MAMMALIAN GALACTOSE-BINDING PROTEINS: STUDIES ON HUMAN AND BOVINE BRAIN GREY MATTER GLYCOPROTEINS RECOGNIZED BY ENDOGENOUS GALACTOSE BINDING LECTIN AND BY HUMAN SERUM ANTI-α-GALACTOSIDE ANTIBODY

A THESIS PRESENTED

by

P.L. JAISON

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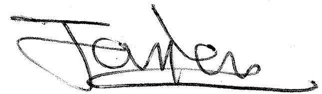
TRIVANDRUM

AUGUST 1994

CERTIFICATE

I, P.L. Jaison hereby certify that I had personally carried out the work depicted in the thesis entitled **"MAMMALIAN GALACTOSE - BINDING PROTEINS: STUDIES ON HUMAN AND BOVINE BRAIN GREY MATTER GLYCOPROTEINS RECOGNIZED BY ENDOGENOUS GALACTOSE BINDING LECTIN AND BY HUMAN SERUM ANTI-α-GALACTOSIDE ANTIBODY".**

Signature: 

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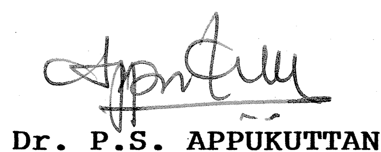

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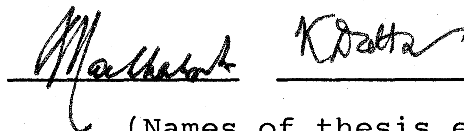

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P.L. JAISON

ABBREVIATIONS

2ME - 2-Mercaptoethanol

AIDS - Acquired immune deficiency syndrome

anti-BBL- Antiserum against bovine brain lectin

anti-Gal-HRP- Horse radish peroxidase labelled antibody to α-galactoside.

anti-BBGP-Antiserum against bovine brain glycoproteins

Anti-Gal or anit-α-Gal - anti-α-galactoside antibody

BBB - Blood-brain barrier

BBGP - Bovine brain glycoproteins

BBL - Bovine brain lectin

BHGP - Bovine heart glycoproteins

ϵBP - IgE binding protein

BSA - Bovine serum albumin

CAM-BBL - Carboxy amidomethylated bovine brain lectin

CAMS - Cell adhesion molecules

CEA - Carcino embryonic antigen

CLGG - Cross-linked guar gum

CNBr - Cyanogen bromide

CNS - Central nervous system

ConA - Concanavalin A.

EAE - Experimental allergic encephalomyelitis

EC - Endothelial cell

HBGP - Human brain glycoproteins

HBL - Human brain lectin

HIV - Human immunodeficiency virus.

HPLGP- Human placental glycoproteins.

HRP - Horse radish peroxidase

IgSF - Immunoglobulin superfamily

LAG - Lactosaminoglycan

Mα Gal - 1-O-methyl α galactoside

MBP - Myelin basic protein

MS - Multiple sclerosis

N-CAM- Neural cell adhesion molecule

PAGE - Polyacrylamide gel electrophoresis

PBS,7.4 - 20mM Sodium phosphate buffer with 150mM NaCl,pH 7.4

PHA - *Phaseolus vulgaris* agglutinin

PMSF - Phenyl methane sulfonyl fluoride

PSA - Polysialic acid

RCA - *Ricinus communis* agglutinin

SPDP- *N*-Succinimidyl 3- (2-pyridyl dithio) propionate

TAG - Terminal α-linked galactose

TEMED- N,N,N',N'- tetramethyl ethylene diamine

THRBC- Trypsinised human red blood cells

TRRBC- Trypsinised rabbit red blood cells

UEA - *Ulex europaeus* agglutinin

WGA - Wheat germ agglutinin

CHAPTER - I

GENERAL INTRODUCTION

INTRODUCTION

I Biochemical studies on human and bovine brain grey matter glycoproteins recognised by endogenous galactose binding lectin

Importance of Lectins

In 1954 Boyd and Shapleigh introduced the term lectin (1). In Latin 'lego' means to select or choose. Lectins are proteins of non-immunoglobulin nature capable of specific recognition and reversible binding to carbohydrates without altering the latter's covalent structure. In other words lectins are carbohydrate binding proteins other than enzymes or antibodies. A large number of lectins have been reported from plants, viruses, bacteria and higher animals. Their ubiquitous occurrence, definitely not being restricted to organisms from only a few branches of the evolutionary tree, fulfills necessary suppositions for a meaningful glycobiological interplay (2-5). Lectins not only distinguish between different monosaccharides, but also specifically bind to oligosaccharides, detecting subtle differences in complex carbohydrate structures. Lectin-carbohydrate interactions satisfy additional requirements expected of a cellular recognition system such as speed and

reversibility. Lectins have been purified from extracts of many tissues and cells by affinity chromatography on immobilised monosaccharides, oligosaccharides or glycoproteins (3, 6-11).

Drickamer (12) has divided the animal lectins into three groups based on structural information and studies on requirements for ligand binding.

a. The C-type lectins containing a carbohydrate recognition domain with 18 conserved residues and exhibiting Ca^{2+}-dependent ligand binding.

b. The S-type lectins which do not require divalent cations, but require reducing conditions for ligand binding and for which amino acid sequence analysis has revealed conservation of 39 residues in the carbohydrate recognition domain.

c. A third group of lectins which do not appear to posses structural similarities to either the C-type or S-type lectins, this last group is referred to as N-(neither C or S) type lectins.

Soluble β -galactoside binding lectins can be extracted without detergents. They are often developmentally regulated and they tend to be secreted, eg., chicken lactose lectin I (2,3,4). These lectins then interact with complementary glycoconjugates found on cell surfaces or in adjacent extracellular materials. Lactose or lactosamine

interacts with (Rat Lectin) RL-14.5, RL-18 and RL-29. Positions 4 and 6 of galactose are apparently critical, as substitution at these positions markedly inhibits binding to all three lectins. β-Galactoside binding proteins also known as 'galaptins' have been described in diverse species and in many tissues (3). These galaptins are characterized by their hemagglutinating activity. The molecular properties of these vertebrate lectins are strikingly similar from chicken to cow to man (3). Observations show that the protein is conserved with sequence identity ranging from 56% between chicken and bovine to 87% between bovine and human (13).

These lectins are predominantly cytosolic (14,15) consistent with the lack of a recognizable signal sequence. However, there is evidence that under certain conditions of growth and differentiation, 14 KDa lectin is externalized (16) or 35kDa lectin is transported to the nucleus (17). The findings that vertebrate lectins occur both in the cytoplasm and on the surface of various cells (9,18,19), suggest that these molecules might play a role in such fundamental phenomena as cell-cell recognition and cellular adhesion (20). These interactions are important during differentiation, tumorigenesis and metastasis (21). It was demonstrated that the 14 KDa lectin is translated on free cytoplasmic ribosomes (22), which is in agreement with

numerous studies documenting the major concentration of these lectins in the intracellular compartment as cytosolic proteins. In adult brain tissue immunolocalisation studies have demonstrated that the soluble lactose binding lectin (S-lac) is always found in the intracellular compartment and is never associated with plasma membrane (23). Such localisation suggests that the lectin can interact intracellularly with soluble ligands. The involvement of endogenous lectins in adhesion has been demonstrated in various types of cells (24). The activity of the 35 KDa galactose-binding lectin, CBP 35, has been investigated in mouse 3T3 fibroblasts in culture where the lectin is found at cell surface and also in the cytoplasm and the nucleus (15). Unphosphorylated CBP 35 is found almost exclusively in the nucleus while most cytoplasmic CBP 35 is phosphorylated (25). This suggests a mechanism where by dephosphorylation of CBP 35 by serum stimulation for example, could target cytoplasmic lectin to the nucleus by exposing a targeting sequence or releasing the lectin from a cytoplasmic anchor. Observations support a model of cell-cell adhesion involving interaction of surface lectins with carbohydrate sequences on the surface of neighbouring cells (26).

Soluble lectins may act as bridges by binding to carbohydrates on apposing cells. Endogenous lectins might be involved in homotypic and heterotypic aggregation. A

β-galactoside-specific lectin is found in the epithelium of the thymus and is postulated to be responsible for holding immature thymocytes in the thymic cortex by binding to galactose residues on the surface of these cells (27,28). On maturation of the thymocytes the galactose residues become masked by attachment of sialic acid, and the cells lose their activity to bind the lectin. They are thus free to migrate to the thymic medulla, where the mature thymocytes reside, or directly enter into the circulatory system. In the "homing" process, the recognition between lymphocytes and the cells of lymphoid organs is based on lectin-sugar interactions (29,30). It was demonstrated that pretreatment of mouse spleen cells with neoglycoproteins containing β-galactosides inhibited cell adhesion to microvenules (31).

A β-galactoside-specific lectin from rabbit bone marrow (32) was isolated by affinity chromatography on immobilized asialofetuin and shown to agglutinate rabbit erythroblasts. This agglutination could be inhibited not only by galactose-containing glycoconjugate but also by Fab fragments of anti-lectin antibodies, providing evidence that the lectin bridges directly between cell surface glycoconjugates (33). Furthermore, lectins may react not only with epithelial cells but also with bacteria present in the lumen of the small intestine (34). Specific recognition

between phagocytes and microorganisms may involve the interactions between lectins and carbohydrates. This type of recognition, followed by a series of events such as endocytosis and killing of the microorganisms, has been termed lectinophagocytosis (35). As interaction with microorganisms is likely to be specific and selective, lectins may shift the microbial balance of the gut, with serious consequences for growth and health (34). Indeed, some of the interferences in cell metabolism may be the result of signals originating from the binding of lectins to membrane glycoconjugates which also function as Class-I receptors for normal physiological luminal factors. As the binding of lectins to epithelium is usually followed by their endocytotic uptake by the brush border cells, Class-II receptors, for which endocytosis is obligatory (36), may also be involved in the lectin-induced information transfer. It has also been reported that the binding to receptors helps to stabilise the lectins against proteolytic break down (37). Immunolocalisation studies in the rabbit suggested that in most tissues, the high concentration of 14 KDa galactoside-binding protein detected during late embryogenesis was due to its increased synthesis by tissue fibroblasts during the extensive tissue reorganisation taking place at this time (38).

A galactoside-binding lectin activity was purified

to homogeneity from bovine, rat and human brain by affinity chromatography (39,40). During the past decade, a number of reports have suggested the actual presence of endogenous lectins in the developing nervous system (41,44,39). The cerebellar soluble lectin (CSL) appears to be secreted during development, like soluble lectins in other systems (9). Such secretion occurs in two regions of the cerebellum, ie, the premigratory zone and white matter (45). This strongly indicates potential involvement in (i) neuroblast migration and (ii) myelination. A β -galactoside specific lectin activity in brain is developmentally regulated, being maximal during postnatal brain development (46), when the galactoside-binding activity is detectable on isolated brain cells by a self aggregation assay (47). A study of the cerebral cortex and corpus callosum during brain development (23) showed the lectin to be predominantly neuronal and to increase from postnatal day 1 to 10, decreasing thereafter.

Studies indicate that the lectin plays a role in the intracellular traffic of molecules in nerves but is not generally externalised by them. During brain development however, lectins on neuroblasts and axonal membranes could be involved in transient neuroblast adhesion, contact guidance migration and fasciculation. Hynes et al have investigated the synthesis of the 14KDa lectin in rat peripheral and central nervous system by in situ

hybridization (48). Lectin mRNA was detected only in neurons. By postnatal day four, mRNA levels in the dorsal root ganglion and spinal cord were much higher than in the brain. Combined with previous immunocytochemical studies (49) these results showed that the lectin is predominantly or selectively expressed in sensory neurons in the peripheral nervous system and in motor neurons in the central nervous system, appearing soon after neuronal differentiation. In some embryos, as well as other developing systems, the lectins are developmentally regulated (50,20,51). Affinity-purified preparations of the blastoderm lectin contain three identifiable polypeptides (52). Two of these polypeptides are the known 14KDa and 16 KDa galactoside-binding lectins that, in the chick embryo, are developmentally regulated (20,53,54,55). The third component that was identified in purified lectin preparations is an apolipoprotein (apo) of plasma very low density lipoproteins, Apo-VLDL-11 (52).

β -galactoside binding soluble vertebrate lectins are divalent cation-independent and are not structurally related to C-type lectins. Many of them require reducing conditions for carbohydrate binding. In the mammalian and chicken lectins this appears to be due to the presence of cysteine residues; hence the term S-type (sulfhydryl type) lectins. However, the lectin from the electric organ of the

electric eel contains no cysteine residues and in this case inactivation is probably due to oxidation of tryptophan residue (56). In the case of the rat 14KDa lectin, it was proposed that intramolecular disulphide bonds are formed leading to a change in the secondary structure (57,58). This conclusion was based on the observations that upon oxidation, decrease in hemagglutinating activity was paralleled by a change in circular dichroism and a decrease in the number of free thiol groups, but without evidence of intermolecular cross-linking as assessed by SDS-PAGE in the absence of 2-mercaptoethanol. Nor was there a change in molecular mass estimated by sedimentation equilibrium experiments. One important new finding is that lectins may contain a second type of binding site that interact with a non-carbohydrate ligand (59). All the S-type galactoside binding lectins share specificity for β -galactose-(1-4)-N-acetylglucosamine structure or N-acetyl lactosamine. The crucial determinants are the hydroxyl groups at positions 4 and 6 of galactose and position 3 of N-acetylglucosamine suggesting that the lectins bind to one side of the preferred conformation of this structure and its derivatives. The N-acetamido group is also important in stabilisng the lectin-ligand interaction (60,61).

The possibility of lactosaminoglycans (LAGs) as the endogenous ligands for this family of lectins is

supported by the photochemical crosslinking of C-14 to a poly-N-acetyl lactosamino-proteoglycan in chicken embryonic skin (62) and is of considerable interest. A major carrier of LAG in the extracellular matrix is laminin, a component of the basement membrane which underlies epithelia and surrounds peripheral nerve and fat cells (63). This large glycoprotein (molecular mass 1000 KDa) has a high carbohydrate content of 12-27%, most of which is LAG, and it binds to the cell surface and to other matrix components as well as to itself. Some of these interactions have been shown to involve specific areas of protein molecule but there have been some recent indications that the LAG chains may also be functionally important, for example in cell spreading and migration (64,65,66). An association of galaptins with laminin in vitro has been described (67) and could be of obvious biological importance as laminin has multiple activities in cell adhesion, differentiation, neurite outgrowth and metastasis (68,69). This heavily glycosylated molecule has multiple functions in the orchestration of cellular interactions which are determined by the structural diversity of its protein and saccharide moieties and mediated by interactions with a number of different cell surface and extracellular matrix components (69). Inclusion of a 67KDa cell surface laminin receptor in the family of soluble vertebrate lectins is based not only

on saccharide binding activities but also on the immunological cross reactivity of the 67 KDa and 14 KDa proteins.

A galactoside binding lectin with a molecular weight of 34,000 (L-34) was reported to be a chimeric gene product with two protein moieties (70). The aminoterminal is proline-glycine rich and composed of 8 tandem repeats homologous with the collagen α_1 chain, where as the carboxy-terminal is derived from a lower molecular weight (14 KDa) galactose-binding lectin and contains the galactoside binding domain (71,72). Interestingly a lectin and repetitive domains are also present in the "homing receptor" which is involved in the dissemination of normal migratory cells ie., lymphocytes (73,74,75). It now seems evident that these proteins must have multiple functions in both domains. This represents the corollary of the thesis that saccharide structures are important carriers of biological information both extracellularly and intracellularly (76). The utilisation of one protein structural motif in multiple activities exemplifies the opportunistic nature of cellular evolution. The cytoplasmic activities of the 14kDa lectin species however remain an enigma. No clues have yet been gained on its regulation or sub cellular distribution. Perhaps as with the identification of CBP 35 as Mac-2, an IgE binding protein

and a laminin receptor, the identification of this abundant protein in another guise will give sudden insight into its functions.

Characteristic changes in lectin expression that coincide with distinct physiological or pathological changes in the life of cells or tissues were observed. β-galactoside-specific surface lectins (14.5 and 34KDa) are present on different murine and human tumor cells including (for eg.) B16 fibrosarcoma and carcinoma. A 14.5 KDa lectin is also found on normal embryonal fibroblasts, where as oncogene-transfected cell clones derived from these cells, as well as established tumor cells, express both the 14.5 and the 34 KDa lectins (71). Lectin expression as measured by the ability of the cells to undergo aggregation in the presence of asialofetuin or by the extend of binding of a monoclonal antibody to the lectin, correlated well with the metastatic potential of the tumor cells. Several lines of evidences strongly suggest that lectins on human and murine metastatic tumor cell surfaces may be involved in the formation of tumour emboli (77,78,79). In addition, the lectins may facilitate adhesion of the emboli to endothelial cells of capillaries. 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. Significantly, highly metastatic melanoma and fibrosarcoma cells that had been treated with the antibody to the lectin before their injection into mice showed decreased metastatic potential (77,78).

The fundamental role of galactoside binding lectins (gal-lectins) and especially of one distinct species with a mol. wt of 34,000 (L-34), in tumor-cell metastasis has been demonstrated (80,81,82). The L-34 lectin is expressed in a wide range of neoplasms, including spontaneous, viral, ultraviolet (UV) and chemically induced tumors (77). Raz et al have shown that three murine transformed cells expressed significantly higher (5 to 50 fold) L-34 probe-hybridizing in mRNAs than their respective normal cell counter parts (70). The ability of 5D7 anti-lectin MAbs to inhibit growth of tumor cells in agarose indicates that there is a relationship between Gal-lectin expression and the suppression of the transformed phenotype (83). The hepatocytes were found to bind a cell line of high metastatic potential (for liver) through galactose and N-acetylgalactosamine specific, lectin like protein with molecular sizes of 52, 56 and 110 KDa (84). On the basis of size, these proteins differ from the galactose and N-acetylgalactosamine specific, hepatic-binding proteins.

Since galactose binding lectins can also bind to

lactosaminoglycan structures, it is possible that an increased affinity for, and interaction with, the basement membrane component, laminin, may contribute to increased metastatic potential of cells bearing high cell surface concentrations of lectins. It is of considerable interest that tumor cells are more susceptible to the actions of killer cells when they are circulating in suspension than when they are adherent to endothelial cells or extracellular matrix components (85). This suggests that the survival of circulatory tumor cells will be enhanced if they can adhere to vascular or subvascular surfaces. Several mechanisms have been proposed to explain the occasional enhancement of metastasis by platelet/tumor embolization (86). The embolus may, for example form a protective 'cocoon' around the tumor cells that offers protection from cytotoxic lymphocytes and natural killer cells (87,88,89). It is known, for example, that platelets can facilitate tumor cell adhesion to extracellular matrix via the platelet glycoproteins lb and llb/llla (90,91,92).

Some investigators have proposed that lectin/ saccharide interactions could be a general mechanism of regulation of cytokine activity. 14KDa, β -galactoside-binding lectin from human placenta and its homologue found in the electric organ of <u>Torpedo electricus</u> (93,94) have been shown to possess immunoregulatory properties, inducing

the release of tumor necrosis factor from macrophages (95) and suppressing experimental autoimmune myasthenia gravis (EAMG) in mice (96). The β-galactoside binding human placental lectin is one of several immunosuppressive molecules (97,98,99) that may prevent maternal recognition of paternal antigens expressed on fetal tissues. Mistletoe lectin (ML-1) at very low doses induces the release of lymphokines and tumor necrosis factor into the circulation and have promising clinical applications in cancer therapy (100).

Importance of Oligosaccharides

Recognition is a central event in a variety of biological phenomena and the first step in numerous processes based on cell-cell interactions, such as fertilisation, embryogenesis, cell migration, organ transformation, immune defense and microbial infection. The information for correct recognition must lie in the expression and organisation of molecules on the cell surface. Almost all cells carry carbohydrates on their surfaces in the form of glycoproteins, glycolipids and polysaccharides (101). Carbohydrates have an enormous potential for encoding biological information (101,102,103). 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, where as in carbohydrates, information is also encoded in the position and anomeric configuration (α or β) of the glycosidic units and in the occurrence of branch points. Among the potential determinants to serve as part of a recognition system, carbohydrate structures of cellular glycoconjugates with their spatial accessibility and remarkable sequence diversity have not remained unnoticed. Although nearly 200 different monosaccharides are found in nature, only 11 are known to occur in glycoproteins (104). Carbohydrates on glycoproteins play an important role in cell biology and development. For some glycoproteins the addition of asparagine (Asn)-linked oligosaccharides are required for glycoprotein transport to the cell surface (105-107), stability and solubility (108), normal conformation and function of the glycoprotein (109), or intracellular trafficking of glycoproteins (110). The oligosaccharide portion can also serve as a ligand for carbohydrate binding proteins (111)

Developmentally regulated changes in oligosaccharide processing have been shown to modulate the activity of specific adhesion mediating glycoproteins. The affinity of binding between NCAM molecules increases during development due to a decrease in polysialic acid sequences

on Asn-linked oligosaccharides in NCAM (112). In the embryonic brain N-CAM is detected as a 200-250 KDa broad band by western blot analysis, while in adults its molecular weight decreases to 120-180 KDa (113). This change is due to a difference in carbohydrate structure. The embryonic N-CAM has an α-2,8 linked polysialic acid an unusual carbohydrate in a protein of vertebrate origin. The polysialic acid in embryonic N-CAM has been shown to reduce the adhesive activity of N-CAM (113).

Polysialic acid (PSA) on the surface of axons regulates patterns of normal and activity dependent innervation. Because axon bundling represents a form of regulated cell-cell adhesion, considerable attention has been paid to the identification of adhesion molecules on axons, and a number of cell adhesion molecules (CAMS) have been found to be associated with their surfaces (114). The long polymer of polysialic acid in a CAM molecule is so large and abundant that its presence can impede the ability of membranes to get close enough for effective receptor-receptor interaction. Thus, a variety of interactions (not only those involving N-CAM itself) can be affected by increases or decreases in PSA levels on the cell surface. A straightforward interpretation of this correlation is that high levels of PSA limit axon-axon interactions and thereby allow nerve fibers in the fast region to branch out over the

muscle surface. Conversely, the lower levels of PSA on nerve fibers in the slow region produce thick fascicles that continue to grow along muscle fibers. The general relationship between PSA, adhesion molecules, innervation and activity is applicable not only to development, but also to repair and possibly to some functions of the adult nervous system. However, it should be noted that PSA is in general lost from the vertebrate nervous system as it matures (115). Observations show that the exposure of galactose residues on the surface of untransformed baby hamster kidney (BHK) fibroblasts (116) or transformed 16C rat dermal fibroblasts (117) by neuraminidase treatment increased intercellular adhesiveness.

Polylactosaminoglycans are high molecular weight carbohydrates and are distinct from usual complex type Asn-linked saccharides by having side chains composed of Galβ 1 --> 4 GlcNAc β 1-3 repeats, which are susceptible to endo-β -galactosidase. Polylactosaminoglycans can carry various antigenic determinants such as I/i and ABO blood groups (118-120). Further the structures of polylactosaminoglycans are often characteristic to different cell types and stage differentiation. Unique structures of poly-N-acetyl-lactosamines in granulocytes and monocytes serve as ligands for adhesive molecules, selectins, in endothelial cells and platelets (121-125).

One of the more consistently observed alteration following neoplastic transformation is a shift toward the synthesis and expression of larger Asn-linked oligosaccharides (126-130). In a number of studies the changes in size has been attributed to an increase in sialic acid content of the structures (129-131). Terminal sialylation of cell surface glycoconjugates has been correlated with increased metastatic potential in a number of murine tumor cell models (132). It has been suggested that increased sialylation may reduce tumor cell adhesion to extracellular matrix proteins (133) and susceptibility to immune destruction (134), phenotypes that may contribute to increased tumor cell autonomy in situ. Transformed cells have been shown (135-137) to have more highly branched Asn-linked glycans due to the addition of β 1-6 linked lactosamine antennae into the trimannosyl core (ie, Gal β - 1-4GlcNAc β 1-6). Because many of the lactosamine antennae are substituted with sialic acid, increased branching may also contribute to the observed increase in sialylation. The β 1-6 linked antenna has been associated with increased malignancy in human thyroid carcinomas (138). It was reported that tumor metastasis in animal models was reduced by the addition of glycosylation inhibitors such as swainsonine and castanospermine (139-141). Since these two inhibitors block the formation of side chains elongating

from C-6 of α-mannose (142), they presumably inhibit poly-N-acetyllactosamine formation. Thus, the inhibition of poly-N-acetyllactosamine synthesis apparently reduces tumorigenicity. Observations indicate that the degree of sialylation increases but fucosylation decreases in highly metastatic cell lines. Increased β 1-6 branching of complex type oligosaccharides has been reported in the conversion of nonmetastatic clones into metastatic ones (143). Oncogene expression related to enhanced metastatic potential was associated to increased β 1-6 branching of Asn-linked oligosaccharides in rat fibroblasts (144). Furthermore inhibitors of Asn-linked oligosaccharide processing such as tunicamycin and swainsonine inhibit lung colony formation by murine melanoma cells injected intravenously (139,145). Swainsonine also inhibits spontaneous metastasis caused by a murine reticulosarcoma (146). Observations show that malignant transformation of both murine and human cells is commonly associated with expression of larger N-linked oligosaccharides (127,147,148).

A remarkable feature of the tumor associated carbohydrate antigens is their close relationship with the classically known blood group antigens (149). It was evident early that carcinoembryonic antigen (CEA) was structurally a very heterogeneous molecule, and that most of this heterogeneity was due to variations in the structure of the

carbohydrate side chains (150,151). Studies using a rat liver perfusion system also showed that CEA was cleared rapidly by the liver and that removal of sialic acid from CEA by neuraminidase treatment increased this rate (152).

The diversity of oligosaccharides makes it possible that lectin-oligosaccharide interactions occur both at the target and the effector cell level. Biochemical studies show that the 67 KDa elastin binding protein is a galactoside binding protein that has functional and immunological similarity to a family of small molecular weight galactoside lectins (153,154). In the absence of carbohydrate, the receptor binds to elastin with high affinity, whereas in the presence of galactoside sugar, receptor affinity is decreased substantially (154) Melanoma and PC 12 cells bound to the unglycosylated laminin, but melanoma cells did not spread, and no neurite outgrowth was observed for PC 12 cells. One interpretation of these experiments is that cell binding to laminin is carbohydrate independent, where as carbohydrate residues are important for cell spreading and neurite extension. In other words, in the presence of carbohydrate, protein ligands still bind to the receptor, but remain bound for only a fraction of the time that they would in the absence of sugar (154). Moreover, perturbation of physiological processes by carbohydrate-specific monoclonal antibodies, carbohydrate

determinants themselves or inhibitors of glycoprotein processing as well as the efficacy of lectins as substrata for cell attachment add further support to the idea that carbohydrate structures are information-bearing elements (155,156,157).

Significance of lectin-carbohydrate interaction in modern biology and medicine

One of the major and long standing goals of neurobiologists is to acquire information on the structure and function of neural membrane components at a molecular level. An understanding of the molecular basis of the cell-surface code, therefore, has implications for intervention in many areas of biology and medicine. To prove carbohydrate dependence of a cell-cell interaction, it is essential to isolate the carbohydrate binding protein and its ligand and establish that they indeed take part in the recognition phenomenon. This should include demonstration of their surface location and of the ability of the purified carbohydrate binding protein and ligand as well as of antibodies to the carbohydrate binding protein and to the ligand, to specifically block the interaction between cells.

Aberrant cell recognition is thought to underlie the uncontrolled cell growth and motility that characterise

neoplastic transformation and metastasis. Embryogenesis is an orderly process involving cell proliferation, cell invasion and cell migration over extracellular matrix, characteristics that appear to be similar to those required of metastatic tumor cells (158,159). Although the reexpression of embryonic carbohydrate structures by tumor cells may simply reflect the expression of earlier genetic programs (160,120), it is also possible that certain structures that are involved in embryonic cell-cell interactions may enhance the malignant potential of tumor cells. Quite apart from normal locomotion, the essential characteristic of the malignant cancer cell is the ability to disseminate widely. This must involve unscheduled locomotion due to loss of normal controls. Without this loss of control of movement, the tumors would be benign. One of the critical events in the progression of normal human cells to tumorigenicity is the escape from the limitations on proliferation imposed by cellular senescence. An understanding of the mechanisms involved in cell mortality should provide essential information about one of the critical and perhaps the rate limiting steps in cancer.

Deletions, additions and alterations in expression of cell surface carbohydrates and secreted glycoprotein molecules produce epitopes recognised by monoclonal antibodies that may be used for new approaches to diagnosis,

prognosis, monitoring and therapy of cancer.

Coupling drugs to carbohydrate binding proteins and/to molecules specifically recognised by membrane bound carbohydrate binding proteins, appears to be a promising tool for targeting drugs to specific site. Recently, Chakraborty et al (161) found that methotrexate conjugated to mannosyl bovine serum albumin was phagocytosed efficiently by murine macrophages through the macrophage mannose receptor. Moreover, in a murine model of experimental visceral leishmaniasis, this drug conjugate was much more effective than unconjugated methotrexate (161).

An intensive effort is also underway to exploit cell surface lectins as targets for the controlled and selective delivery of drugs to malignant cells (79).

Both lectins and monoclonal antibodies have been used to induce mitogenesis and to study its mechanisms; to identify, separate and label cells; and to fulfill a variety of similar roles in diagnostics and therapeutics. Anti-CEA antibodies coupled to cytotoxic agents have been studied as potential therapeutic agents in cell culture and in nude mouse xenografts with human colorectal cancers (162).

The demonstration that the purified β-galactoside-specific lectin can enhance the immunological defense mechanisms at an optimal dose of 1ng/kg body weight offers a reasonable perspective for widespread clinical application

(163,100). Several lectins, most notably lentil lectin, can prolong skin and heart tranplants in rodents (164), and lentil lectin was effective at suppressing graft-versus-host diseases in mice induced by allogenic spleen cell (165). Results of experimental heart transplantation in rodents indicates that succinylated concanavalin A is a more potent immunosuppressive agent than cyclosporine (166). Ricin covalently coupled to monoclonal antibodies to leukemia antigens can be effective reagent for ex-vivo marrow purging (167,168,169), especially useful for autologous bone marrow transplantation. Among the novel therapeutic strategies being developed in response to the AIDS epidemic are human monoclonal antibodies to HIV glycoprotein gp 41 coupled to ricin A-chain (170) and recombinant soluble CD_4 coupled to ricin (171), both of which are effective in vitro. A conjugate of ricin A-chain to a monoclonal anti-T cell antibody has been found effective for acute steroid resistant graft-versus - host disease when administered intravenously to patients following allogenic bone marrow transplantation (172).

The distribution patterns of carbohydrate binding proteins and their ligands on normal and malignant cells are being studied in the hope of finding clear cut differences that may be useful for diagnostic purposes.

II. Importance of human serum α-galactoside binding antibody

The primary function of the immune system is to provide protection against infection by pathogenic microorganisms and against the development and spread of malignant tumors (173). This biological system consists of several lymphocyte subpopulations and macrophages which interact and cooperate to destroy and eliminate all kinds of foreign macromolecules. Most frequently, these complex interactions induce B-cells to differentiate and secrete antibodies which circulate in the blood stream and bind specifically to the invading substance or microorganisms that induced them. Antibody-antigen interactions lead to the destruction of the antigen through several possible effector mechanisms. Certain cytotoxic T-cells have the ability to kill antibody-coated target cells, and antibody binding facilitates antigen ingestion by macrophages and frequently activates the complement system, which is a complex system of blood proteins capable of causing cell lysis. In addition, certain effector cells do not require antibody to destroy antigens, such as certain cytotoxic T-cells specific for given antigens and natural killer (NK) cells. The mechanisms regulating such systems, composed of large numbers of phenotypically distinct cells interacting in a

complex network, are extremely complex. Immune cell cooperations are primarily modulated by the interactions of molecules expressed on the cell surfaces, such as major histocompatibility complex (MHC) class I and II antigens, differentiation antigens, adhesion molecules and various classes of receptors with their appropriate ligands. Molecular recognition phenomena therefore constitute a key element not only in the ability of immune cells to discriminate between self and non-self molecules, but also in the complex cellular and molecular interactions allowing the immune system to ensure highly specific and effective protection against infections.

Biochemical analysis has revealed that most of the regulatory and effector molecules involved in immune responses are glycoproteins. The structural diversity of immunoglobulin (Ig) molecules is greater than that of all other molecules in the vertebrate body. These molecules are glycoproteins of which carbohydrates are usually located in their constant regions of heavy chain (174). These large proteins are assembled in a disulphide cross linked four chain structure. The major serum antibody, IgG, consists of two identical heavy chains of molecular weight approximately 50,000 and two identical light chains of molecular weight 25,000 (175). Sequence comparison of monoclonal IgG proteins indicates that the carboxy-terminal

half of the light chain and roughly three quarters of the heavy chain from the carboxy end show little variation (176,177). The antigen combining site of the molecule is in the first 100 aminoacids of the amino-terminal regions of both light and heavy chains, referred to as V_L and V_H, domains, which show considerable sequence variability. With in these variable regions are short stretches of extreme aminoacid sequence variation that are involved in antigen recognition and are designated as complementarity-determining regions. Proteolytic cleavage of the molecules on the carboxy terminal side of the interstrand disulphide linkage connecting the light and heavy chains generates two Fab molecules, each containing an antigen binding region.

Antibody molecules are found in the earliest vertebrates, but homologous molecules have not been detected in invertebrates. However, when one compares the aminoacid sequence of both the heavy and light chains of immunoglobulins from the earliest vertebrates (178) with antibodies of vertebrates that evolved much later, it becomes evident that such a complex, polymorphic system of molecules that display a high degree of homology across vertebrate species could not have arisen de novo at the time of the emergence of vertebrates. There is overwhelming evidence that antibodies must have evolved from similar, more primitive protein receptor systems.

Analysis of molecular evolution have suggested that immunoglobulin superfamily (IgSF) molecules have evolved from a common ancestral molecule with a single Ig domain (179-181). The diversity in evolution of IgSF molecules can be clearly exemplified in the immune system, within which dozens of distinct membrane glycoproteins have been shown to belong to the IgSF (181).

However, from the demonstration of the saccharide nature of blood group antigens (182) and the observation that certain lectins are able to mitogenically stimulate lymphocytes in culture (183), to the recent discovery that certain molecules involved in lymphocyte homing are cell surface sialic acid-specific lectins (184), the concept has gradually emerged that the carbohydrate moieties of glycoconjugates might act as recognition signals in the immune system (185).

Most natural antibodies seem to display an anti-carbohydrate specificity. They include, for example the anti-blood group A and B antibodies, which interact with Gal NAc α 1 ---> 3 (Fuc α 1 ---> 2) Gal and Gal α 1 ---> 3 (Fuc α 1 ---> 2) Gal, respectively (186,187). They are mostly of the IgM class and are present in only part of the population, according to the blood type.

The anti-T or anti-Thomsen Friedenreich antibodies, which interact with β -galactosyl groups

usually penultimate to terminal sialic acid residues on various cell membranes, are similarly mostly of the IgM class (188, 189).

A natural antibody with anti -α- galactosyl reactivity was first reported by Galili et al. (190).In comparison to many other natural antibodies, antibody to α-galactosyl epitope (Anti-Gal) is found in high titers in all normal human sera regardless of blood group, and it specifically recognizes α -linked galactose residues (190,191) except the fucosylated oligosaccharide groups such as in blood group B antigen. Anti-Gal can recognise galactosides in α(1 ---> 3) linkage and to a lesser extent, those in α (1 ---> 2), α (1 ---> 4) and α (1 ---> 6) linkages (194). Anti-Gal is found exclusively in the sera of humans, apes and Old World monkeys (192). In normal human serum 1% of the circulating IgG is anti-Gal (190,191,193). This is the only natural IgG antibody found to be present in high titers in the serum of every individual.

The anti-Gal differs from all human natural antibodies with known anti-α galactosyl specificity. The anti-blood group B antibody which also displays an anti-α-galactosyl specificity, is present only in A type and O type individuals and is mostly of the IgM class (187). Anti-Gal is present in sera of all blood groups and is mainly an IgG antibody, as indicated by the almost similar titers observed

in maternal and fetal blood. IgM antibodies do not cross the placenta.

The advent of anti-α-Gal accompanied by the near disappearance of Gal-α-1-3 Gal epitope in Old World monkeys, apes and humans is regarded as a milestone in evolution. Structural analysis of glycopeptides and glycosphingo lipids from cell membranes of various species have shown the presence of terminal non reducing Gal α 1-3 Gal residues on glycoconjugate in transformed mouse fibroblasts, Ehrlich ascites cells, and lymphoma cells (195,196,197), rat adenocarcinoma and fibrosarcoma cells (198,199), dog red cells (200), rabbit red cells (201,202,203) hamster kidney cells (204) bovine red cells and thyroid cells (205,206,207, 208). Gal α 1-3 Gal epitopes were found to be abundantly expressed on the red cells of various non primate mammals and on the red cells of New World monkeys. Such epitopes were not detected on the red cells of Old World monkeys, apes or humans. The latter species, however produced large amounts of anti-Gal antibody (192).

The synthesis of the Gal-α-1-3 Gal epitope in the Golgi apparatus of cells of murine (209,210,211), leporine (212,213) and bovine (214) origin has been demonstrated to be catalysed by the enzyme α-1-3 galactosyl transferase in the following reaction:

Galβ1 -> 4GlcNAc-R + UDP-Gal
(N-acetyllactosamine)

(α1 -> 3 galactosyltransferase)
---------------------------------->

Galα1 -> 3 Galβ1 -> 4GlcNAc-R + UDP

The biochemical basis which can account for the diminished expression of Gal α 1 --> 3 Gal epitopes on Old World primates and humans appears to be the suppression of the gene corresponding to α 1 --> 3 galactosyl transferase enzyme. This assumption is based on the following information. First, the terminal α-galactosyl residue of the Gal α 1 --> 3 Gal epitope is commonly found linked to lactosaminyl glycoconjugates (195-199,202-208,215). Second, lactosaminyl glycoconjugates are abundantly expressed on human cells (216-218,187). Therefore, it seems that human cells contain the glycosyl transferases necessary for the synthesis of the core structure of the Gal α 1 --> 3 Gal epitope but lack the α 1 --> 3 galactosyl transferase needed for the synthesis of the epitope.

The findings that human thyroglobulin and human paragloboside molecules can serve as acceptors for α1 --> 3 galactosyl transferase (219) further indicate that the absence of Gal α 1 --> 3 Gal epitopes on human cells is the result of suppressed α 1 --> 3 galactosyl transferase

activity, rather than the lack of acceptor molecules. Galili et al have found that, in the course of physiologic and pathologic aging of human red cells, a cryptic Gal α 1 --> 3 Gal epitope is exposed (190,219,220), suggesting marginal activity of the enzyme.

The distribution of Gal α 1 --> 3 Gal epitopes on cells of various species reflects a unique evolutionary pattern in the expression of this carbohydrate structure. Although Gal α 1 --> 3 Gal epitopes were found to be abundantly expressed on mammalian cells (195-199,202-208,215,221), such epitopes were not detected on cells from nonmammalian vertebrates. The presence of Gal α 1 --> 3 Gal epitopes on fibroblasts of marsupials (Opossum and Kangaroo) suggests that expression of these epitopes on nucleated cells began early in mammalian evolution before the divergence of marsupials. This epitope appears to have been evolutionally conserved thereafter in many mammalian species. Among primates, lemurs express an abundance of this epitope on their cells (Table 1). Lemurs and other prosimians are believed to have diverged from primates more than 60 million years ago. New World monkeys, which also express the Gal α 1 --> 3 Gal epitope, diverged from Old World monkeys 30-40 million years ago (222). In contrast, the Gal α 1 --> 3 Gal epitope was not found on any of the cells of Old World monkeys, apes or humans. Thus it seems

Table I Binding of anti-Gal antibody to nucleated cells of various species

Species (common name)	Cell type	Anti-Gal binding (dilution)
Nonmammalian vertebrates		
Goldfish	Fibroblasts	No binding
Goldfish	Fibroblasts	No binding
Iguana	Fibroblasts	No binding
Quail	Fibroblasts	No binding
Duck	Fibroblasts	No binding
Chicken	Fibroblasts	No binding
Nonprimate mammals		
Opossum	Fibroblasts	1:5
Potoroo (kangaroo)	Fibroblasts	1:10
Armadillo	Fibroblasts	No binding
Mouse	L cells (transformed fibroblasts)	1:5
Mouse	Myeloma cell line (SP/2)	1:20
Mouse	Epithelial cells (lens)	1:10
Rabbit	Fibroblasts	1:10
Rabbit	Epithelial cells (lens)	1:10
Pig	Endothelial cells (aorta)	1:10
Pig	Epithelial cells (lens)	1:5
Cow	Endothelial cells (aorta)	1:10
Cow	Smooth muscle cells (aorta)	1:10
Cow	Thymocytes	1:10
Horse	Fibroblasts (skin)	1:10
Horse	Endothelial cells (aorta)	1:10
Horse	Smooth muscle cells (aorta	1:10
Sheep	Fibroblasts (kidney)	1:10
Dog	Endothelial cells (aorta)	1:10
Mink	Fibroblasts (lung)	1:10
Cat	Fibroblasts (skin)	1:10
Dolphin	Fibroblasts (kidney)	1:10
Bat	Fibroblasts (lung)	1:5

(Contd...)

Table I (Contd...)

Species (common name)	Cell type	Anti-Gal binding (dilution)
Primates		
Prosimians		
Ring-tailed lemur	Fibroblasts (skin)	1:10
Brown lemur	Fibroblasts (skin)	1:10
New World monkeys		
Marmoset	B lymphocyte cell line ($B_{95.8}$)	1:10
Tamarin	Fibroblasts (skin)	1:5
Squirrel monkey	Fibroblasts (skin)	1:5
Spider monkey	Fibroblasts (skin)	1:5
Titi (Callicebus)	Fibroblasts (skin)	1:10
Woolly monkey	Fibroblasts (skin)	1:5
Old world monkeys		
Pig-tailed macaque	Fibroblasts (skin)	No binding
Cynomolgus macaque	Fibroblasts (kidney)	No binding
Patas monkey	Fibroblasts (skin)	No binding
Celebes ape	Fibroblasts (skin)	No binding
Hanuman langur	Fibroblasts (skin)	No binding
Apes		
Gibbon	Myeloma cell line	No binding
Orangutan	Fibroblasts (skin)	No binding
Chimpanzee	Fibroblasts (skin)	No binding
Gorilla	Fibroblasts (skin)	No binding
Man	Burkitt lymphoma cell line (EB_3)	No binding
Man	Myeloid cell line (HL60)	No binding
Man	Carcinoma cell line (HeLa)	No binding
Man	T-125 memia cell line (Be-13)	No binding
Man	Fibroblasts (skin)	No binding
Man	SV-40 transformed fibroblasts	No binding

that the expression of the Gal α 1 --> 3 Gal epitope was suppressed in ancestral lineages of Old World primates less than 30 million years ago before the divergence of apes and Old World monkeys (Fig.1).

A hypothesis which might explain this phenomenon (219) is that this suppression may have resulted from an evolutionary pressure exerted by an infectious agent endemic to the Old World. Such an infectious agent, which probably had a deleterious effect on ancestral Old World primates and which expressed Gal α 1 --> 3 Gal epitopes on its surface, might have driven the evolution of primates toward the development of the capacity to produce high titres of anti-Gal as a protective mechanism and diminished the expression of autologous Gal α 1 --> 3 Gal epitopes. Primates with both the antibody and the autologous Gal α 1 --> 3 Gal epitope would have become extinct as a result of autoimmune processes. In support of this hypothesis, several infectious microorganisms expressing Gal α 1 --> 3 Gal epitopes have indeed been identified among viruses, bacteria and protozoa (223-226). The binding of anti-Gal to the lipopolysaccharide molecules present on *Escherichia coli* and *Klebsiella* indicate the presence of Gal α 1 --> 3 Gal epitope on bacteria of human flora. These bacteria also seem to serve as a constant antigenic source for anti-Gal production.

The anti-Gal was found to present in sera of

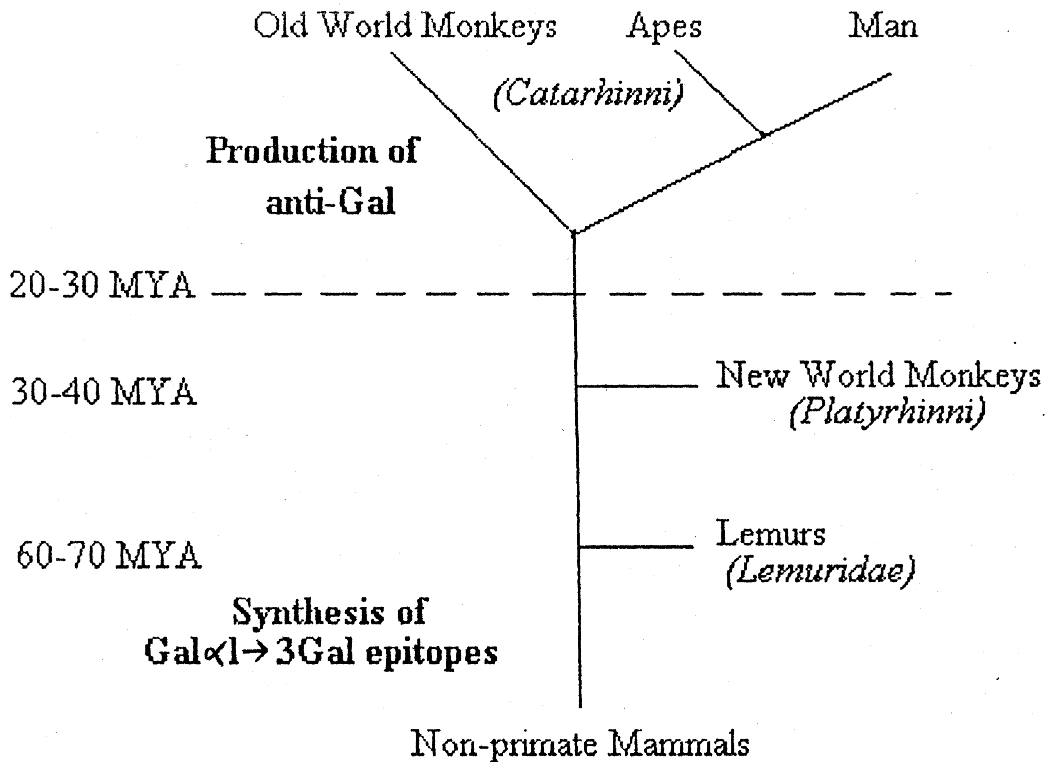


Fig.1. Tentative delineation of the stage in primate evolution in which the synthesis of Galα1-> 3 Gal epitopes ceased and production of anti-Gal began (broken line). The production of anti-Gal in Old World monkeys, apes and humans (Catarhinni) and the absence of detectable Galα1->3 Gal epitopes on cells of these primates suggest that the evolutionary event discussed in the introduction occurred before the divergence of ancestral old world monkey and ape lineages. (MYA=million years ago) (236).

individuals above the age of 4 years in a remarkably high titre ranging between 1:1800 and 1:1600. A marked decrease in the anti-Gal titre was found only in infants of 3-6 months. This is compatible with the total decrease of maternal IgG and the initiation of self IgG synthesis. The unaltered production of the antibody throughout life implies a constant antigenic stimulation by intestinal flora (186,227).

Proposed physiological functions of anti-α-Gal include combating invading parasites that bear α-galactoside epitope and scavenging of senescent red cells in which this epitope so far cryptic, might become accessible (190).

Anti-α-Gal titre was elevated 10-100 fold normal level in Chagas disease, Leishmania, Trypanosoma and inner ear infections (223,228). The marked elevation of the anti-Gal titres observed in individuals with a variety of autoimmune phenomena has suggested that some pathologic autoimmune process may be related to the interaction of anti-Gal with Gal α 1 --> 3 Gal epitopes (223,229-233).

Neoplastic transformation and tumor progression are also frequently accompanied by structural modifications in the carbohydrate moiety of glycoconjugates (234,237). Cell surface carbohydrates can theoretically modulate the metastasizing capacity of tumor cells by acting at least

three different levels of the metastatic process:

a) in the release of tumor cells from primary tumor mass;

b) during blood transportation of metastatic cells;

c) in the attachment of tumor cells to the target tissue.

Alterations in cell surface carbohydrates have been found to severely affect the metastatic potential of experimental tumors (238-240). For example, McCoy et al (239) have observed a strong positive correlation between the expression of α-D-galactopyranosyl (α-D-Galp) residues on murine tumor cells and their ability to metastasize. In addition it has been shown that the glycoprotein laminin accounts for a significant proportion of the total α-D-Galp end groups on highly malignant murine EHS sarcoma cells (241).

Enhanced synthesis of terminal α-linked galactose groups being characteristic of many tumors (241,242), it has also been suggested that anti-Gal functions in an anti-tumor capacity by scavenging tumor cells that bear α-galactosyl epitopes (241). It has been demonstrated that anti-Gal decreased the lung colonization by murine MO_4 cells in C_3H/He syngeneic mice by interacting with α-D-Galp residues located on the surface of MO_4 cells (243).

The causes of the multiple and profound changes in glycosylation during malignant transformation of cells are still hypothetical. They may be linked to the expression or

repression of mRNA inducing the biosynthesis of particular, slightly different polypeptide chains and/or post-transcriptional changes in protein glycosylation. The derepression of gene (s) coding for glycosyltransferses as a result of malignant transformation could also account for these minor structural differences occurring on the same polypeptide chain expressed by both normal and transformed cells. Indeed, important changes in glycosyltransferases during cell transformation have been reported (244), but the conclusions drawn have often been contradictory.

Since as much as 1% of the circulating IgG molecules in humans (anti-Gal) were found to interact with the Gal α 1 --> 3 Gal epitope, both on glycosphingolipids (190,191,193) and glycoproteins (223,243) the study of α-galactosyl epitopes on mammalian cells which are capable of binding anti-Gal, is of particular interest. All these clinical significance advocates the necessity of isolation quantitation and characterisation of this antibody and its glycoconjugates in human tissues. Moreover, anti-Gal holds a high potential as an anomer-specific probe in glycoconjugate structure studies since it exclusively recognises terminal α-linked galactose groups with the known exception of the blood group B oligosaccharide.

OBJECTIVES OF THE STUDY

Carbohydrate recognition is a phylogenetically ancient binding principle for proteins, observed throughout the biological world. Lectins, antibodies and enzymes are the carbohydrate binding proteins widely distributed in almost all living beings. Eventhough these categories of biomolecules differ in their mode of action they recognise specific sugar sequences present on glycoconjugates. Among these carbohydrate binding proteins, investigations on the interaction of lectins and carbohydrate binding antibodies with their endogenous ligands are few.

Eventhough soluble lectins are found in a wide variety of organisms and tissues and their levels appear to be modulated with development, their physiological functions are still not well understood. The findings of a conservation of an antigenic structure through evolution together with the common features displayed by the various galactoside binding lectins can possibly be viewed as manifestations of the important physiological functions of these proteins. Observations indicate that the galactoside binding vertebrate lectins must have multiple functions in amino and carboxy terminal domains. To reveal the functions of these lectins, the interaction with their specific ligands has to be analysed in detail. The objectives of one

part of our study is the identification, isolation and characterisation of the endogenous ligands of bovine and human brain grey matter galactoside binding protein. Our investigations include the following.

I. Demonstration of the sugar-specific interaction between 14 KDa galactose-binding lectin and glycoproteins from bovine brain grey matter using the immobilised glycoproteins as an affinity chromatography matrix.

II. Horse radish peroxidase labeling of BBL through oxidation labile sulfhydryl groups, using a heterobifunctional reagent, N-succinimidyl-3(2-pyridyl dithio) propionate, for identification of bovine brain lectin-interacting glycoproteins of bovine and human brain grey matter.

III. Immobilisation of carboxyamidomethylated bovine brain lectin and isolation of endogenous glycoprotein ligands that sugar specifically bind to the immobilised lectin.

IV. Identification of BBL interacting subunits of bovine and human brain grey matter glycoproteins on western blot.

V. Immunohistochemical localisation of brain lectin binding glycoproteins of normal human brain.

VI. Isolation of individual subunits of brain lectin-binding glycoproteins by electroelution and analysis by reelectrophoresis.

VII. Partial characterisation of endogenous ligands of brain lectin using carbohydrate binding proteins of known specificity.

VIII. A critical analysis on the anomeric specificity of 14KDa galactoside binding bovine lectin.

A number of observations involving an active role of protein-carbohydrate interaction in various biological phenomena inspired investigators to think that the carbohydrate moieties of glycoconjugates might act as recognition signals in the immune system also. The immunological significance of human serum anti-α-Gal (anti-Gal) and the role of Gal α 1->3 Gal epitope in cell sociology have been appreciated on the basis of the findings such as the expression of Gal α 1->3 Gal residues on neoplastic cells, exposure of so far cryptic Gal α 1->3 Gal epitope on human senescent red blood cells and an elevated production of anti- Gal during infections and in a variety of auto immune phenomena. Objectives of the other part of our study were isolation and characterisation of anti-Gal from human plasma as well as identification of anti-Gal-binding bovine and human brain grey matter glycoproteins. For this purpose we used horse radish peroxidase labeled anti-Gal (Anti-Gal-HRP) as a tool for identification of α-galactoside bearing glycoproteins. Our investigations comprised of the following protocols.

I. Isolation of anti-Gal by affinity chromatography on cross-linked guar gum (galactomannan) and Sepharose 4B matrices.

II. Analysis of binding characteristics of anti-Gal

III. Horse radish peroxidase labeling of anti-Gal using a homobifunctional reagent, glutaraldehyde.

IV. Identification of anti-Gal binding bovine and human brain grey matter glycoproteins.

CHAPTER - II

MATERIALS AND GENERAL METHODS

MATERIALS

Soluble guar gum, *Ulex europaeus* agglutinin, *Phaseolus vulgaris* agglutinin, coffee bean α-galactosidase, horse radish peroxidase, fetuin, myosin, β-galactosidase, bovine serum albumin, ovalbumin, trypsin, Freund's complete and incomplete adjuvants, Coomassie brilliant blue G, iodoacetamide, divinyl sulphone, N-hydroxy succinimide, 6-amino caproic acid, Tween-20, 4-Chloro-1-naphthol, diaminobenzidine, dithioerythritol, sodium dodecyl sulphate, acrylamide, N,N'-methylene bisacrylamide, agarose, glutaraldehyde, p-nitrophenyl α-D-galactopyranoside, mannose, galactose, glucose, fucose, lactose, melibiose, raffinose, stachyose, thiodigalactoside, N-acetyl glucosamine, N-acetyl galactosamine, N-acetyl neuraminic acid, 1-O-methyl-α-D-galactoside 1-O-methyl-β-D-galactoside and α-methyl mannoside were obtained from Sigma Chemical Company, USA. Antibodies to human IgA, IgG, IgM and rabbit gamma globulins raised in goat were purchased from Immunodiagnostics Ltd. New Delhi. Sepharose 4B and 6B were the products of Pharmacia Fine Chemicals, Uppsala, Sweden. Nitrocellulose filters (0.45 um) were from Millipore, USA. N-Succinimidyl 3-(-2-pyridyldithio) propionate, Coomassie brilliant blue R-250 were purchased from Pierce Chemical Co. USA. Methyl-α-D-glucopyranoside was obtained from Koch-Light

Laboratories Ltd, England. N,N'-Dicyclohexyl carbodiimide was from ICN pharmaceuticals Inc., N.Y. Triton X-100, Phenyl methane sulfonyl fluoride, Benzamidine hydrochloride and potassium borohydride were obtained from Fluka, Buchs, Switzerland. Other chemicals used were of analytical grade and obtained from local sources. The seeds of *Canavalia ensiformis*, *Ricinus communis*, and *Artocarpus integrifolia* were obtained locally.

GENERAL METHODS

Tissue Collection

Bovine brain and heart tissues were collected from slaughter houses with in two hours of slaughter. Human brain was collected with in 12h post mortum from Forensic Medicine Department, Medical College Hospital, Thiruvananthapuram, avoiding cases of poisoning, neurological disorders and head injury. Meningeal membranes, blood vessels and most of the white matter were removed from brain tissue while keeping the latter at 0-5°C. Grey matter was washed in ice cold PBS 7.4 and kept at - 20°C. After removing fat deposits bovine heart muscle was washed in PBS 7.4 and kept frozen at -20°C before use. Human placenta was obtained from Medical College Hospital, Thiruvananthapuram. Placental tissue was cleaned, washed in PBS 7.4 and kept at -20°C. Human plasma was collected from blood bank, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram.

Preparation of matrices

Soluble guar galactomannan was cross-linked to form an insoluble gel by a modification of the procedure described by Appukuttan et al (245). 10 g Guar gum powder was mixed thoroughly with a finely dispersed emulsion of 2ml epicholorohydrin and 25 ml 3N NaOH until the mixture became

a solid cake. It was then left at 40°C in a water bath for 24h. and then at 70°C for 10h. 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 homogenised in a blender to obtain particles of about 300 μm size. Fine particles were discarded by repeated decantation. Glycine or hydroxylamine was coupled to CNBr activated Sepharose 4B as described by Cuatrecasas and Anfinsen (246) using 50 mg ligand per ml gel in the reaction mixture.

Isolation of anti-α-Gal

All steps were carried out at 4°C. Outdated human plasma was disinfected by incubation at 56°C for 30 minutes. It was then dialysed exhaustively against PBS containing 5mM EDTA and 35 ml was passed through a 1.9 cm x 11 cm column of cross-linked guar galactomannan (CLGG) or Sepharose 4B equilibrated in the same buffer. The column was washed with PBS-EDTA till washings were protein-free and eluted with 0.1M galactose in the same buffer in 3 ml fractions. Protein containing fractions were pooled, concentrated by ultrafiltration through an Amicon PM - 10 membrane and dialysed against PBS to remove galactose. In one batch, each eluted fraction was separately dialysed as above to

determine agglutination titre against trypsinised rabbit erythrocytes and protein concentration. Protein was assayed by the method of Bradford (247) using hen egg ovalbumin as standard.

Effect of pH and ionic strength on anti-α-Gal binding to matrices

To determine variations in anti-α-Gal binding capacity of CLGG with pH, the above chromatography was repeated using 20 ml B+ve human plasma, 5 ml columns of gel and instead of PBS-EDTA, any of the following buffers at 20mM strength, each containing 150 mM NaCl and 2 mM EDTA for different pH values. Sodium citrate-phosphate (pH 5.5), sodium acetate-acetic acid (pH 6.0), sodium phosphate (pH 6.5, 7.0 and 7.5), Tris-HCl (pH 8.0,8.5 and 9.0). Binding capacity of CLGG and Sepharose 4B at different ionic strengths was obtained by chromatography of 20 ml plasma on 5 ml of the respective gel using instead of PBS-EDTA, 20 mM sodium phosphate buffer (pH 7.4) containing 2 mM EDTA and the desired NaCl concentration for dialysis of the plasma before chromatography, for equilibration of the gels and for washing the column after plasma application. Bound protein was eluted with 0.1 M galactose in PBS and was determined after dialysis against PBS to remove sugar.

Preparation of Lactosyl-Sepharose 6B matrix

Lactose was covalently attached to Sepharose using divinyl sulfone as cross-linker. Lactose-divinyl sulfone Sepharose 6B was prepared as described by Dean, PDG et al (248). 20 g (moist weight) Sepharose 6B was washed in distilled water under suction and suspended in 20 ml of 1M Na_2CO_3 pH 11.0. After adding 4 ml divinyl sulfone the suspension was stirred with a magnetic rod for 1h at room temperature. Activated gel thus obtained was washed thoroughly in distilled water and its wet cake obtained by suction filtration over a sintered glass funnel was added to 30ml saturated solution of lactose in 1M Na_2CO_3 and stirred overnight at room temperature as above. The reacted beads were washed successively with

1. 20 ml of 1M Na_2CO_3 pH 11.0
11. 500 ml 0.2 M glycine-HCl pH 3.0 containing 1M NaCl to block unreacted activated groups in the gel.
111. 500 ml of 1 M NaCl and
iv. 500 ml of distilled water.

This lactosyl-Sepharose 6B matrix was equilibrated in PBS 7.4 and packed into a 2 cm x 7 cm chromatographic column.

Preparation of Asialofetuin-Sepharose 6B matrix

Fetuin (10 mg/ml) was dissolved in 0.1N H_2SO_4 and kept at 80^oC in a water bath for 1h. After desialylation the solution was cooled dialysed thrice against PBS at 4^oC. Asialofetuin thus prepared was immobilised to Sepharose 6B which had been activated freshly using CNBr as follows (249). Forty gram Sepharose 6B (wet weight) was washed under suction in distilled water and suspended in 80 ml of 2M Na_2CO_3. Forty ml distilled water was added to the suspension which was kept with constant stirring in ice bath to maintain temperature between $6-8^oC$. Cyanogen bromide (1.6g) dissolved in minimum volume of dimethyl formamide (3 ml) was added to the above gel suspension. This mixture was allowed to stir for 5 minutes at $6-8^oC$. After this activation step, the gel was washed with 80 ml of 0.1 M ice cold $NaHCO_3$ and filtered under suction to a wet cake. This activated gel was added to 320 mg of asialofetuin in 40 ml of 0.1 M $NaHCO_3$ kept at 4^oC and the mixture stirred for 18h. Then 0.5 ml of ethanolamine was added to the coupling mixture and stirring was continued for 2h in order to block the excess CNBr-activated groups. The gel was washed with 1000 ml 0.1M $NaHCO_3$ pH 8.5, 150 ml of distilled H_2O, 1000 ml of acetate buffer pH 5.0 containing 1M NaCl, 150 ml of distilled H_2O and finally equilibrated in PBS 7.4.

Isolation of bovine brain lectin (BBL) or human brain lectin (HBL)

All procedures were carried out at 4°C. 30g of bovine or human brain grey matter was thawed and homogenised using a Polytron homogeniser in 150 ml PBS-2ME containing 0.1 M lactose, 0.2mM phenyl methyl sulphonyl fluoride, 2 mM benzamidine hydrochloride and 2 mM EDTA. The homogenate was brought to pH 7.4 by adding 3 M NaOH solution and stirred for 1 h. The homogenate was sedimented at 14,500g for 20 minutes. Supernatant was brought to 40% ammonium sulfate saturation by slow addition of the salt and then centrifuged at 14,500g. The precipitate was discarded and the supernatant was again subjected to 14,500g after 70% ammonium sulphate saturation. This precipitate was dissolved in minimum of PBS-2ME and dialysed against the same buffer (2 changes).

2 cm x 7 cm lactosyl-Sepharose 6B or Asialofetuin-Sepharose 6B column was equilibrated with PBS-2ME. The dialysed sample from above was subjected to 14,500 g and dissolved proteins were passed through the column. After washing out unbound proteins using PBS-2ME, BBL was eluted using 0.2 M lactose in PBS-2ME, concentrated by ultrafiltration (Amicon PM 10 membrane) and dialysed against the same buffer to remove sugar.

Protein estimation

Protein was estimated according to Bradford (247) or by the method of Lowry et al (250) or by a modification of the method of Lowry et al as described by Dulley et al (251).

Protein estimation by Bradford's dye binding method

Protein was estimated by Bradford's method using bovine serum albumin and ovalbumin as standards.

Reagent: The Coomassie brilliant blue dye 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 preparation was filtered through Whatman No.1 filter paper before use.

The protein estimation was carried out by mixing 1.5 ml of the sample with an equal volume of the reagent and reading absorbance of the mixture at 620 nm.

Protein estimation by Lowry's method

Reagents

a. 2% sodium potassium tartarate

b. 1% copper sulphate

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

d. alkaline copper reagent

1 ml of reagent 'a' and 'b' were mixed at the time of experiment and made up to 100 ml with reagent 'c'. 1 N Folin's reagent

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

Protein was also estimated by a modified Lowry's method, by including 0.5% SDS in the alkali reagent, to estimate protein in presence of the non-ionic detergent Triton X-100 (251).

Carbohydrate estimation

The total neutral sugar was estimated by phenol-sulfuric acid method of Dubois et al in a total volume of 5.5 ml with mannose or galactose as standard (252).

Reagents

a. Sulphuric acid

b. 5% phenol

The sample was made upto 0.5 ml with water and mixed with 1 ml of 5% phenol, to this was added 4ml of chilled sulphuric acid and the mixture vortexed thoroughly. After 15 minutes incubation at 25°C, absorbance was measured at 485 nm.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight range of the bovine and human brain grey matter glycoproteins was determined by SDS-PAGE on 10% slab gels according to the method of Laemmli (254).

Reagents

A. Gel Solution: Fifteen grams of acrylamide and 0.4g 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 coloured bottle.

B. Buffer I: 0.614M Tris/HCl pH 8.8. For one hundred millilitre of the buffer, 164 mg of SDS was dissolved.

C. Buffer II: 0.147 M Tris/HCl pH 6.8. For one hundred millilitre 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 persulfate (15 mg/ml) in distilled H_2O.

For the preparation of 10% separating gel, 6 ml of 'A', 11 ml of buffer I, 0.9 ml of ammonium persulfate and 10 μl of TEMED were mixed at the time of ploymerization. For spacer gel, 1 ml of 'A', 8.5 ml of buffer II, 10 μl of TEMED were mixed at the time of polymerization. Myosin (205 KDa), β-galactosidase (116 KDa) , crystalline bovine serum albumin (67 KDa) and Ovalbumin (45 KDa) were used as standard proteins. The mini slab gel (Hoefer Scientific 7.3 x 8.3 cm) was run at 10mA and the bigger slab gel (12 x 15 cm) at 40mA till the bromophenol blue dye had reached the bottom of the gel. The gels were stained with Coomassie brilliant blue R-250 and destained with methanol: acetic acid: water (1:1.5:17.5, V/V) (268). The gels were also stained with silver nitrate according to the method of Wray et al (267).

Alkaline-PAGE

Alkaline-PAGE at pH 8.3 on 7% tube gel was done as described by Davis (253).

Reagents

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

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

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

D. 20 g acrylamide and 5 g bisacrylamide dissolved in 100ml distilled H_2O.

E. 4 mg riboflavin dissolved in 100 ml distilled H_2O.

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

Separating gel

One part A was mixed with one part C. To this mixture, an equal volume of G was added and mixed together at the time of polymerisation.

Spacer gel

One part B, one part D, one part E and 5 parts distilled H_2O were mixed together at the time of polymerisation.

Reservoir buffer

0.05 M Tris/0.38M glycine, 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 destained with methanol: acetic acid: water (1:1.5:17.5, V/V) (268).

Hemagglutination and its inhibition by sugars or glycoproteins

Trypsinised rabbit or human red blood cell suspension in PBS-2ME was prepared by the method of Lis and Sharon (255). Rabbit red blood cells or human red blood cells were washed in cold PBS 7.4 by centrifugation. A suspension of these cells (5% V/V) in PBS 7.4 containing 0.1% trypsin was incubated at 37°C for 1h. After trypsinisation the cells were washed in cold PBS with or without 2ME.

Minimum haemagglutinating amount of lectin or anti-α-Gal was first determined. For this 50 μl 5% native or trypsinised red blood cell suspension (rabbit or human)

in PBS was mixed with serial two-fold dilutions of the lectin or antibody in 200 μl PBS in 9 mm x 75 mm glass tubes at 25°C. The agglutination titre was noted after 1hr.

To obtain hemagglutination inhibiting concentration of different sugars or glycoproteins serial two fold dilutions of each sugar was equilibrated with twice the minimum haemagglutinating amount of lectin or anti-α-Gal in 200 μl PBS at 4°C for 1h before treatment with TRRBC (5%, 50 μl) and scoring as above.

Production of rabbit antibodies against proteins

BBL isolated on lactosyl-Sepharose 6B matrix was used to immunise experimental rabbits. BBL (300 μg in 1ml PBS 7.4) was emulsified with 1 ml Freunds complete adjuvant and the emulsion was injected intramuscularly (0.5 ml each on four limbs). Subsequent injections were given fortnightly with the same amount of BBL emulsified in Freunds incomplete adjuvant. After three injections immunized serum was collected and total globulins were separated by ammonium sulphate precipitation at 50% saturation. Precipitate was dissolved, dialysed in PBS 7.4 and kept at -20°C. Similar methodology was followed to raise antibody against BBL binding bovine brain grey matter glycoproteins also.

The 186 KDa sub unit of BBL-binding human brain

grey matter glycoproteins was cut out from 10% SDS-PAGE, destained in methanol, crushed and injected to experimental rabbits along with Freunds complete adjuvant. Antiserum raised against this glycoprotein sub unit was collected and stored as described above.

Ouchterlony double diffusion to demonstrate antigen-antibody recognition

Two ml of 1% agarose solution in PBS 7.4 with or without 0.1 M galactose was poured on glass slides 75 mm x 25 mm in size. Antigens and antibodies were loaded in wells made on the 1% agarose gel and kept at 4°C. Precipitin line was observed after 48h.

Isolation and immobilisation of plant lectins

Isolation of Concanavalin A

Concanavalin A (Con A) was isolated from seeds of Canavalia ensiformis (Jack beans) basically as described by Surolia et al (256). Husk was removed from 850g of jack beans after soaking in distilled water. These seeds were homogenised with 1700 ml of 1 M NaCl-0.01 M Tris HCl pH 7.4. The homogenate was stirred for 24h at 4°C and filtered

through glass wool. The mixture was again stirred for 6h with 1200 ml of 1 M NaCl-0.01 M Tris HCl pH 7.4 and filtered through glass wool. The filtrate was made up to 1M acetic acid concentration by adding concentrated acetic acid and stirred for 20 min at room temperature. The mixture was then centrifuged at 14,500g for 20 min. The supernatant was dialysed against 1M NaCl-0.01 M Tris HCl pH 7.4, 1 mM Mg^{2+}, 1 mM Ca^{2+} and 1 mM Mn^{2+}. After dialysis the pH of the sample was made up to 7-7.4 with Tris and NaOH. The sample was centrifuged and the soluble part was passed through 3 cm x 20 cm Sephadex G-50 column. The column was washed with 1 M NaCl-0.01 M Tris HCl pH 7.4. The column was eluted with 0.1 M dextrose in 1 M NaCl-0.01 M Tris HCl pH 7.4, when the protein content of washing was below 75 µg/ml. Fractions having Con A activity were pooled together and dialysed successively against 1M NaCl in distilled H_2O (3changes), distilled H_2O containing Ca^{2+}, Mg^{2+}, Mn^{2+} and finally in distilled H_2O. After dialysis insoluble particles were removed by centrifugation and soluble part was freeze-dried.

Isolation of Ricinus communis agglutinin (RCA)

RCA was isolated from the seeds of Ricinus communis (Castor bean) by the method of Appukuttan et al (245). One hundred grams of castor bean (after removing the

husk) were soaked in 750 ml of PBS 7.4 for 12 h. These seeds were homogenised and stirred for 2 h at 4°C. The homogenate was centrifuged at 14,500 g for 20 min. The supernatant was brought to 65% ammonium sulfate saturation and stirred for 30 min at 4°C. The mixture was centrifuged at 14,500 g for 20 min and the precipitate was dissolved and dialysed against PBS 7.4. After centrifugation at 14,500 g, soluble proteins were passed through a 2.4 cm x 15 cm cross linked guar gum column. The column was washed with PBS 7.4 to remove unbound protein and eluted with 0.15 M lactose in PBS 7.4. Protein- containing fractions were pooled, concentrated by ultrafiltration and dialysed against PBS 7.4. Freeze drying of the protein was done after dialysing in distilled water.

Isolation of Jacalin

Jacalin (Jack fruit seed agglutinin) was isolated from the seeds of _Artocarpus_ _integrifolia_ (Jack fruit seed) by the procedure described by Sureshkumar _et_ _al_ (257). Thirty grams of Jack fruit seeds were dehusked and soaked in PBS 6.5 for 12 h. These seeds were then cut into small pieces and homogenised in 300 ml PBS 6.5 and stirred 2 h at 4°C. Homogenate was centrifuged at 14,500 g for 20 min. Supernatant was subjected to 65% ammonium sulphate

saturation and stirred for 30 min at 4° c. The mixture was again centrifuged at 14,500 g for 20 min and the precipitate was centrifuged to remove the suspended particles and the rest was loaded on a 2.4 cm x 15 cm guar gum column. The column was washed with PBS 6.5 and eluted with 0.1 M galactose in PBS 6.5. Fractions containing protein were pooled and dialysed against PBS 6.5. Before freeze drying the protein was dialysed against distilled water.

ConA, RCA and Jacalin were immobilised on Sepharose 4B (8mg protein per ml gel) by the CNBr method (249).

Preparation of plant lectin-binding animal tissue glycoproteins

To prepare soluble glycoproteins 30 g tissue of bovine or human brain grey matter, bovine heart or human placenta was homogenised in 200 ml PBS containing 0.2 mM phenyl methyl sulphonyl fluoride, 2mM benzamidine hydrochloride and 2mM EDTA, homogenate stirred for 1 h and centrifuged at 100,000 g for 1 h. From supernatant proteins were precipitated at 70% $(NH_4)_2SO_4$ saturation and sedimented by 14,500g centrifugation. The pellet was redissolved in and dialysed against PBS (2 changes). After a further centrifugation at 14,500 g, the supernatant

containing dissolved proteins was saved. This protein solution prepared from bovine brain grey matter, bovine heart or human placenta was used to isolate ConA-binding glycoproteins of these tissues by affinity chromatography on 20 ml ConA-Sepharose column (258). Protein solution was passed through the column equilibrated with PBS 7.4. After washing the column with PBS 7.4 till washings were protein free, bound proteins were eluted using 0.5M methyl α-D-glucopyranoside in PBS 7.4 in 2 ml fractions. Eluted fractions containing proteins were pooled, dialysed against PBS until free from galactose and concentrated by ultrafiltration using Amicon PM-10 membrane.

From 70% ammonium sulphate fraction of bovine or human brain proteins, glycoproteins binding to RCA were also prepared using 20 ml RCA-Sepharose 4 B for 30 g tissue by an adaptation of the procedure described by Surolia *et al* (259). RCA-Sepharose column was eluted with 0.2 M lactose in PBS 7.4. Similarly from 70% $(NH_4)_2SO_4$ fraction of bovine heart proteins, glycoproteins binding to RCA were also isolated. Jacalin binding glycoproteins from 70% $(NH_4)_2SO_4$ fraction of 30 g bovine or human brain glycoproteins were isolated by affinity chromatography on 15 ml jacalin-Sepharose column in PBS 6.5. Bound proteins were eluted from the column using 0.15 M galactose in PBS 6.5, dialysed and concentrated as above.

Immobilisation of animal glycoproteins

ConA binding bovine brain glycoproteins (BBGP), bovine heart glycoproteins (BHGP) and human placental glycoproteins (HPLGP) were immobilised on Sepharose 4B by CNBr method of activation (249), the same procedure as used for immobilizing asialofetuin. BBGP (6.13 mg), BHGP (4.56 mg) and HPLGP (5.35 mg) were immobilised per ml of Sepharose 4 B to prepare BBGP-Sepharose 4B, BHGP-Sepharose 4B and HPLGP-Sepharose 4B matrices respectively.

Preparation of bovine brain proteins recognizing immobilized ConA-binding glycoproteins

All procedures were carried out at 4°C. Each of the Sepharose-immobilised ConA-binding glycoproteins (5ml) prepared as above were packed into columns and equilibrated with PBS-2ME. From bovine brain grey matter homogenised as above for BBL preparation, 70% $(NH_4)_2SO_4$ fraction of proteins was collected, solubilised in PBS-2ME and dialysed against the same buffer. Soluble proteins corresponding to 10 g tissue were passed separately through each of the above columns. After washing out unbound proteins with PBS-2ME, bound proteins were eluted using 0.2 M lactose in the same buffer, concentrated by ultra filtration using Amicon PM-10

membrane and dialysed against PBS-2ME.

Preparation of carboxy amidomethylated BBL (CAM-BBL)

BBL was carboxy amidomethylated during elution from the lactose-Sepharose column, using iodoacetamide in the elution buffer essentially as described by Powell and Whitney (260). All procedures for isolation of BBL on lactosyl-Sepharose matrix were followed till the elution of the bound protein with lactose as described earlier. After washing with PBS-2ME (10 vol.), the column was washed with PBS 7.4 (10 vol.) without 2ME. The BBL was eluted with 0.2M lactose in PBS pH 7.4 containing 0.1M iodoacetamide. Lactose and unchanged iodoacetamide were removed by dialysis against PBS 7.4. After dialysis the protein was concentrated by ultrafiltration using PM-10 membrane.

Immobilisation of CAM-BBL

6-Aminocaproic acid was immobilised through its amino group on CNBr-activated Sepharose 4B (249) using 10mg acid per ml of gel. N-Hydroxysuccinimide ester of the immobilised caproic acid was prepared using dicyclohexyl carbodiimide as described by Cuatrecasas and Parikh (261). For this esterification, 20 ml of 6-aminocaproic acid

Sepharose 4B was washed thoroughly with anhydrous dioxane in a sintered glass funnel under suction and resuspended in 60ml anhydrous dioxane. To this suspension 0.92g of N-hydroxysuccinimide was added, while stirring, to yield a solution with a final concentration of 0.1 M N-hydroxy succinimide. N,N'-Dicyclohexyl carbodiimide (1.648 g) was added to this solution while stirring (final concentration 0.1 M) and stirring continued for 70 min at room temperature (269). The beads were washed successively with 8 volumes of dioxane, 3 volumes of methanol (to remove the precipitated dicyclohexyl urea) and finally in 3 volumes of dioxane. 10ml of this hydroxy succinimide ester-Sepharose 4B was washed with ice cold water and then with PBS. This gel was added to an equal volume of PBS containing CAM-BBL (150 μg/ml) at 4°C. This mixture was stirred for 1h and the reaction was terminated by adding glycine (1 M) and stirring for 2h at 4°C. The CAM-BBL-Sepharose obtained was washed with PBS-2ME at 4°C and packed into a 10 ml column.

Isolation of BBL-binding glycoproteins of bovine brain on CAM-BBL-Sepharose 4B

All steps were carried out at 4°C. The pellet of bovine brain grey matter proteins precipitated by 40% saturation of $(NH_4)_2SO_4$ under conditions described earlier

for preparation of BBL was resuspended in, and dialysed against PBS (2 changes). After centrifugation at 14,500 g the soluble proteins were passed through 15 ml CAM-BBL-Sepharose 4B column equilibrated in PBS. Unbound proteins were washed out in PBS. After the washings were free of protein, bound glycoproteins were eluted using 0.2 M lactose, concentrated by ultrafiltration using Amicon PM-10 membrane and dialysed against PBS to remove the sugar.

Isolation of bovine brain grey matter membrane glycoproteins on RCA-Sepharose 4B and ConA-Sepharose 4B matrices

Bovine brain tissue (30 g) was homogenised by Polytron homogeniser in 150 ml 0.1 M phosphate buffer pH 7.4 containing 0.2 mM phenyl methyl sulfonyl fluoride, 2 mM benzamidine HCl, 2 mM EDTA and 0.1 M lactose. After stirring for 1h the homogenate was subjected to sedimentation at 100,000 g for 1hr. The pellet was extracted in 0.1 M phosphate buffer pH 7.4 containing 0.2 mM PMSF, 2 mM benzamidine HCl, 2 mM EDTA and 1% Triton X-100. After stirring for 1h the extract was subjected to 100,000 g centrifugation for 1 h. Supernatant after thorough dialysis in PBS 7.4, was applied to RCA-Sepharose 4B (2 cm x 7 cm) or ConA-Sepharose 4B (2 cm x 7 cm) matrices. The matrix was washed with PBS 7.4 containing 0.2% Triton X-100. Bound

proteins from RCA-Sepharose 4B and Con A-Sepharose 4B matrices were eluted by 0.15 M lactose and 0.5 M methyl α-D-glucopyranoside respectively in PBS 7.4.

Conjugation of HRP to BBL using the heterobifunctional reagent N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP)

A heterobifunctional reagent N-Succinimidyl 3-(2-pyridyl dithio) propionate was used to conjugate HRP to BBL by a modification of the procedure of Carlsson, J. et al (262). First the aminogroups of HRP were derivatized using SPDP. To a solution of 2.6 mg HRP in 1 ml 0.1 M phosphate buffer pH 7.4, 50 ml of N-succinimidyl-3-(2-pyridyldithio) propionate (20 mM in 99.5% ethanol) was added dropwise and this solution was kept at 23°C for 45 min. Excess reagent was removed by gel filtration on Sephadex G-25 (1 cm x 15 cm) in PBS 7.4 at 4°C and 0.5 ml fractions were collected. Fractions having high concentration of protein were collected and pooled together. The HRP-2-pyridyl disulphide derivative in 1.2 ml PBS 7.4 was mixed with 1 mg BBL dissolved in 1.8 ml PBS 7.4 containing 0.2 M lactose and kept for 4h at 4°C. The resulting conjugate was then dialysed exhaustively against PBS 7.4 to remove lactose.

BBL recognition of glycoproteins on nitrocellulose using BBL-HRP conjugate prepared by the SPDP procedure

Soluble and Triton X-100 extracted bovine brain glycoproteins isolated by affinity chromatography on immobilized RCA and Con A were heated at boiling water bath for two minutes to destroy possible traces of lectin activity that could have leached out of the lectin affinity columns. Two fold serial dilutions of these glycoproteins were dotted on nitrocellulose filters. Highest concentration (3 μg) of each sample was dotted on one nitrocellulose strip which was used as sugar control. These strips were blocked in 0.25% Tween-20 overnight at 22°C. Test strips were incubated in BBL-HRP conjugate (60 μg lectin per ml) in PBS 7.4 containing 0.2% Tween-20 for 2h at 4°C. Control strips were incubated in BBL-HRP conjugate in PBS 7.4 containing 0.2% Tween-20 and 0.2 M lactose for 2h at 4°C. The strips were washed three times in PBS 7.4, 10 minutes each. For detection of conjugate binding washed blots were immersed in peroxidase substrate solution (1 ml 0.3% 4-chloronaphthol solution in anhydrous methanol plus 5 ml PBS and 3 μl 30% H_2O_2) prepared as described by Mastroianni _et al_ (263).

Conjugation of HRP to carbohydrate binding proteins by glutaraldehyde cross linking

HRP conjugates of these proteins were prepared by glutaraldehyde crosslinking more or less as described by Hyderman et al (264). BBL (0.6 mg) was mixed with 1.2 mg HRP in 1ml PBS-2ME in presence of 0.2 M lactose and to this was added 75 μl 1% glutaraldehyde. The mixture was allowed to react for 2h at 25°C and dialysed against PBS 7.4 at 4°C (2 changes). Similar method was followed for the preparation of HRP conjugates of anti-α-gal, jacalin, WGA, RCA, ConA, UEA, PHA etc.

To prepare HRP conjugate of anti-rabbit -γ- globulins raised in goat, 0.5 mg goat antibody was mixed with 1 mg HRP in 0.5 ml of 0.1 M phosphate buffer pH 6.8 and 40 μl of 1% glutaraldehyde was added. The rest of the procedure was as that for BBL-HRP conjugate preparation.

Preliminary characterisation of BBL binding glycoproteins using carbohydrate binding proteins

BBL-binding glycoproteins of bovine or human brain grey matter (2 μg) were dot blotted on 5 mm x 5 mm nitrocellulose strips. After air drying, strips were blocked by keeping them immersed overnight in PBS 7.4 containing

0.2% Tween-20 and 5% BSA at 25°C. These strips were then incubated for 2h at 4°C in 150 µl of peroxidase conjugate solution of Con A, jacalin, WGA, anti-Gal, UEA or PHA (50 µg lectin or antibody per ml) in PBS 1% BSA in the presence or absence of 0.1 M inhibitory sugar. Following sugars were used for inhibition of binding to glycoproteins. α-Methyl mannoside for Con A, galactose for jacalin, NANA and N-acetyl glucosamine for WGA, 1-0-methyl α-galactose for anti-Gal, fucose for UEA and N-acetyl galactosamine for PHA. After treatment with the conjugate, strips were washed thrice in PBS and transferred to peroxidase substrate solution. Positive reactions appeared as well defined blue dots against the white nitrocellulose background.

Detection of BBL-binding or Anti-Gal binding glycoproteins after Western blotting

RCA-binding or jacalin binding bovine or human brain grey matter glycoproteins were resolved by SDS-PAGE slab gel (1.5 mm thick, 10%) according to Laemmli (254) using a mini gel apparatus (Hoefer Scientific). One lane containing molecular weight standards and another containing glycoproteins were fixed in 50% methanol and stained with Coomassie blue. Rest of the gel containing jacalin-binding or RCA binding proteins was electrophoretically transferred

to nitrocellulose sheet according to Towbin et al (265) using transfer buffer consisting of 25 mM Tris, 192 mM glycine and 15% methanol, pH 8.3 and a constant current of 0.8 mA/cm^2 for 2h at 25°C. After blocking by successive 2h immersions in 0.2% Tween 20 and 5% BSA, sheet was incubated for 1h at 4°C in PBS-2ME containing 1% BSA and BBL-HRP (60 μg lectin per ml) or in PBS containing 1% BSA and anti-Gal-HRP (50 μg antibody per ml), a portion in the presence and another in the absence of specific sugar in each case (200 mM lactose for BBL-HRP and 100 mM 1-O-methyl α-galactoside for anti-Gal-HRP). After washing thrice in PBS or PBS-2ME, sheets were checked for bound peroxidase using 4-chloronaphthol as substrate as described above for dot blots. Molecular weight standards used in SDS-PAGE were myosin (205 KDa), β-galactosidase (116KDa), crystalline bovine serum albumin (67 KDa) and ovalbumin (45KDa).

Gelfiltration and HPLC

Gelfiltration of RCA binding bovine and human brain glycoproteins were done on Sepharose 6B column in PBS 7.4 containing 50 mM lactose at 4°C. High performance liquid chromatography (HPLC) was performed on TSK G-3000 SW Column.

Histochemical localisation of BBL binding human brain glycoproteins (HBGP)

Paraffin embedded sections of normal human brain was used for immunohistochemical localisation of BBL binding glycoproteins. Sections were dewaxed in Xylene, hydrated, treated with 3.5% H_2O_2 and 2.5% periodic acid, 0.02% potassium borohydride, 0.25% Tween 20 in PBS and finally in pre-immune serum. Test section was incubated with anti-BBGP and negative control with pre immune serum for 1h. After washing in PBS all sections were incubated with anti-rabbit γ-globulin-HRP for 30 min and washed in PBS. Sections were incubated in substrate solution (Diaminobenzidine 1mg/ml in PBS containing 0.03% H_2O_2) for 5 min and washed in water. After counter staining with hematoxylin, sections were washed, dehydrated and mounted in DPX.

Electrophoretic elution of proteins from gel slices and reelectrophoresis

BBL-binding grey matter glycoproteins of bovine and human brain were subjected to 10% SDS-PAGE as described by Laemmli (254). Electrophoretic elution of glycoprotein sub units was done by the method of Stralfors and Belfrage (266). Gel slices containing the resolved protein sub units

were cut out and thoroughly equilibrated by 4 successive treatments (15-30 min. each) with 1 ml of 25mM Tris, 75 mM glycine buffer, pH 8.8, containing 1% (W/V) SDS, 5 mM dithioerythritol and 40% (W/V) glycerol and heated for two minutes at 100°C. Equilibrated gel slices were divided into small pieces and packed over supporting gels, 9% acrylamide in 25 mM Tris, 75 mM glycine buffer, pH 8.8 containing 0.1% SDS, cast in glass tubes of 5 mm internal diameter. Above this gel pack 0.1 ml of the solution in which the protein is to be recovered, namely 25 mM Tris, 75 mM glycine buffer, pH 8.8, containing 1 mM dithioerythritol and 40% (W/V) glycerol was layered. The tube was carefully filled with 2 M NaCl. Electrophoretic elution was then performed in 50 mM Tris, 150 mM glycine buffer, pH 8.8, for 60 min at 4 mA/tube at room temperature (266). Eluted proteins were recovered from the recovery solution using a 250 µl Hamilton Syringe inserted through the 2M NaCl layer. These proteins were then precipitated by adding anhydrous methanol to 80% concentration and keeping the mixture at -20°C overnight. Precipitate was sedimented at 200 rotations per minute at room temperature and dissolved in 100 mM Tris-glycine pH 8.8. These proteins were again subjected to electrophoresis on 10% SDS-PAGE as described by Laemmli.

Demonstration of immunological cross reactivity between BBL-binding glycoproteins

Western blot of BBL-binding HBGP after 10% SDS-PAGE was prepared as described earlier. Western blot was blocked successively in 0.2% Tween 20 and in 5% BSA for 2h and incubated with antiserum raised against BBGP (30 x dilution) in PBS 7.4 containing 1% BSA and 0.2% Tween 20 for 2h. Negative control was treated with pre immune serum in PBS 7.4 containing 1% BSA and 0.2% Tween 20 for 2h. The strips were washed in PBS 7.4 and incubated with HRP conjugated anti-rabbit-IgG (40x dilution) in PBS 7.4 containing 1% BSA and 0.2% Tween 20 for 1hr. After washing in PBS 7.4 the reacting bands were observed using 4-chloronaphthol as substrate.

Two µg of each of the eluted subunits of BBL-binding HBGP 186 KDa, 158 KDa, 100 KDa and 56 KDa were dot blotted on 5 mm x 5 mm nitrocellulose strips and blocked as described earlier. These strips were incubated with antiserum raised against 186 KDa followed by Anti-rabbit-IgG-HRP conjugate as described earlier. Color was developed using 4-Chloronaphthol as substrate.

α-Galactosidase assay

α-Galactosidase activity was assayed as described by Bossman (333). The standard assay system for α-galactosidase contained 200 μ moles of citrate-phosphate buffer (pH 5.2) or sodium phosphate (pH 7.4) or Tris HCl (pH 8.0), 100 μg of bovine serum albumin, 0.5 μmoles of p-nitrophenyl α -D-galactopyranoside and suitable amount of enzyme in a total volume of 0.5 ml. The mixture was incubated at 37°C for 30 minutes. The reaction was stopped by heating the tubes at 100°C for 30 seconds. The contents after cooling were mixed with 2.5 ml of 0.4 M glycine/NaOH buffer (pH 10.5) and centrifuged for 5 minutes at 2000xg. The colour was measured at 405 nm.

CHAPTER - III

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

I. Bovine and human brain grey matter glycoproteins recognized by endogenous galactoside-binding lectin

Soluble galactoside binding lectins do not require detergents for solubilisation and present a high specificity for saccharides bearing non reducing terminal D-galactose (8).

Presence of a galactoside binding activity in rat and bovine brain extracts was demonstrated by Caron, et al (39). Under our experimental conditions one ml of lactosyl-Sepharose matrix retained 35 μg bovine brain lectin (BBL). On 10% SDS-PAGE this BBL preparation was found as a single band of molecular weight about 14 KDa (Fig. 2). BBL agglutinated native and to a larger extent, trypsinised rabbit erythrocytes. Human brain lectin (HBL) was also isolated on lactosyl Sepharose matrix. In agreement with the earlier report (40), antiserum raised against BBL in rabbits cross reacted with HBL. The β-galactoside specificity of bovine brain lectin was demonstrated by Caron, et al (39). Bovine and human brain lectins belongs to the group of soluble galactoside binding lectins. It has been demonstrated that this group of lectins recognizes oligosaccharides with the terminal non-reducing sequence

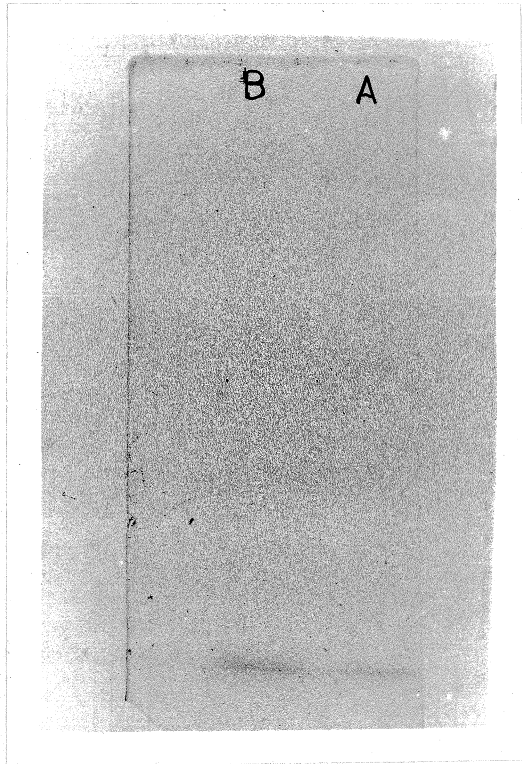


Fig.2. SDS-PAGE on 10% gel of BBL prepared using lactose-Sepharose A = 3 µg, B = 6 µg.

Gal β1-->4 GlcNAc or Gal β1-->3 GlcNAc and their analogues with α1-->2 linked fucose, α1--> 3 linked galactose or α2--> 3 linked sialic acid to the galactose (270, 271, 61).

The existence of a dimeric structure previously observed in case of BBL on gelfiltration under non-reducing conditions (39) was later proved to be due to non covalent associations (40). The aminoacid composition analysis has shown that the predominant aminoacid in BBL is glycine (16.5%). Glutamic acid and aspartic acid together comprise about 26% of total aminoacids (39). Isoelectric focusing in polyacrylamide gel and aminoacid analysis demonstrated that the HBL was an acidic protein. On isoelectric focusing, a single band corresponding to a pI of 3.9 was reported (40). No carbohydrates, either neutral or aminosugars were detected (40) as conjugated to the protein.

The requirement of almost the complete polypeptide sequence of 14 KDa lectin for optimal folding of the carbohydrate recognition domain has been suggested (13). The aminoacid sequences of soluble galactoside binding lectins from human, bovine, chick and rat have significant homologies, but there are systematic differences also. Antiserum raised against HBL was found to cross react with galactoside-binding proteins purified from brain (rat, bovine) and from other organs (amphibian ovary, human placenta) (40). Abbott and Feizi (272) opined that there is

likely to be only one gene for the soluble 14 KDa galactoside-binding lectin in each species. However, there is clearly a family of genes related to that of the 14 KDa lectin, which would include as distinct members the gene for CBP 35 (273) and Clone 1 (14 KDa human lung lectin cDNA clone from a human hepatoma Xenograft grown in mice) (274) and possibly those for other proteins of higher molecular masses that are antigenically cross-reactive with this protein.

Carbohydrate recognition is a phylogenetically ancient binding principle represented throughout the biological world (275, 4, 276). Soluble vertebrate lectins are undoubtedly involved in cellular interactions involving surface glycoconjugates. Galactoside binding soluble lectin is of interest as it is a potent inductor of the embryonic brain cell aggregation. The demonstration that saccharides, specifically inhibit cell reaggregation strongly suggests that at least one of the required components of the cell recognition mechanism is a carbohydrate-binding protein (39).

Multiple sclerosis (MS) is a spontaneously occurring human disease. An experimental model with many features resembling multiple sclerosis is experimental allergic (autoimmune) encephalomyelitis (EAE) (277). Both MS and EAE will produce transient paralysis and focal,

perivascular and sub meningeal areas of chronic inflammation and demyelination. Although a number of differences exist between EAE in various experimental mammals and MS in humans, there are numerous points at which the two conditions appear to parallel one another (277). In MS the inciting event, the antigen inducing the inflammation, the immunoregulatory controls, and the basic pathogenic insult be it infectious or truly autoimmune are all unknown at this time. With EAE, however, a great deal is understood. The antigens capable of causing the illness are myelin basic protein (MBP) or myelin proteolipid protein (PLP).

The tetrapeptide W-G-X-E is conserved among soluble galactoside binding lectins that have been partially or fully sequenced (278). The occurrence of the tetrapeptide sequence in MBP and the antigenic cross-reaction of the lectin with MBP is of considerable immunological interest. The tetrapeptide in myelin basic protein is a part of the main domain involved in the induction of experimental allergic encephalomyelitis (279) having the sequence, Trp^{116}-Gly-Ala-Glu-Gly-Gln-Lys^{122}.

The induction of experimental autoimmune encephalomyelitis in Lewis rats by immunization with guinea pig myelin basic protein (MBP) is partially prevented by the prior or simultaneous administration of recombinant human placental 14 KDa lectin. The lectin, termed recombinant

immunomodulatory lectin, rIML-1, seemed to act by blocking the sensitisation of specific T cells and inducing MBP dependent suppressor cells (63). Monoclonal antilectin antibodies specific for this tetrapeptide epitope bind to both proteins and this sequence has been reported to be part of the major domain responsible for the induction of experimental allergic encephalomyelitis. The significance of tetrapeptide epitope shared between the lectins and myelin basic protein as an autoantigen in autoimmune diseases of man demands further investigation. Myelin basic protein has been shown to bind to certain gangliosides (280,281). In addition the phosphorylation of MBP (at ser^{115}) is inhibited by added gangliosides (282,283). These observations raise the intriguing possibility that (a) there may be carbohydrate binding activity in this part of myelin basic protein, namely $FS^{115}WGAEGQK$ which includes the tetrapeptide epitope and (b) that interaction with carbohydrate may regulate phosphorylation (278).

Eventhough soluble lectins are found in a wide variety of organisms and tissues (9,18-21,8,60,284-290) and their levels appear to be modulated with development (9,46,47) their physiological functions are still poorly understood. The finding of a conservation of an antigenic structure through evolution together with the common features displayed by the various galactoside binding

lectins can possibly be viewed as manifestations of the important physiological function(s) of these proteins (56). A close examination of IgE binding protein (ϵBP) sequence showed that it contains a W-G-K-E sequence and has more than 95% identity with a galactose-binding protein (CBP 35) from mouse 3T3 fibroblasts (273), suggesting that it is probably the rat homologue of CBP 35. The finding that an IgE-binding protein is a member of S-type lectin family is intriguing as it is suggestive that lectin-carbohydrate interactions may mediate some of the effects of IgE. Comparisons of amino acid sequences of chicken, rat, bovine and human 14 KDa lectins and mouse, rat and human ϵBPs revealed conservation of blocks of aminoacids including the single tryptophan. The conservation of this residue across species and lectin types suggests that this particular aminoacid may be critical for activity in S-type lectins (291). Different mechanisms have been proposed to account for the oxidative inactivation of the soluble galactose-binding lectins of the rat and the electric eel. Inactivation of the 14 KDa electrolectin in the electric eel is due to the oxidation of the single tryptophan (W_{70}) to oxindole. Oxidation is prevented by lactose and the fluorescence data and pH dependence of lactose binding suggest the tryptophan residue interacts directly with the saccharide (13,56). Inactivation of the rat lectin on oxidation is probably due to the formation of

intrachain disulphide bonds causing dramatic changes in secondary structure (57,58). Mutagenesis studies on the Chicken-14 lectin and bovine 14 KDa lectin support the thesis that reduced cystein residues are crucial to the maintenance of the overall structure of the lectin (63,13). It is interesting to note that with the plant lectin, Ricinus communis agglutinin (another lactose binding protein), on which X-ray crystallographic data are available, two galactose binding sites have been identified per monomer. Both of these contain an aromatic residue. The domain 1-binding site contains a tryptophan where as the domain 2-binding site has a tyrosine.

Observations indicate that galactoside binding vertebrate lectins of molecular mass (in KDa) 14, 34 and 67 must have multiple functions in amino and carboxy terminal domains (70-75). This represents the corollary of the thesis that saccharide structures are important carriers of biological information both extra and intracellularly (76). The utilisation of one protein structural motif in multiple activities exemplified the opportunistic nature of cellular evolution. The cytoplasmic activities of the 14 KDa lectin species however remain an enigma. Perhaps similar to the identification of CBP-35 as Mac-2, an IgE binding protein and a laminin receptor, the identification of this abundant protein in another guise will give sudden insight into its

functions.

A number of vertebrate galactoside binding lectins have been identified and characterised during the last decade. The next challenging task in this area is the elucidation of the functions of these lectins, which demands both molecular and structural analysis of their interactions with specific ligands. To achieve this goal we have identified, isolated and partially characterised the endogenous ligands of bovine and human brain grey matter galactoside binding protein.

Con A is a broad spectrum plant lectin and is expected to bind more diverse types of glycoproteins than any other lectin (292). Con A binds strongly to high mannose and hybrid type of N-linked oligosaccharides. This lectin also binds biantennary N-linked oligosaccharides with low affinity. Con A-binding glycoproteins, therefore, have been taken as the most representative of total glycoproteins. Affinity chromatography of the total protein extract of bovine brain grey matter on Sepharose-immobilized Con A binding bovine brain grey matter glycoproteins revealed that these glycoproteins sugar specifically retained proteins from the brain extract. Electrophoretic comparison of these proteins with BBL prepared by conventional affinity chromatography on asialofetuin-Sepharose revealed that soluble Con A-binding glycoproteins from bovine brain

retained exclusively the BBL (Fig.3). Also in sugar binding activity, determined by hemagglutination assay, the glycoprotein-binding protein was equivalent to the BBL preparation. Soluble glycoproteins from bovine heart and human placenta were also retained exclusively the BBL from bovine brain homogenate (293). Table II shows similar hemagglutination capacity of BBL isolated on different glycoprotein immobilised matrices. Semiquantitative nature of the agglutination assay may explain minor variations in the minimum amount of BBL-required for agglutination. Incidentally, bovine heart glycoproteins were found to be more efficient retainers of BBL than even bovine brain glycoproteins or asialofetuin. Table II shows the BBL-binding capacity of different glycoprotein-immobilised matrices. Yield of BBL from these columns were in the range of 0.015mg - 0.047 mg per ml of affinity matrix. The above data indicate that heart glycoproteins-immobilised matrix has the potential to be used as an affinity matrix, for large scale isolation of BBL or similar other proteins, due to its high lectin-binding capacity. Soluble glycoproteins isolated from bovine brain and bovine heart tissues inhibited the BBL mediated agglutination of TRRBC. Table III shows the inhibition capacity of glycoproteins. Bovine heart glycoproteins were found to have higher capacity to inhibit BBL mediated agglutination than bovine brain glycoproteins.

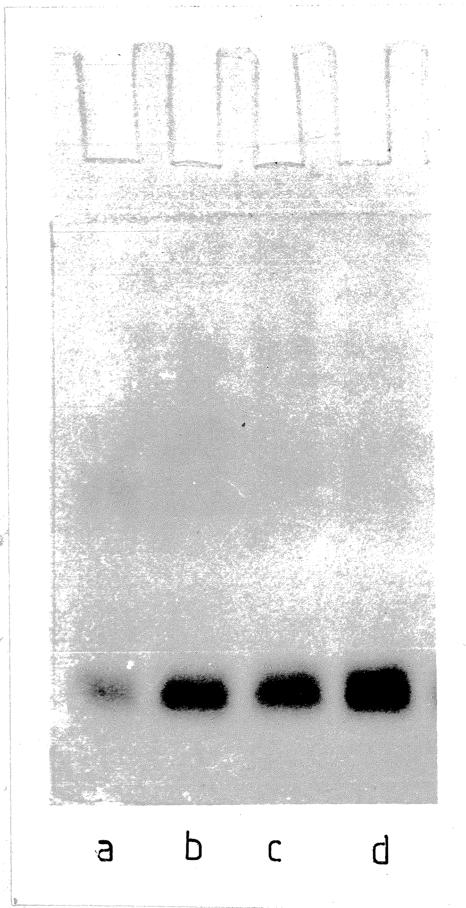


Fig.3. SDS-PAGE on 10% gel of BBL retained sugar-specifically on Sepharose-immobilised asialofetuin (10 μg, a) or Sepharose-immobilised Con A-binding glycoproteins from bovine brain (12 μg, b), bovine heart (12 μg, c) and human placenta (15 μg, d).

Table II BBL-binding capacity of various immobilized glycoproteins and agglutination capacity of BBL samples prepared using them

Glycoprotein immobilized (on Sepharose-4B)	Amount of glycoprotein immobilized (mg/ml gel)	Amount of lectin bound (mg/ml gel)	Minimum amount of BBL required for agglutination (µg)
Bovine brain glycoprotein	6.13	0.029	0.24
Bovine heart glycoprotein	4.56	0.047	0.21
Human placental glycoprotein	5.35	0.015	0.20
Asialofetuin	6.50	0.033	0.19

Table III Inhibition capacity of glycoproteins isolated on Con A-Sepharose matrix

Glycoproteins from	minimum amount (as carbohydrate) required for inhibition (μg)
Bovine brain grey matter	1.125
Bovine heart	0.75

On Ouchterlony double diffusion antiserum raised against BBL in experimental rabbits cross reacted with BBL isolated on animal glycoprotein immobilised matrices. Fig.4 shows the immunological cross reactivity of BBL samples isolated on different matrices. Electrophoretic comparison, hemagglutination assay and immunological cross reactivity confirm that the bound protein is BBL. These results indicate that glycoproteins interacting with the 14 KDa galactoside binding lectin are widely distributed in the mammalian organs and that possibly this lectin is the major protein, if not the only one, that sugar-specifically interacts with the endogenous glycoproteins in vivo (293).

Sulfhydryl groups present on 14 KDa galactoside binding lectins are very susceptible to oxidation. A decrease in hemagglutinating activity was observed upon oxidation possibly due to the formation of intramolecular disulphide bonds (57,58). We exploited these -SH groups of BBL as a site for labeling the protein with horse radish peroxidase (HRP). HRP (EC 1.11.1.7) is a hemoprotein with a molecular weight of 40,000 and is composed of a single polypeptide chain of 308 aminoacids, with four disulphide bridges and eight neutral carbohydrate side chains. Preparation of peroxidase conjugates with low-molecular weight materials or protein antigens results in low coupling yields since native peroxidase (isoenzyme C) has only two or

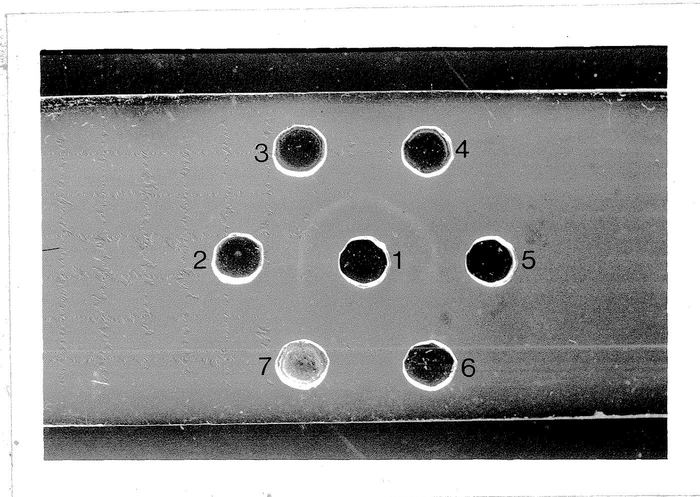


Fig.4. Comparison of the antigenicity of BBL isolated on different matrices.

1. Rabbit antiserum raised against BBL
2. BBL isolated on Lactosyl-Sepharose
3. BBL isolated on BBGP-Sepharose
4. BBL isolated on BHGP-Sepharose
5. BBL isolated on HPLGP-Sepharose
6. PBS 7.4.
7. Pre-immune serum.

three amino groups accessible on the surface of the molecule (294). N-Succinimidyl 3-(2-pyridyl dithio) propionate (SPDP), a heterobifunctional reagent, contains one N-hydroxy succinimide ester moiety and one 2-pyridyl disulphide moiety as reactive functional end groups. The hydroxy succinimide ester reacts with primary amino groups of HRP resulting in the formation of 2-pyridyl disulphide derivative of HRP (262). At pH 7.4 thiol-disulphide exchange reaction between sulfhydryl groups of BBL and 2-pyridyl disulphide derivative of HRP resulted in the formation of an active BBL-HRP conjugate.

BBL-HRP conjugate recognized sugar specifically soluble and Triton X-100 extracted glycoproteins isolated on ConA-Sepharose 4B and RCA-Sepharose 4B matrices. Fig.5 demonstrates this sugar specific binding of the BBL-HRP conjugate. Among the glycoprotein preparations tested RCA-binding soluble glycoproteins were found to contain more receptors for BBL. This BBL-HRP conjugate retained activity upto 45 days at $0^{o}C$.

This method of conjugation not only marked BBL with HRP but also prevented the formation of intramolecular disulphide bonds in BBL. Production of homoconjugates of each of the two proteins can also be avoided by this approach. In addition this conjugate does not require reducing conditions for retaining activity. Thereby we could

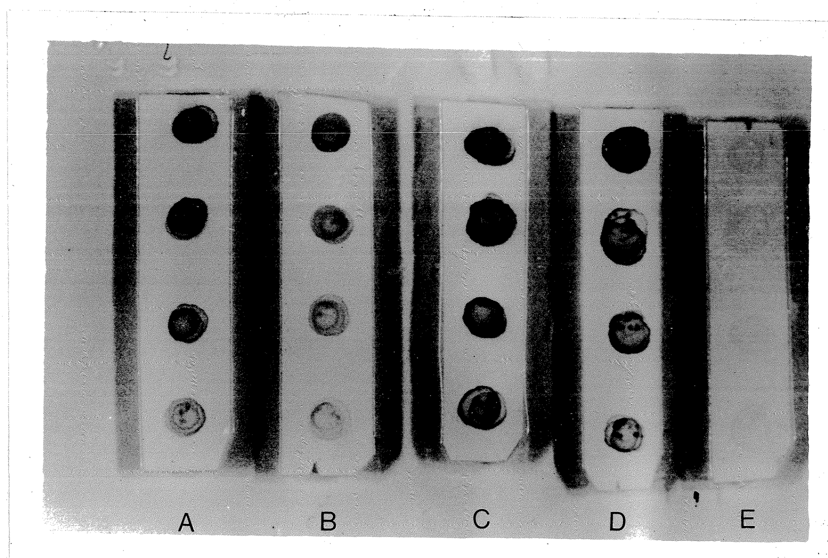


Fig.5. Dot blot assay showing sugar-specific binding of BBL-HRP (SPDP) to brain glycoproteins. Strips A, B, C and D were each dotted with four 2-fold serial dilutions of different glycoprotein samples starting with 3 μg on top dot.

A = soluble Con A-binding bovine brain glycoprotein (BBGP)

B = membrane Con A-binding BBGP

C = soluble RCA-binding BBGP

D = membrane RCA-binding BBGP

In strip E, four dots from top to bottom had the highest glycoprotein concentrations of A, B, C and D respectively, but treated with BBL-HRP (SPDP) in presence of 0.2 M lactose.

synthesise a stable BBL-HRP conjugate for direct detection of glycoconjugates. Application of BBL-HRP conjugate can be extended for detection of glycoconjugates on nitrocellulose and tissue sections. Sulfhydryl groups present on lectins and other proteins may also be considered as a potential site for incorporation of molecules which have diagnostic and therapeutic prospects.

Carboxy amido methylation of BBL, however, has been shown to enhance lectin stability by protecting the essential sulfhydryl groups from oxidation or internal disulphide formation (260). Immobilisation of CAM-BBL through N-hydroxy succinimide derivative of Sepharose 4B (293) was attempted, since this procedure involved protein attachment at pH 7.4 as against the more alkaline, and thus inactivating condition (pH 8.5) for coupling the CAM-BBL to CNBr activated Sepharose (249). From 40% $(NH_4)_2SO_4$ fraction of soluble glycoproteins of bovine brain grey matter, CAM-BBL-Sepharose could sugar-specifically retain several glycoproteins (293) which on SDS-PAGE gave rise to subunits of molecular mass (in KDa) 44, 51, 60, 123 and 186 respectively (Fig.6). The result shows that the goal of immobilising BBL in active form has been achieved by coupling the carboxy amido methylated lectin to N-hydroxy succinimide derivative at near neutral pH. The 40% $(NH_4)_2SO_4$ fraction of proteins was found to be largely free

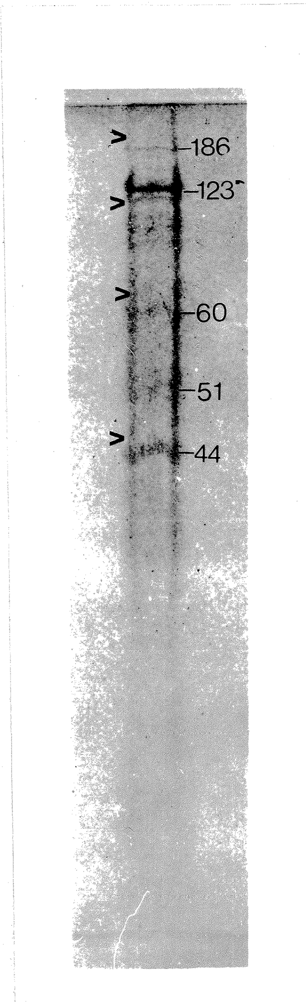


Fig.6. SDS-PAGE in 10% gel of bovine brain grey matter glycoproteins sugar-specifically retained on CAM-BBL-Sepharose column, stained with Coomassie brilliant blue. Position of molecular weight markers are indicated by arrows (myosin 205 KDa; β-galactosidase 116 KDa; bovine serum albumin 67 KDa and ovalbumin 45 KDa).

from endogenous BBL which could interfere with binding to immobilised CAM-BBL. After two cycles of the affinity chromatography the matrix was found to become inactive. Powell and Whitney have already reported a considerable decrease of binding activity after three cycles of chromatography on CAM-rat lung galaptin-Sepharose affinity matrix prepared by CNBr activation (260). For the above reason, we could not use this affinity matrix for quantitative isolation of endogenous glycoprotein ligands of BBL for further studies.

BBL-HRP conjugate prepared by glutaraldehyde cross linking was used as a probe to identify BBL-interacting endogenous glycoproteins resolved by SDS-PAGE and transferred to nitrocellulose sheets. Total brain grey matter proteins were, however too crowded in SDS-PAGE. Moreover, concentration of the BBL-interacting glycoproteins was not high enough to enable their detection by BBL-HRP after SDS-PAGE and electrotransfer in the total protein milieu. This necessitated an initial selection of glycoproteins that were likely to contain BBL-binding sites by affinity chromatography on RCA-Sepharose. Among the widely used plant lectins, RCA which binds terminal β-galactoside groups as well as the polylactosamine units is closest to BBL in sugar specificity (295). After SDS-PAGE and transfer to nitrocellulose sheets, most of the RCA-

binding glycoproteins obtained from soluble bovine brain grey matter was sugar specifically recognized by BBL-HRP (Fig.7), thus justifying the choice of RCA for enrichment of endogenous glycoproteins with respect to BBL-binding ones. The major polypeptides recognized BBL-HRP were of molecular mass (in KDa) 58,87,117 and 186. With one exception (87 KDa), these polypetide subunits corresponded in molecular mass to the larger protein bands obtained using immobilised BBL (Fig.6). Failure of immobilised BBL to bind the 87 KDa subunit could possibly due to a change in affinity consequent to immobilisation as observed for similar lectin from calf heart (296). On the other hand, subtle differences in binding specificities between BBL and RCA might have resulted in the 44 KDa and 51 KDa subunits, recognized by immobilised BBL, although undetectable in RCA-binding glycoproteins.

Glycoproteins binding to immobilised BBL were prepared from the crude protein fraction precipitated by 40% $(NH_4)_2SO_4$ in presence of lactose, since this fraction was found to exclude most of the relatively smaller BBL molecules that could have inhibited glycoprotein binding to BBL-Sepharose. That this selection did not leave out any prominent BBL-binding glycoprotein was obvious from the fact that RCA-binding glycoproteins picked up from nearly total proteins (70% $(NH_4)_2SO_4$ fraction) did not contain any BBL-

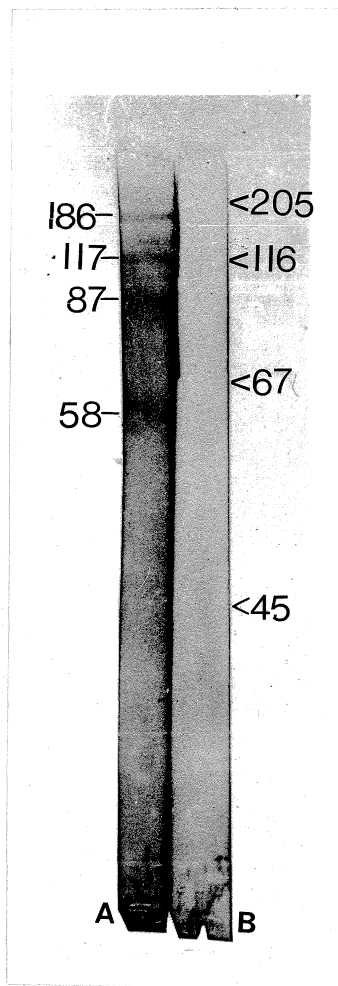


Fig.7. Identification using BBL-HRP of BBL-interacting glycoprotein subunits on Western blot of RCA-binding bovine brain glycoproteins on nitrocellulose sheets.

A = BBL-HRP in PBS-2ME- 1% BSA

B = BBL-HRP in PBS-2ME- 1% BSA containing 100 mM lactose.

Arrows indicate positions of molecular weight markers.

binding glycoprotein lower in size than those retained by BBL-Sepharose.

Similarly, on the Western blot of RCA-binding human brain grey matter glycoproteins BBL-HRP recognized sugar specifically major polypeptides of molecular mass (in KDa) 47,56,100,151 and 186 (Fig.8). Reports on endogenous receptors of animal 14 KDa S-type galactose-binding lectins are only a few. These include the exclusively membrane bound glycoproteins in rat lung (260) and laminin in calf heart (297). From western blot of soluble glycoproteins of human brain, endogenous lectin recognized glycoproteins of molecular mass 38 KDa, 67 KDa and 90 KDa sugar specifically and actin subunits non-specifically (298).

The occurrence of vertebrate lectins in the cytoplasm and on the surface of various cells suggest that these molecules have an active role in cell-cell recognition and cellular adhesion, which are important during differentiation, cell migration, immune defence, microbial infection, tumorigenesis and metastasis (18-21). Localisation of 14 KDa lectins in the intracellular compartment as cytosolic proteins (22,23) also indicates that the lectins can interact intracellularly with soluble ligands. The interaction between endogenous lectins and carbohydrate sequences on the cell surface have been observed in various types of cells (24,26). Soluble lectins

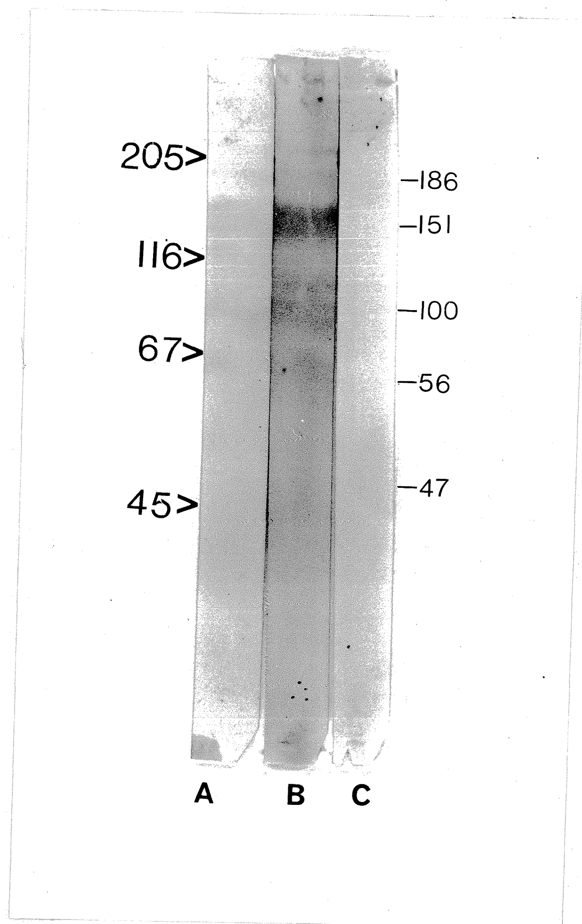


Fig.8. Identification using BBL-HRP of BBL-interacting glycoprotein subunits on Western blot of RCA-binding human brain glycoproteins on nitro-cellulose sheets.

A = stained with Amido black

B = BBL-HRP in PBS-2ME-1% BSA

C = BBL-HRP in PBS-2ME-1% BSA containing 100 mM lactose.

Arrows indicate positions of molecular weight markers.

may act as bridges by binding to carbohydrates on apposing cells. The significance of lectin sugar interaction in biological events such as holding of immature thymocytes in the thymic cortex, the migration of mature thymocytes to the thymic medulla (27,28) and the recognition between lymphocytes and the cells of the lymphoid organs (29-31) have been reported. The increased synthesis of 14 KDa galactose binding protein by tissue fibroblasts during the extensive tissue reorganisation (38) also indicates the possibility of active role of lectin-sugar interactions during late embryogenesis.

Cerebellar soluble lectin (CSL), an endogenous carbohydrate binding protein (299) having a great affinity for mannose-rich N-glycans (300), was shown to be involved in the stabilization of myelin structure (301,302) and in the maintenance of the contact between axons and myelinating cells (302,303). The CSL is synthesised and externalised by the myelinating cells and participates extracellularly in the formation of bridges between glycans of specific surface glycoproteins of the myelinating cells (301,302). Anti-CSL antibodies are found in the cerebrospinal fluid of more than 93% of the multiple sclerosis patients. This presence of anti-CSL antibodies could explain in part the demyelinating processes occurring in CNS myelin of multiple sclerosis patients (304). In CNS dysmyelinating mutants, a strong

decrease in glycoprotein ligands of CSL has been observed (305). The axonal glycoprotein ligand of CSL has been identified as a 31-KDa glycoprotein (305). Moreover, one of the two glycoprotein ligands of CSL found in cultured rat oligodendrocytes had a Mr similar to that of the myelin associated glycoprotein (MAG) (301). In the PNS, glycoproteins having identical Mr values as MAG and Po were the major ligands of CSL (302). These observations suggest that the endogenous lectin, CSL together its glycoprotein ligands, plays a role in stabilization of myelin sheath (306) and cell adhesion mechanisms in the rat sciatic nerve (302). During development the secretion of CSL was found to occur in two regions of the cerebellum, ie. the premigratory zone and white matter (9, 45). This strongly indicates potential involvement in (i) neuroblast migration and (ii) myelination.

A study of the cerebral cortex and corpus callosum during brain development (23) showed the galactoside binding lectin to be predominantly neuronal and to increase from postnatal day 1 to 10, decreasing thereafter. A role in the intracellular transport of molecules in nerves has been attributed for lectin (307). During brain development lectins present on neuroblasts and axonal membranes could be involved in transient neuroblast adhesion, contact guidance migration and fasciculation (63).

Observations have also shown that the lectin is predominantly or selectively expressed in sensory neurons in the peripheral nervous system and in motor neurons in the central nervous system, appearing soon after neuronal differentiation (48,49).

The presence of a second type of binding site, that interact sugar independently in galactoside binding lectins is an interesting observation (59). This additional affinity indicates the potential of lectin to function as a plurifunctional molecule, interacting not only with glycoproteins but also with other cytosolic proteins. Brain actin is localised in dendritic spines at concentrations exceeding those of other neuronal compartments. The sugar independent recognition of actin by HBL was demonstrated by Joubert et al (308). They postulated that at the adult stage, brain lectin could be involved in intracellular binding of cytosolic constitutents. The reversible association of HBL with both actin and glycoconjugates may be one of the complex systems which regulate actin metabolism in brain.

Polylactosaminoglycans are high molecular weight carbohydrates and are distinct from usual complex type Asn-linked saccharides by having side chains composed of Gal β 1--> 4, GlcNAc β 1 --> 3 repeats, which are susceptible to endo-β-galactosidase. It has been

experimentally demonstrated that lactosaminoglycans (LAGs) function as endogenous ligands of S-type galactoside binding lectins (62). A major carrier of lactosaminoglycans in the extracellular matrix is laminin, a component of the basement membrane which underlies epithelia and surrounds peripheral nerve and fat cells (63). Eventhough the binding of laminin to cell surface and to other matrix components involve specific areas of protein molecule, recent investigations have suggested that the LAG chains may also be functionally important, for example in cell spreading and migration (64-66). On the basis of the results from invitro interaction of galactose binding lectins with laminin, investigators opined that laminin may have multiple activities in cell adhesion, differentiation, neurite outgrowth and metastasis (68,69). Metastatic cells bearing high cell surface concentrations of galactoside binding lectins (85) may have an increased affinity for laminin. The resultant attachment to extracellular matrix or endothelial cells will enable the tumor cells to escape from the actions of killer cells which ultimately lead to an enhanced metastatic potential.

Trace amount of RCA found in RCA binding glycoproteins of bovine and human brain grey matter was removed by gel filtration of Sepharose 6B matrix. On Ouchterlony double diffusion antiserum raised against the

RCA binding bovine brain glycoproteins cross reacted with RCA binding human brain glycoproteins.

Fig.9 shows the immunological cross reactivity of BBL-binding BBGP and HBGP. This immunological cross reactivity may be considered as an indication of evolutionary relationship among the glycoprotein ligands of mammalian brain lectin similar to the proposed evolutionary relationship on account of sequence identity observed among soluble 14KDa galactoside binding vertebrate lectins (13,40). Anti-BBGP identified almost all RCA binding human brain grey matter glycoprotein sub units on Western blot after 10% SDS-PAGE. Immunohistochemical localisation of HBGP using anti-BBGP show that the lectin binding glycoproteins are present on the cell surface, nucleus and also in cytoplasm (Fig.10). Role of this group of proteins in various biological processes remains to be established. Expression of the oligosaccharide moieties of these glycoproteins in normal and pathological conditions, especially in brain tumor is an interesting field of study.

Most RCA binding endogenous glycoproteins were recognized by BBL as well. On gel filtration using Sepharose 4B,the entire proteins came out in one peak. These proteins were not found to be resolved by HPLC on TSK 3000 SW column. However BBL-binding endogenous glycoprotein subunits of molecular mass (in KDa) 186,117 and 58 obtained

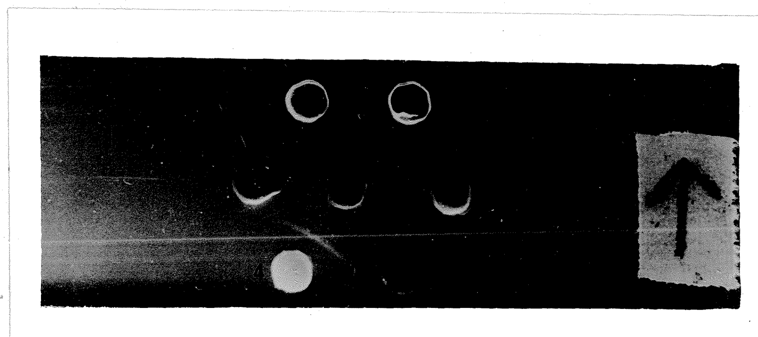


Fig.9. Demonstration of immunological cross reactivity between BBGP and HBGP.

1. Rabbit antiserum raised against BBGP
2. BBL
3. PBS 7.4
4. BBGP
5. RCA
6. HBGP
7. Pre-immune serum.

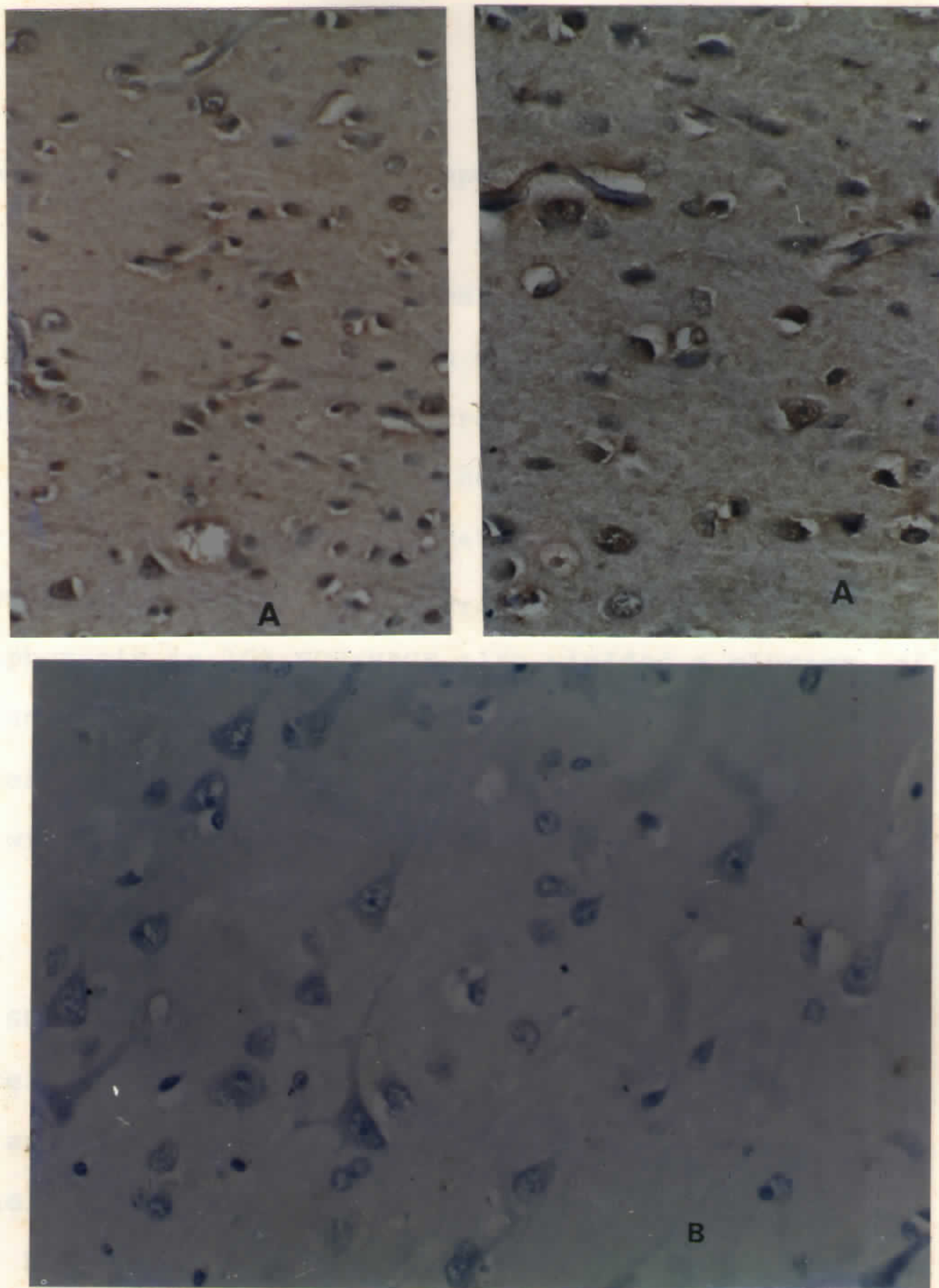
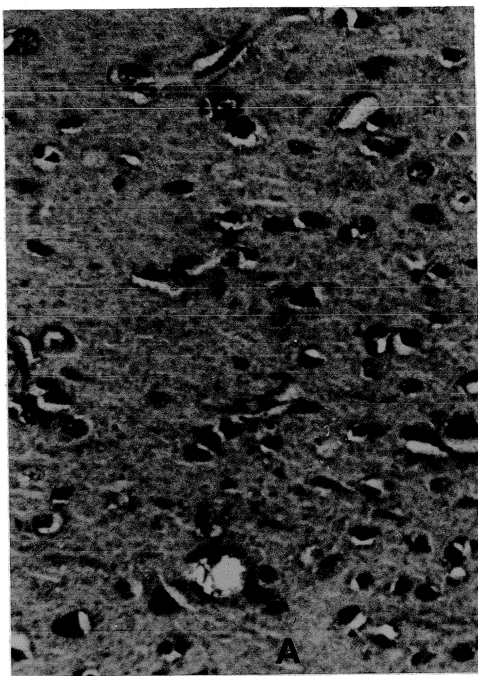
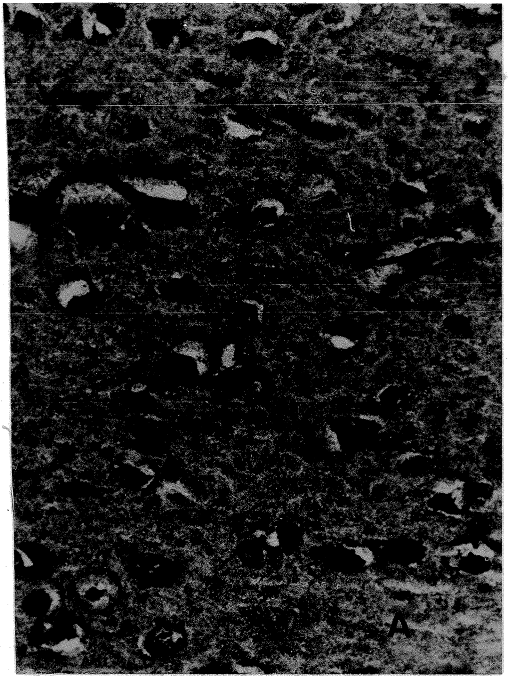


Fig.10. Immunohistochemical localisation of BBL-interacting glycoproteins on human brain tissue sections, stained with DAB and hematoxylin.

A = incubated with anti-BBGP (x250 and x400)

B = incubated with pre-immune serum (x400)





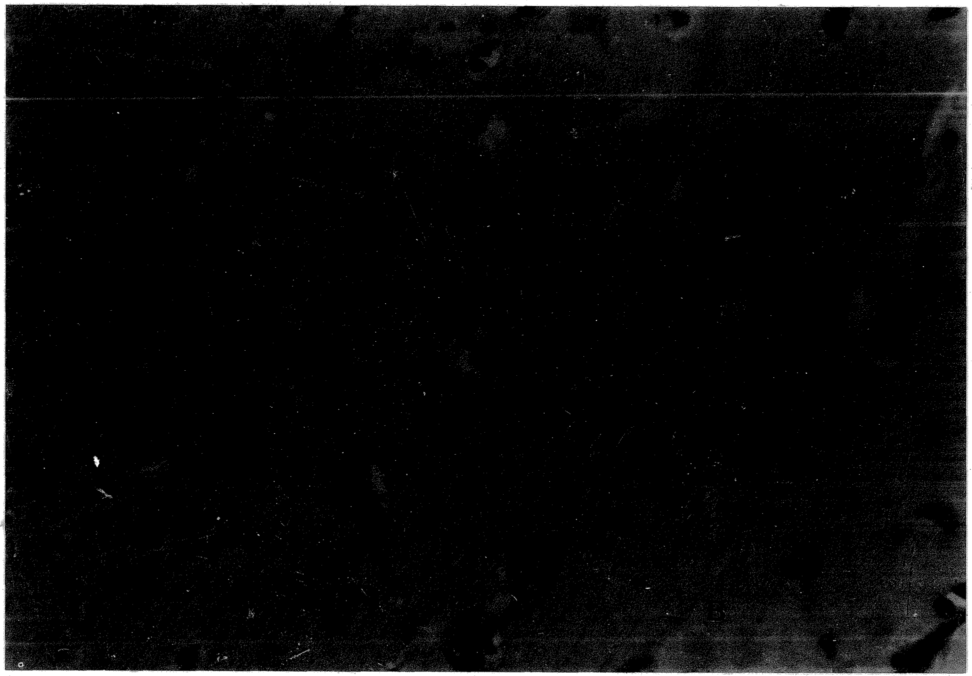


Fig.10. Immunohistochemical localisation of BBL-interacting glycoproteins on human brain tissue sections, stained with DAB and hematoxylin.

A = incubated with anti-BBGP (x250 and x400)

B = incubated with pre-immune serum (x400)

on SDS-PAGE, when electroeluted separately and subjected to SDS-PAGE again, all yielded mainly a group of closely spaced bands corresponding to molecular mass about 56 KDa (Fig.11). Similar observation was made with the 186 KDa, 151 KDa, 100 KDa and 56 KDa glycoproteins from human brain (Fig.12). In addition, 7% PAGE at pH 8.2 of RCA -binding glycoproteins from bovine or human brain grey matter also indicated 3-4 band groups (Fig.13). Each group, on electroelution and reelectrophoresis in 10% SDS-PAGE also yielded a cluster of bands around 56 KDa (Fig.13). Among BBL binding human brain glycoproteins, antiserum against the 186 KDa subunit cross reacted with the 151 KDa, 100 KDa and 56 KDa electroeluted subunits (Fig.14). These results taken together with the molecular mass values of the BBL binding sub units in initial SDS-PAGE suggested that the BBL-binding endogenous glycoproteins are high molecular weight aggregates formed of several sub units of molecular mass around 56 KDa, but incompletely dissociated in initial SDS-PAGE.

Human and bovine brain grey matter glycoproteins recognized by BBL were also recognized by ConA, jacalin, wheat germ agglutinin and though weakly in the human brain glycoproteins, by human plasma anti-Gal antibody (Table IV). Ulex europaeus and Phaseolus vulgaris lectins had no interaction. Results indicate the presence of sugar moieties mannose and/or glucose, terminal α-linked galactose and/or

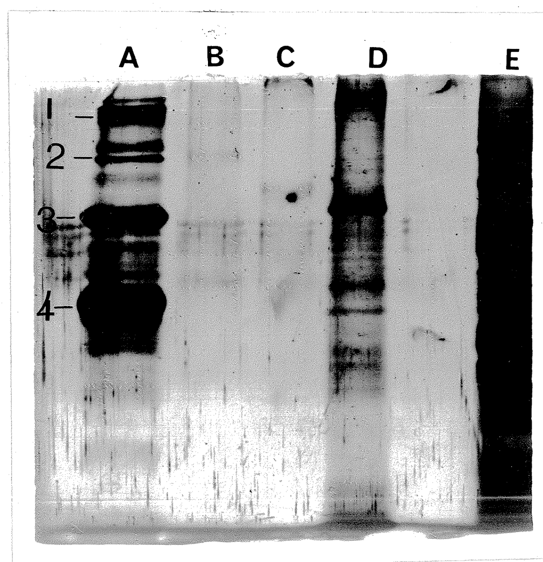


Fig.11. SDS-PAGE on 10% gel of BBL-interacting endogenous glycoprotein subunits electroeluted from 10% gel after SDS-PAGE.

Lanes

A. Molecular weight markers. 1, myosin (205 KDa); 2, β -galactosidase (116 KDa); 3, bovine serum albumin (67 KDa) and 4, Ovalbumin (45 KDa)

B. 186 KDa subunit

C. 117 KDa "

D. 58 KDa "

E. RCA binding total BBGP.

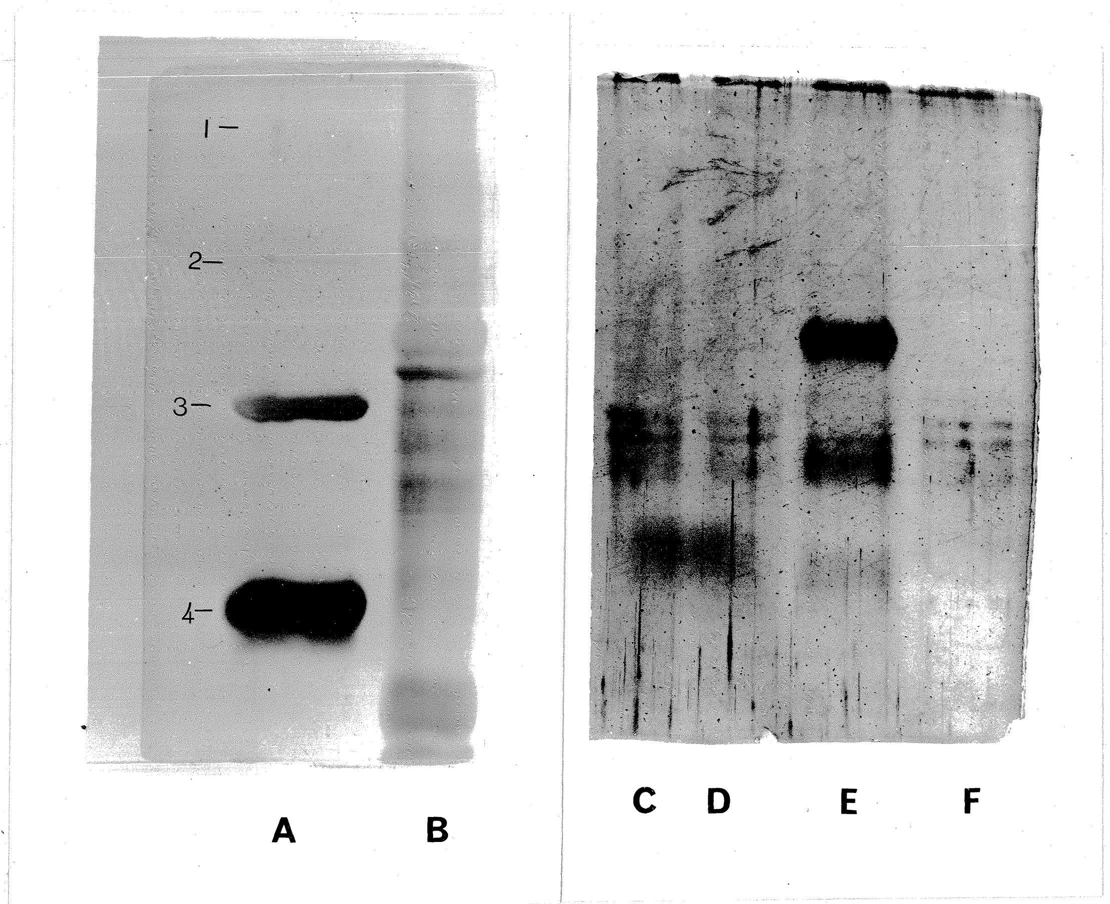


Fig.12. SDS-PAGE on 10% gel of BBL-interacting human brain glycoprotein subunits electroeluted from 10% gel after SDS-PAGE.

Lanes

A. Molecular weight markers. 1, myosin (205 KDa); 2, β -galactosidase (116 KDa); 3, bovine serum albumin (67 KDa) and 4, ovalbumin (45 KDa).

B. RCA binding total HBGP.

C. 186 KDa subunit

D. 151 KDa subunit

E. 100 KDa subunit

F. 56 KDa subunit

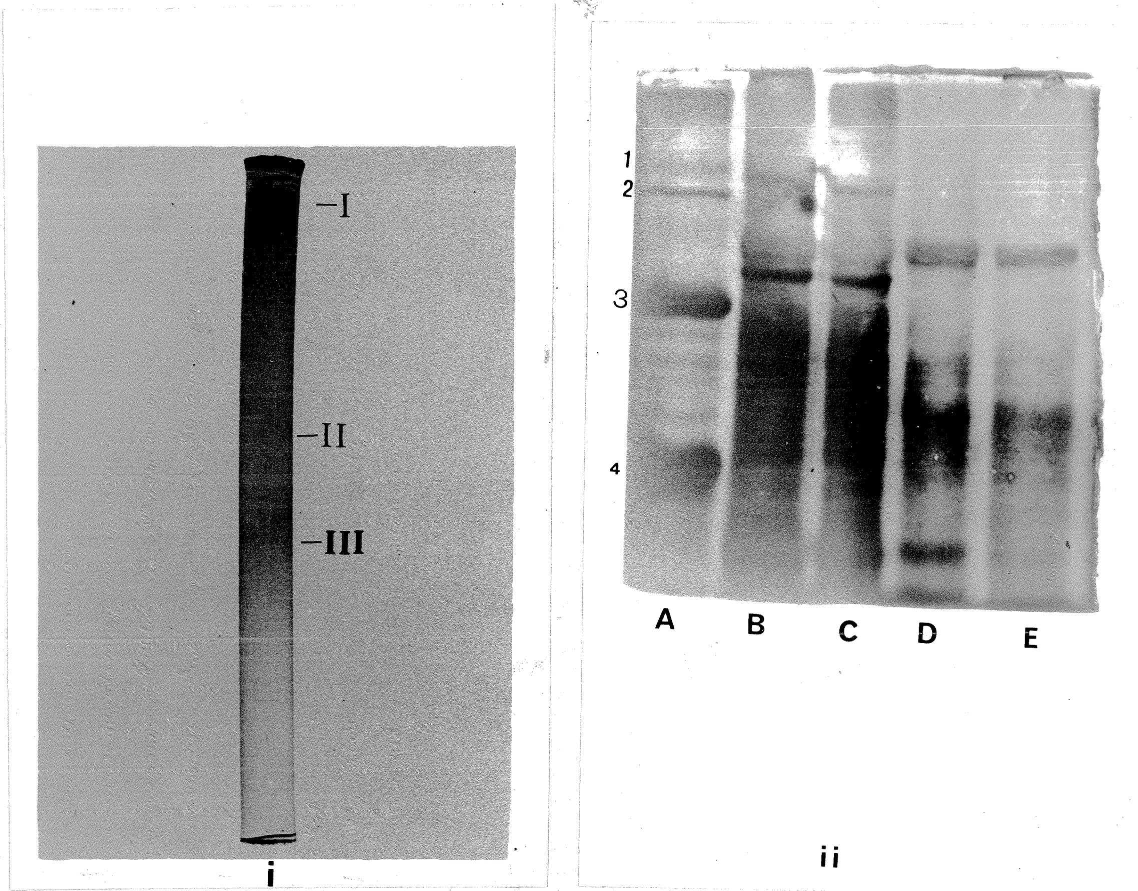


Fig.13. i. Alkaline-PAGE on 7% gel of RCA-binding human brain glycoproteins.

ii. SDS-PAGE on 10% gel of 7% alkaline-PAGE resolved bands after electroelution.

Lanes
A. Molecular weight markers. 1, myosin (205 KDa); 2, β -galactosidase (116 KDa); 3, bovine serum albumin (67 KDa) and 4, ovalbumin (45 KDa)

B. RCA-binding total HBGP

C. Electroeluted alkaline-PAGE band I

D. Electroeluted alkaline-PAGE band II

E. Electroeluted alkaline-PAGE band III

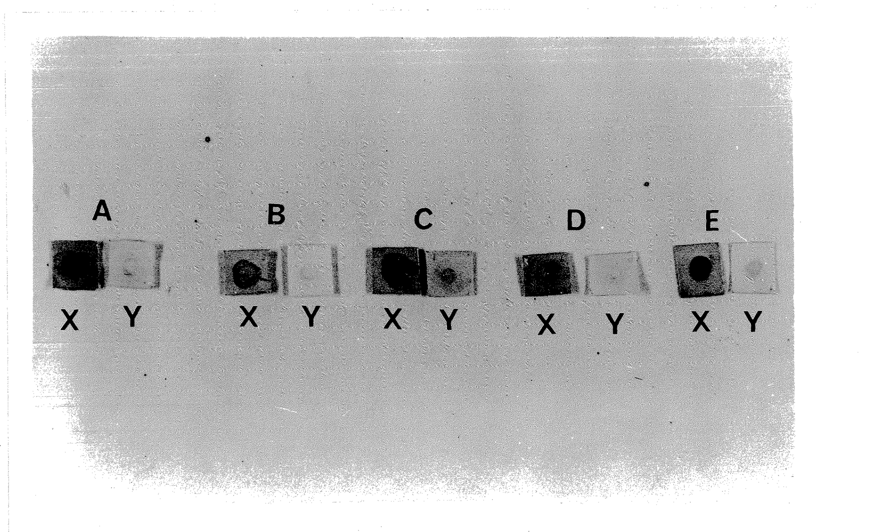


Fig.14. Cross-reaction of rabbit antibody to 186 KDa human brain BBL-binding glycoprotein with other subunits. Electroeluted subunits on dot blots were treated with antiserum to 186 KDa subunit (set X), or with pre-immune serum (set Y), followed by anti-rabbit IgG-HRP conjugate.

A. 186 KDa subunit

B. 151 KDa subunit

C. 100 KDa subunit

D. 56 KDa subunit

E. Total human brain RCA-binding glycoproteins

Table IV Characterisation of RCA-binding BBGP and HBGP using carbohydrate binding proteins of known specificity

Carbohydrate binding protein	BBGP	HBGP	Suggestion
Con A	+++	+++	Presence of mannose and/or glucose, high mannose and/or hybrid type of sugar chains
Jacalin	+++	+++	presence of α-gal and/or Galβ 1-->3 GalNAcα1 --> Ser/Thr.
Anti-α-Gal	+++	+	Presence of α-gal
WGA Test	+++	+++	Presence of N-acetyl neuraminic acid and N-acetyl glucosamine
with NANA	+	+	
with GlcNAc	+	+	
with NANA & GlcNAc	-	-	
UEA	-	-	Absence of Fucose residues
PHA	-	-	Absence of biantennary complex type sugar chains with a bisecting GlcNAc residue

+ Positive interaction - No interaction

T-antigenic structures and N-acetyl glucosamine and/or terminal sialic acid in these glycoproteins. Strong binding of ConA indicates the presence of high mannose and/or hybrid type of sugar chains. Binding of WGA shows presence of N-acetyl neuraminic acid and N-acetyl glucosamine. Strong binding to jacalin indicates the presence of α-galactose and/or Gal β 1--> 3 GalNAc α1--> (T-antigen) (309). Very weak binding of anti-Gal to human brain glycoproteins shows BBL binding grey matter glycoproteins of human brain contains very few α-linked galactose residues. Since Ulex europaeus and Phaseolus vulgaris lectins had no interaction, there may not be detectable amount of fucose residues and biantennary complex type sugar chains with a bisecting GlcNAc residue.

Thyroglobulin is a glycoprotein with a rich distribution of terminal α-galactosyl groups attached to penultimate β -galactosyl groups (221); the remaining β -galactosyl groups are substituted by terminal sialic acid groups. BBL recognized thyroglobulin sugar-specifically and this recognition was abolished by treatment of the glycoprotein with coffee bean α -galactosidase (Fig.15). Desialylated thyroglobulin gave identical results. However, asialofetuin recognition of BBL was not affected by α-galactosidase. Comparison of thyroglobulin to asialofetuin in BBL-binding capacity indicated that

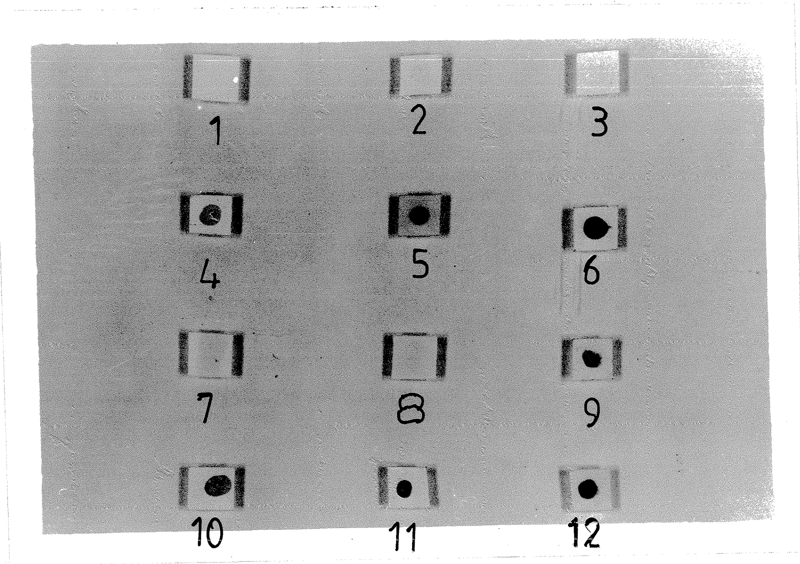


Fig.15. Effect of inhibition with sugar or pretreatment with α-galactosidase on recognition of BBL of bovine thyroglobulin (1, 4, 7, 10), desialylated bovine thyroglobulin (2, 5, 8, 11) and asialofetuin (3, 6, 9, 12). Strips 1 to 6 were dotted with 4 μg proteins, blocked and probed with BBL-HRP in the presence (1, 2, 3) or absence (4, 5, 6) of 100 mM lactose. Strips 7 to 12 were dotted with 4 μg protein after incubation for 1 h in citrate-phosphate buffer pH 5.2 in presence (7, 8, 9) or absence (10, 11, 12) of coffee bean α-galactosidase

α-galactosyl groups are equally recognized by BBL as are β-galactosides, if not better (Fig.15). The failure of α-galactosidase to reduce BBL-HRP binding to AF confirmed that the enzyme α-galactosidase does not cleave terminal β-galactosyl groups.

Laminin is one of the most likely insoluble endogenous receptors of mammalian tissue galactose-binding lectin. Besides being one of the earliest detectable extracellular molecule in the developing organs this glycoprotein has a relatively high carbohydrate content (334). In this context, it is notable that terminal sugar in laminin molecules is α-linked galactose moiety (221). A marked affinity of the 14 KDa lectin for α-linked galactose moieties underline the earlier assumptions (335) that interactions of the lectin with extracellular matrix are mediated by binding to sugar groups in laminin. Though the subsequent sugar group after the terminal α-galactosyl moiety in laminin is a polylactosamine chain, which itself is an efficient candidate for 14 KDa lectin binding (67), the moiety actually involved in lectin binding to intact laminin molecule can reasonably be assumed to be the terminal α-galactosyl group in the light of our present findings. This assumption can be verified using laminin in which the terminal α-galactoside moiety has been modified using for example, galactose oxidase for lectin binding.

Several kinetoplastida infections are accompanied by marked increases in carbohydrate specific anti-laminin antibodies in the serum that have been identified as anti-α-galactoside antibodies (anti-Gal) (232). Anti-heart antibodies in serum that are a known characteristic of certain cardiomyopathies in humans have also been identified as anti-Gal (336). Laminin is also an integral part of the extracellular matrix in the central nervous system (337). Evidences have also been obtained to show that human laminin molecules contain terminal α-linked galactose groups (223). In view of these evidences our present finding that 14 KDa lectin strongly recognizes terminal α-galactosyl groups tend to suggest that this lectin may have a role in sequestering laminin-like molecules from immune recognition and consequent tissue damage.

II Bovine and human brain grey matter glycoproteins recongized by anti-α-Gal

Guar gum is a soluble polysaccharide obtained from the endosperms of the seeds of the plant Cyamopsis tetragonolobus of Leguminosae family. This water soluble gum forms highly viscous solutions in water. Soluble guar gum consists of galactomannan of average molecular mass 210 KDa in which on an average every other mannose of the β 1 --> 4

linked mannan backbone is substituted by a single α 1 --> 6 linked galactose (310). This soluble polysaccharide forms an insoluble three dimensional network after chemical cross-linking with epichlorohydrin (1-Chloro, 2,3 epoxypropane). The high anti-α-Gal binding capacity of CLGG is due to the dense distribution of α 1 --> 6 linked galactose on the mannan back bone. Earlier, CLGG had been used for affinity chromatographic preparation of α-galactoside-binding lectin (245,257) and a plant seed α-galactosidase (311). In this modified procedure of guar gum cross-linking the number of galactose residues taking part in cross-linking are decreased by decreasing the amount of epichlorohydrin. This will provide more unsubstituted α 1 --> 6 linked galactose residues, than by the earlier method of cross-linking (245), for anti-α-Gal binding. In addition lesser degree of crosslinking would also provide a relatively higher effective pore size for the cross-linked guar gum net work of the gel. This would facilitate greater access into the matrix and thereby increased chances for interaction with the galactose moieties for molecules like IgG that are larger in size than the galactose-binding lectin that were earlier purified using cross linked guar galactomannan.

In Sepharose 4B the specific ligand for anti-α-Gal is α 1 --> 3 linked 3,6-anhydro-L-galactose which occurs occasionally in a linear copolymer with

linked mannan backbone is substituted by a single α1 --> 6 linked galactose (310). This soluble polysaccharide forms an insoluble three dimensional network after chemical cross-linking with epichlorohydrin (1-Chloro, 2,3 epoxypropane). The high anti-α-Gal binding capacity of CLGG is due to the dense distribution of α1 --> 6 linked galactose on the mannan back bone. Earlier, CLGG had been used for affinity chromatographic preparation of α-galactoside-binding lectin (245,257) and a plant seed α-galactosidase (311). In this modified procedure of guar gum cross-linking the number of galactose residues taking part in cross-linking are decreased by decreasing the amount of epichlorohydrin. This will provide more unsubstituted α1 --> 6 linked galactose residues, than by the earlier method of cross-linking (245), for anti-α-Gal binding. In addition lesser degree of crosslinking would also provide a relatively higher effective pore size for the cross-linked guar gum net work of the gel. This would facilitate greater access into the matrix and thereby increased chances for interaction with the galactose moieties for molecules like IgG that are larger in size than the galactose-binding lectin that were earlier purified using cross linked guar galactomannan.

In Sepharose 4B the specific ligand for anti-α-Gal is α1 --> 3 linked 3,6-anhydro-L-galactose which occurs occasionally in a linear copolymer with

β1 --> 4 linked galactose as the major constituent (312). Our results suggest that change to an L-configuration or absence of free hydroxyl groups on C3 and C6 positions does not affect anti-α-Gal binding of α1 --> 3 linked galactose. The relatively sparse distribution as well as main chain location of the 3,6 - anhydro-L-galactose moiety in Sepharose might account for its lower binding capacity compared to CLGG.

During affinity chromatography of anti-α-Gal, EDTA was added to exclude a possible binding of the human serum mannose/N-acetyl glucosamine-specific protein to CLGG or to Sepharose 4B. This protein was found to bind to yeast mannan and to Sepharose 4B in a Ca^{2+} dependent manner (313). With 0.1M galactose CLGG-binding proteins from human plasma eluted as a sharp peak in fractions in which protein concentration and agglutination titre were proportional (Fig.16) (314). An identical procedure using Sepharose 4B as matrix gave similar results. Protein samples eluted from CLGG and Sepharose were apparently pure antibodies belonging exclusively to the IgG class as seen from a single band obtained against anti-human IgG on immunodiffusion (Fig.17). Agglutination inhibition studies using common sugars (Table V) revealed that both samples were α-galactoside-specific. 1-O-methyl-α-galactoside, melibiose, raffinose and stachyose were efficient inhibitors whereas 1-0-methyl-β-

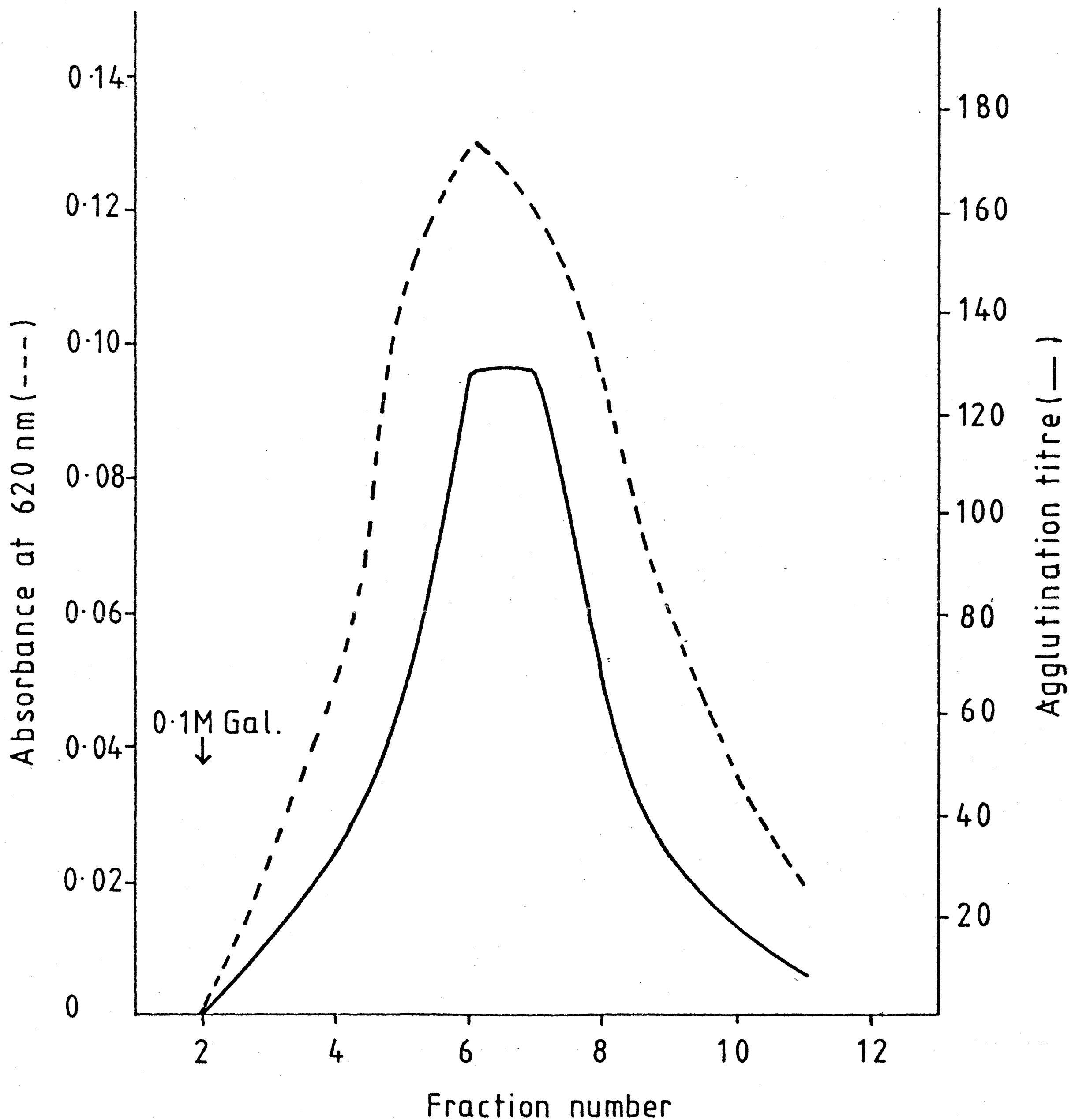


Fig.16. Elution of bound anti-α-Gal from CLGG column using 0.1 M galactose in PBS (------), protein concentration of fractions in terms of absorbance at 620 nm in the Bradford; (______), agglutination titre of each fraction after dialysis, defined as the dilution required to reach minimum haemagglutinating concentration]

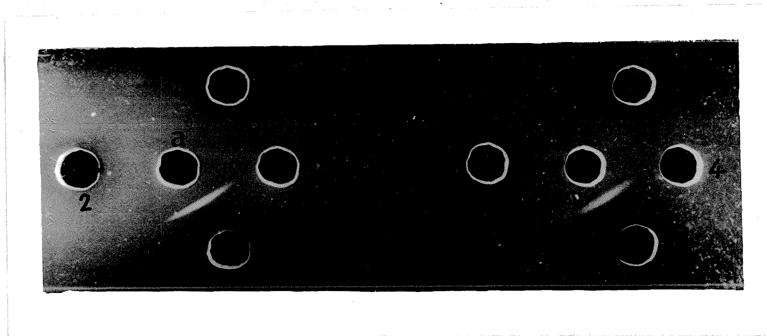


Fig.17. Identification of immunoglobulin type in anti-α-Gal samples prepared on CLGG (a) and Sepharose 4B (b) by Ouchterlony double-diffusion in 1% agarose gel in PBS containing 50 mM galactose at 4°C for 36 h [wells contained anti-α-Gal (centre, 2 μg), anti-human IgG (1), anti-human IgA(2), anti-human IgM (3) and PBS (4) Galactose was used to prevent anti-α-Gal binding to agarose]

Table V Inhibition capacity of common sugars on agglutination of trypsinized rabbit erythrocytes by anti-α-Gal prepared using CLGG or Sepharose-4B

Sugar	Minimum concentration (mM) required to inhibit twice the minimum haemagglutinating amount of antibody	
	Anti-α-Gal from CLGG	Anti-α-Gal from Sepharose 4B
Galactose	3.18	1.59
Glucose	NI(200 mM)	NI(200 mM)
Mannose	NI(200 mM)	NI(200 mM)
N-Acetyl glucosamine	NI(200 mM)	NI(200 mM)
1-O-methyl-α-D-galactoside	0.94	0.94
1-O-methyl-β-D-galactoside	25.50	6.38
Lactose	NI(100 mM)	NI(100 mM)
Meilibiose	0.25	0.25
Raffinose	0.40	0.80
Stachyose	0.40	0.20
Thiodigalactoside	10	2.50

NI, no inhibition up to the concentration given in bracket

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galactoside, lactose and thiodigalactoside were weak inhibitors. Mannose, glucose and N-acetyl glucosamine were non-inhibitors. All the above observations confirmed the identity of the present preparations with anti-α-Gal reported earlier. α-Galactosidase enzyme activity was not detected in anti-α-Gal prepared by either method in assays at pH values 5.2, 6.0, 7.4 or 8.0 using p-nitrophenyl-α-D-galactoside as substrate. Under the conditions employed, the minimum hemagglutinating concentration of either preparation of anti-α-Gal was 0.4 μg/ml.

Minor differences observed in the degree of preference for the α-anomer of galactose between anti-α-Gal samples prepared on CLGG and Sepharose 4B (Table V) may be due to the semiquantitative nature of agglutination inhibition assay. Presence of very minor quantities of variants of anti-α-Gal that possess specificity for α 1 --> 2 or α 1 --> 4 linkage of galactose in addition to that for α 1 --> 3/α 1 --> 6 linkage have been recently proposed by Weislander _et al_ (194). However, even if present in differing proportions in the two samples prepared on different matrices, such variants are not known to differ from the major anti-α-Gal species in anomeric specificity,

Our observation that Sepharose (agarose) as well as its derivatives are capable of binding anti-α-Gal has

obvious implications for chromatography of biomolecules from human plasma or plasma-contaminated tissue in so far as these matrices are the most widely employed for the purpose. Artifactual binding of anti-α-Gal to agarose might explain many anomalous results observed in the past. Contamination of the present preparations with the mannose/N-acetyl glucosamine-binding protein (313, 315) was ruled out since the former were (a) eluted with galactose from affinity columns, (b) inhibited strongly by galactose and not at all by mannose or N-acetyl glucosamine in hemagglutination and (c) non-dependent on Ca^{2+} for sugar binding.

Anti-α-Gal had earlier been purified in three steps including affinity chromatography on melibiose - Sepharose (190). Our procedure, for isolation of anti-α-Gal, is a relatively inexpensive, one step method of purification of anti-α-Gal from human plasma by affinity chromatography either on guar galactomannan insolubilised by cross-linking or on Sepharose 4B.

Variations in anti-α-Gal binding capacities of CLGG and Sepharose 4B with ionic strength of the medium were similar with a peak at 50 mM NaCl and nearly a plateau from 150-500 mM. NaCl (Fig.18). Anti-α-Gal binding to CLGG was even more sensitive to pH than to ionic strength; in 150 mM NaCl binding peaked at pH 8.0 and declined on either side of this value (Fig.19). CLGG could bind a maximum of 0.3 mg

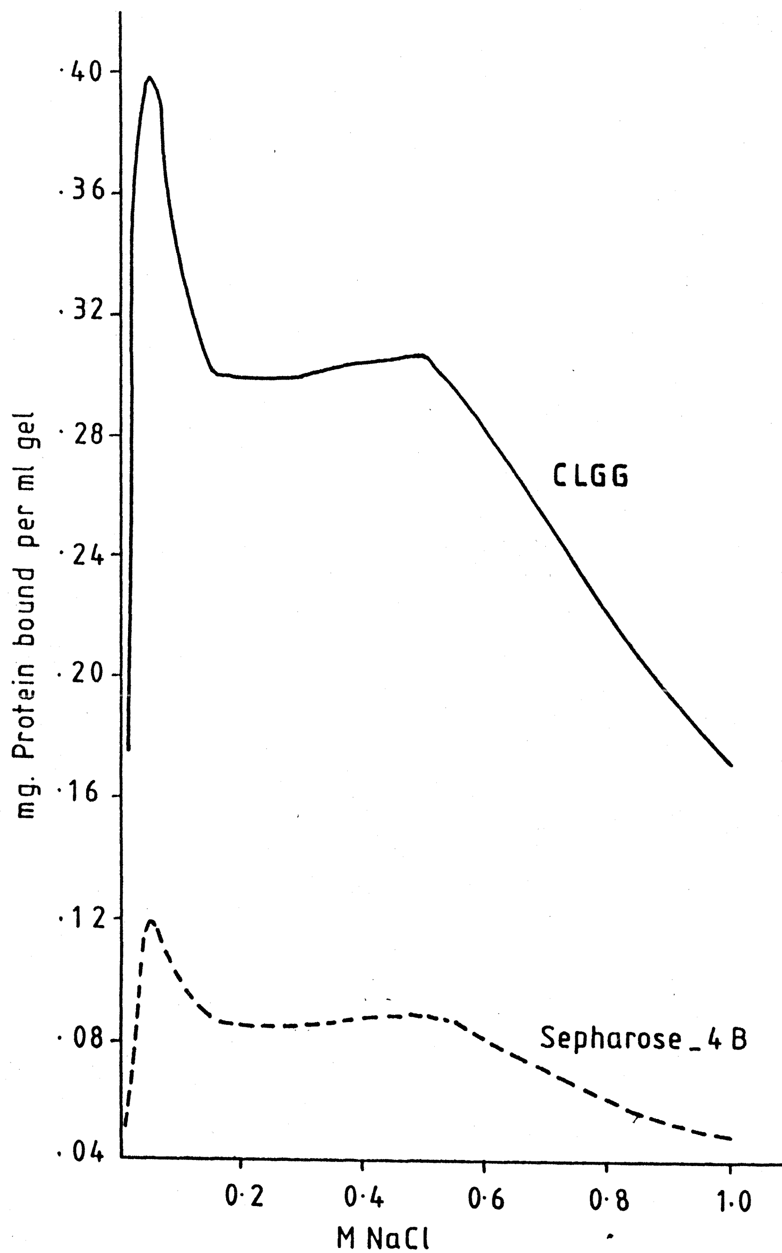

Fig.18. Effect of ionic strength of the medium on galactose-specific binding of anti-α-Gal to CLGG and Sepharose 4B at pH 7.4.

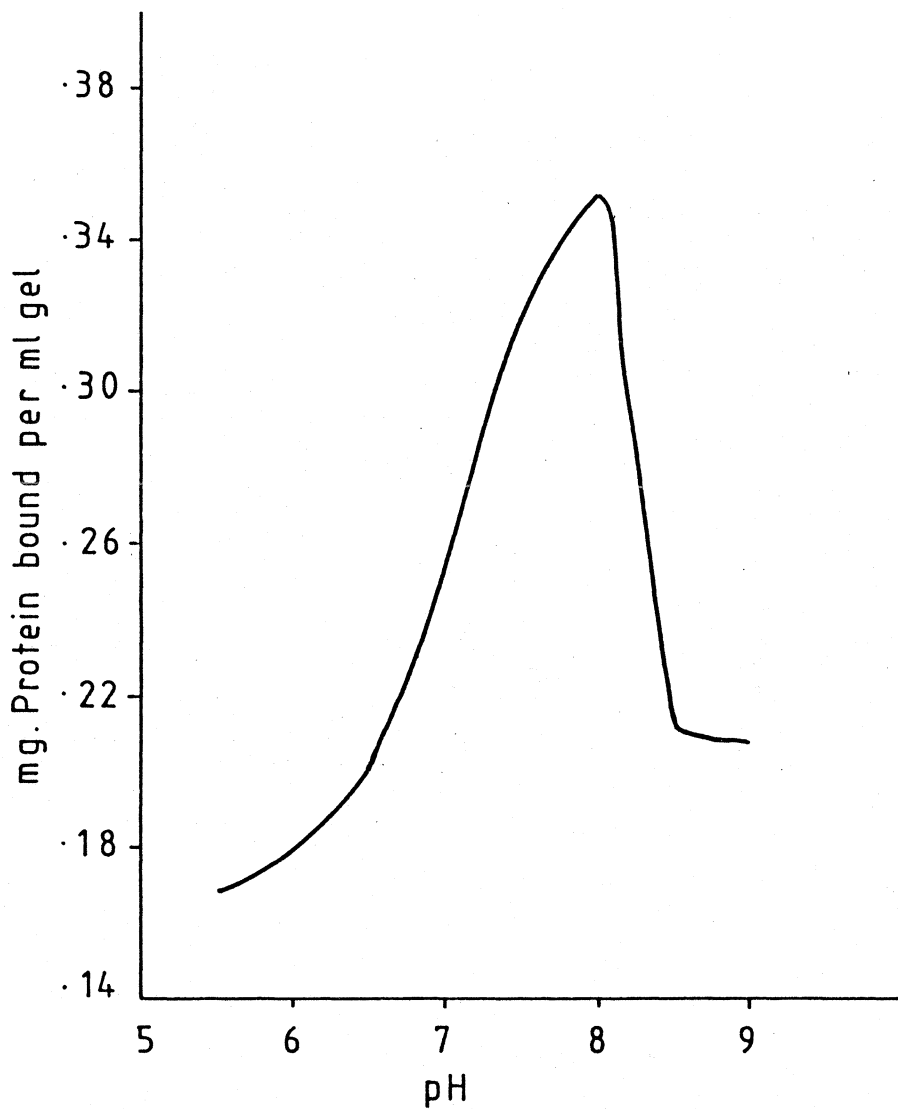

Fig.19. Effect of pH of medium on sugar-specific binding of anti-α-Gal in CLGG at 150 mM NaCl concentration.

anti-α-Gal/ml at pH 7.4 in 150 mM NaCl while Sepharose was only a third as efficient (Table VI). For routine preparation of anti-α-Gal, chromatography on CLGG or Sepharose 4B was performed at pH 7.4 in 150 mM NaCl to maintain conditions close to physiological. Under these conditions the yield of anti-α-Gal from 35 ml plasma varied from 1.5 mg to 3 mg depending on the anti-α-Gal titre of the individual donor. The binding capacity of melibiose-Sepharose matrix used earlier (315) had not been mentioned. But the present results confirm that the matrix (Sepharose) as well as ligand had contributed to it. Table VI also shows that CNBr activation followed by coupling of an inert ligand like hydroxylamine or glycine did not decrease the anti-α-Gal binding capacity of Sepharose 4B. Sugar binding of the anti-α-Gal preparations was not Ca^{2+} dependent since thorough dialysis against Ca^{2+} - free buffers and presence of EDTA did not affect their binding to affinity matrices (Figs.16, 18, 19). Moreover agglutination was also not Ca^{2+} dependent (Table V).

Based on the comparison of aminoacid sequences of immunoglobulins from the earliest vertebrates (178) with that of vertebrates evolved much later, it becomes evident that antibodies must have evolved from similar, more primitive protein receptor systems.Immunoglobulin super family, (IgSF) includes dozens of distinct membrane

Table VI Anti-α-Gal binding capacity of CLGG, Sepharose 4B and Sepharose 4B derivatives at pH 7.4 in 150 mM NaCl

Matrix	Anti-α-Gal binding capacity (mg/ml gel)
CLGG	0.30
Sepharose 4B	0.10
Sepharose 4B-glycine	0.11
Sepharose 4B-hydroxylamine	0.11

glycoproteins (181) which are believed to evolve from a common ancestral molecule with a single Ig domain (179-181). A number of observations showing the active role of protein - carbohydrate interaction in various biological phenomena (182-184) inspired investigators to think that the carbohydrate moieties of glycoconjugates might act as recognition signals in the immune system (185).

Galili et al have postulated that the expression of the Galα1 --> 3 Gal epitope was suppressed in ancestral lineages of Old World primates less than 30 million years ago before the divergence of apes and Old World monkeys (332). The advent of anti-α-Gal accompanied by the near disappearance of Galα-1 --> 3 Gal epitope in Old World monkeys, apes and humans is regarded as a milestone in evolution (192). The expression of Galα1 --> 3 Gal residues on glycoconjugates of various neoplastic cells (195-199) and exposure of this cryptic epitope in human red blood cells in the course of physiologic and pathologic aging indicate the immunological significance of anti-α-Gal and the role of the Galα1 --> 3 Gal epitope in cell sociology. Observation of an elevated production of anti-α-Gal during infections (223,228) and in a variety of autoimmune phenomena have suggested that some pathologic autoimmune processes may be related to the interaction of anti-α-Gal with Galα1 --> 3 Gal epitopes (223,229-233).

It has been shown that the glycoprotein laminin accounts for a significant proportion of the total α-D-Gal end groups on highly malignant murine EHS sarcoma cells (241).

In the brain and spinal cord, the endothelial cells (ECs) lining the blood vessels form tight junctions to make up the blood brain barrier that protects the central nervous system (CNS) from accumulation of toxic substances from the blood circulation. Unlike in other organs, CNS EC lining blocks passage of lymphocytes, immunoglobulins, albumin and other macromolecules. While lipid soluble drugs and metabolites pass the barrier easily, polar molecules except water are largely excluded. There are however, specific carriers or active transport systems across the barrier for glucose and essential metabolites, aminoacids and neurotransmitters. Normal CNS - type ECs demonstrate little, if any, vesicular transport activity, unlike ECs of skeletal muscle, for example. Adjacent ECs forming the brain capillaries are firmly fastened together by numerous belt-like zonula occludens or tight junctions. These two criteria, in addition to a basement membrane and astrocytic end-feet processes located on the abluminal EC surface, collectively describe what is currently accepted as the BBB. The normal BBB differs from skeletal muscle capillary linings functionally because the former has been classically demonstrated to exclude tracer substance, including horse radish peroxidase (316).

Jacalin is a plant lectin from jack fruit seed (<u>Artocarpus</u> <u>integrifolia</u>), which recognizes terminal α-linked galactose and the T-antigenic structure (Gal β 1 --> 3 Gal NAc α -) present in some glycoproteins as part of the O-linked oligosaccharide (318). Anti-α-galacto-side specificity of anti-Gal preparation used here was established by the present observation that in contrast to guar galactomannan, yeast mannan does not bind anti-Gal (Fig.20A). Jacalin binding glycoproteins were selected from bovine or human brain grey matter in order to get a sample enriched in α-galactoside terminals. These glycoproteins were recognized by anti-Gal through the α -galactoside-binding site of the antibody since 1-0-methyl-α-Gal completely inhibited recognition (Fig.20B). Further support to this conclusion was the absence of anti-α-Gal binding to jacalin-binding glycoproteins that had been treated with coffee bean α-galactosidase (Fig.20B).

Asialofetuin is rich in terminal β -linked galactose on its N-linked oligosaccharide chains. (319) and in T-antigenic groups on its O-linked oligosaccharides (320) but has no α-galactoside group. Binding of peroxidase conjugated bovine brain galactose-binding lectin to asialofetuin was unaffected by α-galactosidase treatment (Fig.21), testifying the anomer specificity of the enzyme. Asialofetuin was readily recognized by jacalin through the T-antigenic groups on the glycoprotein, but not at all by anti-Gal (Fig.21). These results also indicated that the recognition of human or bovine brain glycoproteins by

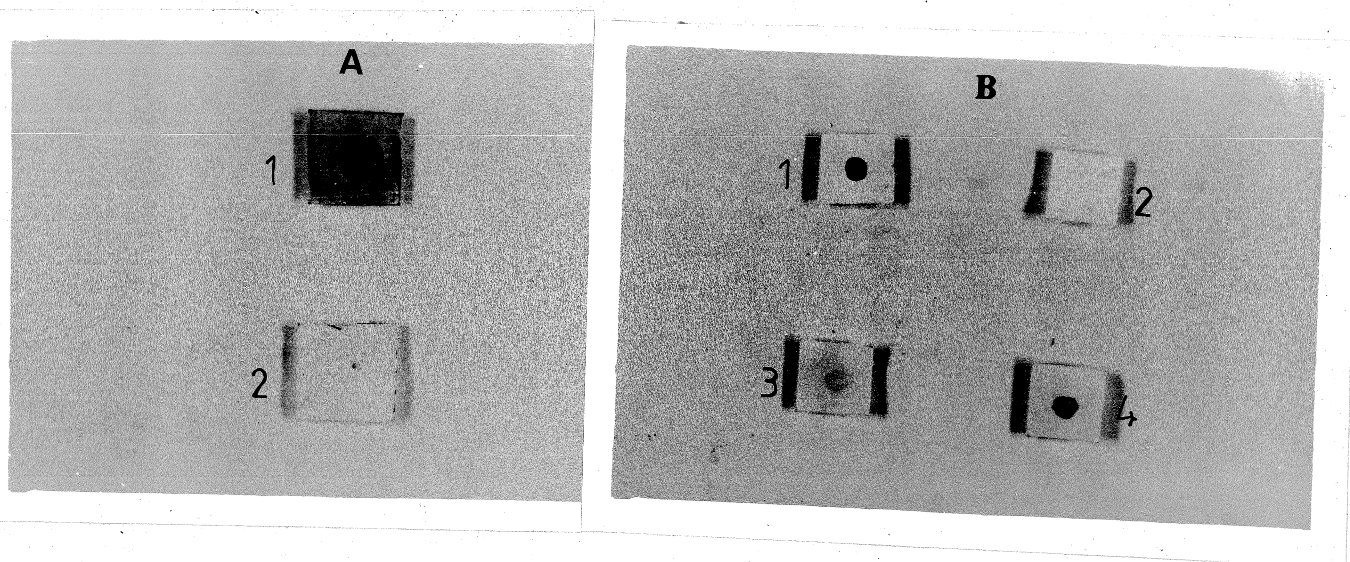

Fig.20. A. Binding of anti-Gal-horse radish peroxidase conjugate to dot blots on nitrocellulose sheets of soluble guar gum (4 μg, 1) and yeast mannan (4 μg, 2).

B. Anti-Gal-peroxidase binding to dot blots of human brain grey matter jacalin-binding glycoproteins (2 μg each). Strips 1 and 2 were treated after blocking, with anti-Gal-peroxidase in absence (1) or presence (2) of 0.1M MeαGal. Strips 3 and 4 were incubated after blocking, in pH 5.2 buffer with (3) or without (4) coffee bean α-galactosidase before conjugate treatment.

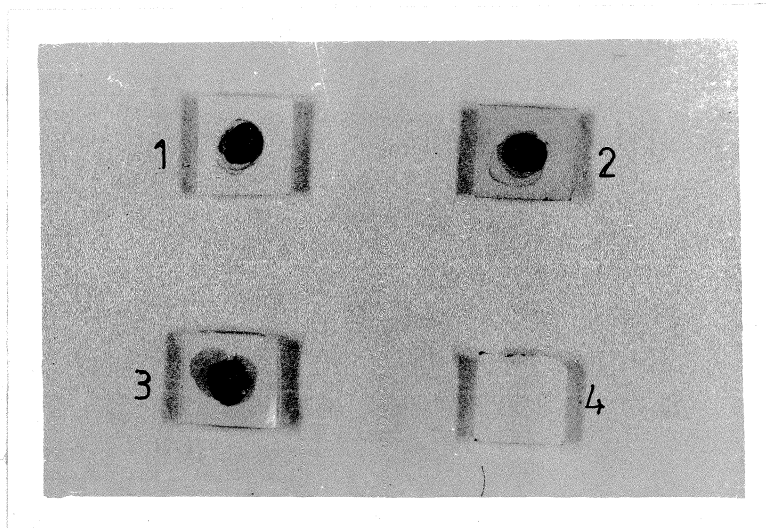

Fig.21. Binding to nitrocellulose-coated asialofetuin (4 μg) of peroxidase conjugates of bovine brain galactoside binding lectin (1,2) jacalin (3) and anti-Gal (4). Before conjugate treatment, strips 1 and 2 were incubated in pH 5.2 buffer in absence (1) or presence (2) of α-galactosidase.

anti-Gal was α-galactoside specific.

Probing of Western blot of jacalin binding bovine brain grey matter glycoproteins with anti-Gal-HRP (Fig.22) revealed that terminal α-linked galactose (TAG) of bovine brain tissue was confined largely to single polypetide of molecular mass 91 KDa (321). On the other hand, on Western blot of jacalin binding human brain grey matter glycoproteins anti-Gal-HRP conjugate recognized five polypeptides containing terminal α-galactoside moiety, having Mr (in KDa) 94, 108, 180, 210 and 230 (Fig.23). Anti-Gal binding to all these polypeptides were inhibited by 1-0-methyl-α-Gal (322).

It is obvious that any mechanism which physically destroys the components of the BBB will render the CNS open to the cellular and molecular constitutents of the blood. This occurs in traumatic or surgical injury, infarction and hemorrhage. In such circumstances the required participants for inflammation are rapidly delivered to the site of injury in gross, non-specifc fashion. In bacterial meningitis, fungal infections and potentially in brain abscess, the infectious agent itself produces material which induces incompetence if not disruption of the BBB. Here again, the initiation of inflammation most probably proceeds via mechanisms identical to those in any other bodily site, although potentially at a slower and less

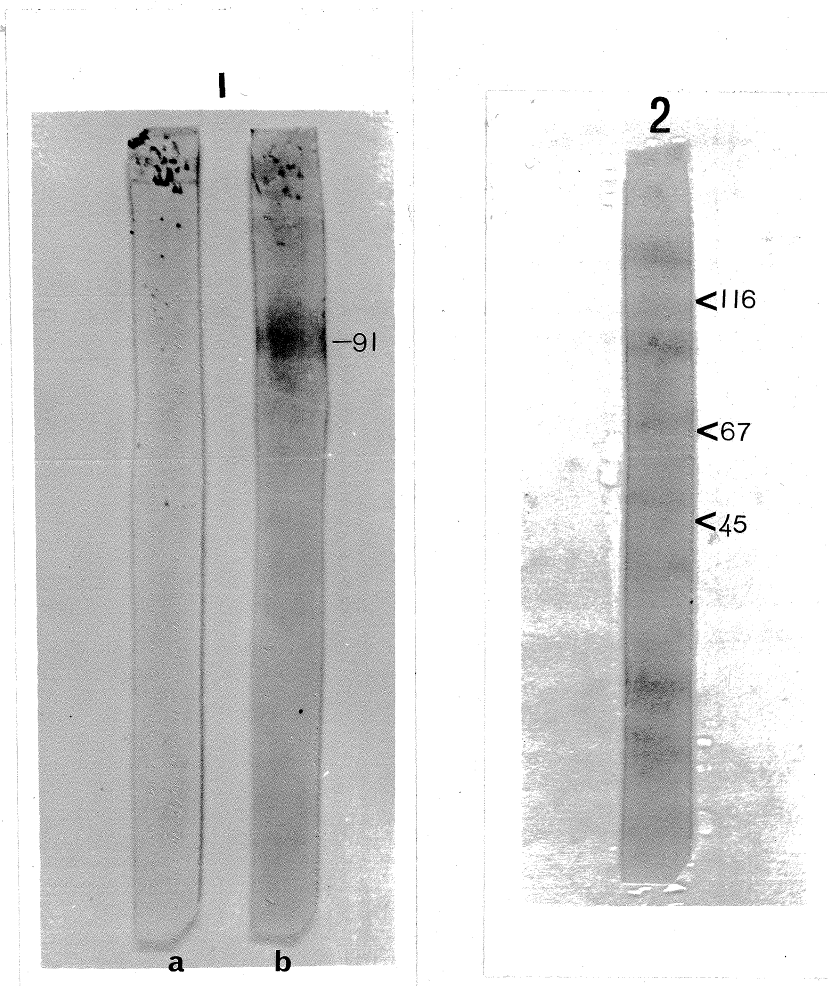

Fig.22. Staining of Western blot of jacalin binding bovine brain glycoproteins using anti-Gal-HRP in presence (1a) or absence (1b) of 0.1M MeαGal or using amido black (2). Arrows mark positions of molecular weight standards in kilo daltons

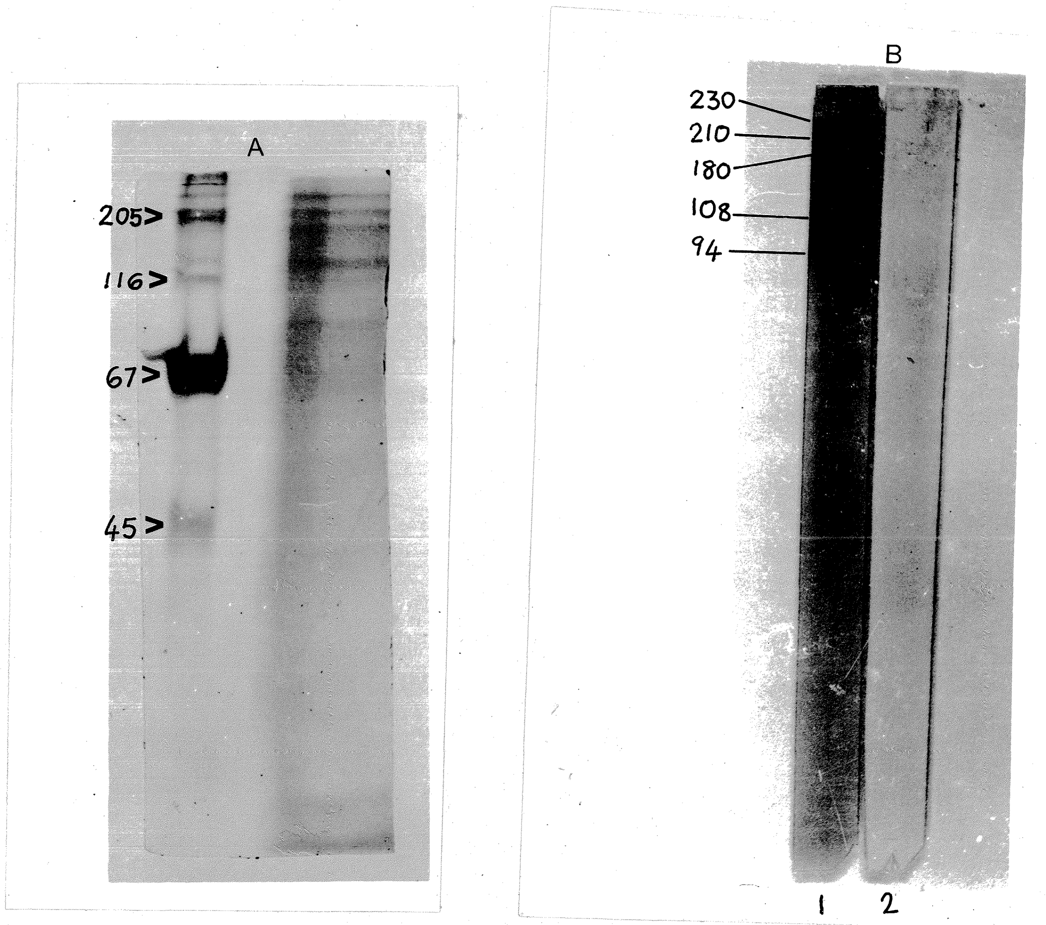

Fig.23. A. SDS-PAGE of jacalin-binding human brain grey matter glycoproteins stained with Coomassie blue. B. Anti-Gal-peroxidase interaction, in absence (1) or presence (2) of 0.1 M MeαGal, with the glycoproteins resolved by SDS-PAGE and electroblotted to nitrocellulose sheet. Arrows indicate positions of molecular weight markers.

efficient pace (277).

Terminal α-linked galactose group as part of O-linked oligosaccharide side chains of glycoproteins had been reported to be present in brain tissues of many lower animals (317). The existence of α-galactosyl epitopes along with a constant titre of anti-α-Gal in human tissues that we have observed in our experiments above was unexpected due to the possibility of immunological imbalance that it might cause (192). One explanation for the coexistence in humans of a prominent serum IgG and of brain glycoproteins with epitopes specific to this IgG, as observed here, is that these glycoproteins are in the normal course protected from exposure to the serum IgG due to sequestration of the brain cells by blood-brain barrier. However, inflammations such as due to cancer or infections could cause breaches in the barrier (323) that are likely to allow an influx of anti-α-Gal into the brain. How far this could precipitate autoimmune disorders due to recognition by anti-α-Gal of the glycoproteins demonstrated here is a clinically important question.

Enhanced synthesis of terminal α-linked galactose groups is characteristic of many tumors (241,242). It has also been suggested that anti-α-Gal functions in an anti-tumor capacity by scavenging tumor cells that bear α-galactosyl epitopes (241). Results of our investigations

indicate the possibility of anti-α-Gal bridging between brain tissue and circulating tumor cells, thus predisposing the brain to metastasis. In view of the abundance of terminal α-galactosides in several bacteria that infect man (324) and the recent demonstration, that anti-α-Gal binds to them (325), a possible role for anti-α-Gal in localizing infectious bacteria in the central nervous system is also worth investigating.

Natural selection was suggested to have favoured synthesis of anti-α-Gal in humans and monkeys presumably to contain invading foreign bodies that bore α-galactoside epitopes (332). Reports of elevation of anti-α-Gal titre consequent to infection (232) and of autoimmune reactions like Grave's disease when α-galactoside synthesis is sporadically resumed in the body (233) support this hypothesis. Ravindran _et al_ have reported that auto antibodies with precise specificity to α-linked galactose are elevated during _Plasmodium falciparum_ malaria (326). Investigations on systemic sclerosis (Scleroderma) patients revealed that about 45% of the patients were found to have anti-α-Gal values above the normal range (327). The seminal fluid normally compartmentalised from the blood, is devoid of anti-Gal reactivity. The seminal fluid of a large proportion of infertile males contained significant anti-α-Gal reactivity, implying transudation of serum IgG molecules probably due to damage in the selective

permeability of the blood genital tract barrier (328). These observations indicate that anti-α-Gal do have a significant role in infection and immunity.

Human food materials of plant and animal origin usually contain biomolecules possessing α 1->6 or α 1->3 galactoside epitopes (333,329). Our results suggest that these molecules could possess high affinity towards anti-α-Gal and thus be possible causative factors for anti-α-Gal production during evolution. Incidentally guar gum which is normally not absorbed through the gut is now increasingly consumed as a food additive due to its viscosity properties or to its hypolipidaemic effect (330). How a possible systemic absorption of such α-galactosides, through an inflammatory breach in the intestine for instance, affects anti-α-Gal titre is a clinically significant question.

Our results also show that purified anti-α-Gal could be a potent tool in glycoconjugate structural studies. So far the most widely used protein probe for TAG has been the lectin GS1 B-4 (331) from *Griffonia simplicifolia* seeds that are not universally available. In comparison, anti-α-Gal is easily prepared by an inexpensive method from a readily available source. Besides, the structural attributes of anti-α-Gal as an immunoglobulin molecule could be additional advantages in its analytical use.

SUMMARY

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Lectins, carbohydrate-recognizing antibodies and enzymes with carbohydrate substrates are three classes of carbohydrate-binding proteins widely occurring in most life forms. Eventhough these biomolecules share the ability to recognize specific sugar sequences their biological functions are entirely different. Intermediate forms of molecules such as those possessing lectin as well as enzyme activity (mungbean lectin), or lectin as well as antibody activity (IgE binding protein) have also been reported. The observation of sugar independent binding of actin by human brain lectin also indicate the plurifunctional property of this lectin (308). The utilisation of one protein structural motif in multiple activities show the opportunistic nature of cellular evolution. Though properties and physiological roles of sugar-binding enzymes and antibodies have been largely explored, such information on lectins is only beginning to emerge. The titre of sialic acid binding lectin carcinoscorpin, from horse shoe crab was found to increase, upon immunisation with its hapten 2-keto-3-deoxyoctonate. This observation indicated that lectins might function as a substitute to the immune system in invertebrates. Available evidences suggest that the biological roles of lectins are in all probabilities played through their

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recognition of complementary sugar residues that may form part of glycoconjugates. It thus becomes imperative to study the complementary endogenous glycoconjugates in order to understand the biological function of any lectin.

In this direction, our study is confined to bovine and human brain grey matter glycoprotein ligands of 14 KDa galactoside binding brain lectin and of human plasma α-galactoside binding antibody.

A number of vertebrate galactoside binding lectins have been identified and characterised during the last decade. But their physiological functions are still not well understood. Recognition is a central event in a variety of biological phenomena and the first step in numerous processes based on cell-cell interactions, such as fertilisation, embryogenesis, cell migration organ transformation immune defense and microbial infection. The diverse carbohydrate structures present on cellular glycoconjugates may function as information for correct cell recognition by carbohydrate binding proteins. The significance of lectin-sugar interaction in induction of embryonic brain cell aggregation (39), holding of immature thymocytes in the thymic cortex and migration of mature thymocytes to the thymic medulla (27,28), the recognition between lymphocytes and the cells of the lymphoid organs (29-31) have been reported.

The cytoplasmic activities of the 14 KDa galactose binding lectin species however remain an enigma. A role in the intracellular transport of molecules in nerves has been attributed for galactoside binding lectin (23,307). Observations have also shown that the lectin is predominantly or selectively expressed in sensory neurons in the peripheral nervous system and in motor neurons in the central nervous system, appearing soon after neuronal differentiation (48,49). The significance of the tetrapeptide epitope, W-G-X-E, shared between soluble galactoside binding lectins and myelin basic protein as an autoantigen in auto immune diseases of man (eg. multiple sclerosis) demands further investigation. Interestingly monoclonal anti-lectin antibodies specific for this tetrapeptide bind to both proteins and this sequence has been reported to be part of the major domain responsible for the induction of experimental allergic encephalomyelitis (63). The role of a mannose binding protein, cerebellar soluble lectin (CSL) and its glycoprotein ligands in multiple sclerosis is also documented (299-303,305,306). The finding that an IgE-binding protein is a member of S-type lectin family is intriguing as it is suggestive that lectin-carbohydrate interactions may mediate some of the effects of IgE (273,291). Several lines of evidences strongly suggest that lectins on human and murine metastatic tumor cell

surfaces may be involved in the formation of tumor emboli (77,78,79). It was also reported that murine transformed cells expressed significantly higher (5 to 50 fold) L-34 probe hybridizing mRNAs than their respective normal cell counterparts (70).

Based on the comparison of amino acid sequences of immunoglobulins from the earliest vertebrates with that of vertebrates evolved much later, it became evident that antibodies must have evolved from similar more primitive protein receptor systems (178). Immunoglobulin superfamily (IgSF) includes dozens of distinct membrane glycoproteins which are believed to evolve from a common ancestral molecule with a single Ig domain (179-181). The advent of anti-α-Gal (anti-Gal) accompanied by the near disappearance of Gal α 1 --> 3 Gal epitope in Old World monkeys, apes and humans is regarded as a milestone in evolution. Anti-Gal is the only natural IgG antibody found to be present in high titers in the normal human serum (190-193). Proposed physiological functions of anti-Gal include combating invading parasites that bear α-galactoside epitope and scavenging of senescent red cells in which this epitope so far cryptic, might become accessible (190). Anti-Gal titre was found to increase 10-100 fold normal level in Chagas disease, Leishmania, Trypanosoma and inner ear infections (223,228). These observations tend to suggest that some

pathologic autoimmune process in man may be related to the interaction of anti-Gal with autologous Galα1 --> 3 Gal epitopes. On highly malignant murine EHS sarcoma cells most of the α-D-gal end groups were found as part of the glycoprotein, laminin (241). The enhanced expression of terminal α-linked galactose in many tumors (241,242) and the ability of anti-Gal to decrease the lung colonization by murine MO_4 cells in C_3H/He syngeneic mice by interacting with α-linked galactose residues located on the surface of MO_4 cells (243), suggested that the anti-Gal functions in an anti-tumor capacity.

The above findings underline the potential of 14 KDa galactoside binding lectin and anti-α-Gal to function as carbohydrate-recognizing proteins in various biological phenomena. An understanding of the molecular basis of these interactions may help in many areas of biology and medicine. In this context the isolation, quantitation and characterisation of these carbohydrate-binding proteins and their glycoconjugates, is indispensable for a better understanding of their physiological functions.

In order to achieve these objectives we isolated and identified endogenous glycoprotein ligands of 14 KDa galactose-binding brain lectin and anti-α-Gal, from bovine and human brain grey matter. For this purpose brain lectin and anti-Gal were labeled with HRP. Partial physico-chemical

characterisation of anti-Gal was also carried out. Carbohydrate-binding proteins of known specificities were employed for preliminary characterisation of oligosaccharide part of the endogenous glycoprotein ligands of brain lectin.

Affinity chromatography of bovine brain grey matter extract on carboxy amidomethylated Sepharose 4B matrix yielded glycoproteins of sub unit molecular mass (in KDa) 44, 51, 60, 123 and 186. Labeling of BBL with HRP through oxidation labile sulfhydryl groups of the lectin resulted in a stable BBL-HRP conjugate. Binding interaction of this conjugate with soluble or membrane-bound bovine brain glycoproteins prepared by affinity chromatography on immobilized RCA or Con A columns indicated that RCA binding soluble bovine brain glycoproteins contain more receptors for BBL than Con A-binding soluble glycoproteins or membrane glycoproteins binding to either lectin. For further studies these soluble glycoproteins were used.

BBL-HRP conjugate (prepared by the method of glutaraldehyde cross-linking) sugar specifically recognized glycoproteins of subunit molecular mass (in KDa) 58,87,117 and 186 on western blot of RCA binding bovine brain grey matter glycoproteins. Similarly, BBL-HRP conjugate recognized major polypeptides of sub unit molecular mass (in KDa) 47,56,100,151 and 186 on western blot of RCA-binding human brain grey matter glycoproteins. Most of the RCA-

binding glycoproteins from bovine and human brains were interacting with BBL-HRP.

Antiserum raised against RCA binding bovine brain grey matter glycoproteins was cross reactive with RCA-binding human brain grey matter glycoproteins. This immunological cross reactivity may be an indication of common determinants shared by RCA binding bovine and human brain grey matter glycoproteins. Such an evolutionary relationship leading to immunological cross reactivity has been suggested also for galactoside binding lectins isolated from various organisms. Immunohistochemical localisation of human brain glycoprotein ligands of brain lectin using antiserum raised against endogenous glycoprotein ligands of bovine brain lectin showed that the lectin binding glycoproteins are present on the cell surface, nucleus and also in cytoplasm.

Separation of individual proteins of total BBL-binding glycoproteins of bovine or human brain grey matter could not be achieved either by gel filtration on Sepharose 4B or by HPLC on TSK 3000 SW column. BBL-binding glycoprotein sub units of bovine and human brain grey matter obtained on SDS-PAGE, when electroeluted and subjected to SDS-PAGE again all yielded mainly a group of closely spaced bands corresponding to molecular mass about 56 KDa. Antiserum against 186 KDa subunit of BBL-binding human brain

glycoproteins was cross reactive with the 151 KDa, 100 KDa and 56 KDa subunits of BBL-binding human brain glycoproteins. These results indicate that the BBL-binding endogenous glycoproteins are high molecular weight aggregates formed of several subunits of molecular mass around 56 KDa, but incompletely dissociated in initial SDS-PAGE.

Characterisation of oligosaccharide part of the BBL-interacting glycoproteins, using carbohydrate binding proteins such as Con A, jacalin, wheat germ agglutinin (WGA), Ulex europaeus agglutinin (UEA), Phaseolus vulgaris agglutinin (PHA) and human plasma anti-Gal antibody, was carried out. Strong interaction of Con A, jacalin and WGA to BBL-binding total glycoproteins of bovine and human brain grey matter indicated the presence of high mannose and/or hybrid type oligosaccharides, α-galactose and/or T-antigen and N-acetyl neuraminic acid and/or N-acetyl glucosamine. Interaction of anti-Gal with BBL-binding bovine brain glycoproteins prepared by RCA-Sepharose chromatography is an evidence for the substantial expression of α-galactose moieties. Very weak binding of anti-Gal to their counterparts in human brain glycoproteins showed that BBL-binding glycoproteins among RCA-specific human grey matter glycoproteins contained very few α -linked galactose residues. UEA and PHA were not interacting with these bovine

and human brain glycoproteins.

A strong sugar-specific interaction of BBL with bovine thyroglobulin was observed. Abolition of this interaction upon α-galactosidase treatment of the glycoprotein and inability of desialylation of native thyroglobulin to enhance BBL-binding, all indicated that α-galactosyl groups are equally recognized by BBL as are α-galactosides, if not better. Evidences had been obtained to show that human laminin molecules contain terminal α-linked galactose groups (223,232). In this context, our present finding that 14 KDa lectin strongly recognizes terminal α-galactosyl groups tend to suggest that this lectin may have a role in sequestering laminin-like molecules from immune recognition and consequent tissue damage.

Guar gum is a soluble polysaccharide of galactomannan, which upon cross-linking with epichlorohydrin forms an insoluble matrix. In our modified procedure of guar gum cross-linking a lesser degree of cross-linking was achieved in comparison to an earlier procedure (245) to decrease the number of galactose residues taking part in cross-linking, in order to provide more unsubstituted α1 --> 6 galactose residues for anti-α-Gal binding. Moreover lesser degree of cross-linking would result in a relatively higher effective pore size and there by increase

the accessibility of larger molecules like immunoglobulins to the galactose residue. In Sepharose 4B the specific ligand for anti-α-Gal is α 1-->3 linked 3,6-anhydro-L-galactose which occurs occasionally in a linear copolymer with β 1-->4 linked galactose as the major constituent.

CLGG and Sepharose 4B have retained anti-α-Gal from human plasma and this antibody was eluted from these matrices using 0.1 M galactose. Agglutination inhibition studies using common sugars revealed that both samples were α-galactoside specific. IgG nature of both samples was evident on immunodiffusion. The minimum hemagglutinating concentration of anti-α-Gal isolated on CLGG or Sepharose 4B was 0.4 μg/ml. Sepharose (agarose) and its derivatives are among the most widely employed matrices for chromatography of biomolecules from human plasma or plasma-contaminated tissue. Our observations suggest that artifactual binding of anti-α-Gal to agarose might explain many anomalous results observed in the past. Our method is a relatively inexpensive, one step purification procedure of anti-α-Gal from human plasma by affinity chromatography. The optimum range of pH for anti-α-Gal binding to CLGG was 7-8 and that of ionic strength for anti-α-Gal binding to CLGG as well as Sepharose 4B was 50-150 mM NaCl. CLGG could bind maximum of 0.3 mg anti -α-Gal per ml at pH 7.4 in 150 mM NaCl, while Sepharose was only a third as efficient. For

routine preparation of anti-α-Gal, chromatography on CLGG or Sepharose 4B was performed at pH 7.4 in 150mM NaCl to maintain conditions close to physiological.

Jacalin binding glycoproteins were isolated, by affinity chromatography on jacalin-Sepharose 4B matrix, from bovine and human brain grey matter in order to get a sample enriched in α-galactoside terminals. On western blot of jacalin binding bovine brain grey matter glycoproteins anti-Gal-HRP identified a single polypeptide of molecular mass 91 KDa. Similarly on western blot of jacalin binding human brain grey matter glycoproteins anti-Gal-HRP recognized five polypeptides having molecular mass (in KDa) 94, 180, 210 and 230.

Terminal α-linked galactose groups as part of O-linked oligosaccharide side chains of glycoproteins had been reported to be present in brain tissues of many lower animals (317). Since there is a substantial titre of anti-α-Gal in normal human individuals, the presence of terminal α-linked galactose residues in the brain may lead to autoimmune reactions. One explanation for this coexistence of antigen and antibody is that brain glycoproteins are in the normal course protected from exposure to the serum IgG due to sequestration of the brain cells by the blood - brain barrier. Sometimes inflammations such as due to cancer or infections could cause breaches in

the barrier (323) that are likely to allow an influx of anti-α-Gal into the brain. In such a condition the probability of developing auto immune disorders due to recognition of the glycoproteins demonstrated here by anti-α-Gal is clinically very significant. Results of our investigations indicate the possibility of anti-Gal bridging between brain tissue and circulating tumor cells, thus predisposing the brain to metastasis. In view of the abundance of terminal α-galactosides in several bacteria that infect man (324) and the recent demonstration that anti-Gal binds to them (325), a possible role for anti-Gal in localizing infectious bacteria in the central nervous system is also worth investigating.

There are observations of enhanced anti-Gal titre during Plasmodium falciparum malaria (326) and about 45% of the patients with systemic sclerosis (scleroderma) (327). The seminal fluid of a large proportion of infertile males contained significant anti-Gal reactivity, implying transudation of serum IgG molecules probably due to damage in the selective permeability of the blood genital tract barrier (328).

Aberrant cell recognition is thought to underlie the uncontrolled cell growth and motility that characterise neoplastic transformation and metastasis. In such circumstances, alterations in expression of cell surface

carbohydrates and secreted glycoprotein molecules produce epitopes recognized by monoclonal antibodies that may be used for new approaches to diagnosis, prognosis, monitoring and therapy of cancer. Coupling drugs to carbohydrate binding proteins and/or to molecules specifically recognised by membrane bound carbohydrate binding proteins, appears to be a promising tool for targeting drugs to specific site. Methotrexate conjugated to mannosyl bovine serum albumin was found much more effective than unconjugated methotrexate, in a murine model of experimental visceral Leishmaniasis (161). Investigations are being done to exploit cell surface lectins as targets for the controlled and selective delivery of drugs to malignant cells (79). Anti-Gal may also be considered as a prospective tool for selective drug targeting to metastatic cells which express α -linked galactose residues on their surface. Anti-Gal can also be used as an anomer-specific probe in glycoconjugate. structure studies since it exclusively recognizes terminal α-linked galactose groups with the known exception of the blood group B oligosaccharides.

The role of carbohydrate binding proteins in regulation of cytokine activity has been demonstrated by some investigators. The 14 KDa, galactoside binding lectin from human placenta and its homologue found in the electric organ of *Torpedo electricus* (93,94) have been shown to

possess immunoregulatory properties, inducing the release of tumor necrosis factor from macrophages (95) and suppressing experimental autoimmune myasthenia gravis (EAMG) in mice (96). The galactoside binding human placental lectin is one of the several immunosuppressive molecules (97,98,99) that may prevent maternal recognition of paternal antigens expressed on fetal tissues. Mistletoe lectin (ML-1) at very low doses induces the release of lymphokines and tumor necrosis factor into the circulation and have promising clinical applications in cancer therapy (100). Several lectins, most notably lentil lectin, can prolong skin and heart transplants in rodents (164), and lentil lectin was effective at suppressing graft-versus-host diseases in mice induced by allogenic spleen cell (165). Results of experimental heart transplantation in rodents indicates that succinylated Concanavalin A is a more potent immunosuppressive agent than cyclosporin (166). Ricin covalently coupled to monoclonal antibodies to leukemia antigens can be effective reagent for ex-vivo marrow purging (167,168,169), especially useful for autologous bone narrow transplantation. Among the novel therapeutic strategies being developed in response to the AIDS epidemic are human monoclonal antibodies to HIV glycoprotein gp 41 coupled to ricin A-Chain (170) and recombinant soluble CD_4 coupled to ricin (171), both of which are effective *in vitro*. A

conjugate of ricin A-Chain to a monoclonal anti-T cell antibody has been found effective for acute steroid resistant graft-versus-host disease when administered intravenously to patients following allogenic bone marrow transplantation (172).

These observations advocate that lectins do have significant functions in cell sociology. Elucidation of distribution patterns of carbohydrate binding proteins and their ligands on normal and malignant cells would pave the way for developing better diagnostic and therapeutic strategies.

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SYNOPSIS

MAMMALIAN GALACTOSE-BINDING PROTEINS: STUDIES ON HUMAN AND BOVINE BRAIN GREY MATTER GLYCOPROTEINS RECOGNIZED BY ENDOGENOUS GALACTOSE BINDING LECTIN AND BY HUMAN SERUM ANTI-α-GALACTOSIDE ANTIBODY

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INTRODUCTION

The enormity of structural diversity possible in oligosaccharides taken together with the cell surface or extracellular location of most glycoconjugates is suggestive of the importance of the latter as carriers of biological information that decides cell-cell and cell-matrix interactions or in short, cell sociology (1). Demonstration in recent years of a large number of plant and animal lectins of diverse sugar specificities underline this hypothesis (2). Of the animal tissue lectins, the most ubiquitous is a 14 KDa galactose-binding lectin (2). Another hemagglutinating galactose-binding protein is the anti-α-galactoside IgG antibody (anti-Gal) that is naturally present in the serum of higher mammals like man, apes and Old World monkeys (3). For elucidating the biological function of these molecules, the glycoconjugate molecule with which they interact endogenously need to be identified. The present work involves identification and initial characterization of human and bovine brain grey matter glycoproteins that are recognized by endogenous galactose-binding lectins and by human serum anti-Gal.

METHODS AND RESULTS

Preparation of lectin and conjugation to peroxidase

The 14 KDa bovine brain galactoside-binding lectin (BBL) was prepared from frozen bovine brain grey matter by affinity chromatography on asialofetuin-Sepharose or lactose-Sepharose. Labeling of BBL with horse radish peroxidase (HRP) as marker enzyme was achieved either by glutaraldehyde cross-linking of the two proteins or by a novel procedure of cross-linking using a bifunctional reagent, N-succinimidyl 3-(2-pyridyl dithio) propionate (SPDP) (4).

Immobilization of BBL and affinity chromatography to isolate endogenous BBL-binding glycoproteins

BBL was carboxyamidomethylated to prevent -SH group oxidation and attached by the N-hydroxy succinimide procedure to the carboxyl group of aminocaproic acid previously coupled to Sepharose by CNBr method. The resulting BBL-Sepharose column retained from bovine brain grey matter extract, a group of glycoproteins which could be eluted with lactose and in SDS-PAGE, gave rise to subunits of molecular mass (in KDa) 44, 51, 60, 123 and 186 respectively.

Identification of BBL-interacting glycoproteins in Western blot

Bovine brain grey matter glycoproteins binding to *Ricinus communis* agglutinin (RCA)-Sepharose were prepared. After SDS-PAGE resolution, RCA-binding glycoproteins electrotransferred to nitrocellulose sheets, when probed with BBL-HRP, indicated four glycoprotein subunits of molecular mass (in KDa) 58, 87, 117 and 186 respectively. Among human brain grey matter glycoproteins BBL-HRP recognized five subunits of molecular mass (in KDa) 47, 56, 100, 151 and 186 respectively.

Isolation of BBL using immobilized endogenous and other glycoproteins

Soluble glycoproteins of bovine brain grey matter (prepared by affinity chromatography on concanavalin A-Sepharose), when immobilized on Sepharose, could sugar-specifically retain from an extract of the same tissue, only one protein, ie, BBL. This was confirmed by immunological and agglutination inhibition studies. Results indicated that BBL was the main protein, if not the only one, that interacted sugar-specifically with tissue glycoproteins in vivo. BBL-binding oligosaccharide groups were also detected, by similar experiments, in bovine heart, and human placenta as well.

Further resolution of BBL-binding subunits by electroelution and reelectrophoresis

Most RCA-binding endogenous glycoproteins were recognized by BBL as well. On gel filtration using Sepharose 4B, the entire proteins came out in one peak. However, BBL-binding endogenous glycoprotein subunits of molecular mass (in KDa) 186, 117 and 58 obtained on SDS-PAGE, when electroeluted separately and subjected to SDS-PAGE again, all yielded mainly a group of closely spaced bands, corresponding to molecular mass about 56 KDa. Similar observation was made with the 186 KDa, 151 KDa, 100 KDa, and 56 KDa subunits from human brain. In addition, PAGE at pH 8.2 of RCA-binding glycoproteins from bovine or human brain grey matter also indicated 3-4 band groups. Each group, on electroelution and reelectrophoresis, in SDS-PAGE also yielded a cluster of bands around 56 KDa. Among human brain BBL-binding glycoproteins, antibody against the 186 KDa subunit cross-reacted with the 151 KDa, 100 KDa and 56 KDa subunits. These results, together with the molecular masses of BBL-binding subunits in initial SDS-PAGE suggested that BBL-binding endogenous glycoproteins are high molecular weight aggregates formed of several subunits of molecular mass around 56 KDa, but incompletely dissociated in initial SDS-PAGE.

Immunohistochemical localization of BBL-binding glycoproteins

Paraffin-embedded sections of normal human brain were treated with rabbit IgG against BBL-binding glycoproteins from the bovine brain grey matter, followed by HRP conjugate of goat anti-rabbit IgG. Glycoproteins were localized on cell surface, nucleus and cytoplasm.

Interaction of glycoproteins with plant lectins

Human and bovine brain glycoproteins recognized by BBL were also recognized by concanavalin A, jacalin, wheat germ agglutinin and, though weakly in the human brain glycoproteins, by human serum anti-Gal antibody. *Ulex europaeus* and *Phaseolus vulgaris* lectins had no action.

Recognition of terminal α-galactosyl groups by BBL

Endogenous glycoproteins that were recognized by jacalin, an α-galactoside-specific lectin, were also recognized by BBL, on dot blots. Also, bovine thyroglobulin which contains numerous terminal, α-galactoside moieties, but hardly any β -anomer (5) was recognized by BBL readily. Moreover, BBL-binding was abolished when the above glycoproteins were treated with coffee bean α-galactosidase

either on nitrocellulose or in solution. These observations suggest that glycoproteins having α-galactoside terminals may also be natural complementary molecules for BBL.

Preparation and characterization of anti-α-Gal:-

Anti-α-Gal was prepared from outdated human plasma by a simple and inexpensive one step procedure of affinity chromatography on a gel matrix prepared by an improvement on the procedure of cross-linking guar galactomannan with epichlorohydrin (6,7). The preparation was identified as anti-α-Gal by immunodiffusion and agglutination inhibition. The pH and ionic strength effects on anti-Gal binding to guar galactomannan were studied. Anti-α-Gal HRP conjugate was prepared by glutaraldehyde cross-linking. Anti-α-Gal was found not to recognize T-antigen groups or terminal β-galactosyl epitopes as present in asialofetuin.

Identification of anti-α-Gal-binding brain glycoproteins:-

Using dot blots and Western blots, bovine brain grey matter glycoproteins (obtained by Jacalin-Sepharose affinity chromatography) were found to contain mainly one 91 KDa subunit that was recognized through its terminal

α-galactoside groups, by anti-α-Gal (8). Among the human brain grey matter jacalin binding glycoproteins, subunits of molecular mass (in kDa) 94, 108, 180, 210 and 230 were recognized by anti-α-Gal (9).

DISCUSSION

Coupling BBL to HRP using SPDP offered an additional advantage of arresting the oxidation of the labile -SH groups in BBL since they were utilized in cross-linking. N-Hydroxy succinimide method of coupling BBL to Sepharose was opted since it involved lectin attachment at near neutral pH to reduce inactivation. To probe for BBL-interacting glycoproteins, RCA-binding glycoproteins were used in order to avoid overcrowding in SDS-PAGE and also since among common lectins, RCA resembled BBL most in oligosaccharide recognition. Occurrence in human brain of glycoproteins recognized by anti-α-Gal was unexpected since in humans, during evolution, synthesis of anti-α-Gal coincided with suppression of such epitopes in most tissue (10). Significance of this observation for autoimmune disorders, tumors and infection is striking and worth investigating.

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Indian patent application No. 578/MAS/91 "A process for preparation of anti-α-Gal from out-dated human plasma" by P.S. Appukuttan and P.L. Jaison.