (RCA-1)

This was done as described by Appukuttan et al. [1977]. *Ricinus communis* seeds (50 g) were peeled and soaked overnight in potassium phosphate buffer, pH 7.2 containing 50 mM NaCl. Then the seeds were homogenized in a blender. The supernatant of homogenate obtained by centrifugation at 12,000 g for 30 min was subjected to 70 % ammonium sulphate saturation and precipitated proteins were redissolved in PBS and

dialysed against the same buffer. The supernatant obtained by centrifugation at 10,000 g was loaded on to a column made of CLGG. After washing away unbound proteins with PBS, bound proteins were eluted with 0.2 M lactose.

Preparation of Plasma membrane enriched fraction of platelets

Platelets were isolated from human blood by the procedure described by Jennings and Philips [1982]. Anticoagulant treated blood was centrifuged at 150 g for 5 minutes at room temperature to obtain platelet-rich plasma (PRP). To PRP, one-tenth volume of 100 mM EDTA was added to prevent platelet activation. The preparation was first centrifuged at 232 g for 10 minutes at 25^{0}C to remove the contaminant erythrocytes. A platelet pellet was obtained at subsequent centrifugation of supernatant at 4530 g for 15 min at 25^0 C. The platelet sediment was dispersed in washing buffer (20 mM PBS pH 7.4,10 mM EDTA) and the platelets were collected after centrifugation at 900 g for 10 minutes. The platelets were lysed in cold hypotonic buffer (7.5 mM phosphate buffer pH 7.3,10 mM EDTA). The lysate was centrifuged at 1, 000,00 g for 1 hour at 4^{0}C to get plasma membrane enriched fraction of platelets as sediment.

For the preparation of jacalin-binding platelet membrane glycoproteins, 1% Triton X-100 was added to the platelet plasma membrane-enriched fraction and the mixture dialysed against PBS 6.5. After

centrifugation at 10,000 g for 30 min to remove any residual matter present this sample was loaded on to a jacalin-Sepharose column equilibrated with PBS 6.5. The column was washed with PBS 6.5 till the washings were protein free and the bound protein were eluted using 150 mM galactose in PBS 6.5 containing 3.5 M potassium thiocyanate. The protein-rich fractions were pooled and dialysed against PBS 7.4 with two changes.

Acid - PAGE

Acid PAGE was done as described by Reisfeld et al. [1962].

❖ Reagents

Solution A	:	1 N KOH 48 ml
(pH 4.3)		Glacial acetic acid 17.2 ml
		TEMED 4 ml
		Made up to 100 ml with water.
Solution B	:	1 N KOH 48 ml
(pH 6.6-6.8)		Glacial acetic acid 2.87 ml
		TEMED 0.46 ml
		Made up to 100 ml with water.
Solution C	:	Acrylamide 30 g
		Bis acrylamide 0.8 g
		Made up to 100 ml with water.

Solution D : Acrylamide 20 g

Bis acrylamide 0.5 g

Made up to 100 ml with water.

Solution E : Riboflavin 4 mg dissolved in 100 ml water.

Solution F : Methylene blue (Tracking dye), 0.005% solution.

Solution G : Ammonium persulphate 2.8 mg/ml in water.

Gel preparation

A. Separating gel (5% acrylamide)

1 part A

1 parts C

1 part H_2O

3 parts G

B. Spacer gel 1 part B

1 Part D

1 part E

5 parts H_2O

Polymerization was achieved under fluorescent light.

Reservoir buffer

0.05 M β-alanine, pH adjusted to 4.5 with acetic acid. Fixing, staining, and destaining were done as descried for alkaline PAGE.

Electroelution

The method described for electroelution of DNA from agarose gel [Ogden and Adams, 1987] was adapted for protein elution from acrylamide gel. For electro elution from alkaline PAGE tube gels, one of the tube gels was fixed, stained and destained to serve as a reference to cut out the required protein bands from other tube gels, which was kept at 4^{0}C after the run. Gel slices containing the required protein band were minced with a scalpel blade and kept in tris-acetate buffer (5 mM Tris; 2.5 mM acetic acid, pH 8) in a dialysis bag and immersed in the same buffer in a horizontal electrophoresis chamber. A current of 100 V was applied across the bag for 3 h. Electro elution was performed at 4^{0}C. Current was reversed for 5 min to detach the protein adhered to the sides of the dialysis bag facing the positive electrode. Contents of the bag was centrifuged at 1000 g and the supernatant containing protein was dialysed against PBS and concentrated by AMICON PM10 ultra filtration membrane.

Preparation of erythrocyte membrane glycopeptides

Outdated human blood was collected and washed thrice, using PBS 7.4 by centrifugation at 400 g, to get erythrocytes free from plasma.

After the third washing and removing the buffy coat 8 ml packed cell was mixed with 72 ml lysis buffer (5 mM pyridine acetic acid buffer, pH 8) and membrane ghosts were pelleted by centrifugation at 1,00,000 g and settled ghosts were washed with the same buffer and again allowed to settle by centrifugation. The pellet obtained was resuspended in 32 ml reaction buffer (50 mM pyridine acetic acid buffer pH 7.9). Freeze thawing and sonication was done to ensure complete lysis. To the lysate 8 mg Pronase and 2 mM $CaCl_2$ were added and incubated at 37^0C for 24 h. After 24 hours 8 mg Pronase and 2 mM $CaCl_2$ were added and incubation continued for another 24 h. Reaction was stopped by heating the sample in boiling water bath for 30 min. Then it was subjected to ultrafiltration using AMICON PM10 ultrafiltration membrane. The filtrate obtained was concentrated to dryness by flash evaporation and resuspended in PBS 7.4 and used for inhibition studies.

For preparation of desialylated erythrocyte membrane glycopeptides neuraminidase treatment of human erythrocyte was done as described earlier and pronase digestion was performed.

CHAPTER III
RESULTS AND DISCUSSION

PART I

Purification of human heart galectin-1

RESULTS AND DISCUSSION

Purification of human heart galectin-1

Human galectin-1 is a dimeric carbohydrate binding protein having subunit molecular weight around 14 kDa. In mammals galectin-1 has a broad tissue distribution, expressed in muscle tissues, spleen, thymic epithelial cells, endothelial cells, lung, brain, heart and the olfactory system [Baum et al 1995 a, b; Puche and Key, 1995; Ahmed et al., 1996; Chadli et al., 1997].

Galectin-1 was purified from human heart (muscle) tissue by affinity chromatography using lactose-Sepharose. Extraction of the lectin from the tissue into buffer is enhanced by the presence of specific sugar, lactose [Waard et al., 1976]. To protect galectin-1 against oxidative inactivation, it was treated with a cysteine-modifying reagent, iodoacetamide, during elution from the affinity resin. Alkylation with iodoacetamide yields carboxamidomethyl-galectin, which is fully active and stable to atmospheric oxygen in the absence of exogenous thiols [Whitney et al., 1986]. Alkylation of lectin not only enabled the affinity chromatography experiments to be performed in the absence of reducing agent but also protected the lectin against inactivation by oxidation of the labile free –SH groups during chromatography, dialysis and storage.

Presence of hapten sugar also helps in protecting the lectin from denaturation and precipitation, especially at higher temperature.

The eluate (HHL-G) obtained from affinity matrix not only contained 14 kDa lectin but also a group of endogenous proteins as evidenced by SDS-PAGE (Fig. 1). The yield of HHL-G from 35 g human heart tissue using 40 ml affinity matrix (lactose -Sepharose) varied from 600-700 μg. The average concentration of galectin-1 in rat lung and intestine was about 30 and 70 μg/g respectively. Galectin-1 can constitute up to 1% of total protein in cells that synthesize it [Allen et al., 1990]. The activity of HHL-G was checked using trypsinized rabbit erythrocytes and the minimum agglutinating quantity was found to be 32 ng (Fig. 2 inset). Rabbit erythrocytes are rich in exposed T antigen and terminal alpha linked galactose (TAG) groups on their surface, which become fully exposed on trypsin-mediated proteolysis. Presence of 1 M NaCl or 1% Triton X-100 in the chromatographic column washing buffer could not prevent the co-purification of proteins along with lectin, suggesting the absence of ionic or hydrophobic interaction as the reason for the binding of co-purified glycoproteins to lactose- Sepharose. But presence of 50 mM lactose in the 70% ammonium sulphate precipitate of heart tissue prevented binding of both galectin-1 as well as endogenous glycoproteins to the matrix indicating the sugar-dependent nature of association of galectin-1 and co-purified glycoproteins.

Separation of galectin-1 from co-purified proteins could not be achieved by gel filtration at atmospheric pressure. Upon HPLC the HHL-G resolved into 3 peaks (Fig. 2) and the lectin activity detected using trypsinised rabbit erythrocytes was confined to peak 2. Minimum agglutination quantity of peak 2 protein considered as HHL was found to be 7.5 ng (Fig. 2 inset). Proteins of peaks 1 and 3 failed to agglutinate trypsinized rabbit erythrocytes even at the maximum amount tried (Fig .2 inset). The purified galectin-1 obtained as peak 2 in HPLC showed a single protein band in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% gel under reducing conditions with electrophoretic mobility corresponding to 14 kDa (Fig. 1). In non-denatured form galectin-1 exists as dimer of molecular weight 28-30 kDa presumably to facilitate clustering of receptors needed for its biological effects [Sharon, 1994]. To study the interactions of human heart galectin-1 (HHL) with various glycoproteins, peak 2 protein was coupled to horse radish peroxidase (HRP). The galectin-1 HRP conjugate was stored in 30% glycerol at 4^0C.

For characterization, proteins in peak 1 and 3 obtained in HPLC were separately collected, coated on microtitre plates and probed with HHL-HRP. These proteins offered much stronger ligands for HHL, even before desialylation, than did asialofetuin to which galectin-1 is known to

bind with high affinity (Table.1). This confirmed that co-purified proteins were glycoproteins, which possessed excellent affinity for HHL. Peanut agglutinin (PNA) is a fairly stable and non-glycosylated lectin, which sharply discriminates between sialylated, and non-sialylated forms of its most powerful inhibitor carbohydrate group Gal β1-3 GalNAc (T antigen) [Lotan et al., 1975; Roque-Barriera and Campos-Neto, 1985]. This lectin has been shown to bind N-linked oligosaccharides poorly [Chacko and Appukuttan, 2001]. Co-purified glycoproteins, especially of peak 1 appear to be rich in non-sialylated (exposed) T antigen since they bind PNA very strongly (Table.1). Proteins in peaks 1 and 3 also contain sialylated T antigen groups since upon desialylation their PNA-binding capacity is enhanced twice and 1.5 times respectively. But HHL-HRP binding to co-purified proteins in peak 1 and 3 was little affected by desialylation (Table.1). Differences in desialylation-dependent enhancement of lectin binding to glycoproteins are also noted between PNA and jacalin both T antigen-specific lectins [Chacko and Appukuttan, 2001]. Since anti-α-Galactoside antibody (Anti-α-Gal) failed to recognize the co-purified glycoproteins (Table.1) it could be concluded that the glycoproteins lack terminal α-linked galactose moiety and that recognition by HHL and PNA was sugar-specific. The abundance of T antigen in co-purified glycoproteins suggested a preference of HHL for this sugar group. The HHL recognition of co-purified glycoproteins was further proven by the sugar- dependent binding of HHL-HRP to heat inactivated HHL-G immobilized on PVDF membrane (Fig.3).

Table 1

HRP-labeled lectin/antibody	Peak 1		Peak 3		Fetuin	
	-N	+N	-N	+N	-N	+N
HHL	0.86 (±0.06)	0.87 (±0.07)	1.14 (±0.16)	1.2 (±0.30)	0.2 (±0.03)	0.27 (±0.03)
PNA	0.77 (±0.08)	1.48 (±0.10)	0.20 (±0.01)	0.33 (±0.01)	0.05 (±0.01)	0.28 (±0.01)
Anti-α-Gal	0.02 (±0.01)	–	0.02 (±0.01)	–	0.02 (±0.01)	–

Recognition of glycoproteins co-purified with HHL, by HHL, PNA and anti-α-Gal. Glycoproteins of peaks 1 and 3, obtained after HPLC of HHL-G and fetuin were coated on polystyrene 96 well microwell plates (80 ng / well) and treated with (+N) or without (-N) neuraminidase, washed and probed with HRP conjugates of HHL (7.5 μg lectin/ml), PNA (3 μg lectin/ml) or anti-Gal (15 μg antibody/ml) for 2 h at 4° C before washing and addition of OPD (details in 'Methods').

Discussion

Endogenous glycoproteins had been shown to co-purify with bovine heart galectin-1 [Appukuttan, 2002], which has got 87% amino acid sequence homology to human galectin-1. It was suggested that when the divalent galectin-1 binds to multivalent endogenous glycoproteins the resulting complexes contain enough sugar binding sites to facilitate

erythrocyte receptor aggregation during hemagglutination by this complex as well as its attachment to lactose-Sepharose during isolation of galectin-1. The bovine heart galectin-1 binding to endogenous glycoproteins co-purified with it during chromatography solely depended on the terminal α-linked galactose moieties on the latter where as these groups are, absent on endogenous glycoproteins co-purified with human heart galectin-1. Both human and bovine galectin-1 contain 134 amino acids but are different at 17 residues resulting in 87 % identity and several differences are non conservative [Abbott et al., 1989; Hirabayashi et al., 1989]. If galectin-1 has an extracellular function then the lability of its binding activity in the presence of oxygen can put stringent restrictions on the latitude of its activity. In tissue and cell extracts there is some evidences that galectin-1 is not free but associated with its biological partners to form high mass complexes and that the spontaneous inactivation of native galectin-1 in the absence of reducing agents can be prevented, if lectin is bound to glycoconjugates [Cho and Cummings, 1995]. This provides a mechanism for the stability of the lectin in the extracellular environment. Present results suggest lectin-carbohydrate recognition as primary reason for association of co-purified glycoproteins with HHL within intact tissue or after homogenization. Co-purified glycoproteins therefore are chosen endogenous complementary glycoproteins for HHL and contain sugar ligands apt for the lectin. Further proof for this is the recognition of coated HPLC-separated

co-purified glycoproteins by purified HHL. In this context the abundance of sialylated as well as free T antigen in co-purified glycoproteins points to a preference for T antigen by HHL.

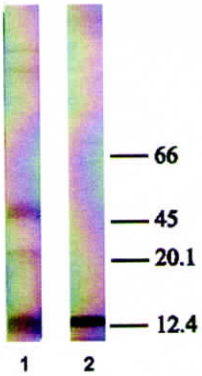


Fig. 1 Sodium dodecyl sulphate poly acrylamide (7.5 %) gel electrophoresis followed by Coomassie blue staining of (1) HHL-G eluted from lactose Sepharose (40 μg) and (2) HPLC purified HHL (25 μg). Positions of standard proteins with their molecular weight in kDa are indicated on the right.

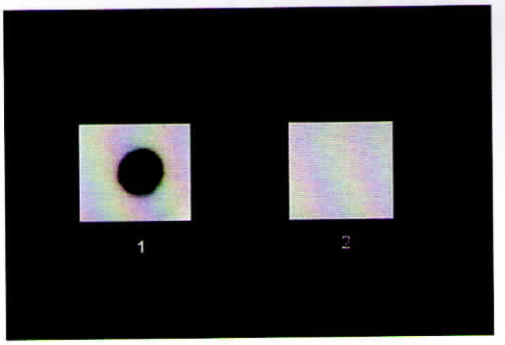
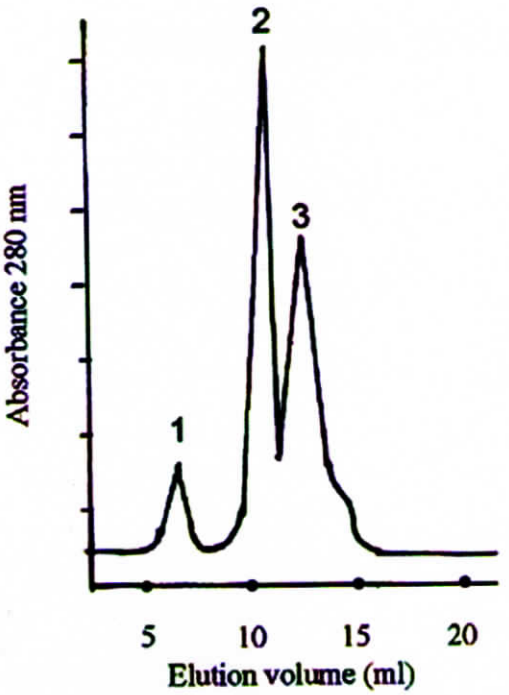
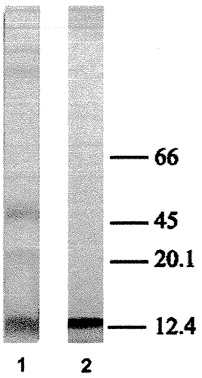


Fig. 3 HHL-HRP recognition of dot-blotted and heat inactivated HHL-G. (1) In the absence and (2) in the presence of 50 mM lactose.



Peak No.	MAQ(ng)
1	>200*
2	7.5
3	>200*
HHL-G	32.0

Fig. 2 HPLC separation of HHL-G proteins in size exclusion column monitored by absorption at 280nm. Relative abundance of proteins in individual peaks varied with tissue sample. HHL-G was pre-HPLC sample containing co-purified glycoproteins along with galectin-1. Inset shows minimum agglutinating quantity (MAQ) of proteins from peaks. *: Maximum amount of protein tried.



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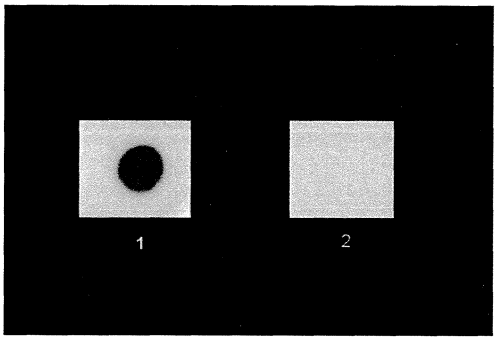

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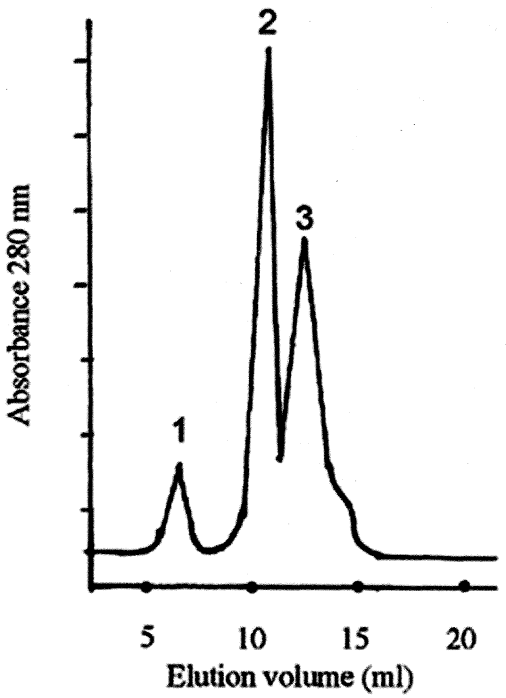

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PART II

Demonstration of T antigen specificity of human heart galectin-1

Demonstration of T antigen specificity of human galectin-1

The affinity of HHL towards T antigen (Galβ 1→3 GalNAc) as part of O-glycan in contrast to that towards LacNAc (Galβ 1→4 GlcNAc) structure as part of N-glycan is examined with emphasis on endogenous glycoprotein, IgA1 which is the only immunoglobulin having T antigen structure. IgA is present in normal human serum at about one fifth of the concentration of IgG and catabolized around five times faster than IgG [Heremans, 1974]. IgA is the most abundant immunoglobulin in secretions. Human IgA occurs in two isotypic forms IgA1 and IgA2. Although the importance of IgA in mucosal secretions is well established, it is now clear that in humans much of the IgA is secreted directly into the blood and never reaches the mucosal surfaces [Kutteh et al., 1982]. Human serum IgA is predominantly monomeric IgA1, which is produced in the bone marrow, while in external secretions most of the locally produced IgA is polymeric [Kett et al., 1986; Mestecky and McGhee, 1987]. IgA1 contains two potential N-linked glycosylation sites and five O-linked sites in the hinge region while IgA2 lacks O-linked sugars.

Purification of IgA1 samples

IgA1 that binds to Sepharose-immobilized jacalin (IgA1S) was isolated from jacalin-binding plasma glycoproteins (JPGP). For the

separation of JPGP (prepared as described under "Methods"), they were subjected to both acid and alkaline PAGE electrophoresis. In alkaline PAGE the JPGP resolved into distinct components (Fig. 4) but in acid PAGE the resolution as well as the mobility of these glycoproteins was less (Fig .5). To ensure that those glycoproteins which were moving in acid PAGE were also moving in alkaline PAGE the glycoproteins which had already moved into the acid page gel were electroeluted and subjected to alkaline PAGE where it was found that the glycoproteins which were moving in acid PAGE were also moving in alkaline PAGE (Fig .5). Thus all members of JPGP had moved and got resolved from each other on alkaline PAGE.

Constituents of JPGP were separated by alkaline PAGE before electroelution under non-denaturing conditions (Fig. 4). Coomassie blue staining of one marker gel indicated five protein bands. From corresponding slices of non-denatured gels proteins were electroeluted and concentrated by ultrafiltration. Polystyrene microwell coatings (100 ng per well) from each of the five bands were probed with anti-human IgA-HRP. Proteins in IgA-containing bands (1 and 2; Fig 4) in alkaline pH electrophoresis were pooled as IgA1S and others as non-IgA1S serum O-glycosylated proteins. IgA1 that binds to polystyrene-immobilized jacalin (IgA1P) was isolated from the naturally occurring dextran-binding human immunoglobulin (DIg) as described under "Methods".

To study the T antigen specificity of HHL, differentially N- and O-linked glycoproteins were used as inhibitors of HHL binding to polystyrene-coated asialofetuin. Among the glycoproteins tested, the two IgA1 samples, IgA1S and IgA1P were excellent ligands for HHL being respectively about 2.65 and 12 times better than asialofetuin in terms of sugar content (Table.2). But human serum IgG, which contains only N-linked oligosaccharides failed to inhibit HHL. Being an anti-polysaccharide antibody IgA1P is likely to posses a higher ratio of polymeric to monomeric IgA [Johnson et al., 1996] than in total serum IgA and this may explain its higher HHL inhibitory capacity compared to IgA1S.

Human serum IgG (Sigma Chemicals, U.S.A.) contained traces of IgA (Fig. 6) and was purified by passing its solution in PBS, pH 6.5 (2 mg/ml) through a 5 ml jacalin-Sepharose 4B column in the same buffer at 4^{0}C. Unbound fraction (free from IgA as shown by enzyme-linked immunosorbent assay [ELISA]) was used as IgG (Fig .7).

Even without desialylation, PNA-binding glycoproteins from bovine and human heart muscle were almost as effective and jacalin-binding human erythrocyte membrane glycoproteins nearly thrice as effective as asialofetuin in inhibiting HHL (Table.2). Serum glycoprotein-rich fraction that contains all immunoglobulin types also inhibited HHL though less effectively than did asialofetuin, which contains both N- and O-linked oligosaccharides (Table.2). However, peripheral N-linked oligosaccharides

Table 2

Glycoprotein / sugar	Concentration (μg neutral sugar /ml) required for 50 % inhibition.
Human IgG	>100*
Non IgA1 O-glycosylated serum proteins	9
Human IgA1S	1
Human IgA1P	0.22
Non-IgA1 DIg	20
Human serum glycoprotein-rich fraction	6.5
Peripheral N-linked oligosaccharides of human serum glycoprotein-rich fraction[1]	175
Asialofetuin	2.65
Peripheral N-linked oligosaccharides of asialofetuin[1]	>160*
PNA-binding human heart glycoproteins	2.17
PNA-binding bovine heart glycoproteins	2.92
Jacalin-binding human erythrocyte glycoproteins	0.84
Lactose	180

Inhibition of HHL binding to polystyrene well-coated asialofetuin by various glycoproteins / sugars. Serial two-fold dilutions of glycoproteins / sugars were incubated with HHL-HRP (7.5 μg lectin/ml) for 1h at 4°C and the mixture was added to microwell-coated asialofetuin for incubation at 4°C and binding assay as described under "Methods". Mean of triplicate trials shown. S.D. was less than 10% of mean. [1]: prepared using jack bean α-mannosidase as described. *Maximum concentration tried.

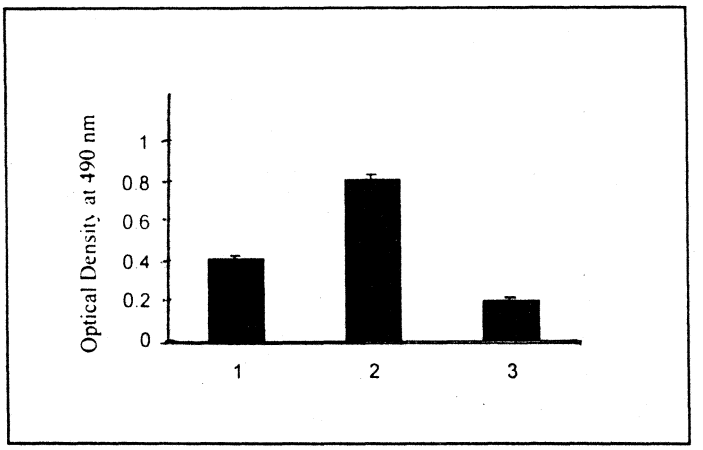


Fig. 6

AHIgA-HRP, AHIgG-HRP and jacalin-HRP recognition of IgG (Sigma, USA). IgG immobilized (500 ng/well) on polystyrene microwell plates were blocked with PBS containing 0.5 % Tween-20 and probed with (1) AHIgA-HRP (2) AHIgG-HRP and (3) jacalin-HRP.

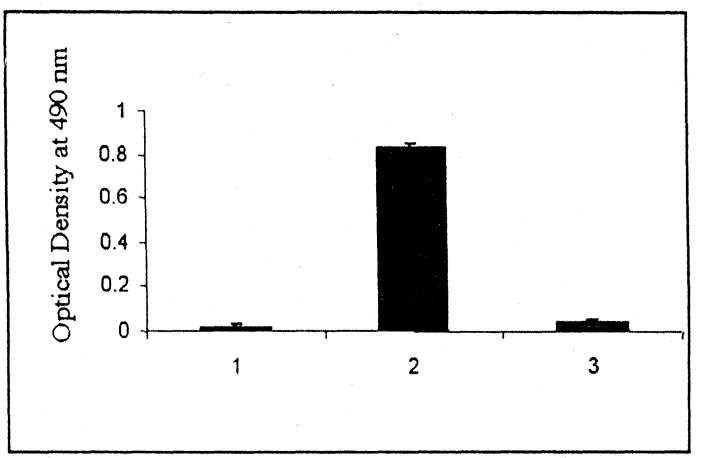


Fig. 7

AHIgA-HRP, AHIgG-HRP and jacalin-HRP recognition of IgG (Sigma, USA) purified using jacalin-Sepharose. Purified IgG immobilized (500 ng/well) on polystyrene microwell plates were blocked with PBS containing 0.5 % Tween-20 and probed with (1) AHIgA-HRP (2) AHIgG-HRP and (3) jacalin-HRP.

of asialofetuin or of serum glycoprotein-rich fraction (released by jack bean α-mannosidase, which cleaves internal α- mannose linkage with their LacNAc group unaffected) did not inhibit HHL even at the maximum concentration tried (Table.2). Given the marked specificity of PNA and jacalin for T antigen these results indicate that galactose moieties as part of T antigen structures at the termini of O-linked oligosaccharides such as present in IgA1 or fetuin are very strong ligands where as galactose moieties which occur as part of sialylated or free LacNAc terminals of O-linked oligosaccharides are poor ligands for HHL. Notably a much poorer inhibition by non-IgA1 O-linked serum glycoproteins compared to IgA1 is seen from Table.2.

Preference of HHL for terminal alpha linked galactose moiety

The affinity of HHL towards terminal α- linked galactose in contrast to that towards its β-anomer was checked using sugars. It was found that 1-O-methyl α-D-Gal and para nitro phenyl α-D-Gal were nearly twice and 8 times better inhibitors than their corresponding β-anomers respectively (Table.3). Among the glycoproteins tested, bovine thyroglobulin, which contained terminal alpha linked galactose moiety, was nearly as good as human IgA1 in inhibiting HHL (Table.3).

Table 3

Glycoprotein / sugar	Concentration (μg neutral sugar /ml) required for 50 % inhibition.
1-O-methyl α-D-Gal	8,424
1-O-methyl β-D-Gal	168,00
Para nitro phenyl α-D-Gal	850
Para nitro phenyl -β-D-Gal	6800
Bovine thyroglobulin	1.56

Inhibition of HHL binding to polystyrene well-coated asialofetuin. Serial two-fold dilutions of glycoprotein / sugars were incubated with HHL-HRP (7.5 μg lectin/ml) for 1 h at 4°C and the mixture was added to microwell-coated asialofetuin for incubation at 4°C and binding assay as described under "Methods". Mean of triplicate trials shown. S.D. was less than 10% of mean.

Effect of removal of peripheral sugars of N-linked oligosaccharides of fetuin.

Bovine fetuin is a globular glycoprotein containing three each of both N- and O-linked oligosaccharides amounting to a total carbohydrate content of 22.6 % [Nilsson et al., 1979]. The terminal sugars on each of the six oligosaccharide units is N-acetyl neuraminic acid (sialic acid, NANA). On the complex type N-linked oligosaccharide units removal of sialic acid moiety exposes Gal β1-4 GlcNAc (LacNAc) disaccharide on the periphery while on the O-linked oligosaccharide chain desialylation exposes T antigenic group. [Green et al., 1988; Spiro and Bhoyroo, 1974].

Desialylation followed by selective removal of peripheral N-linked oligosaccharides of fetuin coated on polystyrene plates using jack bean α-mannosidase partially affected its capacity to bind RCA1, which is specific for both N-and O-linked oligosaccharides (Fig. 8). But the α- mannosidase treated fetuin was as good as native fetuin in capturing HHL and jacalin indicating that N-linked oligosaccharides of fetuin contributes little towards HHL binding and that T antigen which solely accounts for the galactose moieties in O-linked oligosaccharides is the lectin-binding saccharide group in fetuin.

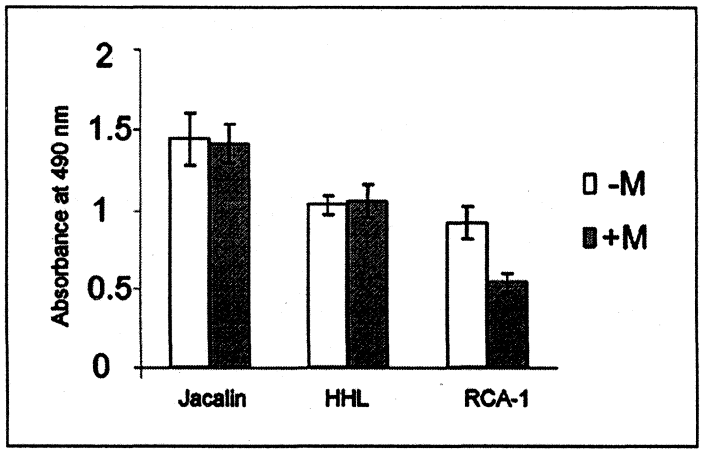


Fig. 8

Effect of removal of peripheral sugar units from N-linked oligosaccharides of fetuin using jack bean α-mannosidase on its recognition by HHL and other lectins. One μg fetuin coated on microtitre plate well was treated with (+M) or without (-M) jack bean α-mannosidase before addition of HRP conjugates of lectins (7.5 μg lectin/ml), followed by detection of bound lectin conjugate using OPD as described in the "Methods". Mean ± SD of triplicate trials shown.

Effect of removal of peripheral N-linked oligosaccharides of IgA1.

The preference of HHL for galactose in O-linked oligosaccharides was also demonstrated in human IgA1 using dot blotted IgA1S sample. Desialylation followed by alpha mannosidase treatment of IgA1S sample fully abolished its capacity to bind ConA where as binding of HHL or jacalin was unaffected (Fig. 9). The N-linked oligosaccharide terminal fraction released by α-mannosidase from IgA1S contains the sugar chain from periphery to the α-linked mannose including LacNAc groups intact. However this oligosaccharide was a poor inhibitor of HHL, though it inhibited ConA (due to the presence of α-linked mannose units) proving that single LacNAc group, as part of N-linked oligosaccharides is a poor ligand for HHL (Fig. 9 inset).

Hemagglutination inhibition of IgA1S, IgG and Fetuin

Affinity of HHL for glycoproteins was also quantitated in terms of inhibition of HHL-mediated hemagglutination at 25°C. Twice minimum agglutinating quantity of HPLC-purified HHL was preincubated with two-fold serial dilutions of glycoproteins before addition of trypsinized rabbit erythrocytes to a final concentration of 1%. Results (Table. 4) agree with the findings above since IgA1S was nearly 12 times better inhibitor than fetuin while IgG was not an inhibitor.

Table 4

Glycoprotein	MIQ (μg)
IgA1S	12.5
IgG	> 150
Fetuin	150

Inhibition of hemagglutination of HHL by IgA1S, IgG and fetuin. Serial two fold dilutions of glycoproteins were incubated with twice the minimum agglutinating amount of HHL for 1 h at 4° C before addition of trypsinized rabbit erythrocytes as described under "Methods". MIQ: Minimum inhibitory quantity of glycoprotein, expressed as mean of triplicate trials; S.D. < 10 % of mean.

Interaction of HHL with O-glycan containing glycoproteins of human plasma

The jacalin-binding plasma glycoproteins resolved into components by alkaline electrophoresis (Fig.4), were separately electroeluted under non-denaturing conditions and immobilized on polystyrene wells. These glycoproteins were readily recognized by HHL-HRP. The most prominent band recognized by HHL-HRP was second (IgA1 rich band) (Fig.10). This is in line with the earlier observation that IgA1 is the prominent plasma glycoprotein recognized by HHL. Upon desialylation HHL-HRP recognition of these glycoproteins increased (Fig.10) remarkably. ConA-HRP readily recognized these glycoproteins (Fig.11) while PNA-HRP recognized them only after desialylation (Fig.12). Purification of these glycoproteins using immobilized jacalin confirms the presence of T antigen in their O-linked oligosaccharide sequence while requirement of desialylation for recognition by PNA-HRP indicates that the T antigen

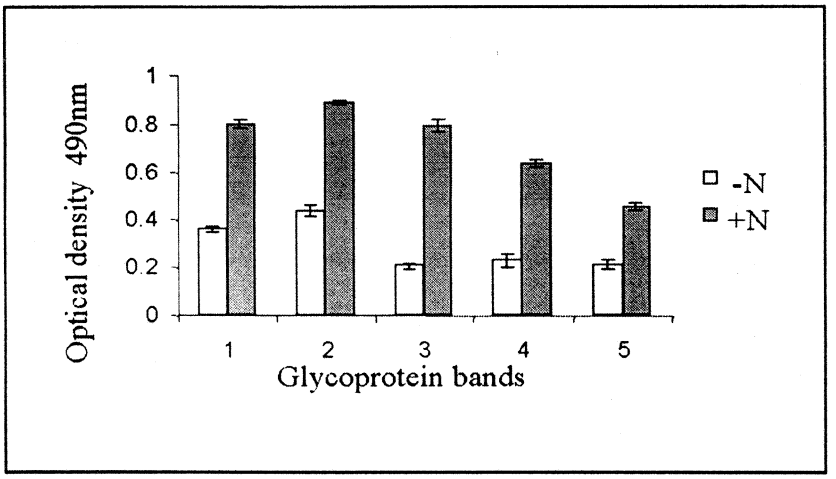


Fig. 10

HHL-HRP recognition of JPGP resolved into components by alkaline pH (8.3) electrophoresis in 5% polyacrylamide gel. Proteins corresponding to bands (1,2,3,4,& 5) (200 ng/well) immobilized on microtitre plates were blocked using PBS containing 0.5% Tween 20, treated with neuraminidase ,probed with HHL-HRP, colour developed using OPD and measured at 490 nm.

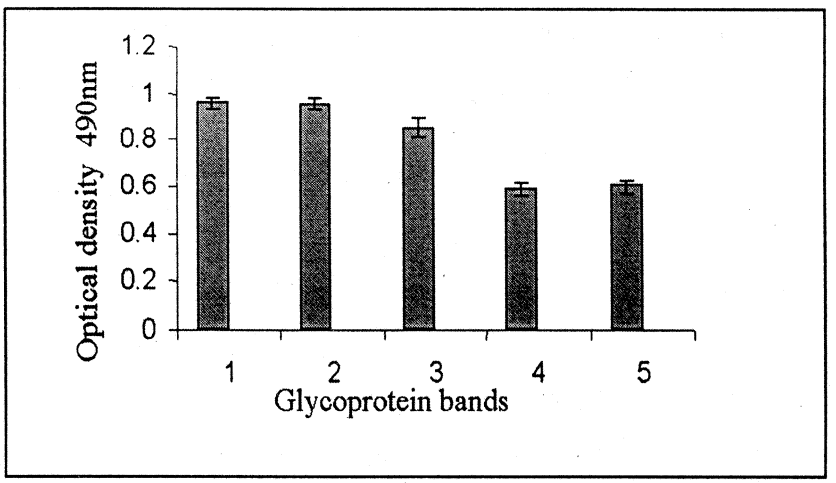


Fig. 11

Concanavalin A recognition of JPGP resolved into components by alkaline pH (8.3) electrophoresis in 5% polyacrylamide gel. Protein corresponding to bands (1,2,3,4,& 5) (200 ng/well) immobilized on microtitre plates were blocked using PBS containing 0.5% Tween 20 and treated with Con A. Bound Con A was assayed with HRP as described under "Methods" using OPD.

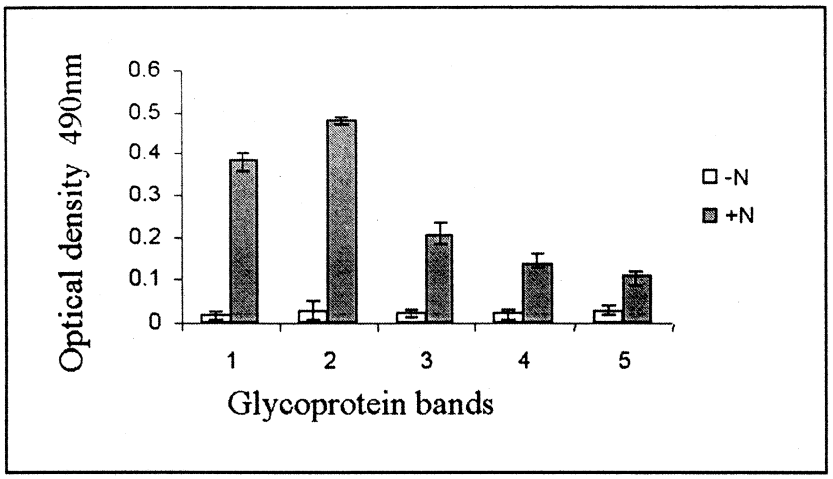


Fig. 12

PNA-HRP recognition of JPGP resolved into components by alkaline pH (8.3) electrophoresis in 5% polyacrylamide gel. Protein corresponding to bands (1,2,3,4 & 5) (200 ng/well) immobilized on microtitre plates were blocked using PBS containing 0.5% Tween 20, treated with neuraminidase, probed with PNA-HRP, colour developed using OPD and measured at 490 nm.

moieties are invariably covered. More significantly, HHL-HRP recognition shows that HHL can recognize sialylated oligosaccharide structures as well and that the difference in reactivity between sialylated and desialylated galactose units varies among glycoproteins. Thus desialylation does not enhance reactivity of fetuin as remarkably as it does that of serum glycoproteins. HHL-HRP recognition of jacalin-binding plasma glycoproteins was also proven by western blot. Western blotted JPGP were sugar specifically recognized by HHL-HRP (Fig. 13). HHL-HRP recognition increased upon desialylation of the Western blot (Fig .13).

Purification of HHL using immobilized jacalin-binding plasma glycoproteins

The jacalin-binding plasma glycoproteins (JPGP) which is rich in IgA1 as well as desialylated jacalin-binding plasma glycoproteins

(DJPGP) were coupled to cyanogen-bromide activated Sepharose 4B at the rate of 4 mg/ml gel. Both the matrices captured galectin-1 from human heart tissue homogenate as did lactose Sepharose 4B (Fig. 14). But the yield of HHL purified by DJPGP-Sepharose was about three times higher than the HHL purified by JPGP-Sepharose (Table. 5). However, desialylated human IgG when immobilized on Sepharose failed to purify HHL from tissue extract.

Table 5

Glycoprotein immobilized (on Sepharose 4B)	Amount of lectin bound (mg/ml gel)
JPGP	0.008
DJPGP	0.023

Effect of desialylation of glycoproteins on their recognition by HHL.

Since many infectious pathogens secrete the enzyme, neuraminidase to which human tissue glycoconjugates are susceptible, the effect of bacterial neuraminidase-mediated desialylation of a few glycoproteins on their capacity to bind HHL was studied. Results (Table .6) reveal that following desialylation of IgA1S and IgA1P in polystyrene plate-coated form using *Clostridium perfringens* neuraminidase, HHL binding to these glycoproteins increased tremendously, their desialylated derivatives being respectively 92% and 154% more efficient than the native IgA1 samples. In contrast, desialylation-dependent increase in binding of

HHL was marginal for fetuin. Non-IgA1 O-glycosylated serum glycoproteins were weaker ligands than IgA1 for HHL in their polystyrene-coated form as well. Results in Table 6 also show that human IgG as well as non-O-glycosylated serum glycoproteins were much poorer than IgA1 or other O-glycosylated glycoproteins in capturing HHL.

Table 6

Glycoprotein	Absorbance 490 nm		Percentage increase in HHL binding on desialylation.
	-N	+N	
IgA1S	1.21 ±0.10	2.32 ± 0.2	92
IgA1P	1.00 ± 0.12	2.54 ± 0.3	154
Non-IgA1S O-glycosylated serum proteins	0.31 ± 0.02	0.60 ± 0.03	95
Fetuin	0.61 ± 0.05	0.72 ± 0.06	19
Human IgG	0.12 ± 0.02	0.13 ± 0.02	8
Non-O-glycosylated serum proteins	0.11 ±0.02	0.12 ±0.02	9

Micro plate well-coated glycoproteins (250 ng for IgA1P and 500 ng for others) was treated with neuraminidase (100 μg / ml) followed by HHL-HRP (7.5 μg lectin/ml) and binding measured using OPD as described in "Methods"

Discussion

With its carbohydrate-recognition domain spanning almost the entire monomer polypeptide sequence [Harrison, 1991] the physiological

role of animal galectin-1 is very likely to be based on its recognition of complementary carbohydrate groups offered by glycoconjugates present on microbial or tumor cell surface, or on circulating or endogenous macromolecules. The strong inhibition of HHL by glycoproteins such as IgA1, PNA- and jacalin-binding human and bovine glycoproteins and fetuin, all rich in T antigen, but not by their N-linked oligosaccharides or by human IgG, which lacks the T antigen sugar group suggests the T antigen specificity of HHL. Sustained HHL binding to fetuin and IgA1S from which N-linked oligosaccharides were selectively removed, efficacy of O-glycosylated serum glycoproteins in contrast to that of IgG in Sepharose-immobilised form in capturing HHL and the very strong inhibition of hemagglutination of HHL by IgA1 but not by IgG are further evidences for a strong preference of the lectin for T antigen rather than for N-linked sugars of these glycoproteins. In support of this conclusion, sugar-specific recognition of autologous brain gangliosides, which contain terminal T antigen, by bovine brain galectin-1, was reported [Kannan and Appukuttan, 1993]. It had also been shown [Appukuttan, 2002] that bands recognized by jacalin from Western blots of human serum glycoprotein-rich fraction were the same as those recognized by bovine heart galectin-1 which shares about 90% sequence homology with HHL [Harrison, 1991].

Galectin-1 came to be called a β-galactoside-binding lectin primarily because the disaccharides lactose and LacNAc, its best inhibitors known were β-galactosides [Zhou and Cummings, 1993]. Results here demonstrate poor galectin-1 binding to oligosaccharides possessing single LacNAc only and do not contradict reports of strong poly N-acetyl lactosamine chain recognition by the lectin [Zhou and Cummings, 1993] though most serum and cell surface glycoproteins bear N-linked oligosaccharides of exclusively the former category. Occurrence of poly N-acetyl lactosamine sequence in human O-linked oligosaccharides is rare and limited to activated T cells and leukemia cells [Fukuda, 2000]. Significantly, unlike lactose, lactosyl-BSA was poor ligand for the two T antigen-binding lectins PNA [Chacko and Appukuttan, 2001] and galectin-1 [Appukuttan, 2002]. On the other hand conjugation to proteins markedly elevated lectin binding by T antigen as exemplified by O, NN blood group substance (M.W. 550,000) which was 250 times more active towards PNA than was T antigen disaccharide as such, compared on the basis of epitope concentration [Lotan et al., 1975].

Evidences obtained here for strong recognition of T antigen more so in non-sialylated state by human galectin-1 offer possible explanations for several reported findings on mammalian galectin-1 as well as suggest mechanisms for its physiological function. Firstly abundance of exposed

(non-sialylated) T antigen as well as of galectin-1 being distinguishing features of tumor cells [Springer, 1984; Meromsky et al., 1986] extensive galectin-1-T antigen interactions may contribute towards the well known capacity of tumor cells to aggregate and to get anchored on endothelial cell surfaces where galectin-1 is known to be present [Baum et al., 1995b]. Moreover anti-galectin-1 antibody prevented colonization and metastasis of tumor cells in vitro as well as in vivo [Meromsky et al., 1986]. T antigen though widely distributed in the human body is mostly covered by at least one sialic acid substitution probably to keep immunological harmony with a naturally occurring serum antibody against free T antigen (anti-T). Since human galectin-1 agglutinated autogenous erythrocytes after their desialylation, this lectin possibly distinguishes tumor and microbial cells from host cells in terms of non-sialylated surface T antigen.

Secondly the observation that IgA1 is the most prominent serum glycoprotein interacting with tissue galectin-1 is significant to both the normal biology of serum IgA1 and IgA1-mediated immune pathology. Incidentally, though IgA is the most synthesized and most turned over immunoglobulin type in serum, biological fate of IgA1 subtype that makes up more than 85% of total IgA is much less clear than that of IgG or IgA2 [Monteiro and Van de Winkel, 2003]. Clearance via the hepatobiliary route mediated by asialoglycoprotein receptors accounts for a very minor fraction

of turnover of IgA1 unlike in the case of IgA2 though serum half life of both are similar [Kerr, 1990]. Moreover, FcαR1 receptors resident on neutrophils and macrophages bind IgA transiently and with comparatively very low affinity [Wines et al.,1999]. Thus a role for galectin-1, well expressed and widely distributed in human tissues including the endothelial cells, in sequestering the most prominent T antigen-bearing serum glycoprotein, IgA1, seems very likely.

Immune inflammatory events accompanying infections may involve galectin-1 recognition of IgA1 since neuraminidase or sialidase, an enzyme secreted by many infectious pathogens [Corfield et al., 1981; Corfield, 1992] may desialylate serum IgA1 and its immune complexes leading to their deposition in tissues containing galectin-1 since formation of immune complexes does not engage the carbohydrate side chains of IgA1. Elevated levels of sialidase activity have been observed in human serum during infection with sialidase producing bacteria or viruses. Most streptococci isolated from patients with glomerulonephritis were found to produce a sialidase activity able to hydrolyse immunologically relevant substrates [Mosquera et al., 1985] and sialidase activity together with free sialic acid has been detected in the serum of patients with acute post streptococcal glomerulonephritis [Rodrigues-Iturbe et al., 1981]. By desialylating serum glycoproteins especially immunoglobulins, this enzyme

promotes the formation of autologous immune complexes [Rodrigues-Iturbe et al., 1981; Mosquera and Rodrigues-Iturbe, 1986] probably via an interaction with naturally occurring galactosyl specific antibodies [Springer, 1984; Lambre et al., 1985].

Even in the possible event of desialylated IgA1 getting recognized by serum anti-T antibody, the latter being comprised mostly of pentameric IgM, the resulting complexes could still bear unoccupied T antigen groups of IgA1 that may be recognized by galectin-1. Indeed circulating immune complexes have been noted as a major factor predisposing patients to cardiovascular disorders [Mustafa et al 2000]. High serum IgA has been correlated with complications of diabetes that are mostly accompanied by vascular damage [Rodriguez-Segade, 1996]. More compelling evidences are emerging for T antigen-dependent IgA1 deposition in IgA nephropathy. Molecular pathology of this highly prevalent disorder that often accompanies respiratory infections is still unclear even as IgA1 has been shown to be the sole immunoglobulin isotype getting deposited in the glomerular mesangium [Mestecky et al., 1986]. Henoch-Schonlein purpura (HSP), a variant of IgA nephropathy marked by skin lesions apart from renal damage is also characterized by deposition of IgA1 aggregates or IgA1 immune complexes in cutaneous capillaries resulting in inflammation [Scheinfeld et al., 2003]. Interestingly, a recent report demonstrated that in

contrast to serum IgA1, mesangial deposit of IgA1 was enriched in desialylated T antigen [Iwase et al., 2002]. Similarly O-linked oligosaccharides of serum IgA1 from children with acute HSP are deficient in sialic acid compared to serum IgA1 from control children [Saulsbury, 1997]. Presence of galectin-1 in mesangial cells and in cells around capillaries being very likely, IgA nephropathy and HSP are apt models to investigate the role of galectin-1-mediated IgA1 sequestration in immunopathology.

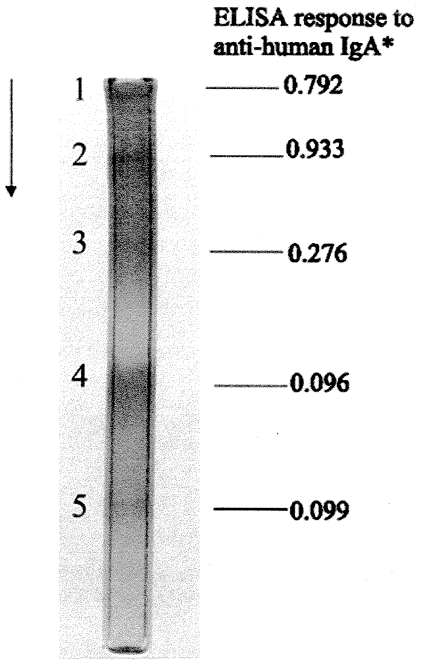


Fig. 4 Alkaline pH (8.3) electrophoresis in 5% polyacrylamide gel of jacalin-binding human plasma glycoproteins (JPGP) with Coomassie blue staining (left). Non-denatured proteins corresponding to bands were electro-eluted and coated on microwells (100 ng/well) and response to anti-human IgA measured using HRP conjugate of the latter (right). Proteins in bands 1 and 2 were pooled as IgA1S.
*: Absorbance at 490 nm.

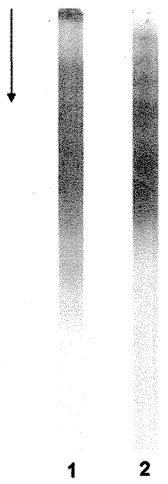
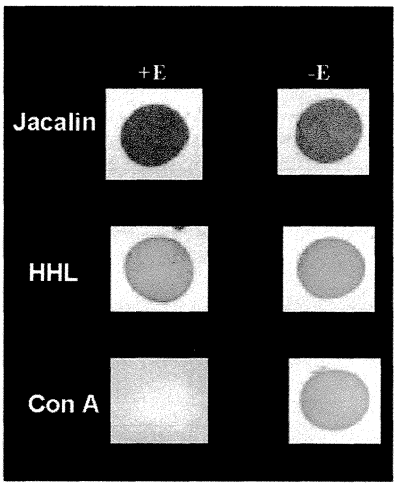


Fig. 5 (1). Acid pH (4.5) electrophoresis in 5% polyacrylamide gel of jacalin-binding human plasma glycoproteins (JPGP) (50 μg) with Coomassie blue staining. (2) Proteins (50 μg) which had entered into acid PAGE electrophoresis were electroeluted and subjected to alkaline pH (8.3) electrophoresis in 5% polyacrylamide gel with Coomassie blue staining.



Peripheral N-linked IgA1S oligosaccharides required (μg / ml) for 50% inhibition of lectins.

Con A	HHL	jacalin
4.66(± 0.75)	>150*	>150*

Fig. 9 Effect of removal of peripheral N-linked sugar units of IgA1 using α-mannosidase on its recognition by jacalin, HHL and Con A. Dot blotted IgA1S (obtained as described in fig. 4) (2 μg) was desialylated by neuraminidase before overnight treatment with (+E) or without (-E) jack bean α-mannosidase followed by probing with HRP conjugates of jacalin, HHL or Con A using 4-chloronaphthol substrate as described under "Methods". Inset: Demonstration that exclusively the mannose containing outer sugars of N-linked oligosaccharides are released by α-mannosidase from IgA1S. Mean (±SD) of triplicate trials given. Details are under "Methods". *Maximum concentration tried.

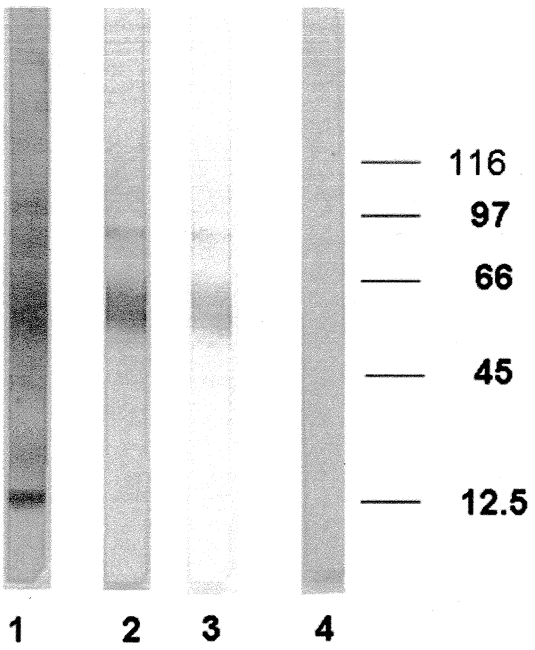


Fig. 13 Recognition by galectin-1 of total jacalin-binding plasma glycoproteins (JPGP) transferred by western blotting to PVDF membrane. Strip (1) staining with amidoblack; (2) & (3) HHL-HRP staining after (2) and before (3) neuraminidase treatment. Strip (4) was sugar control of strip 2. Positions of standard proteins with their molecular weight in kDa are indicated on the right.

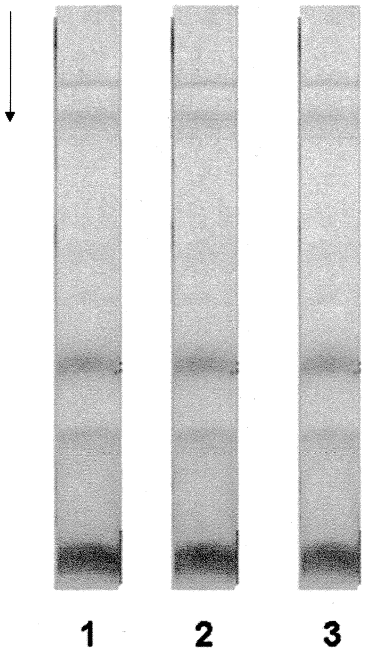


Fig.14 Sodium dodecyl sulphate poly acrylamide (7.5 %) gel electrophoresis followed by Coomassie blue staining of (1) human heart proteins binding sugar specifically to Sepharose-4B-immobilized desialylated jacalin binding human plasma glycoproteins (40 μg), (2) human heart proteins binding sugar specifically to Sepharose-4B-immobilized native jacalin binding human plasma glycoproteins (40 μg) and (3) HHL-G eluted from lactose Sepharose (40 μg)

PART III

Human heart galectin-1 interaction with enzyme modified human erythrocytes, erythrocyte membrane glycoproteins and erythrocyte glycopeptides

Human heart galectin -1 interaction with enzyme - modified erythrocytes

Human heart galectin-1 agglutinated trypsinized human erythrocytes irrespective of blood types but not normal erythrocytes. Trypsinisation of erythrocytes under controlled conditions exposes sugar groups on cryptic membrane protein domains for recognition by lectins. Minimum agglutinating amounts HHL-G and HHL were found to be 250 ng and 62.5 ng respectively (Table.7). The specific agglutinating activity of HHL was thus 4 times higher than that of HHL-G. Galectin-1-induced hemagglutination of trypsin-treated human erythrocytes was inhibitable with lactose indicating sugar-specific interaction as the reason for hemagglutination. Lactose was hundred times more potent than galactose in inhibiting galectin-1 mediated agglutination (Table.8). Melibiose and 1-O-methyl α-Gal inhibited up to 15 mM. α-anomers of sugars such as 1-O-methyl α-Gal and PNP-α-Gal were respectively twice and four times better inhibitors of galectin-1 compared to their β-anomers (Table.8). The increased inhibitory activity of p-nitro phenyl galactosides over their corresponding methyl galactosides suggests that the hydrophobic interaction between aglycon and galectin may also occur [Williams et al., 1979]. But Raffinose and Stachyose were nearly as inhibitory as 1-O methyl α-Gal. Glucose or mannose was not inhibitory even at the maximum concentration tried (Table. 8).

Table 7

	MAQ (ng)	
	HHL-G	HHL
THRBC	250	62.5
NHRBC	2000	800

Comparison of minimum agglutinating quantities (MAQ) of HHL-G and HHL using trypsinized (THRBC) and neuraminidase treated (NHRBC) human erythrocytes.

The human heart galectin-1 was also found to agglutinate neuraminidase treated human erythrocytes. But the minimum agglutinating amount of galectin-1 required was nearly thirteen times higher than what is required for trypsin treated human erythrocytes (Table.7). Sialic acid residues of glycoproteins largely account for the negative charge on human erythrocytes, which is a repulsive force opposing receptor aggregation on cell surface as also cell-cell adhesion. Neuraminidase treatment removes terminal sialic acid (N-acetyl neuraminic acid; NANA) moieties from both O- and N- linked oligosaccharides resulting in (a) exposure of the hitherto covered T antigen or LacNAc structure and (b) reduction in the repulsive negative charges. Both these effects are expected to contribute to increased agglutinability of cells. As discussed earlier HHL-G is a multimolecular lectin-glycoprotein complex between HHL and powerful endogenous glycoprotein ligands in sugar-dependent association. Presence of non-lectin protein in large quantities as well as inhibition of the lectin by these

glycoproteins would predict a far higher reduction in agglutinability in HHL-G compared to HHL, than seen in table 7 data. This data also indicates the possibility that even more than two sugar-binding sites are available on the surface of the HHL-G complex to contribute to high specific agglutinating activity.

Table 8

Sugar	MIC (Minimum inhibitory concentration) (mM)
Lactose	0.15
Galactose	15
1-O- methyl α-Gal	15
1-O-methyl β-Gal	30
PNP-α-Gal	2.4
PNP-β-Gal	9.6
Melibiose	15
Raffinose	19.2
Stachyose	19.2
Glucose	> 100
Mannose	> 200

Hemagglutination inhibition assay of HHL. Serial two fold dilutions of sugars were incubated with twice the minimum agglutinating amount of HHL for 1 h at 4°C before addition of trypsinised human erythrocytes as described under "Methods". Minimum inhibitory concentration (MIC) of sugars, expressed as mean of triplicate trials; S. D< 10% of mean.

Galectin-1 interaction with erythrocyte membrane glycoproteins

In the human erythrocyte membrane, glycoproteins comprise about 10% of total proteins (Winzler, 1970). Glycoproteins of human erythrocyte ghosts were solubilized with 1% Triton X-100 since non- ionic detergents do not affect significantly the ability of immobilized lectins to bind glycoproteins and thus do not interfere in the affinity chromatography of membrane constituents [Lotan et al., 1977]. The yield of HHL obtained from lactose-Sepharose affinity column was not high enough to enable its immobilization in active form to CNBr-activated Sepharose for the purpose of isolating HHL interacting glycoproteins from human RBC. This necessitated an initial selection of glycoproteins that were likely to contain HHL binding sites from human erythrocyte membrane by affinity chromatography. Among readily available lectins jacalin was closest in sugar specificity to HHL so that jacalin-binding erythrocyte membrane glycoproteins (JRBCGP) were prepared using immobilized jacalin as described under 'Methods' instead of HHL-binding RBC membrane glycoproteins. Human RBC membrane glycoproteins contain sialic acid as their terminal sugar. The penultimate sugar is either β(1→4) linked galactose that forms part of an N-acetyl lactosamine structure or β(1→3) linked galactose as part of O-linked Thomsen-Friedenreich antigen (Gal β(1→3) GalNAc). The JRBCGP were readily recognized sugar specifically by HHL-HRP (Fig. 15). These glycoproteins could inhibit the binding of HHL-HRP to immobilized asialofetuin. They were more than 200 times better than lactose as inhibitor in this respect (Table 9).

Table 9

Glycoprotein/Sugar	Minimum inhibitory concentration (μg neutral sugar / ml)
JRBCGP	0.84
Lactose	180

HHL inhibition capacity of JRBCGP. Serial two-fold dilutions of glycoprotein/sugar were incubated with HHL-HRP (7.5 μg lectin/ml) for 1 h at 4°C and the mixture was added to microwell-coated asialofetuin for incubation at 4°C and binding assay as described under "Methods". Mean of triplicate trials shown. S.D. was less than 10% of mean.

For further characterization JRBCGP were subjected to 5% alkaline PAGE at pH 8.3 (Fig.16). The five protein bands obtained upon staining with Coomassie brilliant blue were separately electroeluted under non-denaturing conditions. When coated on micro titre plate, they were readily recognized sugar- specifically by HHL- HRP (Fig. 17). Desialylation of these glycoproteins using neuraminidase hardly enhanced HHL-HRP binding showing that presence of terminal sialic acid is no hindrance to HHL recognition of these glycoproteins (Fig. 17). As expected, however, PNA-HRP recognized erythrocyte membrane glycoproteins only after desialylation (Fig.18). Recognition of these glycoproteins using ConA-HRP and jacalin-HRP shows the presence of N-linked (Fig.19) as well as O-linked oligosaccharide structures (Fig. 20), respectively on these glycoproteins. Desialylation followed by treatment with jack bean β-

galactosidase which cleaves only the β1→4 linked galactose moieties reduced HHL-HRP recognition only slightly indicating the marginal role of LacNAc group penultimate to sialic acid in HHL recognition (Fig. 21).

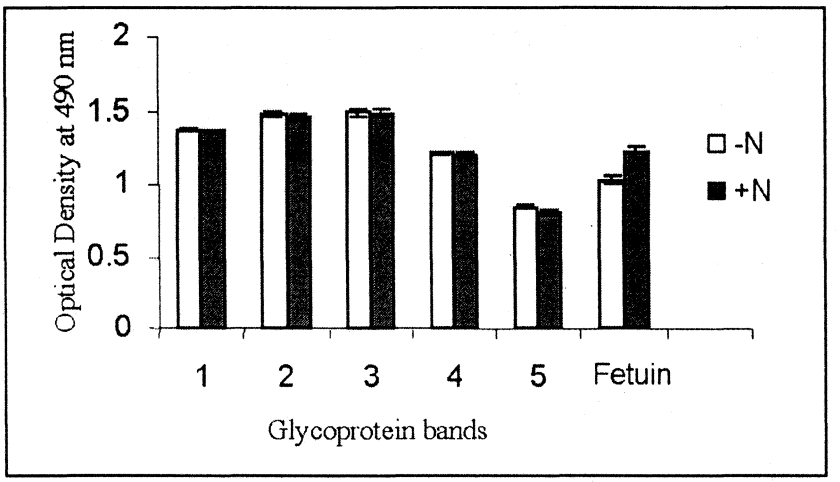


Fig. 17

HHL-HRP recognition of JRBCGP resolved into components by alkaline pH (8.3) electrophoresis in 5% polyacrylamide gel. Protein corresponding to bands (1,2,3,4,& 5) of fig. 16 (100 ng/well) immobilized on microtitre plates were blocked using PBS containing 0.5% Tween 20, treated with neuraminidase ,probed with HHL-HRP and colour developed using OPD and measured at 490 nm.

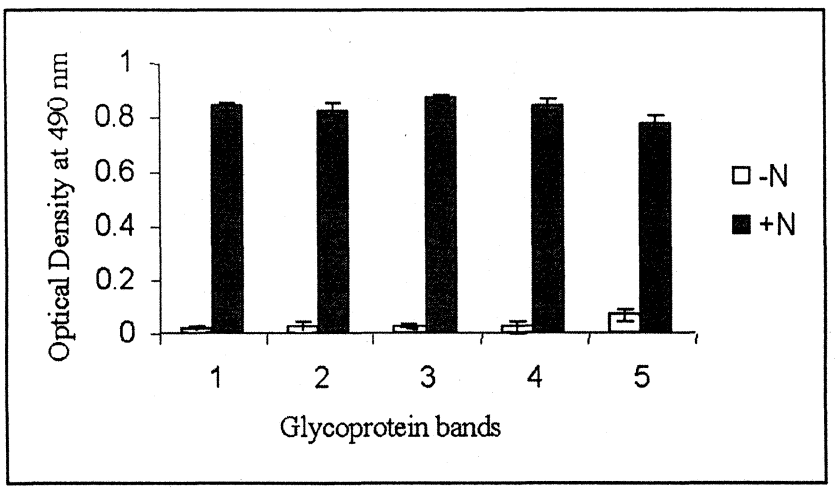


Fig. 18

PNA-HRP recognition of JRBCGP resolved into components by alkaline pH (8.3) electrophoresis in 5% polyacrylamide gel. Protein corresponding to bands (1,2,3,4,& 5) of fig.16 (100 ng/well) immobilized on microtitre plates were blocked using PBS containing 0.5% Tween 20, treated with neuraminidase, probed with PNA-HRP and colour developed using OPD and measured at 490 nm.

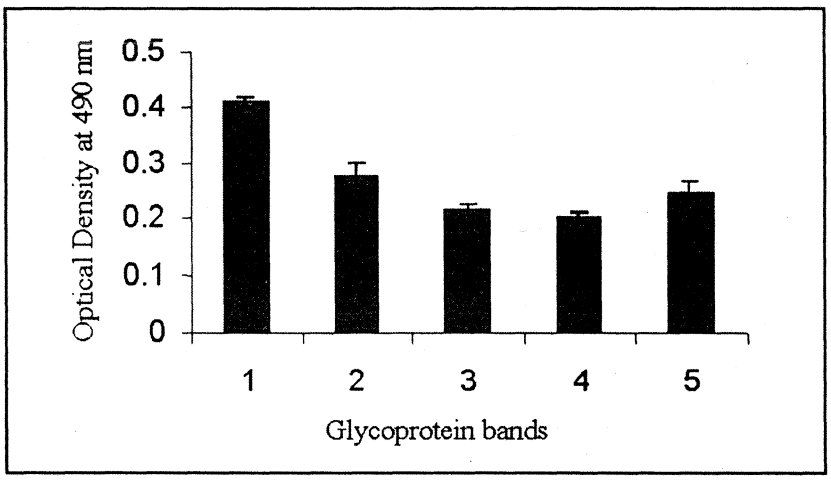


Fig. 19

Concanavalin A recognition of JRBCGP resolved into components by alkaline pH (8.3) electrophoresis in 5% polyacrylamide gel. Protein corresponding to bands (1,2,3,4,& 5) of fig.16 (100 ng/well) immobilized on microtitre plates were blocked using PBS containing 0.5% Tween 20 and treated with Con A. Bound ConA was assayed by addition of HRP. Colour due to bound HRP was developed using OPD and measured at 490 nm.

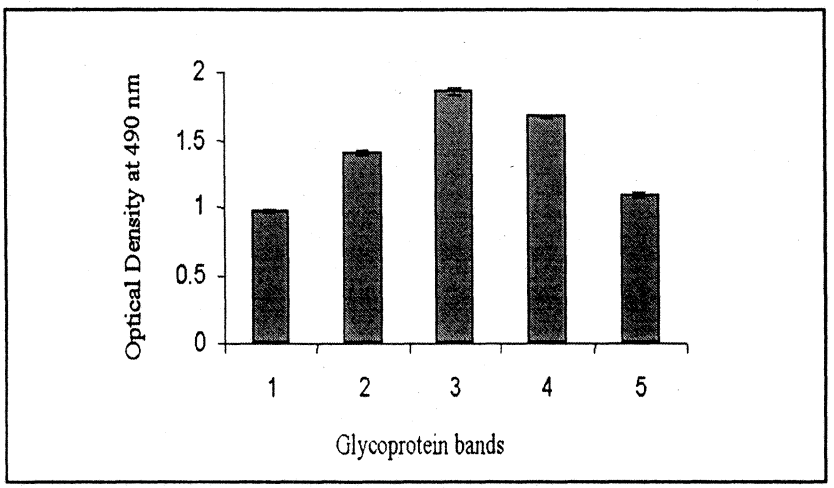


Fig. 20

Jacalin-HRP recognition of JRBCGP resolved into components by alkaline pH (8.3) electrophoresis in 5% polyacrylamide gel. Protein corresponding to bands (1,2,3,4,& 5) of fig.16 (100 ng/well) immobilized on microtitre plates were blocked using PBS containing 0.5% Tween 20, probed with jacalin-HRP and colour developed using OPD and measured at 490 nm.

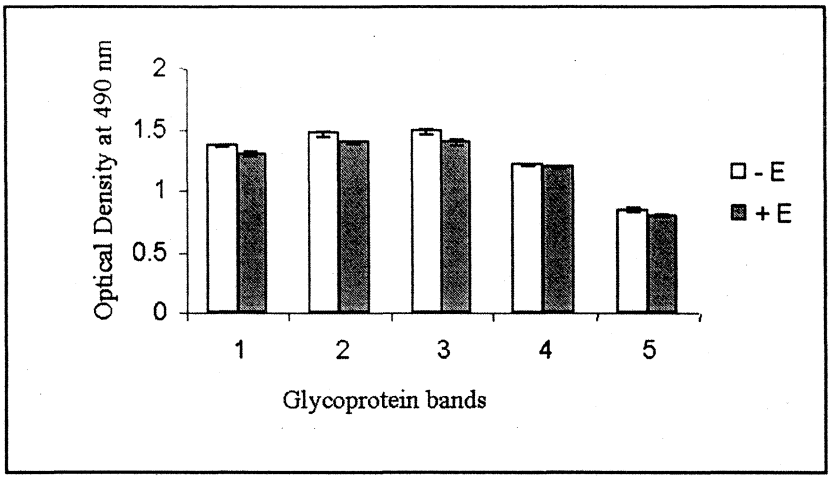


Fig. 21

Contribution of β (1→4) linked galactose in LacNAc group of N-linked oligosaccharides towards HHL-HRP binding to erythrocyte membrane glycoproteins resolved into components by alkaline pH (8.3) electrophoresis in 5% polyacrylamide gel. Protein corresponding to bands (1,2,3,4&5) of fig.16 (100 ng/well) immobilized on microtitre plates were blocked using PBS containing 0.5% Tween 20, treated with neuraminidase followed by jack bean β- galactosidase. Resulting glycoproteins were probed with HHL-HRP and colour developed using OPD measured at 490 nm.

The molecular weight of glycoprotein bands was determined by SDS PAGE 7.5% (Fig. 22). Upon SDS-PAGE under reducing conditions glycoprotein in band 1 give rise to subunit of molecular weight 71 kDa, band 2 to subunit of 105 kDa and band 3 to subunits of 91 kDa, 73 kDa, 60 kDa, 51 kDa and 37 KDa.

The 73 kDa and 37 kDa bands in band 3 glycoprotein correspond in size to the dimeric and monomeric forms of glycophorin A respectively. Since glycophorin A is the most heavily O-glycosylated erythrocyte membrane glycoprotein, it is reasonable to assume that band 3 contains glycophorin A. Glycophorin A is a molecule, which migrates as a non-disulphide linked homodimer (77 kDa) in equilibrium with a monomeric

form (39 kDa) in SDS-PAGE under both reducing and non-reducing conditions [Tomita et al., 1993]. The diffused appearance of the bands apparently due to increased glycosylation, supports this inference. Molecular weight of band 4 and band 5 could not be determined due to insufficient yield. HHL-HRP recognition of jacalin-binding erythrocyte membrane glycoproteins was also proven by western blot. Western blotted JRBCGP were readily recognized by HHL-HRP and the reaction was sugar-reversible (Fig. 23).

Human heart lectin binding to Sepharose immobilized jacalin-binding erythrocyte membrane glycoproteins.

Jacalin-binding erythrocyte membrane glycoproteins were coupled to Sepharose 4B at the rate of 2 mg /ml gel by CNBr activation. One ml 50% suspension of JRBCGP-Sepharose 4B was made in PBS and a 50% suspension of activated Sepharose 4B was taken as control. After both gels were settled at 4^0C, 100 µl clear supernatant was removed from each and replaced by 100 µl HHL (500 µg protein/ml), the mixture was stirred and kept at 4^0 C for 2 hours with occasional stirring. After centrifugation at 1500 rpm for 10 minutes at 4^0 C the supernatant was checked for agglutination activity with trypsinised human erythrocytes. It was found that HHL was completely captured by JRBCGP-Sepharose, since the supernatant was devoid of agglutination activity (Table.10). The supernatant from activated Sepharose 4B-treated HHL gave agglutination activity equal to that of untreated HHL.

Table 10

HHL treated with	Agglutination +ve upto dilution (times)
JRBCGP-Sepharose	Nil
Activated Sepharose	16
No gel	16

Serial two-fold dilutions of gel treated or untreated HHL (50 μg/ml) were tested for agglutination with trypsinised human RBC as described under "Methods".

From the jacalin-binding erythrocyte membrane glycoproteins the peripheral N-linked sugars removed using alpha mannosidase were found to be poor inhibitors of human galectin-1, and good inhibitors of Concanavalin A (Table.11) further proving that N-linked oligosaccharides on JRBCGP do not contribute towards HHL binding to them.

Table 11

Lectin	Peripheral N-linked Oligosaccharides of JRBCGP required for 50 % inhibition of lectins. (μg/ml)
Con A	5
HHL	>100

Serial two fold dilutions of oligosaccharides were incubated with Con A (5 μg/ml) or HHL-HRP (7.5 μg lectin /ml) before addition to immobilized dextran or asialofetuin respectively. HRP (5 μg/ml) was added to ConA wells. Bound lectin activity was assayed using OPD as described under "Methods".

Galectin-1 interaction with erythrocyte membrane glycopeptides

Human heart galectin-1 causes sugar-dependent agglutination of trypsinised human erythrocytes. Moreover jacalin-binding erythrocyte membrane glycoproteins offer very efficient ligands for human heart galectin-1. So low molecular weight glycopeptides were prepared from human erythrocytes under the assumption that they may contain efficient galectin-1 ligands.

Glycopeptides derived from human erythrocytes by pronase digestion (prepared as described under "Methods"), contained epitopes complementary to human heart lectin since they strongly inhibited the binding of HHL-HRP conjugate to immobilized asialofetuin and were 90 times better than lactose in this respect (Table.12). Desialylated erythrocyte glycopeptides (prepared as described under 'Methods") also inhibited galectin-1 to the same extent as native erythrocyte glycopeptides did (Table.12). But the glycopeptides prepared from glycoproteins such as invertase or horse radish peroxidase, which are not galectin-1-binding glycoprotein ligands, failed to inhibit the lectin even at the maximum amount tried (Table.12). Since erythrocyte-derived glycopeptides were low in molecular weight (obtained by PM 10 ultrafiltration so that molecular weight is less than 10 kDa) it was not possible to immobilize them on polystyrene plates for characterization. The yield of glycopeptides from 1 ml packed erythrocytes was found to be 200 μg in terms of carbohydrate.

Table 12

Glycopeptide /Sugar	Minimum inhibitory concentration (μg neutral sugar / ml)
Erythrocyte glycopeptide	2
Desialylated erythrocyte glycopeptide	2
Lactose	180
Invertase glycopeptide	> 116
HRP glycopeptide	> 50

Inhibition of HHL binding to polystyrene well-coated asialofetuin by glycopeptides/ sugar. Serial two-fold dilutions of glycopeptides / sugar were incubated with HHL-HRP (7.5 μg lectin/ml) for 1 h at 4°C and the mixture was added to microwell-coated asialofetuin for incubation at 4°C and binding assay as described under "Methods". Mean of triplicate trials shown. S.D. was less than 10% of mean.

Discussion

Agglutination of red cells requires a cross-linking of cells so that a minimum of two binding sites (valencies) on the cross-linking protein for the respective erythrocyte surface ligand is mandatory. Erythrocytes being rich in distribution of saccharide ligands born by glycolipids as well as glycoproteins, lectin-erythrocyte interaction escalates in all directions around the red cell to form a network resulting in agglutination. Detailed investigation on the mechanism of agglutination and cell surface events accompanying this phenomenon had been undertaken using primarily Concanavalin A as agglutinin. It was revealed that

agglutination of erythrocytes by di-or multivalent lectin involves events far beyond the lectin binding to single ligands on two red cells on either side of the lectin. A crucial requirement is aggregation (patching) of glycoconjugates at the point of contact on both of the neighboring red cells that are cross-linked. This requires mobility of the glycoconjugate molecule bearing the sugar ligand on the cell surface. As a result, multi point contacts are established using more than one lectin at one point of contact, to stabilize the cross-linking.

It is reasonable to assume that the greater receptor mobility for reasons outlined above may also be facilitated by protease treatment of RBC, in addition to exposing hitherto inaccessible sugar ligands by cleavage of protein chains. Galectin-1 being widely distributed in the human body cells including endothelial cells, the implications of galectin-1 attachment to modified erythrocytes are many. Senescent erythrocytes and those subjected to sheer stress are known to possess altered topology akin to those of protease treated cells. There is also striking similarities between protease treated erythrocytes and certain tumor cells [Burger, 1969; Sela et al., 1970]. Changes in agglutination of cells have been observed during differentiation and following transformation [Brown and Hunt, 1974].

It is also notable that since macrophages are known to secrete proteases [Reiko and Werb, 1984], prolonged or repeated passage of erythrocytes through areas where, macrophages are concentrated, such as organs of reticulo endothelial system, could provide an in vivo mechanism

for cumulative proteolytic alteration of the cell surface. Thus proteolytic cleavage of protein components on the cell surface provides a means of ligand exposure. Erythrocyte deformability is important in the removal and subsequent destruction of abnormal or senescent red blood cells from circulation. Since galectin-1 is present in the plasma, erythrocytes modified by proteases or due to senescence are liable to be recognized by this lectin suggesting a role of the lectin in the removal of senescent erythrocytes from circulation. Galectin-1-mediated agglutination of trypsinized erythrocytes can also be compared with the sequestration of erythrocytes in various pathological conditions.

Jacalin-binding erythrocyte membrane glycoproteins offered very efficient ligands for human galectin-1. On the red cell surface, the T antigen [Gal β1-3 GalNAc] is mostly carried by the O-glycosyl protein glycophorin A [Anstee, 1981]. Glycophorin A is a major sialoglycoprotein of human erythrocytes and is highly glycosylated with 1 N-linked and 15 O-linked oligosaccharides. In the case of erythrocyte membrane glycoproteins the terminal sialic acid offered no hindrance in HHL binding. Decreased HHL binding to neuraminidase-treated human erythrocytes compared to trypsin treated cells supports this observation. The inertness of N-linked oligosaccharides of jacalin-binding glycoproteins towards galectin-1 as well as absence of any change in galectin-1 recognition of desialylated JRBCGP

upon jack bean β-galactosidase treatment suggested that N-linked oligosaccharides had little role in galectin-1-binding of jacalin-binding erythrocyte membrane glycoproteins.

Galectin concentration can change in a variety of neoplastic cells and their surface expression correlates with metastatic potential of some cells [Lotz et al., 1993; Raz et al., 1988]. Cells in which galectin-1 is over-expressed lose contact inhibition while pre-incubation of metastatic cells with monoclonal anti-lectin antibodies abolished their binding to form lung tumor colonies [Lotan et al., 1985]. It is known that prevention of tumor cells from interacting with each other or with their microenvironment can suppress tumor growth and metastasis

Erythrocyte membrane glycopeptides were prepared for use as inhibitors of galectin-1 for preventing tumor spread since some saccharides that inhibit galectins in vitro were found to have anti-tumor effects [Inohara and Raz, 1994; Glinsky et al., 1996]. Glycopeptides were expected to offer two advantages over simple sugars; firstly oligosaccharide sequences are always found to be much stronger lectin-binding ligands than small sugars and secondly glycopeptides are easily soluble compared to their membrane glycoprotein precursors. Search for synthetic glycoconjugates capable of blocking pathological cell-cell recognition is also reported [Paulson, 1996]. From the point of view of a possible therapeutic potential, erythrocyte

derived glycopeptides offer other major advantages such as i) ease of availability of erythrocytes from discarded blood as well as after plasma separation and ii) being non-immunogenic in humans. A major consideration in this context will be the blood group antigen activity of glycopeptides. Further investigations are required to assess the extent to which blood group antigens, if present on these glycopeptide oligosaccharide sequences, interacts with host anti-blood group antibodies. Such interferences may require use of group-specific glycopeptides as therapeutic agents.

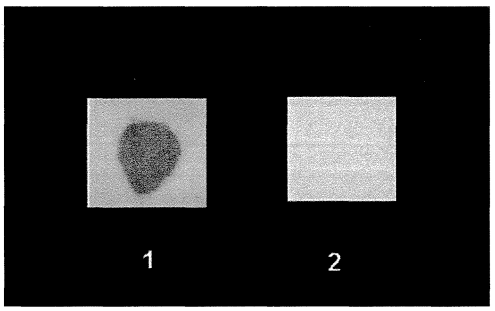


Fig.15 HHL-HRP recognition of jacalin-binding erythrocyte membrane glycoproteins (JRBCGP). Dot blotted JRBCGP (2 μg) probed with HHL-HRP without sugar (1) and with sugar(2).

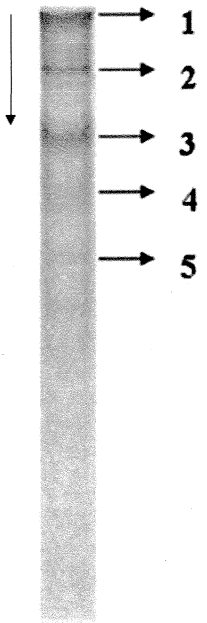


Fig.16 Alkaline pH (8.3) electrophoresis in 5% polyacrylamide gel of jacalin-binding human erythrocyte membrane glycoproteins (JRBCGP) (50μg) with Coomassie blue staining.

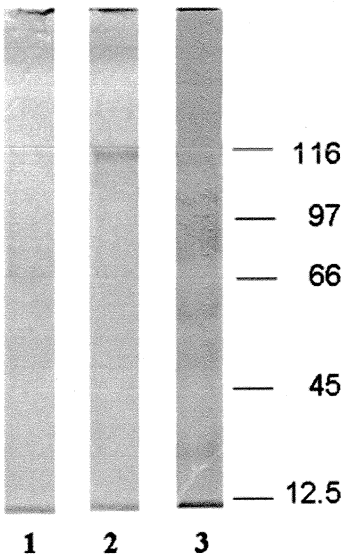


Fig. 22 Molecular weight determination of jacalin-binding erythrocyte membrane glycoproteins resolved into components by alkaline PAGE. SDS-PAGE electrophoresis followed by Coomassie blue staining of glycoproteins in bands 1-3 of fig.16 (marked 1-3 above). Positions of standard proteins with their molecular weight in kDa are indicated on the right.

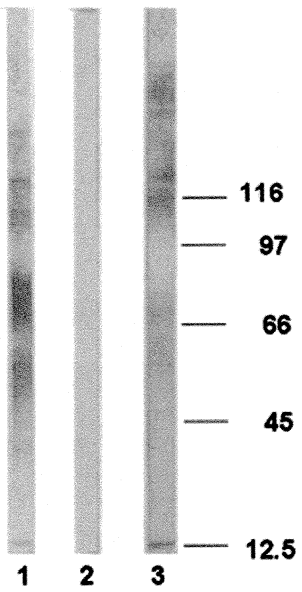


Fig. 23 HHL-HRP binding to Western blot of total jacalin-binding erythrocyte membrane glycoproteins on PVDF membrane. (1) & (2): probing with HHL-HRP in the absence (1) and presence (2) of 50 mM lactose and (3): amidoblack staining. Positions of standard proteins with their molecular weight in kDa are indicated on the right.

PART IV

Human heart galectin-1 interaction with platelet and lymphocyte membrane glycoproteins

Interaction of galectin-1 with platelet membrane glycoproteins

Plasma membrane-enriched fraction of platelets upon SDS-PAGE under reducing conditions revealed the presence of 18 protein bands on staining with Coomassie brilliant blue R-250 (Fig. 24). When they were transferred on PVDF, the most prominent band recognized by jacalin-HRP was of molecular weight 117 kDa (Fig.25). PNA-HRP recognized the same band only after neuraminidase treatment of the western blot (Fig.25). HHL-HRP could sugar specifically recognize the 117 kDa band before and after desialylation, the latter being more effective (Fig.26). Con A interaction with 117 kDa band was weak though it strongly recognized other bands, especially one of 105 kDa (Fig. 25).

From the plasma membrane-enriched fraction of platelets the galectin-1- interacting glycoprotein was picked out using jacalin-Sepharose matrix (prepared as described under "Methods"). Jacalin-binding platelet glycoproteins coated on microtitre plates were recognized sugar-specifically by HHL-HRP since 50 mM lactose could inhibit the binding of HHL-HRP to these glycoproteins (Fig.27). Desialylation of these glycoproteins increased HHL-HRP binding. But PNA-HRP recognized these glycoproteins only after desialylation indicating that these glycoproteins are sialylated (Fig. 27). HHL-HRP recognized both sialylated as well as non-sialylated versions of platelet glycoproteins, with greater affinity for desialylated versions (Fig.27).

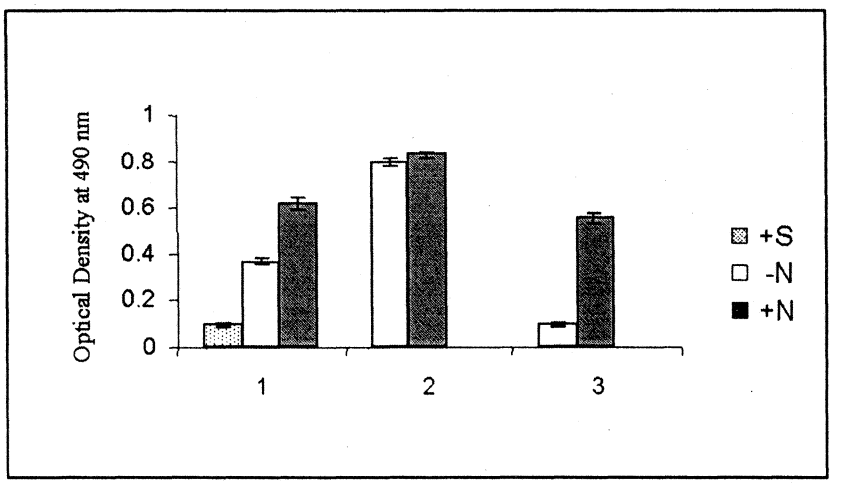


Fig. 27

Effect of desialylation of jacalin-binding platelet membrane glycoprotein on its recognition by HHL, PNA and jacalin. Jacalin-binding platelet membrane glycoproteins (2 μg/well) immobilized on microtitre plates were blocked with PBS containing 0.5 % Tween 20, treated with neuraminidase and probed with (1) HHL-HRP, (2) Jacalin-HRP and (3) PNA-HRP, colour developed using OPD and measured at 490 nm.

Discussion

The reported molecular weights of GP IIbα are 110 kDa, 112 kDa, 130 kDa and 132 kDa. The molecular weight determined from SDS-PAGE as well as interaction with the lectin, jacalin suggests the possibility that the platelet membrane glycoprotein interacting with galectin-1 is platelet glycoprotein IIbα. The abundance of T antigen structure on GP IIbα and its interaction with jacalin had been reported previously [Madhan et al., 1999]. The most abundant platelet membrane glycoprotein is the β_3-integrin GP IIb/IIIa (60 000 to 100 000 per platelet and 1% to 2% of the total platelet protein), which is a heterodimer consisting of an α-subunit (GP IIbα) and a β-subunit (GP IIIa) [Plow, 1992]. Glycoprotein IIb/IIIa is one of the primary

mediators of platelet aggregation. The binding of HHL to the glycoprotein recognized by jacalin and PNA rather than to strongly ConA-binding N-glycosylated glycoproteins reaffirms the dominant role of O-glycans as HHL ligands.

Platelets play a fundamental role in atherogenesis and development of ischemic complications [Ross, 1993; Fuster et al., 1992; Willerson, 1989]. A remarkable observation in atherosclerosis is the deposition of components of serum-derived platelets and leucocytes at the sub endothelial region of a plaque. The exact mechanism of platelet anchoring on the vessel wall is unknown. Galectin-1 has been reported to be present on endothelial cell (EC) surface [Baum et al., 1995b]. Under normal physiological conditions platelet anchoring at ECs is absent, but desialylation of platelet membrane surface by neuraminidase is a modification that may lead to the anchoring of platelets by human heart galectin-1 since galectin-1 recognizes desialylated platelet membrane glycoproteins with increased affinity. Since many pathogenic bacteria and viruses secrete neuraminidase, desialylation of platelets may accompany infections and render their surface glycoproteins more susceptible to galectin-1 binding. Thus a possible mechanism for platelet deposition is through binding to galectin-1, which has been shown to reside on endothelial cells as well. Serum sialidase and asialotransferrin levels in coronary heart

disease was found to be higher than control group supporting this assumption [Sonmez et al.,2000]. The level of sialic acid residues in platelets was found to be significantly lower in old age, diabetes, and lymphoma cases in comparison to controls. In addition to pathogen-mediated desialylation of platetets, free radical-mediated desialylation of platelets had also been reported [Goswami and Koner, 2002].

Interaction of galectin-1 with lymphocyte membrane glycoproteins.

The lymphocyte membrane-rich fraction prepared as described under "Methods" was transferred to PVDF membrane. This western blot of total lymphocyte membrane glycoproteins was probed with jacalin- HRP. The most prominent bands recognized were of molecular weight 49 kDa and 12.5 kDa (Fig.28). Their recognition by jacalin ensured the presence of T antigen on them. However, PNA-HRP and HHL-HRP recognized 49 KDa and 12.5 kDa bands only after desialylation of the western blot and this interaction was sugar-specific (Fig.28 & Fig.29). Probing with ConA on the other hand revealed that there were several N-glycan-containing glycoproteins on lymphocyte membrane.

Discussion

Galectin-1-mediated apoptosis of activated T lymphocytes was reported and it was mediated through T cell glycoprotein CD 45. It was

also suggested that CD 45 expressed on activated T cells might contain polylactosamine sequences, which were supposed to be the preferred ligands for galectin-1. Since polylactosamine groups are often found on N-linked oligosaccharides, which are known to bind Con A as well, the poor overlap between Con A- and HHL-binding subunits found here does not support the above conclusion. Polylactosamine groups as part of O-linked oligosaccharides are rare and detected only on activated T lymphocytes [Fukuda, 2000]. On the other hand the perfect correlation of mobilities of jacalin-binding subunits with those of HHL-binding subunits suggested in the light of data presented in earlier chapters that O-linked oligosaccharides containing T antigen could be the HHL ligands in lymphocytes.

Changes in neuraminidase activity also accompany T cell activation and may unmask glycan ligands by removing sialic acid [Galvan et al., 1998]. Increased binding of galectin-1 to desialylated lymphocyte membrane glycoprotein suggests the possibility of galectin-1-mediated anchoring of lymphocytes at selected sites.

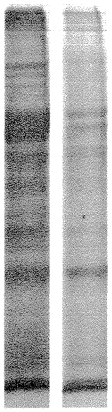


Fig.24. Sodium dodecyl sulphate poly acrylamide (7.5 %) gel electrophoresis followed by Coomassie blue staining of platelet membrane glycoproteins (1) 40 μg and (2) 20 μg per well.

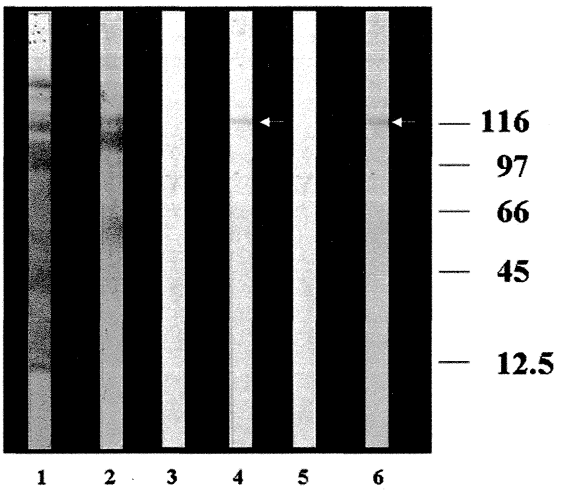


Fig 25. Recognition by plant lectins of total platelet membrane glycoproteins transferred by western blotting to PVDF membrane. 1, amidoblack staining; 2, staining with Con A/ HRP; 3 staining with PNA-HRP; 4, staining with PNA-HRP after neuraminidase treatment ; 5, sugar control of 4 and 6, staining with jacalin-HRP. Positions of standard proteins with their molecular weight in kDa are indicated on the right.

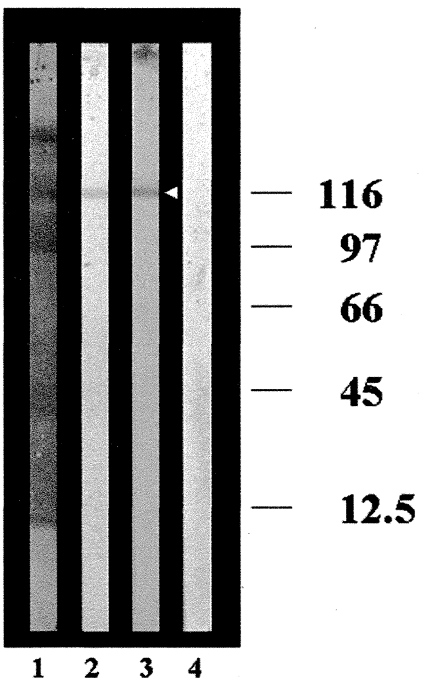


Fig. 26. HHL-HRP recognition of western-blotted total platelet membrane glycoproteins. 1, amidoblack staining; 2, HHL-HRP staining; 3, HHL-HRP staining after neuraminidase treatment and 4, sugar control of 3. Positions of standard proteins with their molecular weight in kDa are indicated on the right.

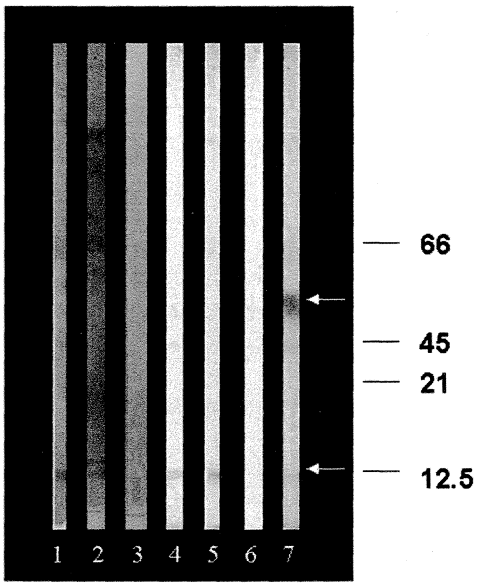


Fig 28. Recognition by plant lectins of total lymphocyte membrane glycoproteins transferred by western blotting to PVDF membrane. Strip (1): staining with Amidoblack. Strips (2&3): staining with Con A, without sugar (2) and with sugar (3). Strips (4,5&6): staining with PNA-HRP, before (4) and after (5) neuraminidase treatment and sugar control of 5 (6). Strip (7): staining with jacalin-HRP. Positions of standard proteins with their molecular weight in kDa are indicated on the right.

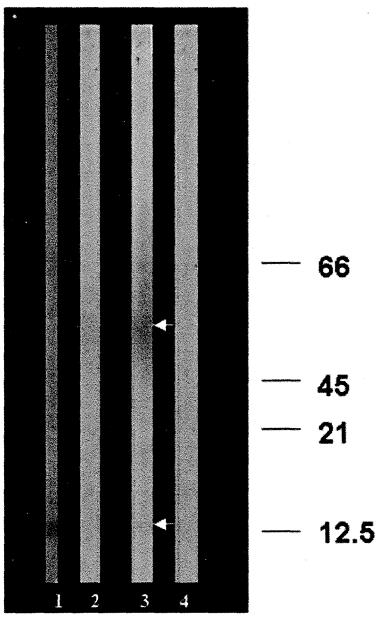


Fig 29. HHL-HRP recognition of western-blotted total lymphocyte membrane glycoproteins. Strip (1): amidoblack staining. Strips (2,3,&4): staining with HHL-HRP, before (2) and after (3) neuraminidase treatment and sugar control of 3 (4). Positions of standard proteins with their molecular weight in kDa are indicated on the right.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Summary and Conclusions

Galectins, a growing family of galactoside-binding lectins are extensively conserved and distributed in the animal kingdom. Originated more than 800 million years ago and derived from an ancestor gene, their common basic molecular properties and affinity for β-galactoside binding have been maintained throughout evolution. At present about 14 mammalian galectins have been identified and found distributed in a variety of tissues. Galectin-1 the earliest described and most widely distributed member of the family is a homodimeric protein with a carbohydrate recognition domain of 134 amino acids. The precise in vivo functions of galectin-1 are currently unclear because targeted disruption of the galectin-1 gene in null mutant mice resulted in the absence of major phenotypic abnormalities perhaps because of compensatory phenomena. Their developmentally regulated expression in a wide variety of cell types and capacity for multiple interactions with carbohydrate ligands make them important factors influencing cell–cell and cell–matrix interactions. In the present work human heart galectin-1 interactions with glycoproteins on circulating cells like erythrocytes, platelets and lymphocytes as well as with IgA1 ,its most prominent ligand in the plasma are studied. In these studies particular emphasis was given to examining galectin-1 affinity towards T antigen as part of O-glycan in contrast to that towards LacNAc structure as part of N-glycan.

Human heart galectin-1 was separated by high pressure liquid chromatography from endogenous glycoproteins co-purified with it during affinity chromatography. These glycoproteins offered excellent ligands for HHL binding and were far superior in binding galectin-1 compared to asialofetuin, which is one of the well known and powerful galectin-1 ligands. Abundance of both exposed as well as covered T antigen on endogenous glycoproteins indicated that galectin-1 may have preference for T antigen structure and galectin-1 function may be mediated through these high affinity endogenous glycoproteins.

In enzyme linked lectin assay and hemagglutination inhibition assay, human IgA1, which is the only immunoglobulin having T antigen structure, bovine fetuin, and other O-glycosylated T antigen-bearing glycoproteins bound to the lectin efficiently in contrast to IgG which is not O-glycosylated. Peripheral N-linked sugars of powerful galectin-1 ligands such as asialofetuin and IgA1 were inert towards galectin-1. Desialylated or polymeric IgA was better inhibitor than monomeric IgA1. These data suggest HHL to be specific to O-glycosidically linked sugars. Enzymatic removal of peripheral N-linked sugars of IgA1 as well as fetuin reduces / abolishes their recognition by Con A and RCA1 but not that by jacalin or HHL further suggesting the T antigen specificity of HHL. Galectin-1 recognizes galactose as part of sialylated or free T antigen, which is terminal

part of O-linked oligosaccharides, but not galactose as part of single N-acetyl lactosamine which is terminal moiety in most of the N-linked oligosaccharides. The only carbohydrate binding protein in human heart tissue interacting with O-glycosylated plasma glycoproteins appears to be galectin-1 since the immobilization of the latter captured only HHL from heart tissue homogenate. The exceptional affinity of human galectin-1 for IgA1 suggests a possible role for this lectin in the uniquely high serum turn over of this antibody as galectin-1 abundant in tissues can mediate sequestration of IgA1 from circulation. Increased IgA1 deposition in tissues in nephropathies, atherosclerosis and diabetes may be mediated by the IgA1 affinity of galectin-1, which is ubiquitous in tissues. Further, the enhancement of IgA1 binding of galectin-1 by microbial neuraminidase-mediated desialylation of the antibody may explain the reported role of infections in precipitating the above disorders. In hemagglutination inhibition assay α-anomers of galactose were better inhibitors compared to β-anomers as has been observed in the case of other T antigen binding lectins such as jacalin and PNA. Bovine thyroglobulin, a glycoprotein containing terminal α-linked galactose was as good as IgA1 in inhibiting galectin-1.

Galectin-1-mediated sugar-specific agglutination of human erythrocytes suggested the presence of galectin-1 ligands on erythrocytes.

More convincing evidences for HHL ligands on erythrocytes were (i) HHL binding to micro-plate coated bands electrophoretically resolved from jacalin-binding RBC glycoproteins (ii) complete capture of HHL from solution by immobilized jacalin-binding erythrocyte glycoproteins and (iii) effective inhibition of HHL by glycopeptides derived by pronase digestion of erythrocyte membrane glycoproteins.

Since senescent / transformed RBC resembles trypsinized RBC in cell surface changes, galectin-1 recognition of the latter suggest a role of the lectin in the fate of aged / transformed RBC. Galectin-1 ligands on RBC bind the lectin regardless of their sialic acid cap while in IgA1 desialylation leads to marked increase in galectin-1 binding. Differential response of galectin-1 to desialylation may be attributed to the differences in change of microenvironment around the galactose moiety brought about by desialylation in various glycoproteins.

Platelet glycoprotein IIbα, which is reported to be of molecular weight in the range of 110-132 kDa and to contain O-linked oligosaccharides is likely to be the galectin-1 ligand on platelets. The glycoprotein subunits on lymphocytes recognized by HHL are of molecular weight 49 kDa and 12.5 kDa. Since desialylation remarkably increases HHL binding to platelet and lymphocyte membrane glycoproteins, these results also show that

desialylation of membrane surface may be a mechanism to modulate galectin-1 binding to platelets and lymphocytes.

Some saccharides such as lactose, which possess galectin-1 ligands, have been used as anti-cancer agents in animal models by virtue of their galectin-1 inhibitory activity. Our results indicate that pronase-derived erythrocyte glycopeptides may be tried for their potential as prophylactic / therapeutic anti-cancer agents since they were potent hapten inhibitors of HHL.

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SYNOPSIS

BLOOD GLYCOCONJUGATE RECOGNITION BY TISSUE CARBOHYDRATE-BINDING PROTEINS: GLYCOPROTEIN RECOGNITION BY HUMAN HEART GALECTIN-1

SYNOPSIS OF THESIS SUBMITTED

by

SANGEETHA.S.R

for Ph.D Degree

in

BIOCHEMISTRY

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

THIRUVANANTHAPURAM

Introduction

Structure-specific protein carbohydrate interaction is now appreciated as a major mode of biological recognition. The enormous structural variations possible in oligosaccharide sequences, in comparison with oligonucleotides or proteins, render glycoconjugates potential carriers of varied biological information to be recognized by cognate biomolecules. Lectins, which are proteins of non-immune origin that recognize and bind to corresponding sugar residues without altering the structure of the latter, have been detected in most plants and animals and are believed to be major participants in biological recognition. The most prevalent carbohydrate binding protein in animal tissues is the galactose binding lectin of molecular weight around 14 kDa or galectin-1.

Galectins are believed to mediate cell-cell and cell-matrix interactions during development, inflammation, apoptosis and tumor metastasis. However, neither the detailed mechanisms of their function(s) nor the identities of their natural ligands has been unequivocally elucidated. Elucidation of function of galectin-1 primarily involves identification and characterization of its endogenous cognate glycoconjugates.

Objectives of the present study

1. Purification of human heart galectin-1 and characterization of sugar-specifically bound endogenous glycoproteins that co-purify with the lectin during affinity chromatography.

2. Demonstration of T antigen (Gal $\beta 1 \rightarrow 3$ Gal NAc) specificity of human heart galectin-1.

3. Study of interaction of human galectin-1 with erythrocyte and erythrocyte membrane glycoproteins.

4. Preparation of human erythrocyte membrane glycopeptides and demonstration of their galectin-1 inhibition.

5. Identification of galectin-1-interacting glycoprotein subunits on human platelet and lymphocyte membranes.

Results

Human heart galectin-1 was purified from heart muscle tissue extract using lactose-Sepharose affinity matrix. The eluate (HHL-G) obtained from lactose-Sepharose affinity column not only contained 14 kDa galectin-1 but also a group of endogenous glycoproteins as evidenced by SDS-PAGE. Endogenous co-purified glycoproteins were separable from the lectin by HPLC, but not by gel filtration under atmospheric pressure. HHL-G upon HPLC resolved into three peaks in which the lectin activity was solely confined to peak 2 (HHL),

which upon SDS-PAGE gave a single band having molecular weight 14 kDa. But proteins in peaks 1 and 3 failed to give agglutination with trypsinised rabbit erythrocytes even at the maximum amount tried. Proteins corresponding to peaks were immobilized on polystyrene wells and probed with various lectin-HRP conjugates. Peaks 1 and 3 were rich in glycoproteins with exposed as well as covered T antigen structure. To study the interaction of galectin-1 with various glycoproteins the lectin was covalently coupled to horseradish peroxidase (HRP).

Sugar specificity of the lectin was determined using various sugars and glycoconjugates, which were used in inhibiting 1) binding of HHL-HRP to immobilized asialofetuin on polystyrene microtitre plates and 2) lectin-mediated hemagglutination of trypsinised rabbit erythrocytes. Demonstration of T antigen specificity of human galectin-1 was done with emphasis on human serum glycoprotein IgA1, which is the only O-glycosylated immunoglobulin having T antigen structure. Human IgA1 was purified from O-glycosylated serum glycoproteins as well as from dextran-binding immunoglobulin. The former was termed as IgA1 (S) and the latter as IgA1 (P). In contrast to other immunoglobulin types including IgG, human IgA1 offered powerful ligands for galectin-1 as seen from inhibition of HHL-HRP binding to asialofetuin. Between the two IgA1 samples IgA1 (P) was better inhibitor compared to IgA1 (S). Non-IgA1 (S) and non-IgA1 (P) samples offered poor galectin-1 ligands

compared to IgA1 (S) and IgA1 (P). Peanut agglutinin (PNA)-binding human and bovine heart glycoproteins that are rich in O-linked sugars were as good as asialofetuin in inhibiting HHL-HRP. Peripheral N-linked sugars of powerful HHL-binding glycoproteins such as asialofetuin and IgA1 (S) and of total human immunoglobulin fraction are however not inhibitory. Towards inhibition of HHL-mediated agglutination of trypsinised rabbit erythrocytes IgA1 (S) was better than fetuin while IgG was not an inhibitor. These results suggest HHL to be specific to O-glycosidically linked sugars, which often terminate in T antigen. Enzymatic removal of peripheral N-linked sugars of IgA1 as well as of fetuin abolishes / reduces their recognition by concanavalin A and *Ricinus communis* agglutinin (RCA1), but not by jacalin or HHL, further suggesting T antigen specificity of HHL. Human plasma jacalin-binding glycoproteins when immobilized after desialylation binds only HHL from human heart tissue extract. Desialylation sharply increases HHL affinity towards IgA1, unlike that towards fetuin or RBC glycoproteins. In agglutination inhibition assay, α-anomers of galactose were better inhibitors compared to β-anomers as has been observed in the case of other T antigen binding lectins such as jacalin and PNA. Bovine thyroglobulin, a glycoprotein containing terminal α-linked galactose was as good as IgA1 in inhibiting galectin-1.

Human heart galectin-1 agglutinated trypsinised as well as neuraminidase- treated human erythrocytes irrespective of blood types, the

minimum hemagglutinating concentration of lectin being lower for the former. Purification of galectin-1 from co-purified glycoproteins increased its agglutination activity about four- fold. The galectin-1 mediated agglutination of human erythrocytes was sugar specific; lactose was 100 times better inhibitor compared to galactose. HHL-binding glycoproteins from erythrocyte membrane were isolated by jacalin chromatography of detergent-solubilized membrane proteins followed by electrophoretic separation at pH 8.3 and electroelution of individual proteins. HHL-reactivity of these glycoproteins was demonstrated after coating them on polystyrene microwells, using HHL-HRP. The jacalin-binding erythrocyte membrane glycoproteins immobilized on Sepharose completely captured galectin-1 from its solution. HHL recognizes both desialylated and non-desialylated erythrocyte glycoproteins to the same extent. Jacalin-binding erythrocyte membrane glycoproteins were 200 times better than lactose as inhibitor of galectin-1. HHL-HRP recognition of RBC glycoproteins was also proven by western blot.

Since erythrocyte membrane glycoproteins offer very powerful ligands for galectin-1, low molecular weight glycopeptides from them were prepared using pronase digestion followed by ultra filtration using PM 10 (AMICON) membrane. Glycopeptides thus obtained were far superior to lactose as HHL inhibitor. The desialylated as well as non-desialylated erythrocyte glycopeptides inhibited the lectin to the same extent. Glycopeptides prepared

from non-galectin-1-binding glycoproteins such as invertase and HRP were non-inhibitory.

Total platelet glycoproteins were resolved by SDS-PAGE and immobilized by Western blotting on PVDF membrane. The glycoprotein subunit from platelet membrane recognized by PNA, jacalin and galectin-1 was the same and had molecular weight 117 kDa. The galectin-1 binding to platelet membrane subunit 117 kDa increased upon desialylation of the latter. The galectin-1 binding to platelet membrane glycoprotein isolated by jacalin chromatography almost doubled following desialylation of the latter. The glycoprotein subunits recognized by HHL on Western blot of lymphocyte membrane protein were of molecular weight 49 kDa and 12.5 kDa and recognition increased substantially upon desialylation.

Discussion

The co-purification of endogenous glycoproteins along with galectin-1 suggests formation of carbohydrate-dependent complexes between the two. Though stoichiometry of galectin-1 glycoprotein complexes are not determined, it can be envisaged that such complexes consisting of several divalent lectin and multivalent glycoproteins would have unoccupied sugar binding sites on their peripheral lectin molecules to facilitate binding of the complex to immobilized lactose. Abundance of both exposed as well as covered

T antigen on endogenous glycoproteins indicated that galectin-1 may have preference for T antigen structure since these are the chosen glycoproteins with apt ligands for the lectin .It is likely that galectin-1 function is mediated through high affinity endogenous glycoproteins.

More convincing evidence for T antigen preference of galectin-1 is that IgA1, the only O-glycosylated immunoglobulin is also the sole immunoglobulin recognized by the lectin. The observation that IgA1 is the most prominent serum glycoprotein interacting with tissue galectin-1 is significant to both the normal biology of serum IgA1 and to IgA1-mediated immune pathology. The physiological fate of serum IgA1, the most biosynthesized immunoglobulin, is less clear compared to that of IgG or IgA2. Since galectin-1 is abundant in tissues it is likely to have a role in the sequestration of IgA1 from circulation. This assumption is supported by the fact that galectin-1 is the only heart tissue protein that binds sugar-specifically to immobilized O-glycosylated serum glycoproteins. Since the recognition of IgA1 by HHL-HRP increases after desialylation of the former, it is quite possible that IgA1-containing immune complexes get trapped in tissues by virtue of tissue galectin-1. Incidentally many pathogenic microbes secrete neuraminidase, which can desialylate IgA1 in physiological conditions. IgA nephropathy, diabetes and atherosclerosis are characterized by tissue deposition of IgA1, mostly its desialylated analogue.

Several studies have indicated a role for galectin-1 in tumor cell aggregation and colonization. The present demonstration of T antigen specificity of galectin-1 has an important bearing on tumor biology since expression of both galectin-1 and T antigen is reported to be increased in tumor cells compared to normal ones so that galectin-1 - T antigen interaction is a possible mechanism for tumor cell aggregation and host tissue localization. The present results also throw up the possibility of using T antigen-containing glycopeptides as anti-cancer therapeutics.

Since galectin-1 receptors on erythrocytes get exposed upon trypsinization a role for galectin-1 in sequestering senescent / transformed erythrocytes is worth investigating as transformed / senescent erythrocytes resemble trypsinised erythrocytes in surface changes.

The far higher affinity of erythrocyte membrane glycopeptides compared to lactose, for galectin-1 observed in the present work suggests their potential as inhibitors of tumor cell adhesion and colonization for which lactose had been used in the past. Erythrocyte glycopeptides, besides being self-antigens can be prepared in large quantities.

The sugar-specific and desialylation-dependent attachment of human galectin-1 to platelet and lymphocyte membrane offer exciting challenges

regarding sugar-dependent mechanisms of their adhesion to other tissues, especially as the ligands are limited to one or two glycoproteins.

Conclusions

Galectin-1 is indicated to have a cardinal role in the biological fate of IgA1 and / or its immune complexes, especially following their desialylation. Results also suggest a crucial role for O-linked oligosaccharides ending in T antigen in lectin-dependent biological recognition in normal physiology, immune recognition, bacterial adhesion and tumor cell colonization.

PUBLICATION

Publication

Sangeetha.S.R and Appukuttan P.S "IgA1 is the premier serum glycoprotein recognized by human galectin-1 since T antigen (Gal β1 $\rightarrow$3 GalNAc) is far superior to non-repeating N-acetyl lactosamine as ligand" 2005, International Journal of Biological Macromolecules, 35, 269-276.