

**STUDIES ON MEMBRANE-BOUND AND SOLUBLE
GLYCOCONJUGATES RECOGNIZED BY BOVINE BRAIN
 β -GALACTOSIDE BINDING LECTIN: GANGLIOSIDES FROM
GREY MATTER AND GLYCOPROTEINS FROM BRAIN STEM**

A THESIS PRESENTED

by

KANNAN V.M.

to

THE DIVISION OF NEUROCHEMISTRY IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

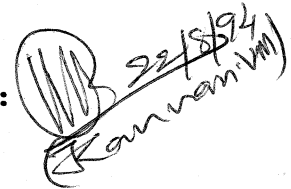
SREE CHITRA TIRUNAL INSTITUTE FOR
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TRIVANDRUM

AUGUST 1994

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Thesis entitled

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RECOGNIZED BY BOVINE BRAIN β -GALACTOSIDE BINDING
LECTIN: GANGLIOSIDES FROM GREY MATTER AND
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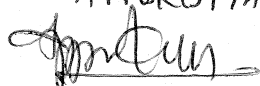
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ABBREVIATIONS

BBL	-	Bovine brain 14 KDa galactose-binding lectin
BSA	-	Bovine serum albumin
CNS	-	Central nervous system
Con A	-	Concanavalin A
EDTA	-	Ethylenediamine tetraacetic acid
Fuc	-	Fucose
Gal	-	Galactose
Glc	-	Glucose
GalNAc	-	N-acetylgalactosamine
GlcNAc	-	N-acetylglucosamine
HRP	-	Horse radish peroxidase
IgG	-	Immunoglobulin G
Man	-	D-Mannose
2-ME	-	2-mercaptoethanol
NeuNAc	-	N-acetylneuraminic acid
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	0.2 M potassium phosphate buffer containing 0.15 M NaCl, pH 7.4.
PMSF	-	Phenylmethane sulfonylfluoride
RCA	-	<u>Ricinus communis</u> agglutinin
SDS	-	Sodium dodecyl sulfate
TEMED	-	N,N,N',N'-tetramethylenediamine
TRRBC	-	Trypsinized rabbit red blood corpuscle
WGA	-	Wheat germ agglutinin

CHAPTER I

INTRODUCTION

INTRODUCTION

LECTINS

Lectins are carbohydrate-binding proteins other than enzymes or antibodies (1). Lectins have been identified and isolated from viruses, bacteria, yeasts, plants, invertebrates and vertebrates (2). Plants are the source of majority of lectins isolated and characterised so far. Of late many animal lectins have also been isolated and studied. In general, animal lectins are more labile than plant lectins.

Lectins differ in their sugar specificity. Among the animal lectins, β -galactoside specific lectins are the predominant and are widely distributed in vertebrate cells (3). Galactoside-binding lectins have been reported from bacteria to higher animals including man. Murray et al. identified a galactoside-binding lectin on the cell surface of Fusobacterium nucleatum (4). In the last decade, many soluble β -galactoside binding lectins have been detected in vertebrate tissues. Rat, bovine and human brain contain a lectin belonging to this family (5,6). Calf heart contains a very similar, but not identical, membrane associated lectin (7). The β -galactoside binding lectin from bovine muscle has a subunit molecular mass of 14 KDa and recognizes,

oligosaccharides with the terminal non-reducing sequence Gal β -1-4 GlcNAc or Gal β -1-3 GlcNAc and their analogues with α -1-2 linked fucose, α -1-3 linked galactose or α -2-3 linked sialic acid to the galactose (7). A lectin very similar to the β -galactoside binding lectin from bovine brain, in subunit and native molecular weights, saccharide specificity and pI has been reported from the amphibian (Bufo) ovaries (8). There are multiple proteins in the range 14-200 KDa which are antigenically related to 14 KDa β -galactoside binding lectin (9). Studies of Abbott et al. revealed that there is only one gene for the 14 KDa lectin in man (10). But Barondes et al. indicated the presence of at least three variants of the gene encoding 14 KDa lectin in human tissue (11). From rat and bovine brain a β -galactoside binding soluble lectin (subunit molecular weight 15,000) was purified and characterised by Caron et al. (12). A similar lectin (sub unit molecular weight 14,500) was purified and characterised from human brain by Dominique Bladier et al. in 1989 (13). Another lectin with a subunit Mr of 14,000 and a pI of 4.9 was characterised from human placenta (14).

Lectins bind sugars by non-covalent forces. The binding is specific; a lectin binds one or more sugars or oligosaccharide structures with a defined structure. The specificity is not absolute, so that each lectin has a

spectrum of sugars or sugar sequences which it can recognize. Thus once the specific sugar of the lectin is known, it is possible to inhibit the interaction of a lectin with its glycoconjugate by incubating the lectin with its specific hapten sugar before treatment with the glycoconjugate. But it is important to note that a specific oligosaccharide structure involved in the binding of the lectin to a glycoconjugate might be quite different from the best monosaccharide inhibitor (15). Rat or bovine brain lectin is inhibited by D-galactosamine and D-galactose. But N-acetyl-D-galactosamine is not an inhibitor. These observations indicate that a free hydroxyl group or a free amino group at C-2 is required for monosaccharide to inhibit these lectins (12).

Recently it was reported that lectins, in addition to the carbohydrate binding site, may contain a second type of binding site that interacts with a non-carbohydrate ligand (16). For example, human brain lectin strongly reacted with endogenous proteins ranging in Mr. from 14×10^3 to 67×10^3 . Among these proteins, 41 and 43 KDa molecules were recognized by the lectin even in presence of lactose. The protein to which human brain lectin binds through a carbohydrate-independent interaction was found to be actin (17,18).

Evidences from mutagenesis and proteolysis studies

suggest that almost the complete polypeptide chain is necessary for integrity of the carbohydrate recognition domain of bovine soluble 14 KDa β -galactoside-binding lectin. In general, lectins are broadly divided into two groups namely the C-type (calcium dependent) and S-type (sulphydryl dependent) lectins. The former require Ca^{2+} for their activity and are mostly membrane-bound. The latter are -SH group dependent and require reducing agents like 2-mercaptoethanol to prevent the oxidation of free -SH groups that are essential for their carbohydrate binding activity. In rat lectin (14 KDa) it is proposed that oxidative inactivation is due to a change in the secondary structure as a result of intramolecular disulphide bond formation (19). The 14 KDa β -galactoside binding lectin belongs to the S-type and requires a reducing agent like 2-mercaptoethanol for its stability. Presence of the haptan sugar (Lactose) also helps in protecting the lectin from denaturation and precipitation, especially at higher temperature.

Reports concerning the cytological localization of lectin from various sources differ widely. Studies show that 14 KDa galactose binding lectin is predominantly cytosolic, consistent with the lack of a recognizable signal sequence. However, there is evidence to show that under certain conditions of growth and differentiation, they (14 KDa

lectin molecules) are externalized (20) or transported to the nucleus (21). Endogenous β -galactoside binding lectins have been localized at the surface of different viable cultured tumor cells and after fixation and permeabilization, in intracellular pools (22). The surface distribution was in the form of microclusters suggesting that the membrane-associated lectin molecules were laterally mobile and subject to rearrangement by exogenous ligands (mAbs or glycoproteins) (22). The human brain lectin is more abundant in grey matter than in white matter. This suggests that human brain lectin is predominantly in neuron soma, as confirmed by immunohistochemical studies (13).

Many studies have revealed that during development, there occurs change in expression of galactose-binding lectin and its cognate glycoconjugates. For example, the membrane and cytosolic glycoconjugates of rat brain bear appropriate β -galactoside moieties which may be recognized by an endogenous lectin expressed in tissue itself in relation to brain maturation (23). Moreover, the endogenous β -galactoside binding lectin with sub unit molecular weight of 43,000-45,000 was found to be secreted into the extracellular matrix of early embryo in Xenopus laevis (24). Further, the role of endogenous lectins in development is shown by the involvement of β -galactoside specific lectin in adhesion among cells of early chick blastoderm (25). It has

been shown that chicken lactose lectin I is expressed at very high levels in embryonic chick muscle, but at lower levels thereafter (26). This lectin is secreted around developing skeletal muscle fibres (26). Developmental regulation of lectin expression has been reported in the case of rat brain (27,28) lectin as well as in rat lung lectin (29), the maximum concentration of the latter coinciding with the peak of alveolarization of the lung.

In the last decade many soluble β -galactoside binding lectins have been detected in vertebrate tissues (29,30). With a few exceptions, such as a lectin found in hepatocytes which apparently clears asialoglycoprotein from circulation, the function and endogenous ligands of most vertebrate lectins remain unknown (17). However, surface glycoconjugates are involved at all levels of cellular interactions, and carbohydrate recognition is a phylogenetically ancient binding principle represented throughout the biological world. It is reasonable to assume that soluble vertebrate lectins may be involved in such recognition events. It is however, important to note that lectin-carbohydrate binding need not be the only or the main determinant of specificity in the interactions of the brain cell with its environment (11). Hepatic receptor-mediated endocytosis after recognition of asialoglycoproteins, homing of cells like the hematopoietic stem cells or lymphocytes,

egg-sperm binding, interaction among cells of immune system (31), controlling of cellular proliferation (32), etc. are examples of processes involving lectin-carbohydrate interaction in higher vertebrates. In addition, lectins have been implicated, in (a) the initiation of viral, bacterial, mycoplasmal and parasitic infections, (b) the targeting of cells and soluble components, (c) fertilization, (d) the metastatic distribution of malignancies and (e) growth and differentiation (2, 33-43). It is documented by in vitro studies that Entamoeba histolytica adheres to human colonic mucus, colonic epithelial cells and other target cells via a galactose-binding surface lectin (44). It has been shown that an endogenous carbohydrate binding protein called cerebellar soluble lectin (CSL) (45) having a great affinity for mannose-rich N-glycans (46), was involved in stabilization of myelin structure (47,48) and in the maintenance of contact between axons and myelinating cells (48,49). The CSL is synthesized and externalized by the myelinating cells and participates in the formation of bridges between glycans of specific glycoproteins of the myelinating cells (47,48). Thus, CSL serves to stabilize myelin and the formation of these bridges can be inhibited by small concentrations of anti-CSL Fab fragments (47) resulting in myelin decompaction. Further more, anti-CSL antibodies are found in the cerebrospinal fluids of more

than 93% of the multiple sclerosis patients. In CNS demyelinating mutants a strong decrease in glycoprotein ligands of CSL has been observed (50,51). The axonal glycoprotein ligand of CSL has been identified as a 31 KDa glycoprotein (48,52). There are reports in favour and against the role of lectin in myotube fusion. It has been reported that the addition of 15 mM thiodigalactoside to L6 cells (derived from rat skeletal muscle) prior to their differentiation prevented myotube formation (53). In contrast to this 50 mM thiodigalactoside failed to prevent myotube formation in primary muscle cell cultures of chick embryo skeletal muscle and rat embryo skeletal muscle (54). The influence of lectin on immune system is illustrated by the finding that nanogram quantities of the β -galactoside-specific lectin from mistletoe exhibited immunomodulatory potency and enhanced cytokine secretion in vitro and in vivo (55). Some of the lectins are mitogenic, that is they stimulate the proliferation of lymphocytes. Examples include Concanavalin A, β -galactoside binding lectin from chick embryo, kidney (56) etc.

A family of carbohydrate-binding proteins or lectins, termed selectins, has been discovered and shown to be involved in many steps in lymphocyte recirculation and inflammatory responses. L-selectin is expressed constitutively by lymphocytes, E-selection is expressed by

activated platelets and endothelial cells (57).

The human 14 KDa β -galactoside binding lectin was expressed in E. coli cells by insertion of cDNA into a plasmid carrying a tag promoter. This facilitated the easy availability of large quantity of lectin (58).

The amino acid sequence of β -galactoside binding lectin from different sources shows extensive sequence homologies. The sequence identity of β -galactoside binding protein ranges from 56% between chicken and bovine to 87% between bovine and human (59). The tetrapeptide sequence W-G-A/S-E/D is found in β -galactoside binding animal lectins as well as in myelin basic protein, which shows antigenic cross-reactivity with the lectin. It is important to recall that the tetrapeptide in myelin basic protein is a part of the major domain responsible for induction of experimental allergic encephalomyelitis (7).

There are evidences to show that alteration in lectin expression is associated with malignant transformation. Reports show that the expression of endogenous tumor cell surface lectin is associated with transformation and metastasis (60). Allen et al. reported lactose-inhibitable lectin (sub unit Mr 14,000) in different types of malignancies and in a variety of normal human tissues (61). In addition, quantitative analysis of cell surface lectins by flow cytometric analyses of anti-

lectin antibody binding revealed that among related sublines, variants or clones of murine B16, K-1735 melanoma or UV-2237 fibrosarcoma, those exhibiting a higher metastatic potential express more lectin molecules on their surface (62). Immunoblotting of various bovine tissues and human lymphocytes with the monoclonal antibody 36/8 to the bovine lectin revealed multiple antigenically cross reactive proteins in the range 13-200 KDa. The relative proportion of these proteins change in transformed and stimulated lymphocytes (63,64). The β -galactoside-binding proteins are often found in embryonic tissues and also in tumorigenic cells (65) and they are supposed to have important functions in some onco-developmental events (58). Recently it was shown that mistletoe lectin showed not only immunomodulatory role but anti-metastatic activity as well in BALB/c mice (66). Endogenous lectin might be involved in specific recognition and adhesion between tumor cells and host cells in vivo in at least two ways. Those present on surface of certain normal host cells such as platelets, lymphocytes, macrophages, hepatocytes, endothelial cells and some extracellular matrix (ECM) components might bind tumor cell surface glycoconjugates and promote heterotypic aggregation and adhesion of tumor cells to capillary walls and basal lamina. Secondly tumor cell-surface lectins can recognize and bind complementary glycoconjugates on the surface of

other tumor cells and mediate homotypic aggregation or bind to glycoconjugates on the surface of host cells to mediate heterotypic aggregation or attachment to endothelial cells or to ECM (67) (Fig.A).

Another area where lectins find application is in the elucidation of structure and investigation of functions of cell membranes. Lectins have been used as a tool to identify changes in surface carbohydrates accompanying many pathological states. Peanut agglutinin (PNA) and Ricinus communis agglutinin (RCA) have been used to identify the expression of a surface antigen on the surface of infective but not in non-infective promastigotes of Leishmania major (68). Similarly lectin bearing (Con A or WGA) liposomes differentially binds to normal and transformed fibroblasts (69).

GLYCOCONJUGATES

The discovery of animal lectins evoked the search for their endogenous glycoconjugate ligands. Lectins in combination with their endogenous ligands have been ascribed a role in cell to cell communication (87). The observation that malignant transformation, where a disruption of cell to cell communication occurs, is accompanied by changes in expression of lectins (95,96) and their endogenous

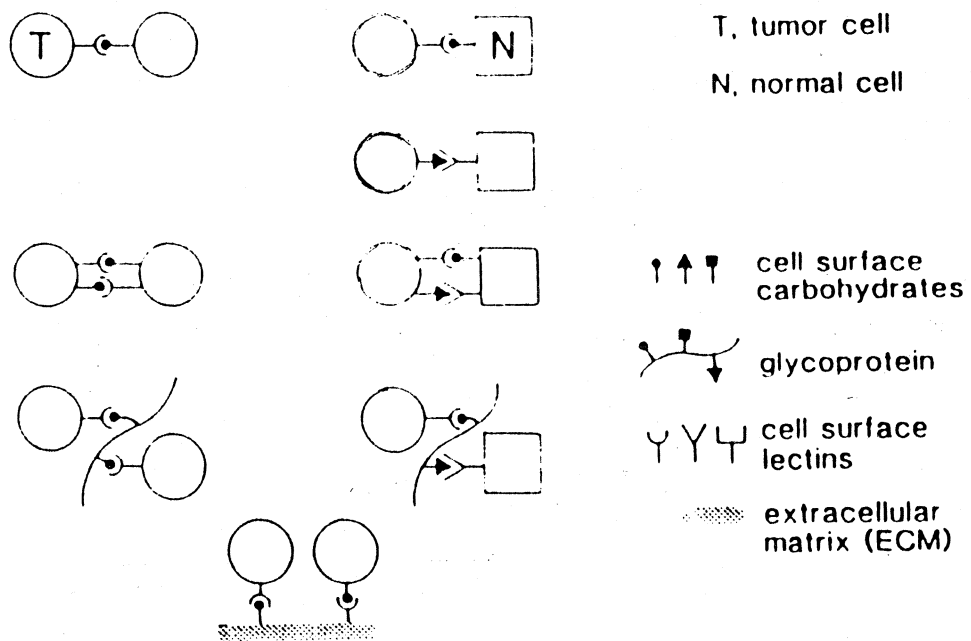


Fig.A.

A schematic representation of the mediation of cell adhesion by endogenous cell surface lectins.

Adapted from Lotan R and Raz A (1988) J. Cellular Biochemistry 37, 107-117.

glycoconjugates (92,93,94) reinforces this contention. The structural diversity of oligosaccharides renders the glycoconjugates vast repositories of biological information. From three different aminoacids it is possible to get only a maximum of six different tripeptides whereas three different monosaccharides in pyranose form can give rise to a maximum of 1056 different types of trisaccharide structures (70). As a result lectins in combination with glycoconjugates provide an efficient biological recognition system. The antibody-like specificity of lectins augments the accuracy of this recognition system. Therefore the lectin-glycoconjugate interaction can be viewed as a secondary information storage system, the primary being the information stored in the base sequence of DNA.

Cell surface carbohydrates have been implicated as cell-cell recognition molecules (71-76). Surface glycoconjugates on one cell may bind to complementary carbohydrate binding proteins (lectins) on another cell, resulting in specific adhesion. Such interactions may underlie the binding of bacteria to intestinal epithelium (77), aggregation of slime mold cells (78), aggregation of sponge cells (79), yeast mating (80), sperm-egg binding (81), interaction between cells in the immune system (82-84) and the recognition between cells during embryonic development (85). Bazzaro and Roseman (86,87) demonstrated

that Dictyostelium discoideum cells adhere, via separate cell surface receptors, to glucose-, mannose-, and GlcNAc gels. Binding to the latter sugar is developmentally regulated, while contact of the cells with glucose containing gels can block formation of multicellular aggregates. It appears that carbohydrate-derivatized surface conveys signals which profoundly modify the social behaviour of these cells (88). Also it is found that toxicity due to lectins like Griffonia simplicifolia 1-B4 and wheat germ agglutinin toxicity is mediated by soluble lectins that bind to the specific receptors, get internalized and trigger programmed cell death (apoptosis) (89). Glycoconjugate-lectin interactions have been postulated to participate in the establishment of metastatic lesions (90,91,92). In various tumor cell types, qualitative or quantitative changes in the glycoconjugate (93,94,95) or lectin patterns (96,97) have been reported to correlate with their metastatic potency.

Various methods were adopted to study the interaction of glycoconjugates with lectin. Cell adhesion to glycolipids immobilized on silica gel thin-layer chromatography plates or on polyvinylchloride plates was examined by Siwank-Hill et al. (98) and Blackburn (99,100) respectively, while studies with glycoprotein were performed on derivatives of polyacrylamide gels (101) or

nitrocellulose (102) that contain the immobilized glycoprotein as matrix.

GLYCOLIPIDS

Glycoconjugates comprises glycolipids and glycoproteins. The most predominant glycolipids are glycosphingolipids. All sphingolipids contain, as their backbone, sphingosine or a related long chain sphingoid base, an amide-linked fatty acid and a polar head group (hydroxyl for ceramide, phosphoryl choline for sphingomyelin or carbohydrate residues for glycosphingolipids). The most predominant sphingoid base is sphingosine (4-trans-sphingenine). The amino group of sphingoid base is attached to a long chain fatty acyl group (that can be saturated or monounsaturated depending on factors such as, cell type, diet or age) to form ceramide (Fig.B).

The most complex sphingolipids are glycosphingolipids, which include neutral lipids containing from one (cerebroside) to more than twenty sugar residues, and acidic glycosphingolipids containing one or more sialic acid residues (gangliosides) or sulfate esters (sulfatides). A large degree of heterogeneity exists in the carbohydrate moiety with respect to the composition, sequence, number, and linkage of the sugar residues.

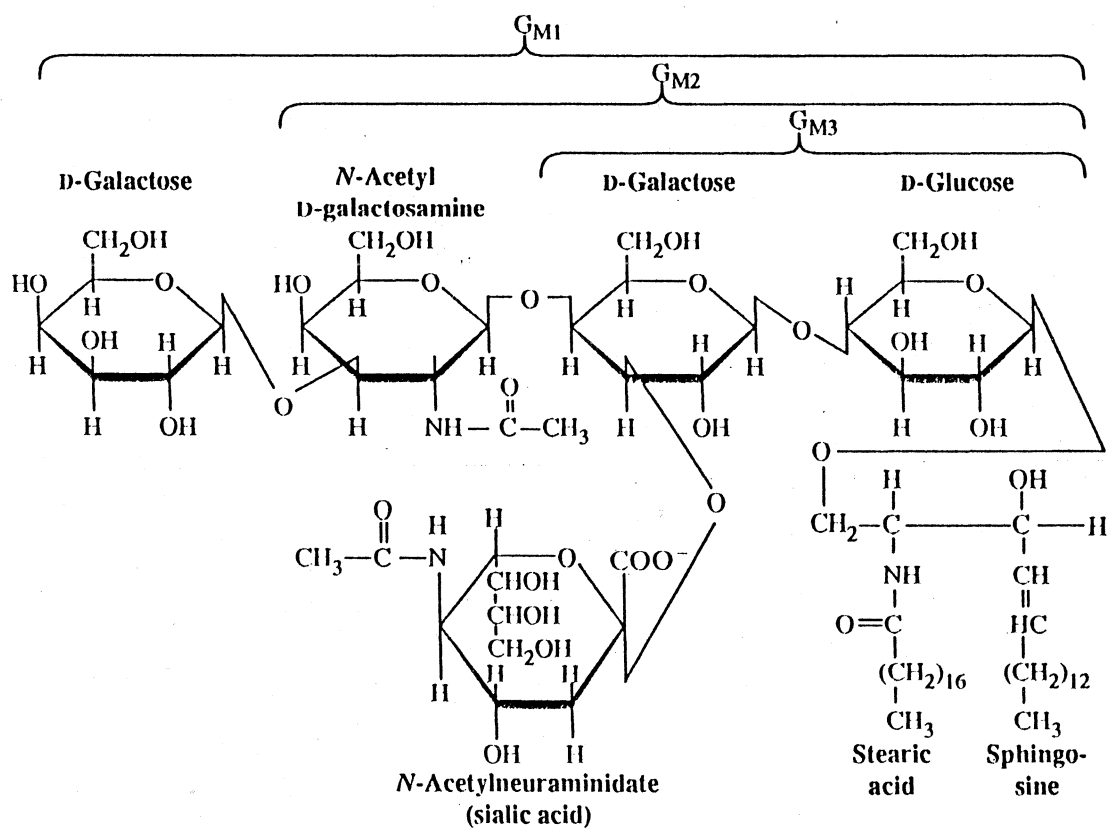


Fig.B

Structure of gangliosides GM1, GM2 and GM3

Adapted from Glycoconjugate J. (1992) 9, No.3, Chapman & Hall 2-6 Boundary, London.

Gangliosides are named according to the number of sialic acid residues as well as the number of neutral sugar residues present in the molecule. They are designated as mono, di, tri or tetra sialogangliosides corresponding to the number of sialic acid residues. Although differences in the ceramide portion of molecules certainly exist (such as its chain length and presence of double bonds), the characteristic feature taken into consideration in naming, is the composition of the carbohydrate chain. There are several systems of nomenclature of which the most commonly used is that of Svennerholm in which the number of sialic acid residue is designated by "M" (mono), "D" (di), "T", "Q" and "P". The rest of the molecule is designated by a number related to the length of the carbohydrate chain. The number is given by the formula $(5-n)$ where 'n' is the number of neutral sugar residues. Thus for example a ganglioside containing one sialic acid and two neutral sugar residue are designated as GM₃ [G-for ganglioside, M-monosialo and 3 is $(5-2)$] (Fig.B).

Sphingolipids have long been implicated in diverse cellular functions, including cellular communication, transformation, proliferation, differentiation and modulation of receptor function (103, 104, 105, 106). It is known that glycosphingolipids are receptors for viral and bacterial toxins and can serve as mediators for cell-cell or

cell-substratum recognition (107). The sialic acid containing glycosphingolipids namely gangliosides are receptors of many toxins like cholera toxin (108) tetanus toxin (109) Botulinum neurotoxin (110), E. coli enterotoxin (111), Staphylococcal alpha toxin (112) and is known to inhibit the hemolytic activity of the hemolysin of Vibrio parahemolyticus (113). Many different types of gangliosides have been isolated and characterized. Recently (1993) a de-N-acetylated GM1 was purified from bovine brain by Kazuya et al. (114). In general, the brain grey matter is richer in gangliosides than white matter (115).

Although the majority of gangliosides reside in the outer leaflet of the cell membrane, some are found in blood serum and other body fluids where presumably, they are present as protein-bound complexes or membrane fragments. Some ganglioside binding proteins like cholera toxin which binds specifically to GM1 ganglioside, causes remarkable modification of the GM1 distribution in membrane which may lead to formation of channels also (116). Gangliosides are also known to modify the physicochemical properties of the membrane where they are inserted, such as fluidity (117, 118) or thermotropic properties (119) and affect the activity of a number of enzymes (120) GM1, one of the major gangliosides in the mammalian brain, when exogenously administered is known to penetrate the blood brain barrier

(121) and get incorporated into the neuronal membrane (122). Gangliosides being amphipathic molecules forms micelle in aqueous medium, with their carbohydrate groups projecting out. The critical micellar concentration for the mixed ganglioside was found to be approximately 10^{-8} M by gel filtration, equilibrium dialysis and band centrifugation (123).

Recent results from various laboratories suggest that gangliosides could affect cell growth regulation by modifying growth factor receptors directly (104, 124, 125, 126) or by modifying the activity of novel protein kinases within the cell (127-129). Another possibility is that the products of sphingolipid turnover could mediate cellular responses (130). With the discovery that sphingosine, a metabolite of membrane sphingolipids, inhibited potently and specifically protein kinase C activity in vitro, another link between sphingolipids and signal transduction emerged (105, 131, 132, 133).

Gangliosides are receptors for bacterial toxins and viruses and can function as tumor antigens (134). It is known that cholera toxin binds to GM1 ganglioside and, wheat germ agglutinin has a high affinity for glycoproteins with N-acetyl glucosamine residues. Both cholera toxin and wheat germ agglutinin are transported efficiently in all peripheral neurons. Preincubation of 125 I-cholera toxin with

monosialoganglioside GM1 completely blocked the retrograde axonal transport of the toxin (135).

Gangliosides can suppress the immune response in vitro (136) and can inhibit the growth of fibroblasts (137). In the latter case, the added gangliosides inhibited the interaction of growth factors with their receptors as well as the receptor associated tyrosine Kinase activity. On the other hand, gangliosides seem to stimulate cell growth and differentiation also. The ganglioside GQ1b, when added to certain neuroblastoma cell lines, increases both cell number and the length and number of neurites (138). Gangliosides especially GM1, promote neuritogenesis in both primary neurons and neuroblastoma cells (139). The binding of B-subunit of cholera toxin to GM1 on surface of thymocyte leads to its proliferation (140). The in vivo studies in neonatal brain shows that GT1b ganglioside prevents the initial effects of tetanus toxin namely, protein kinase-C activation as well as the late effect of down regulation of the enzyme (141). The hypothesis that some of the neural retina cell surface receptors are capable of binding to gangliosides is supported by the observation that rapid and specific cell-adhesion occurred when neural retina cells were incubated on surface adsorbed with various gangliosides but not on surface adsorbed with various neutral glycosphingolipids, phospholipids or sulfatides (142).

Gangliosides are (a) capable of promoting functional recovery after traumatic and ischemic lesions to the peripheral and central nervous system (CNS) and of reducing cerebral edema (in laboratory animals) (b) capable of inducing increased dendrite formation and cell vitality in neuronal cell cultures and (c) involved in the immunology of several types of neuroectodermal tumors and employable as determinants for the production of monoclonal antibodies useful in therapy (143). Recently there are evidences to suggest that glycosphingolipids, including gangliosides, are potential target antigens in various neurological disorders, such as multiple sclerosis (144, 145) and polyneuropathy associated with immunoglobulin MM - protein (146,147,148). Glycolipids are targets for immunoglobulins in some human demyelinating diseases also (149,150,151). They constitute an important class of target antigens in Chagas disease (152,153). In an experimental model of human African trypanosomiasis in sheep, an immunoglobulin reactivity towards CNS glycolipid (galactocerebroside) was observed (154). The antisera raised against galactocerebroside have been shown to demyelinate CNS in vitro (155-158). Several types of neuropathies have been considered to be of autoimmune type. Autoantibodies against glycosphingolipids have been demonstrated in patients with several neurological disorders (146,150,159-161). There is

increasing evidence that serum antibodies directed against GM1 ganglioside occur in high titre in patients with motor neuron diseases (MND) (150,161-170). Antibodies to GM1 and GD1b, two gangliosides of the nervous tissue, have been reported to be associated with MND. High-titred antibodies against GM1 are also reported in patients with motor neuropathy with or without conduction block (160,161,164). Antibodies are also found against GM1 ganglioside in sera of patients with Guillain Barre syndrome and chronic inflammatory demyelinating polyneuropathy (171). It is assumed that infection by Campylobacter jejuni serotype 19 (PEN 19) which has the GM1 oligosaccharide structure (in lipopolysaccharide) induces autoantibody to GM1 ganglioside, and elicits Guillain Barre syndrome (172).

The monoclonal antibodies (MAbs) directed towards carbohydrate groups of gangliosides are useful in elucidating the structural changes as well as alteration in expression of gangliosides associated with malignant transformation. Such MAbs could be valuable tools to study the effect of perturbation of normal endogenous ganglioside-lectin interactions. It has been shown that melanoma cell lines derived from a patient's metastatic lesions have a marked increase in the expression of the disialoganglioside GD2 and GD3 compared to cell lines derived from primary melanoma lesions of the same patient (173). It is also

documented that pretreatment of M21 melanoma cells with anti-GD2 antibody resulted in 92% inhibition of attachment to a matrix of bovine endothelial cells laid down on tissue culture plastic. The inhibition suggested that these gangliosides were involved in the initial phase of the cell attachment process (174). It has also been observed that monoclonal antibodies against GD3 directly induced T-cell proliferation (240). Thus the presence of anti-carbohydrate antibodies directed towards gangliosides in the sera of patients assumes importance especially when the same oligosaccharide groups on gangliosides serve as site for attachment of the antibody and endogenous lectins.

When a mixture of the four major gangliosides derived from normal tissue was administered intramuscularly in patients with diabetic neuropathy, both neurological signs and symptoms, and instrumental parameters (vibratory perception threshold and conduction velocity of the sural nerve) improved considerably in a dose-dependent manner (175). The observed changes in elemental composition (K^+ , Na^+ , Ca^{2+} & Cl^-) evoked by ganglioside treatment of diabetic rats might reflect the ability of these substances to stimulate Na^+ , K^+ - ATPase activity and might be related to the mechanism by which gangliosides improve functional deficits in experimental diabetic neuropathy (176). It is also demonstrated that daily injection of GM1 (30 mg/Kg)

could enhance the recovery or ability to solve spatial reversal learning that is lost after bilateral lesions of the caudate nucleus (177). Ganglioside treatment also reduces cerebral edema (178) and prevents progressive neuronal damage following trauma by means of complex intracellular mechanisms (activation of membrane bound enzymes) (179). In addition, exogenously administered gangliosides increase seizure thresholds also (180). GM1 ganglioside has been shown to be effective in the treatment of acute spinal cord injury (181) and also to ameliorate effects of experimental Parkinsonism in primates (182).

It has been shown that exogenous gangliosides at concentrations found in serum inhibit the Concanavalin A-induced mitogenic response of mouse thymocytes. These observations and the elevated levels of gangliosides in the sera of tumor-bearing animals, suggest that gangliosides shed by tumor cells could be involved in the generalized immunosuppression observed in such animals (183). The CSF samples from seven meningioma patients were found to contain significantly increased concentrations of gangliosides GD3, one of the major gangliosides in meningioma tissue specimens. Sulfatide was significantly increased in 6 of 7 patients (184). The 3'-iso LM1 ganglioside was not detected in normal grey matter or white matter or in the normal corpus callosum, but in one of three breast cancer

metastases (to brain) and in one stomach cancer (185). Normal melanocytes, the precursors of melanoma, predominantly contain GM3 gangliosides, while GD3 ganglioside increase only after neoplastic transformation (186-188). During the progress from low to high metastatic variants, there is a shift in the ganglioside GM3: GD3 ratio.

Genetic disorders (inborn errors of metabolism) leading to deficiency of enzymes which degrade gangliosides leads to gangliosidosis (accumulation of the substrate ganglioside of the respective enzyme). The deficiency of β -hexosaminidase leads to GM2 gangliosidosis, three forms of which are identified. They are Tay Sachs disease caused by the deficiency of β -hexosaminidase A, Sandhoff disease caused by the deficiency of both β -hexosaminidase A and B, and AB variant caused by the defect of a non-enzyme protein (GM2 activator protein) (189).

Our knowledge about the ganglioside binding endogenous proteins is rather limited. Bovine serum albumin is known to bind gangliosides. In rat reperfusion experiments, the washing off of GM1 loosely bound to the surface of liver cells was faster in presence of BSA (190). Another endogenous protein is the myelin basic protein which has been shown to bind certain gangliosides (191,192). Recently two ganglioside-binding proteins have been isolated

from calf brain. The subunit molecular mass of these proteins are 11-12 KDa and 28 KDa. Component II (28 KDa) is a glycoprotein. When examined by reverse-phase HPLC each component separated into two major closely migrating peaks. The amino acid sequence of three of these peaks (one peak from component I and both peaks from component II) showed as far as the sequences were established, identity with the sequence of ubiquitin. It is hypothesized that the protein may be instrumental in intracellular trafficking of gangliosides (193). Another protein which binds to gangliosides is laminin. This interaction is important because the role of laminin in cell-adhesion process in nervous system is well documented (194).

Gangliosides are abundant in the nervous system, especially the gray matter. Besides being membrane receptors for bacterial and viral toxins, gangliosides are also involved in regulation of cell growth by modulation of cell growth factor receptors, modulation of cell substratum adhesion receptors, regulation of membrane bound enzymes and neuritogenic and neuronotrophic functions (195). However, the possibility of an endogenous ganglioside binding ligand binding to it through the sugar residues has been poorly investigated. Also it is not known whether any of these endogenous ganglioside binding ligands could

trigger any of the array of activities associated with gangliosides.

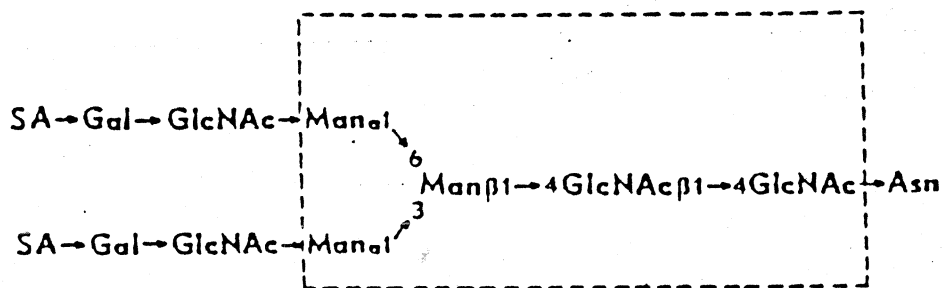
GLYCOPROTEINS

Glycoproteins are a heterogeneous group of macromolecules in which oligosaccharide structures are attached to the protein core. Based on the type of linkage of the oligosaccharide with the protein, generally, glycoproteins are divided into two groups. (1) The O-linked glycans where the GalNAc of the oligosaccharide forms linkage with the hydroxyl group of serine or threonine of the protein moiety (2) the N-linked glycans in which the GlcNAc residue of the oligosaccharide is linked to the amide nitrogen of an asparagine residue in the protein component. Asn-linked oligosaccharides share a common pentasaccharide core structure as shown in Fig.C.

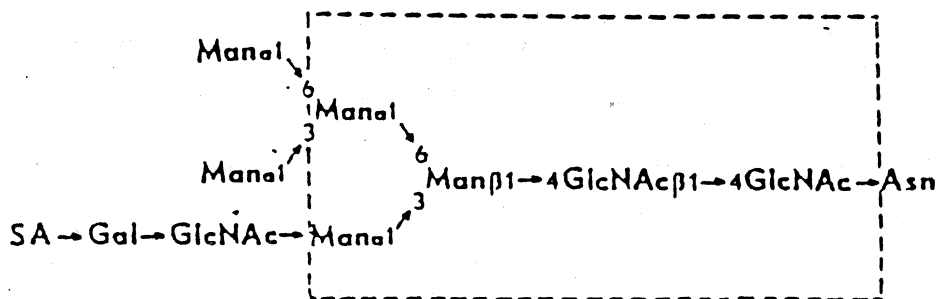
Based on the structure of outer branches, N-linked glycans are divided into three. (a) High-mannose type - in which there are 2-6 additional residues linked to the $(\text{Man})_3 (\text{GlcNAc})_2$ core. (b) Complex type which contains sialyllactosamine sequence and presents two other commonly found substituents, namely a fucose $\alpha(1-6)$ linkage to the innermost GlcNAc residue in the core and a 'bisecting' GlcNAc-linked $\beta(1-4)$ to the β -linked mannose residue. The



I. HIGH-MANNOSE TYPE



II. COMPLEX TYPE



III. HYBRID TYPE

Fig.C

Structures of the major classes of asparagine-linked oligosaccharides. The box delineated by the broken line indicates the common pentasaccharide core structure.

Adapted from Elbein AD (1984) CRC. Crit. Rev. Biochem. 16, 21-49.

complex type glycans may be further modified by the addition of extra branches on the α -mannose residues or by the addition of extra sugar residues that elongate the outer chains. In most cases the complex type chains have two, three or four outer branches or antennae, but units with five outer branches have also been found on avian ovomucoids (196) (c) The hybrid type possesses features of both high-mannose and complex - type glycans. Majority of them have the 'bisecting' GlcNAc β (1-4) Man β -structure (197). All N-linked oligosaccharides share a common core structure since they are formed from the same biosynthetic precursor lipid linked glycan (198).

Approximately 85-90% of the carbohydrates in the brain glycoproteins are N-glycosidically linked, the remainder being found as O-linked glycans. Again most of the N-linked oligosaccharides in nervous tissue glycoproteins are of the complex type (199). The O-linked glycans of neural tissue consists of the core disaccharide Gal β (1-3) GalNAc-, with out any modification or with sialic acid substitution at C-3 of Gal and/or C-6 of GalNAc. Rat brain O-glycans contains α -anomer of the core disaccharide which appears to be specific to nervous tissue (200).

The attachment of different types of oligosaccharides to identical protein core resulting in variation in the oligosaccharide part alone is common in

glycoproteins. This variation in carbohydrate group of glycoproteins produced by differing substitution of sugar residues on a basically similar core structure is called microheterogeneity. For example, the lone carbohydrate unit of ovalbumin is not identical in all the molecules of a single preparation (201).

The significance of the glyco-part of the glycoproteins in their biological activity vary considerably. Many enzymes are glycosylated proteins; but deglycosylation in most cases does not lead to the loss of enzyme activity (202). But in the case of the placental glyco-hormone human chorionic gonadotropin (HCG), removal of carbohydrate leads to loss of ability of the hormone to stimulate adenylate cyclase while maintaining its ability to stimulate steroidogenesis (203,204). In HCG, pituitary gonadotropin and thyroid stimulating hormone (TSH), core glycosylation is required for proper folding and assembly of α - and β - subunits during biosynthesis. Carbohydrate processing and terminal glycosylation appears to be under the control of several endocrine factors that regulate intracellular trafficking and release of these hormones (205). The other important functions postulated to be related to the carbohydrate part of glycoenzymes and glyco-hormones include secretion and transport, resistance to proteolysis and maintenance of conformation (202). In human

immunodeficiency virus (HIV-I), the envelope glycoproteins (rgp 120 and rgp 160) have been shown to specifically interact with N-acetyl glucosaminyl core of natural and synthetic oligosaccharide structures (206).

Cell surface glycoproteins play significant roles in central nervous system cell sociology (207-209). Cell surface carbohydrates undergo remarkable alteration during development and differentiation resulting in appearance of novel glycoproteins (210,211). Several cell surface glycoproteins have been identified for their roles in cell adhesion interactions of neurons, glial cells and matrices essential for development (212-214). An appreciable reduction in the level of 23 KDa, 18 KDa and 16 KDa glycoprotein ligands for the cerebellar soluble lectin (CSL) in myelin deficient (mld) mutant while the CSL concentration remained unaffected showed the importance of these proteins in myelin compaction (51) Myelin-associated glycoprotein (MAG) and Po are two myelin glycoproteins that are unique to the nervous system. MAG (Mr 100,000) is the major myelin glycoprotein of the CNS whereas it is a minor protein constituent of myelin sheaths of both CNS and PNS (215). Because of its localization in periaxonal membrane in PNS and CNS myelin, MAG is thought to be involved in glia-neuron and glia-glia interactions (215). Myelin-associated glycoprotein is a component of myelin sheaths and is

believed to be involved in the interaction between myelin-forming oligodendrocytes and axons (216). Cerebrospinal fluid of multiple sclerosis patients contains antibodies against this protein (217). An immortalized optic nerve-derived cell line transfected with cDNAs encoding the two isoforms of myelin associated glycoprotein, showed enhanced cell-adhesive properties, indicating a role for MAGs in cell-adhesion. A further finding which supports this is the observation that MAG was preferentially localized at the junction between cells, in confluent cultures (218).

Three groups of glycoproteins of molecular mass 65-73 KDa, 52-63 KDa and 43-48 KDa have been identified from foetal neuronal and glial surfaces. These glycoproteins exhibited anomalous behaviour on SDS PAGE, indicating the existence of a gradation of mutually interconvertible protein-SDS aggregates in dynamic equilibrium with one another (219).

Ultrastructural localization of binding sites for plant lectin shows that in rat liver cell surface membrane, lectin-binding sites are found only on extracellular surface (220). This indicates the cell surface location of glycoconjugates.

Transformation-associated changes are observed in the case of glycoproteins also. For example differences in lectin-binding to glycoproteins were observed with different

sublines of murine B16 melanoma. Altered glycosylation of membrane glycoproteins associated with human mammary carcinoma is documented by Hiraizumi et al. (221). The expression of glycosylation of Thy-1 (glycoprotein) is altered when thymocytes mature into immunocompetent cells and after malignant transformation. Similarly, when whole cells have been analysed by lectin binding, changes in cellular glycoproteins have been observed after malignant transformation (222). It is reported that in meningioma patients tumor-associated glycoprotein is present as detected by the lectin Ricinus communis I. This glycoprotein is present in meningioma tissue specimens also, but not in normal brain tissue (184).

The feasibility of the application of neoglycoprotein-conjugated liposomes as drug-targeting devices, based on carbohydrate-protein interaction has been shown in vivo in Ehrlich solid tumor-bearing mice (223).

The involvement of lectin-glycoconjugate interaction in many biological phenomena necessitates the study of lectins and their endogenous ligands - the glycolipids and glycoproteins. Endogenous lectin-glycolipid interaction has been poorly investigated and the reports concerning endogenous lectin-glycoprotein interaction is very scanty. In 1990, Adalid et al. reported a few glycoproteins from human brain ranging in Mr from 41×10^3

to 67×10^3 that interacted with human brain 14 KDa galactose binding lectin (17). Lectin may interact with endogenous membrane bound or soluble ligands. Considering the sugar specificity of bovine brain lectin, gangliosides are probable candidates as they contain β -galactosyl groups. Thus we studied the sugar-specific interaction of bovine brain lectin with endogenous membrane bound ligands (gangliosides from grey matter) and soluble ligands (glycoproteins from brain stem). We showed that a lectin very similar to the lectin from cerebral cortex is present in the brain stem as well. In a search for the endogenous soluble ligands of brain 14 KDa galactose - binding lectin, we found that soluble glycoproteins from brain stem interacts sugar specifically with this lectin.

OBJECTIVES OF THE STUDY

Lectin-glycoconjugate interaction has been shown to be involved in many physiological phenomena. Surface glycoconjugates and lectins are known to participate in the binding of bacteria to intestinal epithelium (77), aggregation of slime mold cells (78), aggregation of sponge cells (79), Yeast mating (80), sperm-egg binding (81), interaction between cells in the immune system (82-84) and the recognition between cells during embryonic development (85). Qualitative or quantitative changes in glycoconjugate (93,94,95) or lectin patterns (96,97) have been reported to accompany malignant transformation and metastasis.

Mammalian brain contains a 14 KDa galactose-binding lectin. Elucidating the physiological function of this lectin would require identification and characterization of its endogenous glycoconjugate receptors. Though the 14 KDa galactose-binding lectin is synthesized without a signal peptide for export to noncytoplasmic regions, it is believed to be externalized (20) by other means, so that the protein is detectable on the cell membrane and the extra-cellular matrix, in addition to the cytoplasm. Treatment of brain tissue with soluble galactosides such as lactose greatly enhances extraction of the tissue lectin into aqueous buffers. The above

observations indicated that an array of cognate glycoprotein and glycolipid molecules may be instrumental in the function of this lectin in vivo.

The main objectives of the study include

- (a) Identification of bovine brain lectin interacting endogenous membrane-bound glycoconjugates (gangliosides) from grey matter and the study of lectin-ganglioside interaction in vitro. Expression of gangliosides undergoes alterations during malignant transformation and metastasis (273). Anti-ganglioside antibodies especially those directed at their carbohydrate moieties have been implicated as causative agents in many neuropathies of unknown etiology (150,161,164,171). Moreover, administration of exogenous gangliosides has been shown to improve clinical conditions in a host of neurological disorders (176,177,178). These observations and the fact that ganglioside sugar structure did not preclude their recognition by the 14 KDa galactose binding lectin led us to examine recognition of ganglioside sugar by this lectin, since such recognition may play a role in the physiological effects of ganglioside molecules in normal and pathological states.

(b) Identification and characterization of bovine brain lectin-interacting soluble glycoconjugates (glycoproteins) from brain stem. The study of the interaction of bovine brain lectin (BBL) with soluble glycoproteins from brain stem is important in view of the reduced ganglioside concentration in brain stem compared to grey matter and the absence of detectable BBL-interacting membrane-bound glycoproteins in brain stem.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

Bovine serum albumin, ovalbumin, trypsin (type II), D-galactose, D-glucose, D-mannose, N-acetyl glucosamine, N-acetyl galactosamine, N-acetyl neuraminic acid, Coomassie brilliant blue G, Tris, Sodium lauryl sulphate, acrylamide, N,N'-methylene bis acrylamide, N,N,N',N'-tetramethyl ethylene diamine, iodoacetic acid, guar gum, thiobarbituric acid, neuraminidase (from Clostridium perfringens), horseradish peroxidase, 4-chloro-1-naphthol, fetuin, Ulex europaeus agglutinin, wheat germ agglutinin, Phaseolus vulgaris lectin, anti-rabbit gammaglobulin, glutaraldehyde, molecular weight markers (myosin, β -galactosidase, ovalbumin), agar and thin layer chromatography plates (precoated) were purchased from Sigma chemicals company, St. Louis, USA. Coomassie brilliant blue R-250 was obtained from Pierce Chemicals Company, Rockford, USA. Tween-20, phenylmethane sulphonyl fluoride and benzamidine hydrochloride, were purchased from Fluka, Buchs, Switzerland. Silica Gel G (for thin layer chromatography) was obtained from E.Merck (India) Limited, Bombay. Nitrocellulose was from Schleicher and Schuell, Sepharose gels and DEAE-Sephadex were from Pharmacia, LKB Biotechnology, Uppsala, Sweden. 2-mercaptoethanol was from Romali and lactose was from Loba Chemie Bombay. All other

reagents used were of analytical grade.

Bovine brain tissues was obtained from local abattoir, cleaned and kept frozen at -20°C until use.

GENERAL METHODS

Anti α -galactoside antibody of IgG type present exclusively in human and old world monkey sera was prepared from human plasma by affinity chromatography on cross linked guar galactomannan gel (224).

Coupling of asialofetuin to Sepharose 4B by cyanogen bromide method

Fetuin (10 mg/ml) desialylated by heating to 80°C in 0.1 N H_2SO_4 for 1 h was dialysed to remove acid. Asialofetuin was coupled to Sepharose 4B by the cyanogen bromide method (225). Sepharose-4B (40 gram-wet weight) was suspended in 80 ml of 2 M Na_2CO_3 . Then 40 ml of distilled water was added to the suspension and the mixture was cooled in an ice bath to reach temperature range of $6-8^{\circ}\text{C}$. Cyanogenbromide (1.6 g) dissolved in minimum volume of dimethylformamide (≈ 3 ml) was added to the above gel suspension. This mixture was allowed to stir for 5 minutes at $6-8^{\circ}\text{C}$. The gel thus activated, was washed with 80 ml of

0.1 M NaHCO_3 buffer (ice cold), pH 8.5 and dried under suction. To this activated gel was added 320 mg of asialofetuin in 40 ml of 0.1 M NaHCO_3 buffer, pH 8.5, kept at 4°C and allowed to stir for 18 h. Then 0.5 ml of ethanolamine was added and the gel was stirred for 2 h (to block the excess CNBr activated groups). The gel was washed with 1000 ml of 0.1 M NaHCO_3 , pH 8.5, 150 ml of distilled water, 1000 ml of acetate buffer, pH 5.0, containing 1 M NaCl, 150 ml of distilled water and finally equilibrated in PBS.

Ricinus communis agglutinin (mixture of RCA I and RCA II) was also coupled to Sepharose 4B by the above procedure (225) at the rate of 8 mg protein per ml gel.

Coupling of lactose to Sepharose 4B

Lactose was coupled to Sepharose 4B using divinyl sulphone as cross-linker (226). Sepharose beads were activated as follows. Sepharose 4B (10 g wet weight) was suspended in 10 ml of 1 M Na_2CO_3 (pH 11.0) and 2 ml of divinylsulphone was added to it and the mixture stirred for 1 h at room temperature. Then the beads were washed with distilled water. To the wet cake of activated gel, lactose (0.2 M) in 1 M Na_2CO_3 was added and stirred for 24 h at room temperature. The reacted beads were washed successively with

1 M Na_2CO_3 (pH 11.0), 0.2 M glycine-HCl, pH 3.0 containing 1.0 M NaCl (500 ml), 1.0 M NaCl (500 ml) and finally with water (500 ml).

Protein was estimated by the method of Bradford (227) or Lowry (228). In Bradford method, 1.5 ml of the dye solution (0.06% Coomassie brilliant blue G-250 in 3% perchloric acid w/v) was mixed with 1.5 ml of the protein solution and the intensity of colour developed was measured immediately at 620 nm. Bovine serum albumin and ovalbumin were used as standards for proteins and glycoproteins respectively.

Neutral sugar was estimated by phenol-sulphuric acid method (229). To 0.5 ml of the sample, 1 ml of 5% phenol in water was added. Then 4 ml of concentrated H_2SO_4 was added to the centre of the liquid in one lot, the mixture stirred and after 15 min colour read at 485 nm. Galactose was used as a standard for neutral sugar estimation of gangliosides.

Quantitation of individual gangliosides was done by estimating the sialic acid (230) as follows.

Reagents

1. Resorcinol stock solution, 2 g in 100 ml of water.
2. Copper sulphate, 0.1 M aqueous solution.

3. Concentrated hydrochloric acid.

Resorcinol stock solution (10 ml) was added to 80 ml of concentrated HCl containing 0.25 ml of copper sulphate, and the total volume is brought to 100 ml with water. The mixture is allowed to stand for 4 h at room temperature, then stored at 4°C.

Procedure

Two milliliters of the above reagent mixture are added to 2 ml of sample, tubes are capped securely and heated for 15 min at 100°C. After heating coloured solutions were quickly cooled, extracted with 4 ml of n-butyl acetate-1-butanol(85:15), and placed in ice for 15 min. After brief centrifugation the colour intensity of the upper organic phase was measured at 580 nm.

Isolation of Ricinus communis agglutinin

This was done as described by Appukuttan et al. (231). Briefly, Ricinus communis seeds (50 g) were peeled and soaked overnight in potassium phosphate buffer, pH 7.2 containing 50 mM NaCl. Then the seeds were homogenized in a blender. Subsequent steps were carried out at 4°C. The homogenate was centrifuged at 12,000 g for 30 min and

supernatant collected. Proteins were precipitated at 70% ammonium sulphate saturation and the precipitate collected. The precipitate was dissolved in PBS was dialyzed against PBS, the clear solution loaded on to a gel made of cross-linked guar galactomannan (2 cm x 20 cm). After washing away unbound proteins with the above buffer, bound proteins were eluted with 0.2 M lactose.

Isolation of Jackfruit seed agglutinin (Jacalin)

Jackfruit seed agglutinin (jacalin) was isolated by an adaptation of procedure of Suresh Kumar et al. (232). Ten gram dehusked seed was soaked in 20 mM potassium phosphate buffer, containing 150 mM NaCl, pH 6.5 overnight. The seeds were homogenized in 70 ml of potassium phosphate buffer, pH 6.5 and stirred for 3 h at 4°C. The supernatant was made to 70% ammonium sulfate and the precipitated protein recovered by centrifugation at 16,000 g for 20 min. The precipitate was, dissolved in 10 ml of potassium phosphate buffer, pH 6.5 and dialysed against the same buffer before loading on to cross-linked guar galactomannan column (2 cm x 15 cm). The bound proteins were eluted with 150 mM galactose. The fractions containing jacalin were pooled and concentrated.

Preparation of protein-horse radish peroxidase conjugate

Horseradish peroxidase was coupled to proteins (lectin or antibodies) by the glutaraldehyde method (233). To the protein solution in PBS, HRP was added (Protein-HRP, 1:2 w/w) and to 1 ml of this mixture 75 μ l of 1% glutaraldehyde (25% stock solution diluted with water) added. The mixture was kept at room temperature for 2 h for the coupling to take place. When lectin was coupled to HRP, the hapten sugar (lactose for BBL, for example) was also added to the incubating mixture at a concentration of 0.2 M to stabilize the lectin. (Rate of denaturation of the lectin is less in presence of the hapten sugar). Then the conjugate was dialyzed against PBS (two changes) at 4°C. The conjugate was stored in ice or at -20°C if the conjugate was not prone to inactivation on freezing.

Isolation of bovine brain lectin (BBL) from bovine brain tissue.

For BBL isolation, the procedure employed was roughly as described by Caron et al. (234). Bovine brain grey matter was separated by removing patches of white matter from the cerebral cortex. Bovine brain stem was removed and

the attached blood vessels were cleaned off and kept at -20°C until use. Bovine grey matter (70 g) or a single whole brain stem was minced and homogenized separately in 250 ml PBS containing 0.2 mM phenyl methane sulphonyl fluoride, 0.2 mM benzamidine hydrochloride, 2 mM ethylene diamine tetra acetic acid, 0.1 M lactose and 5 mM 2-mercaptoethanol and stirred for 1 h and centrifuged at 16,000 g for 20 min. The supernatants were subjected to 70% ammonium sulphate precipitation with stirring for 1 h. Afterwards the precipitate was collected by a similar centrifugation as above and dissolved in minimum buffer (about 30 ml), dialysed against PBS-2ME (2 changes) to remove lactose. After centrifugation at 16,000 g for 20 min, the supernatant was loaded on to lactose-Sepharose column or asialofetuin-Sepharose previously equilibrated with PBS-2ME. Unbound proteins were removed by washing with PBS-2ME and bound proteins were eluted with 0.1 M lactose in PBS-2ME in fractions of 2 ml. Fractions were checked for the presence of protein by mixing 0.05 ml with an equal volume of Coomassie brilliant blue dye (0.06% dye in 3% perchloric acid w/v) solution. Protein containing fractions were pooled, concentrated by ultrafiltration and dialysed against PBS-2ME to remove lactose wherever necessary.

Preparation of bovine brain gangliosides

Acetone powder of the tissue was prepared from 100 g bovine brain grey matter as described by Morton (235). From the acetone powder gangliosides were extracted (by the method of Folch-Pi et al. (236)) by homogenization in chloroform-methanol (2:1, 2.1 liters) using a Polytron homogenizer. To the homogenate after filtration (through a Whatman No.1 filter paper using a Buchner funnel), 400 ml of 0.1 M KCl were added, mixture stirred for 30 min with heating at 55°C and upper phase removed using a separating funnel. The lower phase was extracted twice successively using 800 ml theoretical upper phase (chloroform-methanol-0.074% KCl, 3:48:47) each time. The combined upper phase containing gangliosides was concentrated by flash evaporation and dialysed against deionized water (2 changes) and dried by lyophilization at -80°C. Base treatment of the resulting ganglioside and subsequent chromatography on a silica gel column (2.3 cm x 12 cm) as well as further purification using ion-exchange chromatography on DEAE-sephadex A-25 column were performed as described by Ledeen and Yu (230)

DEAE-Sephadex chromatography

DEAE-Sephadex A-25 (20 g) was soaked in 300 ml solvent B (Methanol-chloroform-0.8 M aqueous sodium acetate, 60:30:8) overnight and then washed with solvent A (Methanol-Chloroform-water, 60:30:8) three times and packed in a column containing a small volume of solvent A and a supporting bed of fine glass beads on top of a glass wool plug. Further, the resin was washed with solvent A to remove all sodium acetate. The lipid (100 mg) dissolved in solvent A (100 ml) was applied on top of the gel very slowly. Then the column was washed with two column volumes of solvent A to elute all the uncharged and zwitterionic lipids. The bound gangliosides were then eluted with three column volumes of solvent B.

Base treatment and dialysis

The fraction containing gangliosides were concentrated by flash evaporation. The residue was solubilized with mild sonication in 0.1 N methanolic sodium hydroxide (50 ml). After warming at 35-40°C for 2-3 h, the mixture was quickly evaporated under reduced pressure to remove methanol. The remaining mixture was transferred to a dialysis tubing and the container rinsed with a total volume of 20-30 ml water. One milliliter of 0.5 M EDTA (tetrasodium salt) is added to eliminate the divalent cations. Dialysis

was carried out at 4°C against water (6 changes) and the contents dried by lyophilization.

For desialylation, gangliosides were dissolved in 0.1 N H₂SO₄ heated at 80°C for 1 h. After thorough dialysis against water, the samples were dried by lyophilization.

Affinity electrophoresis

Affinity of BBL for gangliosides was tested in terms of the capacity of gangliosides to arrest the mobility of BBL during electrophoresis in polyacrylamide gel at a pH 8.2. Electrophoresis in 7% polyacrylamide gel at a pH 8.2 and 4°C by the method of Davis (237) was employed using bromophenol blue as tracking dye and Coomassie brilliant blue for staining. Affinity electrophoresis was done in glass tubes of 9 cm length and 0.5 cm diameter. For incorporation in the gel, gangliosides or sugars were dissolved in ammonium persulfate solution (catalyst for gel polymerization) prior to its addition to acrylamide solution. The final concentration of gangliosides or sugars in the gel was half that in ammonium persulphate solution since it was mixed with acrylamide solution in a 1:1 ratio. A current of 3 mA per tube was applied. After electrophoresis, the gels were fixed in 12.5% trichloroacetic acid.

Inhibition of lectin-mediated hemagglutination

Rabbit erythrocytes were trypsinized by the procedure of Lis and Sharon (238). Blood was collected in anticoagulant dextrose medium. The sample of blood was diluted, 10X, with PBS and centrifuged. The supernatant was removed and cell washed twice in PBS and centrifuged. Trypsin (type II) was weighed and dissolved in PBS (1 mg/ml solution). This solution was centrifuged to remove undissolved materials. To 4 ml of trypsin solution, 0.2 ml of RBC (pipetted out with a wide mouth tip) added and the mixture kept at 37°C in a water bath for 1 h. After washing the RBC thrice with PBS, cells were made up to 5% suspension of trypsinized RBC.

Agglutination was assayed at 25°C in 10 mm x 75 mm round bottomed glass tubes in a total volume of 0.25 ml containing 1% erythrocytes. To 0.2 ml of lectin solution, 0.05 ml of 5% trypsinized rabbit RBC (TRRBC) in PBS was added and mixed. The suspension was mixed once again after 10 min and agglutination was noted after 45 min. Minimum agglutinating concentration of the agglutinin was determined using its serial two fold dilutions in 0.2 ml with the above system. For inhibition assay, serial two fold dilutions of gangliosides along with BSA (4 times in excess of

gangliosides) in total volume of 0.15 ml were treated with the minimum hemagglutinating concentration of agglutinin added in 0.05 ml and incubated for 45 min at 4°C. Then 0.05 ml 5% TRRBC was added and the mixture kept at room temperature. The suspension was mixed once again after 10 min. Agglutination titre was recorded after 45 min and from this the minimum concentration of the ganglioside to inhibit the agglutination was noted.

Detection of BBL in the gel after affinity electrophoresis

Bovine brain lectin (10 µg) was subjected to electrophoresis through each of two ganglioside-impregnated (2 mg ganglioside per ml gel) alkaline PAGE tube gel. The ganglioside concentration was sufficient to abolish the lectin band due to 10 µg BBL as judged by staining one of the tube gels. The other unfixed tube gel was minced and homogenized in 10 ml PBS and kept overnight at 4°C. After centrifugation at 1000 g, the supernatant was collected, 6 µl of which was loaded on to nitrocellulose strip and probed with anti-BBL-serum (raised in rabbit) followed by HRP conjugated anti-rabbit IgG as described in dot blotting. A control was also processed identically except that preimmune serum was used instead of anti-BBL-antibody.

Extraction and quantification of ganglioside from the polyacrylamide tube gel

Ganglioside impregnated gel before or after alkaline PAGE was sliced and homogenized in PBS using a polytron homogenizer. The homogenate was centrifuged at 1000 g and supernatant was collected. The extraction was repeated two to three times and the combined supernatant was dialysed exhaustively against water and concentrated by lyophilization. Quantification of the extracted ganglioside was done by colorimetric determination of neutral sugar.

Thin layer chromatography (TLC)

Analytical TLC: Analytical TLC was done as described by S.K. Kundu (239). Precoated TLC plates were activated by heating at 110°C for 1 h. Plates were cooled to room temperature in a vacuum desiccator. Then lipids (50-200 µg) were applied in chloroform-methanol (1:1) and allowed to dry in a vacuum desiccator for 45 min. Plates were developed using a suitable solvent system (chloroform-methanol-water 55:45:10 v/v/v containing 0.02% w/v $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ for gangliosides and chloroform-methanol-water 60:35:8 v/v/v for neutral glycosphingolipids.) Bands were visualised by

exposing the plates to I_2 vapour or by spraying resorcinol spray reagent (239).

TLC Overlay technique:

The procedure of J.L Magnani et al. was used (241). Glycolipids were separated on TLC plates (precoated). The plates after development were soaked, in PBS containing 1% polyvinyl pyrrolidone for 10 min. The plates were then layered with the HRP-conjugated lectin and incubated for 2 h or overnight in a humid chamber. Colour was developed by soaking the plates in substrate solution (4-chloronaphthol) after giving three washings in PBS.

Alternatively, the above technique was modified as follows. The developed plates were layered over with lectin or lectin coupled to HRP. Prior to colour development, the bound lectin or lectin-HRP was transferred from the TLC plates to nitrocellulose sheets by apposing the plates to nitrocellulose sheets soaked in PBS containing the hapten sugar of the lectin (this is to reverse the binding of lectin-HRP to the glycolipids). For the efficient transfer, the plates were pressed against nitrocellulose sheets and kept in this position for 1 h by placing a standard weight (1Kg) over the plate. Then nitrocellulose sheet was removed and soaked in substrate solution for colour development or

in case where unlabelled lectin is layered, after transferring the lectin to nitrocellulose, the sheets blocked in 0.2% Tween 20 for 2 h and then incubated with anti-lectin antibody coupled to HRP or anti-lectin antibody followed by second antibody coupled to HRP. In between each incubation step and also before colour development, nitrocellulose sheets were washed thrice in PBS. Colour was developed with 4-chloronaphthol as substrate.

Microtitre Plate well adsorbed ganglioside-BBL Interaction using ELISA and Enzyme linked lectin assay.

Glycolipids were coated on to polyvinyl chloride/polystyrene microtitre plate wells. For this, glycolipids were dissolved in 50% ethyl alcohol and 50 μ l of this solution added to each well and the solvent allowed to evaporate at 37°C. The wells were blocked by incubating with 5% BSA in PBS (0.2 ml) for 2 h at room temperature. Hundred μ l HRP-coupled lectin or lectin (30 μ g/ml) was added to each well and incubated overnight at 4°C. Wherever unlabelled lectin was used, the lectin incubation was followed by a similar incubation with anti-lectin antibody coupled to HRP (10 μ g/ml) for 2 h at 4°C. Wells were washed three times with (200 μ l each) PBS in between each incubation step and also before colour development. Colour was developed with

the substrate orthophenylene diamine according to the manufacture's instructions (Sigma Chemicals Co, USA). The reaction was terminated after 15 min by adding 50 μ l of stopping solution. The colour intensity was measured using an ELISA reader.

Liposome Preparation

Liposomes were prepared by Huang's method (242) using egg lecithin, cholesterol and gangliosides. Briefly, 15 mg egg lecithin, 4 mg cholesterol and 3 mg native mixed gangliosides were dissolved in 15 ml of chloroform-methanol, 2:1, in a 50 ml round bottom flask. The solvents were evaporated under reduced pressure to get a thin film. The film was dried overnight in a desiccator and then suspended in PBS and sonicated for 2 h under nitrogen at 4°C. The liposome suspension was stored at 4°C in contact with nitrogen. Liposomes containing desialylated gangliosides were also prepared as described above. But here, only two-third the concentration of ganglioside used for liposome containing native gangliosides were used so as to keep the molar ratio of ganglioside comparable.

Interaction of individual gangliosides with brain lectin

Interaction of brain lectin with individual ganglioside species (GM1, GD1b, GM2, GD1a and GT1b) was studied in terms of the ability of each ganglioside to retain BBL during alkaline polyacrylamide gel electrophoresis. Each ganglioside sample in 50 μ l PBS was mixed with BBL (3 μ g in 10 μ l PBS) and the mixture incubated for 2 h at 4°C. The amount of ganglioside used was as follows. GM1 (79 μ g), GD1b (42 μ g), GM2 (38 μ g), GD1a (102 μ g) and GT1b (23 μ g). For calculating the amount of ganglioside used, sialic acid was estimated (230); from the weight of sialic acid and knowing the chemical formula of each ganglioside, the weight of individual gangliosides were calculated. After incubation, the mixture was loaded on to polyacrylamide tube gel (9% acrylamide) and subjected to electrophoresis at 4°C. Only the unbound lectin moved down and the intensity of the lectin band was measured using a gel scanner. A control was performed by incubating the ganglioside with BBL in presence of lactose for GM1 ganglioside and GT1b ganglioside. A reference standard for BBL was done by running 3 μ g of BBL in a tube gel.

Isolation of soluble RCA-binding glycoproteins from brain stem

Bovine brain stem (50-70 g) as a whole was homogenized using a Polytron homogenizer in PBS containing 0.2 mM phenylmethane sulphonyl fluoride, 2 mM Benzamidine hydrochloride and 2 mM ethylene diamine tetra acetic acid. It was then stirred for 1 h at 4°C and centrifuged at 100,000 g for 1 h. The supernatant after dialysis against PBS was loaded on to RCA-Sepharose column (2.3 cm x 10 cm) previously equilibrated with PBS. After removing the unbound protein by washing with PBS, the bound proteins were eluted with 0.1 M lactose in PBS and collected in fractions of 2 ml. Proteins containing fractions, as judged by mixing 100 µl of sample with 100 µl of Coomassie brilliant blue dye solution (0.06% dye in 3% perchloric acid), were pooled and concentrated by ultrafiltration using an Amicon PM10 membrane.

Dot blotting

Nitrocellulose strips (5 mm x 5 mm) were blotted with 2 µl PBS containing the appropriate amount of protein or glycoprotein (2-8 µg) and allowed to dry in air and then blocked with either 0.2% Tween 20 or 5% BSA in PBS for 2 h at room temperature. The strips were incubated for 2 h with

lectin (30 $\mu\text{g/ml}$) or the HRP conjugate of lectin (30 μg lectin/ml) or antiserum (50% ammonium sulphate fraction; 10 $\mu\text{g/ml}$) in 0.05% Tween 20 or 1% BSA in PBS at 4°C. Wherever strips were incubated with the unlabelled lectin, the bound lectin was followed using HRP-conjugated anti-lectin antibody. A control for antibody binding was performed with preimmune serum. For lectin binding, the control strip was incubated with lectin preincubated with its hapten sugar (0.1 M). Strips were washed thrice in PBS in between incubations and before colour development. Colour was developed by incubating the strips for 5 min in 0.2 ml of 4-chloronaphthol substrate (1 ml 0.3% 4-chloronaphthol in anhydrous methanol plus 5 ml PBS and 3 μl 30% H_2O_2). A well defined blue dot represented positive interaction.

Polyacrylamide gel electrophoresis

(a) Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were resolved by SDS-PAGE under reducing conditions in 8% acrylamide (for western blotting) and 10% acrylamide (for subunit molecular weight determination and electroelution) as described by Laemmli (243). For preparative and analytical purpose, SDS-PAGE was done using vertical slab gel of 1.5 mm thickness in a minigel unit

(8.3 x 7.3 cm - Hoefer scientific). A 3% spacer gel was layered over the separating gel.

Gel containing 3% (spacer gel) 8% or 10% acrylamide were prepared from a stock solution of 30% by weight of acrylamide and 0.8% by weight of N,N-bis methylene acrylamide. Separating gel of 10% acrylamide concentration was prepared by mixing.

- (a) 6 ml acrylamide (stock solution)
- (b) 11 ml buffer (0.614 M Tris-HCl adjusted pH 8.8 with HCl containing 0.164% SDS w/v)
- (c) 0.9 ml ammonium persulfate (15 mg/ml) and
- (d) 0.02 ml TEMED

The Spacer gel was prepared by mixing

- (a) 1 ml acrylamide (stock solution)
- (b) 8.5 ml buffer (0.147 M Tris, pH adjusted to 6.8 with HCl, containing 0.108% SDS w/v)
- (c) 0.5 ml ammonium persulfate (15 mg/ml) and
- (d) 0.01 ml TEMED

Protein sample containing 1% SDS, 10% glycerol and 5% 2-ME was heated in a boiling water bath for 2 min and cooled before loading on to the gel. Bromophenol blue was used as tracking dye.

Electrophoresis was carried out using a buffer system (chamber buffer, pH 8.3) containing 0.025 M Tris, 0.192 M glycine and 0.1% SDS, at a constant current of 15

mA. The gel was fixed in 50% methanol containing 0.075% formaldehyde for 45 min.

The gel was stained for protein by treating with Coomassie brilliant blue R-250 staining solution (44 ml methanol, 44 ml H₂O, 12 ml glacial acetic acid containing 0.125 g Coomassie brilliant blue R-250) for 45 min and destained by heating in the destaining solution (50 ml methanol, 75 ml glacial acetic acid and 875 ml H₂O). The staining and destaining were done according to Weber and Osborn (244)

Alkaline polyacrylamide gel electrophoresis (Alkaline PAGE)

Alkaline PAGE was done by the method of Davis (237). For tube gels, glass tubes of 9 cm length and 0.5 cm diameter were used. Vertical mini slab gel (8.3 cm x 7.3 cm) was used for alkaline slab gel electrophoresis.

Reagents

Solution A: 1 N HCl - 24 ml

(pH 8.8-9.0) Tris - 18.1 g

TEMED - 0.12 ml

Made upto 100 ml with water

Solution B: 1 N HCl - 48 ml

(pH 6.6-6.8) Tris - 5.98 g

TEMED - 0.46 ml

Made upto 100 ml with water

Solution C: Acrylamide - 28 g

Bis acrylamide - 0.735 g

Made up to 100 ml with water

Solution D: Acrylamide - 20 g

Bis acrylamide - 0.5 g

Made up to 100ml with water.

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

Solution G: Ammonium persulphate 1.4 mg/ml

Gel preparation

Separating gel: To the mixture of solution A and C
(1 part A and 1 part C) Solution G added in 1:1 ratio

Spacer gel

1 part Solution B

1 part Solution D

1 part Solution E

5 parts water

Spacer gel polymerized in presence of light

Reservoir buffer (pH 8.3)

Tris - 6 g

Glycine - 28.8 g

Made upto 1 litre with water

Bromophenol blue was used as the tracking dye. Sample was loaded in 10% glycerol. Gel was fixed in 12.5% trichloro acetic acid for 45 min and stained for protein by keeping in Coomassie brilliant blue dye solution (44 ml methanol, 44 ml water, 12 ml glacial acetic acid and 120 mg Coomassie brilliant blue R-250) for 45 min. Destaining was performed as described in SDS-PAGE. Alkaline PAGE done in slab gel was fixed in 50% methanol containing 0.075% formaldehyde.

Acid-Polyacrylamide gel electrophoresis (Acid PAGE)

Acid PAGE was done in vertical slab gel apparatus (8.3 x 7.3 cm-Hoefer Scientific) as described by Reisfeld et al. (245) in 7.5% acrylamide gel.

Reagents

Solution A: 1 N KOH - 48 ml

(pH 4.3) Glacial acetic acid - 17.2 ml

TEMED - 4 ml

Made up to 100 ml with water

Solution B: 1 N KOH - 48 ml

(pH 6.6-6.8) Glacial acetic acid - 2.87 ml

TEMED - 0.46 ml

Made up to 100 ml with water.

Solution C: Acrylamide - 30 g

Bis acrylamide - 0.8 g

Made upto 100 ml with water.

Solution D: Acrylamide - 20 g

Bis acrylamide - 0.5 g

Made up to 100 ml with water

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

Solution G: Ammonium persulfate, 2.8mg/ml in water

Solution F: Methylene blue, 0.005% solution

(Tracking dye)

Gel preparation

A. Separating gel (7.5% acrylamide)

1 part A, 2 parts C and 1 part H₂O mixed and to this mixture is added solution G in 1:1 ratio.

B. Spacer gel

1 part B

1 part D

1 part E

5 parts H₂O

polymerized under fluorescent light

Reservoir buffer: 0.05 M β -alanine, pH adjusted to 4.5 with acetic acid. Fixing, staining and destaining was done as described for alkaline PAGE.

Electroelution

The method described for electroelution of DNA from agarose gel (246) was adapted for protein elution from acrylamide gel. In the case of slabgel SDS-PAGE, soon after electrophoresis, a portion of the slab gel was fixed, stained and destained to serve as a reference to cut out the required protein bands from the other portion of the gel which was kept at -20°C after the run. Similarly, for electroelution from alkaline PAGE tube gels, one of the tube gels was used as a reference. A correction factor for the shrinkage of unfixed gel kept at -20°C was applied using the formula.

$$\text{Position of the protein band in unfixed gel} = \frac{\text{Length of the unfixed gel}}{\text{Length of the fixed gel}} \times \text{Position of the band in the fixed gel}$$

Gel slices containing the required protein band were minced with a scalpel blade and kept in Tris-acetate buffer (5 mM Tris; 2.5 mM, acetic acid, pH 8.0) in a dialysis bag and immersed in the same buffer in a horizontal electrophoresis

chamber. A current of 100 V was applied across the bag for 3 h. Electroelution was performed at 4°C for alkaline PAGE tube gel slices and at room temperature in the case of SDS-PAGE slab gel slices (this was to prevent the SDS from precipitating at low temperature). Current was reversed for 5 min to detach the proteins adhered to the sides of dialysis bag facing the positive electrode. Contents of the bag was centrifuged at 1000 g and the supernatant containing the protein was concentrated by lyophilization. In the case of SDS-PAGE separated proteins, SDS was removed by precipitation of proteins by methanol (20 volumes of methanol added and kept at -20°C overnight and then centrifuged at 1000 g to get the protein pellet). The precipitated protein was redissolved in minimum PBS.

Identification of BBL-interacting proteins by Western blotting

The RCA binding glycoproteins were separated by SDS-PAGE in 10% gel under reducing conditions as described by Laemmli (243). A portion of the gel containing molecular weight markers and glycoproteins was fixed in 50% methanol and stained with Coomassie brilliant blue. The glycoprotein in the other protein of the gel was transferred electrophoretically to nitrocellulose according to Towbin

et al. (247) using transfer buffer consisting of 25 mM Tris, 192 mM glycine and 15% methanol, pH 8.3 and a current of 0.8 mA/cm² for 2 h at 25°C. A portion of the nitrocellulose was stained with amidoblack to make sure that proteins are transferred from the gel to nitrocellulose. The rest of the sheet was blocked in 0.2% Tween 20 for 2 h, dried and stored at 4°C. Just before use, the sheets were further blocked in 5% BSA in PBS for 2 h at room temperature. The sheets were incubated with BBL-HRP (50 µg/ml) in 1% BSA in PBS for 2 h and washed thrice with PBS before colour development with 4-chloronaphthol (as described in dot blotting)

Molecular weight determination

Subunit molecular weight of proteins were determined by SDS-PAGE under reducing conditions. Molecular weight standards used were myosin (205 KDa) β -galactosidase (116 KDa), BSA (66 KDa), and ovalbumin (45 KDa). Mobility of the protein was compared to the mobility of standards in either 8% or 10% acrylamide concentrations. Molecular weight was calculated from a plot of log molecular weight verses the relative mobility of the standards. Average of the molecular weight obtained using 8% acrylamide gel and 10% acrylamide gel was calculated.

Raising antibodies to electroeluted proteins

Antiserum to the electroeluted proteins were produced in mice by repeated intradermal injection of 10 μ g protein sample in 100 μ l PBS. First injection was given after mixing the protein with Freund's complete adjuvant (1:1 v/v). For subsequent injections, protein was mixed with Freund's incomplete adjuvant (1:1 v/v) keeping an interval of 14 days between injections. Presence of antibody in the serum was ascertained by immunodiffusion (Ouchterlony double diffusion) and dot blot analysis. Dot blot analysis was done using HRP conjugated antiserum prepared as described earlier.

Neuraminidase treatment

Nitrocellulose strip (5 mm x 5 mm) was blotted with the protein in 2 μ l PBS. After blocking with 5% BSA in PBS for 2 h, the strip was transferred to 150 μ l of citrate-phosphate buffer (0.5 M, pH 5.0) containing 1% BSA and neuraminidase enzyme (0.8 units, Clostridium perfringens, type V) and then incubated at 37°C for 2 h. The strip after washing thrice with PBS, was kept in boiling water for 2 min to stop the enzyme activity and then incubated with HRP conjugated lectin for 2 h. The strip was washed thrice

and colour developed using 4-chloronaphthol as substrate. Control strip was processed in a similar fashion except that no enzyme was added in the incubation buffer.

Carboxymethylation of glycoproteins

Carboxymethylation of soluble RCA binding glycoproteins was done as described by Gurd (248). One milligram of the protein in 1 ml of 0.1 M NaHCO_3 containing 20 mM of 2-ME was flushed with nitrogen, sealed with parafilm and incubated for 1 h at 37°C. One milliliter of 200 mM iodoacetic acid in 0.1 M NaHCO_3 (pH adjusted to 8.5 with NaOH) was added to the protein solution, flushed with nitrogen, sealed and incubated for 2 h in dark at 37°C. Then the solution was thoroughly dialysed against PBS at 4°C.

High performance liquid chromatography (HPLC)

(a) Gel filtration

The glycoproteins were subjected to gel filtration using an HPLC gel filtration column, G4000 SW. Peaks were monitored with the help of a detector (UVICORD-II) and fractions were collected using a fraction collector.

(b) DEAE-Chromatography

This was carried out using the HPLC ion exchange column TSK DEAE -3 SW and LKB Broma HPLC system. Peaks were monitored on a UVICORD-II detector and fractions collected using LKB Redi Rac fraction collector. The column was operated at a pressure of 20 bar and a flow rate of 0.5 ml/min. A linear gradient of sodium acetate (0.1 M) was used for elution (0% sodium acetate to 100% sodium acetate over a period of 30 min).

CHAPTER III

RESULTS AND DISCUSSION

PART I

**Sugar specific interaction of bovine brain lectin
with endogenous gangliosides**

Many proteins like cholera toxin (108), tetanus toxin (109), botulinum neurotoxin (110) are known to bind gangliosides. But the number of endogenous ganglioside - binding proteins characterised so far is very limited. They include serum albumin (190) and myelin basic protein (191,192). Being membrane components of cells, with their carbohydrate groups projecting out, gangliosides are most likely to be involved in signal transduction through recognition of their sugar moieties by extracellular molecules. The presence of autoantibodies to gangliosides in the sera of patients with neurological disorders (160,161,164,171) and the effect of monoclonal antibodies towards gangliosides in altering the behaviour of tumor cells (174) has been shown by many investigators. The binding of brain lectin to gangliosides is significant in the above context, especially when antibodies concerned are directed against the carbohydrate groups of gangliosides. Inflammatory states such as cancer, infection etc. are known to cause breaches in the blood-brain barrier there by facilitating entry of serum IgG into the brain parenchyma

(320). Even otherwise intrathecal synthesis of IgG antibodies in brain has been observed (321). Gangliosides are sialoglycosphingolipids and contain galactose residues in their oligosaccharide structure. Therefore they are likely to interact with 14 KDa galactose-binding lectin which is widely distributed in mammalian brain. Thus we tried different methods like affinity electrophoresis, hemagglutination inhibition etc. to examine whether bovine brain lectin (BBL) interacts with endogenous gangliosides.

Affinity electrophoresis to show the interaction of BBL with gangliosides

Affinity electrophoresis has been employed by various workers in such studies as the interaction between phosphorylase and glycogen, α -amylase and starch, Concanavalin A and sugars and dextran-specific myeloma protein and dextrans (249). In this method, one of the components is immobilized within the gel and the other one which is likely to interact with the former is allowed to move down through the gel under the influence of an electric field.

A prerequisite for use of affinity electrophoresis as a tool to study binding of ligands to proteins is that the proteins should not be denatured under the conditions of

electrophoresis. In our trials when BBL run in alkaline PAGE at pH 8.2 in 7% gel was eluted from the gel to PBS either by passive diffusion or by electroelution, the eluted lectin continued to be active in sugar recognition, as demonstrated by hemagglutination (data not shown). The withdrawal of sulphhydryl reagents such as 2-ME for the duration of electrophoresis and electroelution also did not detectably affect the activity of the lectin.

In our experiments, we incorporated bovine brain ganglioside mixture (2 mg/ml) in the polyacrylamide tube gel during polymerization by dissolving the gangliosides in the catalyst (ammonium per sulfate) solution. Normally, on electrophoresis at pH 8.2, BBL moved as a distinct band. But this movement as a band was abolished when ganglioside was present in the gel (Fig 1). This retention of BBL by gel impregnated ganglioside was sugar-dependent since lactose 50 mM, when present in the gel, restored the BBL band, while sucrose(50 mM) was ineffective.

Desialylated gangliosides (2mg/ml) when incorporated in the gel, instead of native gangliosides, also gave similar results (Fig.2) to native ganglioside mixture.

Dependence of the interaction between ganglioside and lectin on ganglioside concentration was also tested. When ganglioside concentration increased from gel to gel

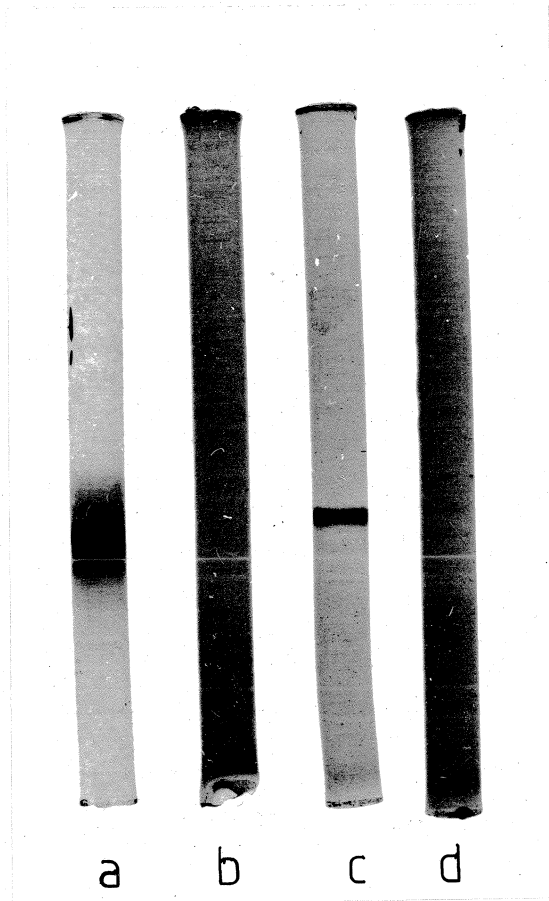


Fig.1. Affinity electrophoresis of BBL at pH 8.2 in 7% polyacrylamide gel containing native bovine brain grey matter gangliosides [All gels except gel a contained gangliosides (2 mg/ml). Sugars incorporated were lactose (c - 50 mM) and sucrose (d - 50 mM). BBL (10 μ g) was run in all gels]. (Details are given in text)

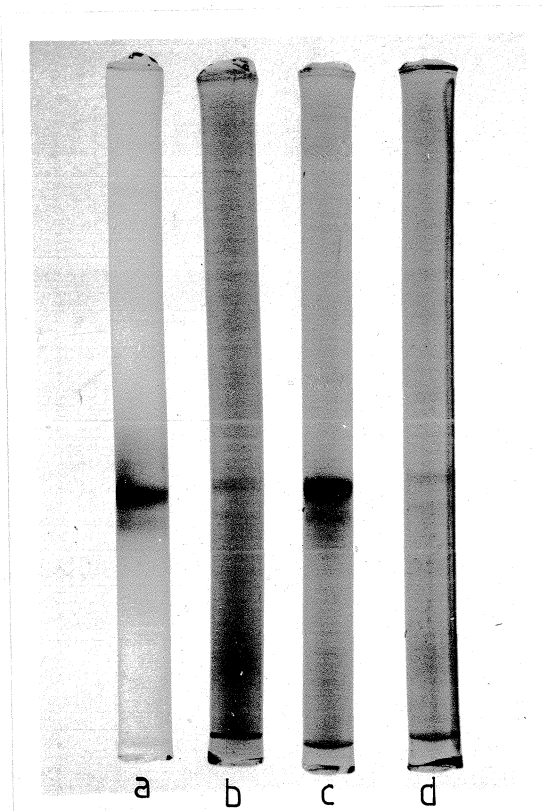


Fig.2. Affinity electrophoresis of BBL at pH 8.2 in 7% polyacrylamide gel containing desialylated bovine brain grey matter gangliosides (b,c,d - 2 mg/ml), lactose (c - 50 mM) and sucrose (d - 50 mM) [BBL (10 μ g) was run in all gels]. (Details are given in text).

(10-2000 $\mu\text{g/ml}$) there was a proportionate decrease in the intensity of the BBL band (Fig.3).

Dependence of the reversibility of the ganglioside lectin interaction on concentration of inhibiting sugar was found out by incorporating different concentrations of lactose. When lactose concentration was increased from gel to gel (0.2 mM to 50 mM), keeping the ganglioside concentration constant (2 mg/ml), we observed a proportionate increase in the restoration of the BBL band (Fig.4). The BBL binding to gangliosides was almost fully reversed at 50 mM lactose concentration. This shows the dependence of the sugar reversibility of the interaction on lactose concentration. The lack of reversibility of binding in presence of sucrose (50 mM) confirms the sugar specificity of the interaction as well as disproves the involvement of solute effect as a cause for the reversibility. The decrease in intensity of lectin band with increase in concentration of ganglioside may be due to the attachment of a fraction of the lectin to the gangliosides while the rest moves down to give a less intense band. Thus when ganglioside concentration was raised to 2mg/ml there was complete retention of lectin by the gangliosides. The retention rather than a retardation may be possibly due to high affinity binding offered by the cluster of sugar residues on the surface of the ganglioside

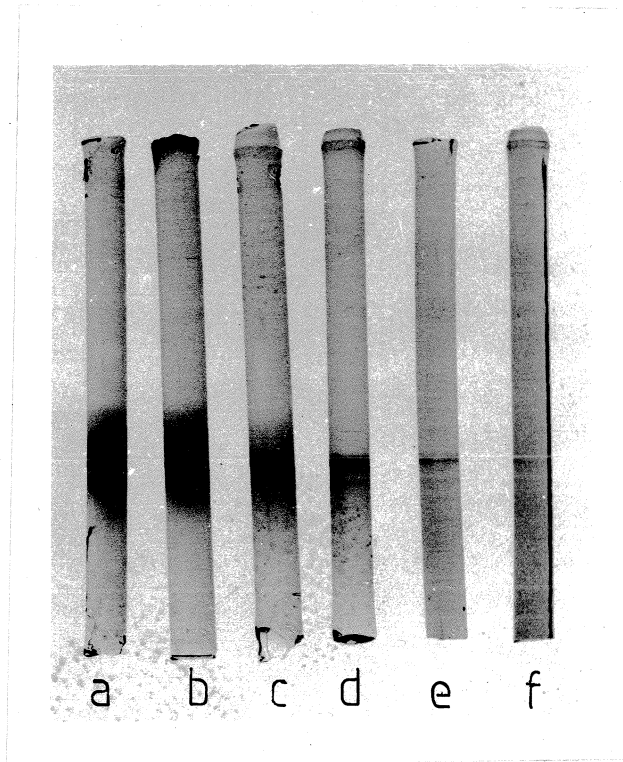


Fig.3. Dependence of retention of BBL on ganglioside concentration in electrophoresis at pH 8.2 in 7% polyacrylamide gel [Concentrations of gangliosides incorporated in gels ($\mu\text{g/ml}$) were: a, 0; b, 10; c, 50; d, 100; e, 1000; and f, 2000. BBL run in each gel was 10 μg]. (Details are given in text).

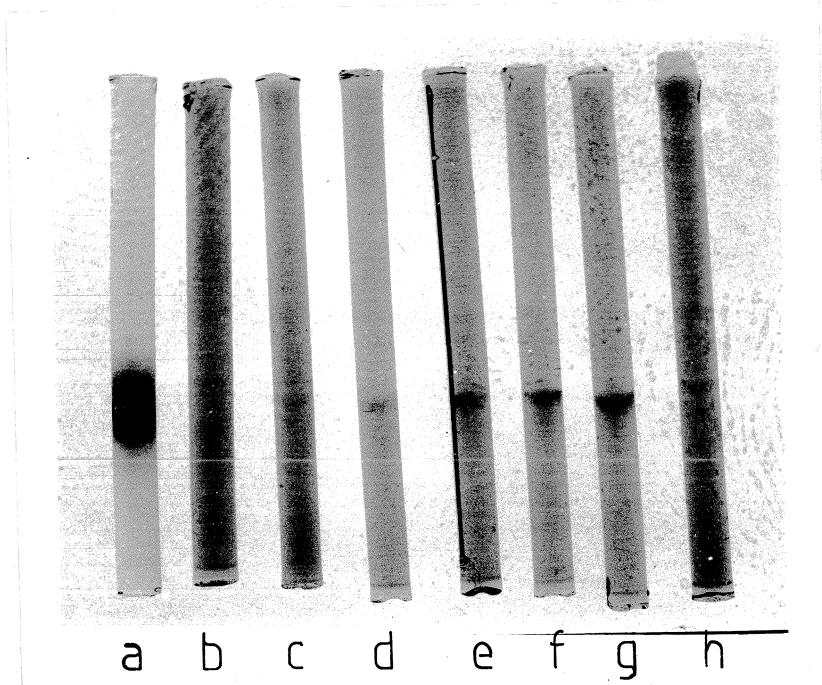


Fig.4. Effect of increasing concentrations of lactose in reversing ganglioside-mediated retention of BBL in electrophoresis in 7% polyacrylamide gel at pH 8.2 [Gel-incorporated ligands were: native gangliosides (gel b to h, 2 mg/ml), lactose (c, 0.2 mM; d, 2 mM; e, 5 mM, f, 10 mM; g, 50 mM and sucrose (h, 50 mM). In all gels 10 μ g BBL was run]. (Details are given in text).

micelle. Baenziger and Fiete in their study of interaction of immobilized Con A with glycopeptides showed that glycopeptides with association constants in the range of $4.5 \times 10^6 \text{ M}^{-1}$ to $25 \times 10^6 \text{ M}^{-1}$ were retained by Con A-agarose column, while glycopeptides with the association constants in the range of $0.3 \times 10^6 \text{ M}^{-1}$ to $4.0 \times 10^6 \text{ M}^{-1}$ were not retained but retarded on a Con A-agarose column (250).

The high affinity of BBL towards gangliosides in the polyacrylamide gel may be due to the formation of smaller micelle within the gel. In aqueous medium mixed ganglioside micelle could be 300 KDa big (251) but the pore size of the cross-linked 7% polyacrylamide may not exceed 200 KDa (252). This could necessitate formation of smaller micelle and thus would cause more efficient retention of BBL. Notably, many glycosidases act on ganglioside micelle only after they are disintegrated into smaller micelle by detergents (253).

Affinity electrophoresis could not be performed at the physiological pH of 7.4 due to very low mobility of BBL and the formation of multiple bands at this pH.

Detection of BBL in the ganglioside impregnated gel after affinity electrophoresis

When the supernatant from a PBS homogenate of the polyacrylamide gel containing impregnated ganglioside and

through which BBL was run for affinity electrophoresis was dot-blotted on nitrocellulose strip and probed with anti-BBL antibody (raised in rabbit) followed by anti-rabbit IgG-HRP, it was observed that BBL was actually retained in the gel by the gangliosides (Fig.5). Control gel (ganglioside alone) when processed in a similar fashion did not give any positive interaction. This result positively proved that gangliosides bound and retained BBL during affinity electrophoresis.

Estimation of gangliosides present in the gel after affinity electrophoresis

Gangliosides are negatively charged at pH 8.2 of the affinity electrophoresis. Therefore there is a chance that gangliosides may also move out of the gel during electrophoresis. But ganglioside molecules, being amphipathic, form micelle in aqueous medium above their critical micellar concentrations which for mixed gangliosides ranges from 7×10^{-9} M to 2×10^{-8} M (254). Since the concentration of mixed gangliosides used in affinity electrophoresis in our study exceeds this range ($>10 \mu\text{g/ml}$), gangliosides incorporated in the polyacrylamide gel were in the micellar form. These micelles have molecular weights in the neighbourhood of 300 KDa (251) and therefore at the

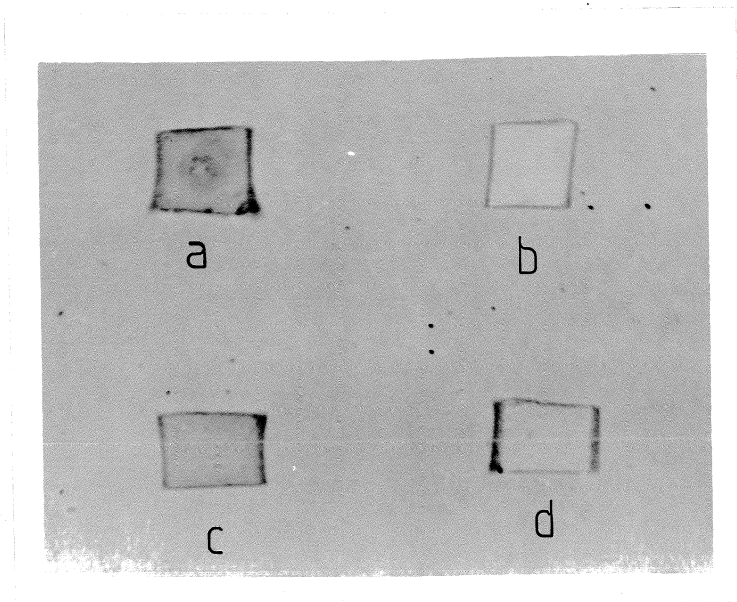


Fig.5. Recovery of BBL retained by ganglioside in affinity electrophoresis gel. Dot blots of concentrate of PBS extract of polyacrylamide gel containing ganglioside after electrophoresis of either BBL sample (a) or sample buffer (b) was probed with rabbit anti-BBL serum followed by anti-rabbit IgG-HRP. Control blots c and d had samples of a and b respectively, but treated with preimmune rabbit serum.

acrylamide concentration used here (7%), could be immobilized in the pores of the gel (pore size may not exceed 200 KDa (252)) during electrophoresis though they are negatively charged. This assumption was verified by estimating ganglioside in the gel after affinity electrophoresis.

The ganglioside impregnated gel after affinity electrophoresis was cut into pieces without fixing and the slices were homogenised. After centrifugation, the neutral sugar content of the supernatant was estimated and the amount of ganglioside present calculated by comparing with the neutral sugar of a known amount of ganglioside. It was found that 83% of the ganglioside originally added was recoverable from the gel after affinity electrophoresis. This data confirmed that ganglioside was retained in the gel in an immobilized state.

Gel porosity is not affected appreciably by ganglioside incorporation

To establish that incorporation of ganglioside does not reduce the porosity of acrylamide gel to the extent that mobility of any protein is affected, 10 μ g of ovalbumin was subjected to electrophoresis through the ganglioside incorporated gel (2 mg ganglioside per ml gel). As shown in

Fig.6, the mobility of ovalbumin was not affected by the incorporated ganglioside. But there was a slight decrease in the intensity of ovalbumin band in the ganglioside impregnated gel compared to the control (gel without ganglioside). This may be due to the earlier reported capacity of albumin to bind gangliosides (255). However this ganglioside - ovalbumin interaction was not sugar-dependent as lactose could not reverse the binding, unlike the interaction of brain lectin with gangliosides. It is known that binding of albumin to gangliosides is sugar independent (255). Such binding may be mediated through hydrophobic interaction of these proteins with the lipid-portion of ganglioside.

Gangliosides did not undergo chemical modification under the conditions of affinity electrophoresis

On TLC, the pattern of mixed gangliosides extracted from polyacrylamide gel after electrophoresis was found to be similar to that of the original gangliosides (Fig.7). Thus the structural integrity of the gangliosides remained unaffected under the conditions of gel polymerization or electrophoresis.

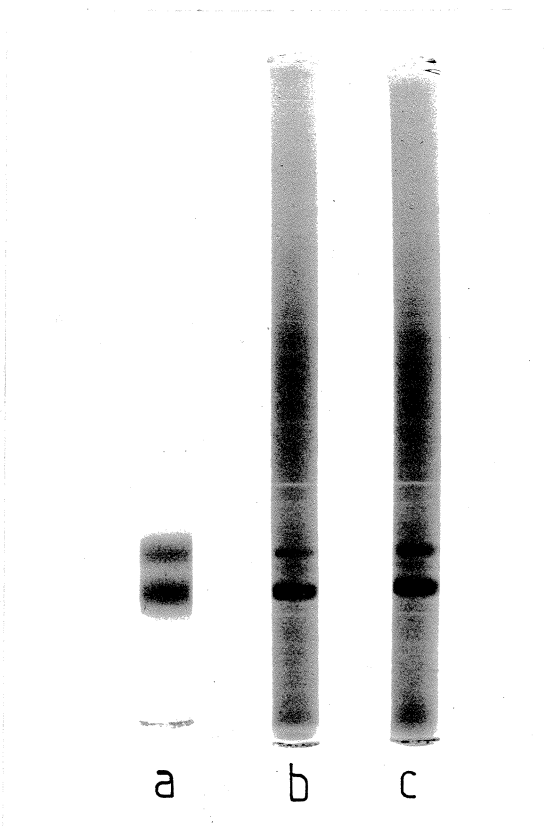


Fig.6. Electrophoresis of ovalbumin (10 μ g) at pH 8.2 in 7% polyacrylamide gel (a) without gangliosides (b) containing 2 mg/ml bovine brain grey matter gangliosides (c) containing 2 mg/ml bovine brain grey matter gangliosides and 50 mM lactose. (Details are given in text).

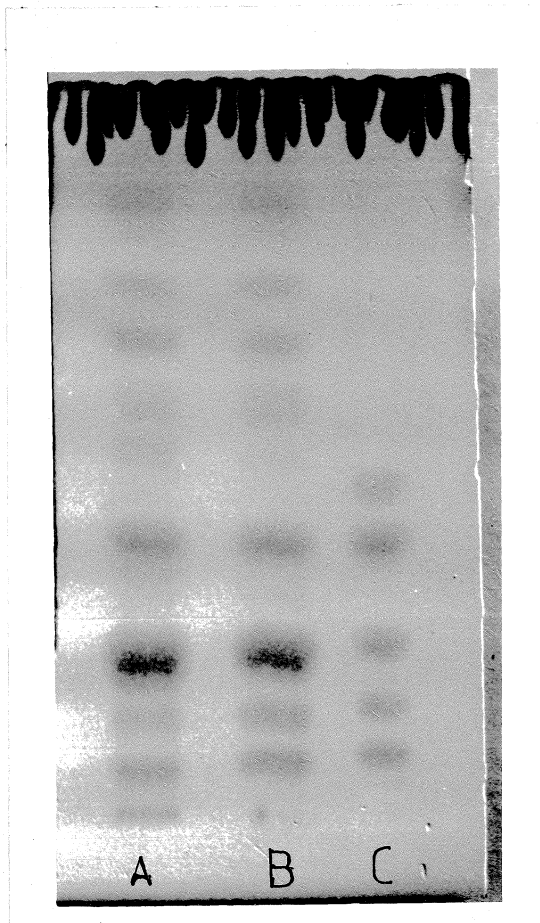


Fig.7. TLC pattern of gangliosides before and after affinity electrophoresis. Lane A native ganglioside mixture (200 μ g); Lane B-ganglioside mixture extracted from polyacrylamide gel after affinity electrophoresis, lane C-standard gangliosides from top to bottom GM2, GM1, GD1a, GD1b, GT1b.

BBL-interacting glycoproteins are absent in the ganglioside sample.

The possibility of ganglioside sample extracted from acetone powder to Folch upper phase being contaminated by traces of glycoproteins could not be ruled out. To check for a possible contribution of contaminating glycoproteins in ganglioside mixture towards BBL-binding, nitrocellulose strips dot blotted with the ganglioside sample were probed with BBL-HRP conjugate. Results (Fig 8) show that binding of the conjugate to 24 μg of mixed ganglioside was negative. In contrast in presence or absence of the same amount of ganglioside, 6 μg of asialofetuin strongly bound the conjugate. This positive control made sure that ganglioside no way interfered with the recognition of glycoprotein by BBL. It is clear from this observation that BBL-binding glycoproteins were absent or undetectably low in the ganglioside sample used for affinity electrophoresis. So the ganglioside-binding of BBL was solely due to the presence of ganglioside and contaminating glycoproteins, if any, did not contribute towards BBL-binding.

BBL-ganglioside interaction is sugar mediated

The hydrophobic part of the lipid may interact with the hydrophobic patches on protein molecule. The number

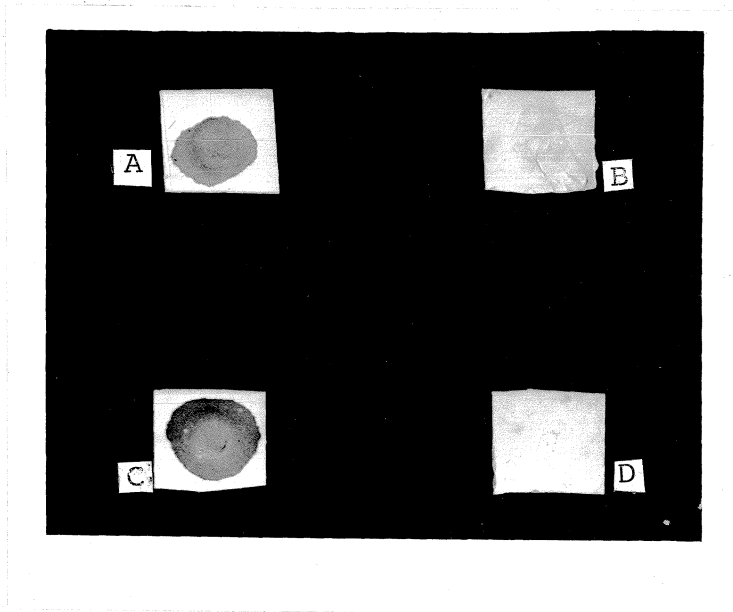


Fig.8. BBL binding to nitrocellulose-coated ganglioside and asialofetuin [nitrocellulose strips (5 mm x 5 mm) were blotted with 6 μ g asialofetuin (A,B), 24 μ g gangliosides (D) or a mixture of 6 μ g asialofetuin and 24 μ g ganglioside (C), dissolved in PBS - 2 ME. Strips were then blocked using 5% BSA overnight, and treated with BBL-HRP corresponding to 5 μ g BBL in PBS - 2 ME containing 1% BSA for 1 hr, in the absence (A,C,D) or presence (B) of 50 mM lactose. After three washings, 5 min each in PBS - 2ME, strips were stained with 4-chloronaphthol]. (Details are given in text).

and distribution of hydrophobic patches depends on the conformation of the protein. It is known that the binding of ligand to protein lead to changes in conformation of the protein. Kaar Sholm et al. reported the ligand-induced conformation changes in folate-binding protein (256). Opinions differ as to the effect of sugar on lectin conformation. Clerch et al. reported that the presence of ligand may not induce a large change in protein folding (the circular dichorism spectrum of the rat 14 KDa lectin did not alter appreciably in the presence of lactose (323)). But the results of Abbott and Ten Feizi suggests that there is a general tightening up of the protein folding, following ligand binding (59).

In the case of BBL-ganglioside interaction, there is a possibility that BBL may interact with ganglioside through a hydrophobic binding (between the hydrophobic part, namely the ceramide moiety of ganglioside and hydrophobic regions on BBL) and getting detached from the ganglioside owing to a change in conformation of BBL induced by the hapten sugar (example; lactose). To check this possibility, GM1 ganglioside was mixed with excess (200 μ g) of RCA (RCA is known to interact with GM1 by binding to the sugar groups on GM1 (257)) and then incubated with BBL and loaded on to 9% polyacrylamide tube gel. On electrophoresis at pH 8.2, BBL moved as a band whereas free GM1 stayed almost at

the top of the gel. As is evident from the Fig.9 when BBL was incubated with GM1 (3 μ g) and then loaded on the gel prepared as above, most of the BBL remained at the top of the gel along with GM1. But when GM1 was preincubated with excess of RCA (200 μ g) and then incubated with BBL (3 μ g), the BBL band appeared almost similar in intensity to that of a control gel electrophoresis in which the loaded sample consisted of BBL preincubated with lactose (0.1 M) for 1 h and then with GM1. No interaction between BBL and RCA was observed as BBL moved down as a band when the incubated mixture of these two proteins was loaded. These results indicate that BBL-ganglioside interaction is sugar mediated as BBL failed to bind GM1 when the sugar moiety of ganglioside was occupied by RCA. Thus we ruled out the possibility that BBL got bound to ganglioside through some hydrophobic interaction and got detached from ganglioside by a change in conformation of BBL induced by the presence of lactose.

Inhibition of BBL-mediated hemagglutination by gangliosides

Bovine brain lectin, like many other lectins, agglutinates trypsinized rabbit red blood corpuscles (TRRBC). Preincubation of the lectin with the interacting glycoconjugates abolishes the agglutination. As the sugar

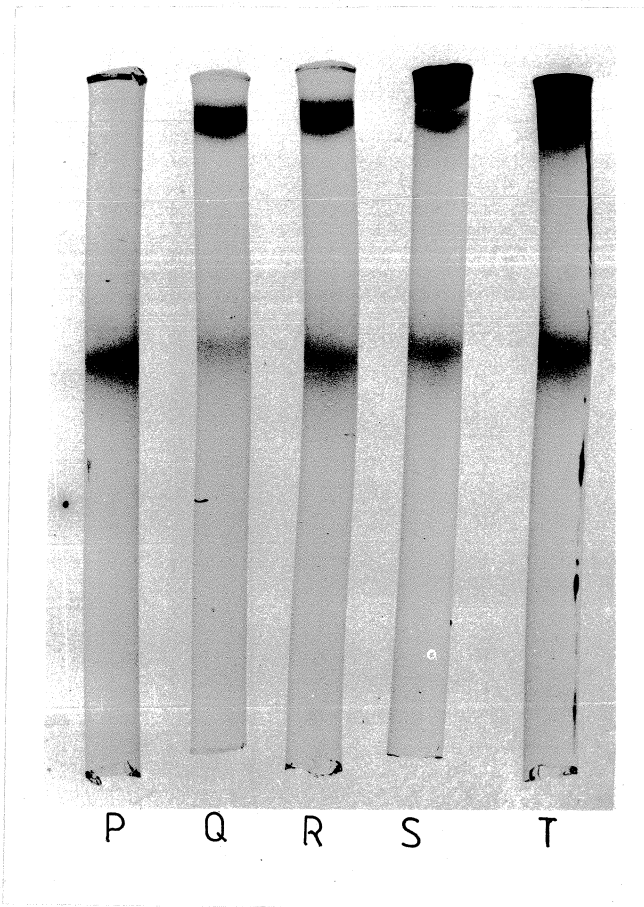


Fig.9. Demonstration of the sugar-dependence of the interaction between ganglioside and BBL by affinity electrophoresis of the lectin along with GMI ganglioside that had been preincubated with RCA, in 9% polyacrylamide gel at pH 8.2. Gels were stained with Coomassie blue after run. Samples applied to gels were: P-BBL (3 μ g): Q-BBL incubated with GMI, R-BBL incubated with GMI in presence of 0.1 M lactose, S-BBL incubated with GMI which had been previously incubated with RCA, T-BBL incubated with RCA. Details are in the text.

binding sites of the lectin is occupied by the added glycoconjugate, it can no more bind to the glycoconjugates on the RBC surface to produce agglutination. Many lipids, including gangliosides also agglutinates trypsinized rabbit red blood corpuscles. This lipid-mediated and sugar-independent agglutination could be prevented by the presence of BSA (255). Study of inhibition by gangliosides of BBL-mediated agglutination of TRRBC in presence of BSA (4 times in excess of gangliosides) revealed that both native and desialylated mixed gangliosides inhibited agglutination to the same extent (Table 1). The minimum concentration of ganglioside required to inhibit minimum hemagglutinating concentration of BBL was 0.0312 and 0.0331 (mg/ml expressed in terms of galactose) respectively for native and desialylated gangliosides. Mixed gangliosides further purified by DEAE-Sephadex chromatography were nearly five times as effective as untreated gangliosides thus indicating that non-ganglioside glycolipids possibly present in the ganglioside sample hardly contributed towards inhibition. The inhibition of BBL by gangliosides was sugar-specific since untreated and desialylated gangliosides at concentrations, respectively, 23 and 42 times needed for inhibition of BBL could not inhibit agglutination by human serum anti- α -galactoside antibody. This antibody is known to recognize exclusively the terminal galactose moiety.

Table 1 Inhibition by gangliosides of agglutination of TRRBC by BBL and anti- α -galactoside antibody

Agglutinin	Concentration of gangliosides* required to inhibit minimum hemagglutinating amount		
	Native ganglioside	DEAE purified ganglioside	Desialylated ganglioside
BBL	0.0312	0.0063	0.0331
Anti- α - galactoside antibody	NI(0.72)	-	NI(1.32)

* Expressed in terms of neutral sugar content (mg/ml) assayed using galactose as standard by the phenol-sulphuric acid method

NI, no inhibition up to the concentration tried, given in parenthesis

linked α -anomerically, unlike the bovine gangliosides in which galactose is β -linked (258). Thus, the agglutination inhibition data also supports the sugar specific interaction of BBL with gangliosides.

Interaction of BBL with individual gangliosides

The high cost of individual gangliosides prevented us from studying interactions of BBL with individual gangliosides by affinity electrophoresis through ganglioside impregnated gels. Instead, the effect of preincubation of BBL with ganglioside on the mobility of the incubated lectin was studied by non-denaturing electrophoresis under alkaline conditions. The affinity of BBL for individual ganglioside was measured in terms of the degree of retention of BBL by the ganglioside molecules that stay nearly at the top of the gel. This was quantitated by measurement of intensity of the fast moving protein band corresponding to unretained BBL using a gel scanner. BBL (3 μ g) was incubated with the individual gangliosides (GM1-79 μ g, GD1b-42 μ g, GM2-38 μ g, GD1a-102 μ g) in a total volume of 60 μ l PBS at 4 $^{\circ}$ C and then loaded on to 9% polyacrylamide tube gel and subjected to electrophoresis at pH 8.2 (Fig.10). BBL (2.75 μ g) was incubated with GT1b (23 μ g) and subjected to electrophoresis as above (Fig.11). Depending on the degree of interaction

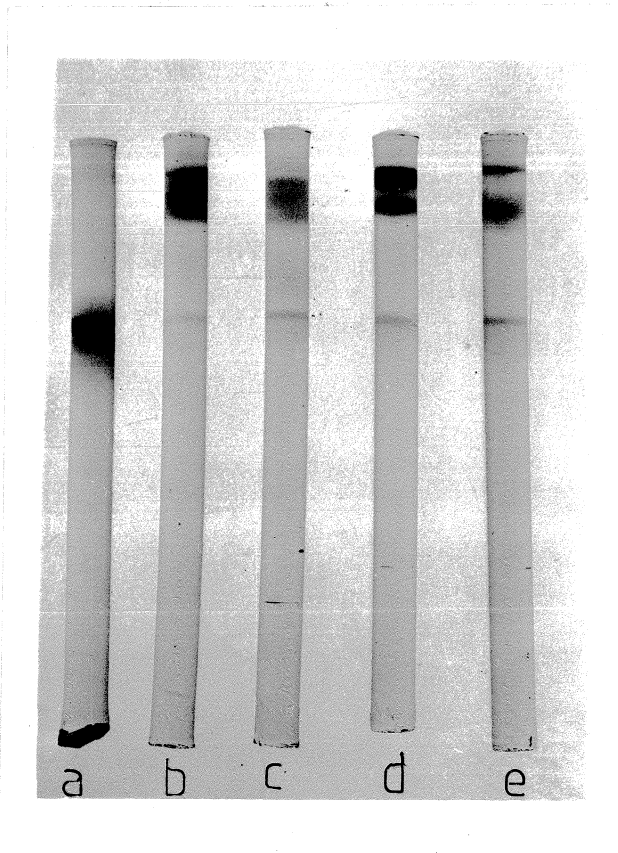


Fig.10. Affinity electrophoresis of BBL mixed with individual gangliosides, through alkaline pH polyacrylamide gel (7%; pH 8.2). BBL (3 μg) was incubated for 2h at 4 $^{\circ}\text{C}$ with ganglioside in 50 μl PBS and loaded for electrophoresis at 4 $^{\circ}\text{C}$. Gangliosides used were: a-nil; b-GD1a (102 μg) c-GD1b (42 μg); d-GM1 (79 μg); e-GM2 (38 μg). Gels were stained with Coomassie blue.

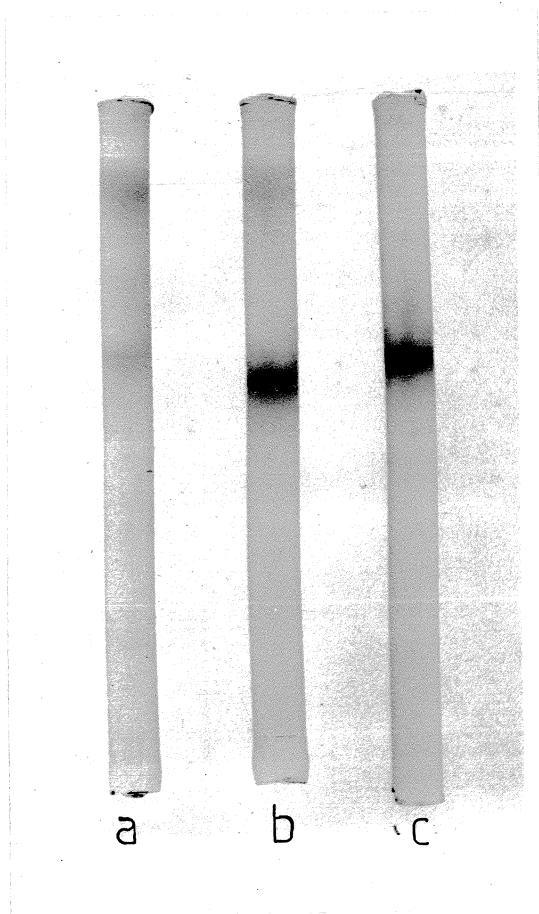


Fig.11. Effect of lactose on affinity electrophoresis of BBL in presence of ganglioside GT1b in 7% polyacrylamide gel, pH 8.2 at 4°C. BBL (2.75 μ g) was incubated with 23 μ g GT1b in the absence (a) or presence (b) of lactose for 2h before loading to gel. C-BBL alone. Gels were stained with Coomassie blue. Details are in the text.

between the ganglioside and BBL, more or less of BBL moved down as a band (Figs.10 & 11). After fixing and staining, the intensity of the lectin band was measured using a gel scanner and compared with that of the same initial amount of BBL that was not incubated with the ganglioside. From the percentage of BBL retained and the concentration of ganglioside used, the percentage of BBL retained per nanomole of each ganglioside was calculated. Results given in table 2 indicate that, of the five gangliosides used, GT1b was found to be the best in interacting with BBL, followed by GD1b, GM2, GM1 and GD1a in the order of decrease in degree of interaction.

The trisialoganglioside, GT1b which strongly interacts with BBL has a terminal sialic acid and the penultimate sugar is galactose linked (β 1-3) to GalNAc. Galactose-linked β (1-3) had been shown to be equal to galactose β (1-4), as in lactose, in affinity towards 14 KDa β -galactoside binding lectin (259). In GD1b as well as in GM1 there is a terminal galactose (β 1-3) linked to GalNAc (230). Lectins recognize not only terminal sugar moieties but inner sugar groups as well in the oligosaccharide sequence. Moreover, the penultimate sugar in the oligosaccharide sequence also influences the binding of lectin to the terminal sugar as demonstrated in the case of calf β -galactoside-binding lectin for which lactose and Gal

Table 2 INTERACTION OF GANGLIOSIDES WITH BBL

Ganglioside	Gandliosides in n moles	Percentage of BBL retained	Percentage of BBL re- tained/ n mole of ganglioside
GD _{1a}	55.00	98.62	1.793
GD _{1b}	23.00	95.37	4.150
GM ₁	51.00	91.66	1.797
GT _{1b}	10.62	75.33	7.090
GM ₂	27.00	93.98	3.480

β 1-3 GalNAc are significantly poorer haptens than lactosamine (Gal β 1-4 GlcNAc). GM2 has no terminal galactose but GalNAc linked (β 1-4) to Gal (235). An explanation for the high affinity of BBL for GM2 compared to that for GM1 is not obtained from considerations of the structural differences between the two gangliosides or the known behaviour characteristics of BBL. Factors other than the chemical structure may also play an important role in deciding the degree of interaction. These include the orientation of the oligosaccharide groups in the micelle, the number of monomers entering into the formation of a micelle and thus the size of the micelle. These parameters may differ from one ganglioside to another. Therefore the configuration of the micelle is also important in deciding the degree of interaction with BBL as gangliosides are in a micellar form under the conditions of the experiment.

Ganglioside-incorporated liposomes

Mixed gangliosides, both native and desialylated, were incorporated in liposomes. Lectin-mediated aggregation of these liposomes were studied by following increase in absorbance on addition of lectin to the liposomes at 320 nm. Liposome suspension with an absorbance in the range of 0.5 to 0.8 was taken in a 1ml cuvette and 20 μ g of BBL or 100 μ g

of RCA was added. Absorbance of the suspension was measured immediately. Sugar control was performed using lectin incubated for 45 min with 50 mM lactose. In our trails using RCA or BBL we could not get any significant increase in absorbance of the liposome suspension on addition of lectin (results not given).

Gangliosides on TLC plates are not recognized by BBL

Gangliosides were separated on precoated TLC plates. Plates, after blocking with 1% polyvinyl pyrrolidone, were layered over with BBL-HRP conjugate or BBL. Then the BBL or BBL-HRP bound to the glycolipids on TLC was transferred to nitrocellulose sheets by apposing a wet nitrocellulose sheet previously dipped in PBS containing 0.2 M lactose on to the TLC plate. When the TLC plate was layered with BBL (not conjugated to HRP), after transferring the BBL to nitrocellulose, the lectin on the nitrocellulose was followed using anti-BBL-antibody coupled to HRP. In either case colour was developed with peroxidase substrate 4-chloronaphthol. In this way when TLC separated gangliosides were allowed to interact with BBL on TLC plate, no interaction was obtained. But by the same procedure RCA-HRP gave positive interaction with desialylated gangliosides on TLC plates (data not shown). This shows that gangliosides on TLC plates were not recognized by BBL.

Microtitre plate-adsorbed gangliosides are also not recognized by BBL

When gangliosides were immobilized on microtitre plate wells and probed with BBL-HRP or BBL followed by anti-BBL antibody coupled to HRP, we could not get any positive interaction between the lectin and the ganglioside (data not shown). But RCA-HRP recognized gangliosides (desialylated mixed gangliosides or GMI) on microtitre plate wells. These results indicate that microtitre plate adsorbed gangliosides were not recognized by BBL.

From the results of TLC overlay and microtitre plate well coating experiments it appears that gangliosides when immobilized on to a solid support were not recognized by BBL. This points towards the possibility of requirement for a micellar arrangement for the interaction between gangliosides and BBL to take place. It is notable that micellar disposition of gangliosides were obtained under conditions in which experiments such as agglutination-inhibition, affinity electrophoresis, incubation of the gangliosides in solution with BBL followed by electrophoresis in alkaline pH polyacrylamide tube gel etc. It is unlikely that gangliosides form micelles when bound to silica gel on TLC plates or when adsorbed on to microtitre plate wells. Probably this may be the reason why gangliosides are not recognized by BBL when present on TLC

plates or on microtitre plate wells. Similarly GM1 dot-blotted on nitrocellulose sheets was recognized by RCA-HRP but not by BBL-HRP. Some enzymes which act on gangliosides like hexosaminidase requires a serum factor for their activity (189,260). Whether any such serum factor could facilitate binding of BBL to adsorbed gangliosides was tested by incubating dot blots of ganglioside with BBL-HRP in presence of human serum (2X diluted in PBS) for 2 h. However no binding of BBL to nitrocellulose-bound ganglioside was observed.

Blackburn and Schnaar reported that cells bind readily to polyacrylamide-bound aminoethyl-GalNAc even at 0°C but the plastic well adsorbed glycolipids could not bind the cells even at 4°C (261). The difference was attributed to the relative mobility of the ligands. The mobility of the aminoethyl glycoside in a hydrated gel would not be expected to be reduced greatly at lower temperature (261). This emphasises the role of mobility of the ligand in binding. Therefore the difference in mobility of the ganglioside in adsorbed and micellar form may influence the correct alignment of ganglioside with the binding site on BBL.

Adsorbed gangliosides bound cells even at 4°C on removal of calcium from the medium, but the extent and strength of binding however, was much lower than that at 37°C (142). But in our experiments with dot blots of

ganglioside incubated at 4°C or 37°C with BBL-HRP for 2 h, no binding was observed in either case. Therefore the lack of binding cannot be attributed to lower temperature

Another possibility which could be envisaged was that the blocking agents used (BSA or Tween 20) could form complexes with gangliosides on nitrocellulose, microtitre plate or on TLC so that BBL could not bind this complex. But in agglutination inhibition experiments, the added BSA (to prevent lipid mediated agglutination) formed complexes with gangliosides which are in the micellar form and could still bind BBL. Moreover blocking nitrocellulose sheets containing coated ganglioside GM1 with BSA did not inhibit RCA-binding to the ganglioside. Another difference between the adsorbed form and micellar form is that the latter offers a cluster of sugar residues on its surface which may lead to increased interaction with BBL. It has been reported that liposomes incorporated with synthetic mannose containing glycolipids were sugar specifically aggregated by Con A (262). This study revealed that vesicle aggregation is highly dependent on the density of the sugar moieties on the surface of the membrane (262). Studies of Li and Pestronk pointed to the influence of membrane environment on antibody binding to terminal carbohydrate groups on GM1 (263). Many authors have shown the interaction of carbohydrate binding proteins with adsorbed glycolipids. Cell adhesion to glycolipids

immobilized on silica gel thin layer chromatography plates was studied by Swank-Hill et al. (98). Hamilton et al. used immune-thin layer chromatography in which antibodies to gangliosides were layered over TLC-separated gangliosides for the analysis of ganglioside composition of 20 human malignant melanomas (264). In the above studies positive binding of cells or antibodies respectively to TLC-separated gangliosides was obtained.

However, in our experiments, BBL failed to recognize the adsorbed gangliosides though in micellar form gangliosides were easily recognized by the lectin.

The role of glycolipids and complementary binding proteins in cell to cell interaction is well documented. For example E-selectin on endothelial cell bind leukocytes that contain carbohydrate structures mainly on their glycolipid surface molecules (265). In addition to such glycolipid protein interactions, glycolipid-glycolipid interactions are also known to participate in cell adhesion as well as spreading and motility of the cell lines as shown by Kojima and Hakomori (266).

Gangliosides are membrane glycolipids that occur at high concentration in the nervous system, especially in the grey matter. They have long been implicated in cell growth regulation (267). Gangliosides could bring about this either by modifying growth factor receptors directly

(104,124-126) or by modifying the activity of novel protein kinases (127-129). Gangliosides have been detected in cell adhesion sites (268). The involvement of gangliosides in cell adhesion is further supported by the observation that addition of exogenous gangliosides at the initial stage of plating inhibits cell adhesion on plates (268-270). The specific binding of proteins to cell surface gangliosides leads to redistribution of these molecules and in many cases elicits physiological changes within the cell. Typical example is the redistribution of sialoglycolipid receptor on binding of cholera toxin at the lymphocyte membrane(271)

The binding of laminin to brain gangliosides (194) and inhibition of laminin-neuron interaction by gangliosides is of significance in that brain is rich in gangliosides and laminin is known to be involved in cell to cell contact and in cell migration. Cell migration is very decisive in the nervous system considering the extensive neural connections present in the brain. Human galactose-binding protein (hL-31) binds to the poly-N-acetyl lactosamine sequences of laminin and this interaction is inhibited by lactose (272). It will be interesting to investigate how ganglioside laminin interaction is affected by BBL as the BBL can interact with both gangliosides and laminin. The physiological consequences of such a triangular interaction among BBL, laminin and ganglioside would be both exciting

and useful to study.

Gangliosides assume importance in malignant transformation in two ways. Firstly, it is reported that gangliosides undergo qualitative and quantitative changes during transformation (273). This alters their normal interaction with endogenous lectins. This can affect biological events or equilibria that are dictated by lectin-ganglioside interaction. Secondly, changes in lectin expression is also observed in malignancy (96,97). Therefore change in expression of one or both of these complementary molecules may be disastrous in view of their involvement in cell-cell or cell-extracellular matrix interaction. Marked changes in the core structure of the cell surface glycolipids occur as the embryonal carcinoma cells differentiate; thus globoseries structures rapidly diminish and are replaced by lactoseries and then by ganglioseries glycolipids (274). Gangliosides have been related to invasiveness and tumor associated gangliosides have been characterized in both tumor tissues and established tumor cell lines. It is illustrated that ganglioside GD3 is found in cellular lipid extracts of T-cell acute lymphoblastic malignancies (T-ALL) but not detectable by resorcinol staining in extracts of non-T-acute lymphoblastic leukemia blasts (non-T-ALL). Binding experiments using an antibody to GD3 , R24 confirmed this observation (275). The observed

alterations in gangliosides and galactose binding lectins in malignant transformation is significant in the light of our finding that gangliosides are recognized by β -galactoside binding lectin from brain, in vitro. Many tumors shed gangliosides. For example neuroblastoma shed ganglioside GD2 (276). It is known that transport of serum gangliosides and glycolipids is by lipoproteins, mainly LDL (66%) followed by HDL (25%) and VLDL (7%) (277).

Antibodies against gangliosides are found in the sera of patients with neurological disorders like motor neuron diseases (160), motor neuropathy (163), Guillain-Barre Syndrome (161), chronic inflammatory demyelinating polyneuropathy etc. The interaction of the endogenous lectin with gangliosides in vivo may also influence the fates of these autoantibodies.

Gangliosides are sialylated glycosphingolipids. Sialic acid is present in some glycoproteins also. Terminal sialic acid residues of glycoproteins and glycosphingolipids are important in diverse ways such as increased half life of circulating sialoglycoproteins, blood clot formation, interaction of hormones with their target cells, neurotransmission, cell-cell interaction and cellular transformation (278). Most of the functions of sialic acid can be divided into two groups. Sialic acids either are recognized themselves or they mask other molecular and

cellular recognition sites (279). Terminally sialylated gangliosides are important receptor binding sites for viruses and are able to mediate virus attachment (280), a prerequisite for virus penetration into host cell. The biological importance of removing the sialic acid was shown by the classical experiment of Ashwell and Morell in which serum glycoproteins from which terminal sialic acid moieties were removed resulting in exposure of galactose moieties as terminal sugar were easily captured by hepatocytes from circulation via a galactose specific hepatic receptor (281). Moreover erythrocytes and lymphocytes are bound by macrophages from liver, spleen and peritoneum at a rate which is proportional to the amount of sialic acid removed (282). Sialidase-treated erythrocytes are engulfed by macrophages in liver and spleen whereas sialidase-treated lymphocytes reappear in circulation after trapping in liver and spleen. This seems to be due to resialylation of glycoconjugates on lymphocyte surface. Thus it is involved in a reversible cellular recognition, which may also be valid for other cell systems (283). The high degree of sialylation found in some tumor types in comparison to be corresponding normal cells, and the extremely low sialylation observed in certain other tumor cells, indicate that sialylation status of glycoconjugates strongly influence the behaviour of malignant cells in an organism

with regard to immunological properties, growth rate and metastatic potential of the tumor (284). It is also observed that in general, increased levels of sialylation of surface-exposed carbohydrates is a necessary condition for the manifestation of invasiveness and tumorigenicity (285)

In glycoproteins, desialylation generally increases affinity for β -galactose binding lectins including BBL. However, in our studies, desialylation which renders more β -galactoside groups terminal, failed to augment BBL binding of gangliosides in affinity electrophoresis and agglutination inhibition. Whether charge effects of removing sialic acid moiety adversely affected ganglioside deployment on micelle so as to offset the expected increase in BBL-binding consequent to desialylation remains to be examined. Another reason for this behaviour may be that, unlike glycoproteins, desialylation of glycolipids from brain, under the conditions used, is far from complete (286) and in our trials was nearly 46%. In our experiments the affinity of BBL towards individual gangliosides could not be correlated with the presence or absence of terminal sialic acid. In fact the most strongly interacting ganglioside GT1b has a terminal sialic acid moiety in addition to two other internally substituted sialic acid moieties.

Gangliosides, in addition to serving as receptors for many bacterial toxins and viruses, are known to bind a

few endogenous proteins also. A calcium dependent ganglioside-binding protein was isolated from a soluble cytosol fraction of mouse brains and was shown to be calmodulin. It is suggested that this binding may be a general mechanism for regulating calmodulin-dependent enzymes with consequent cellular response, such as cell differentiation (287). Other proteins which bind to ganglioside include serum albumin (190), lipoproteins (254), myelin basic protein (191,192), 11-12 KDa and 28 KDa (sequence identity with ubiquitin) proteins from calf brain and laminin (194). Ganglioside-protein interactions are important in infection, toxicity, transport of molecules, cell migration etc. Presence of lectin-like receptor for gangliosides on neuronal membrane was indicated (288). Caron et al. had shown that asialofetuin and GM1 ganglioside at very low concentrations (4×10^{-4} mM and 2.5×10^{-5} mM respectively) inhibited hemagglutination by rat brain and bovine brain galactose binding lectins (5) though no other information on this interaction had been furnished. However this study had not taken into consideration the effect of ganglioside-mediated agglutination. Nor was this observation pursued further by any investigator. Very recently, after our studies on BBL-ganglioside interaction had been completed and part of it reported, interaction of human brain lectin to GM1 studied on a high performance affinity

column containing immobilized GM1 (lyso-GM1) was reported (289). Here biotinylated human brain lectin (HBL) was injected into the high performance affinity column composed of lyso-GM1 coupled to Sepharosil beads coated with DEAE-dextran. The bound proteins were eluted with a pH gradient or with lactose. In these experiments only half of the molecules were adsorbed during the chromatography, reflecting a low affinity between the partners inspite of the high mobility of GM1 owing to the presence of a spacer arm. As a probable reason for this low affinity, the authors cited a repulsive effect of sialic acid or an inhibitory effect of NaCl (present in the buffer). However, the authors had not examined the effect of biotinylation of the lectin on its binding to ganglioside. Our studies reveal that not only GM1 but other gangliosides (GT1b, GD1b, GM2, GD1a) also sugar specifically interacted with 14KDa β -galactoside binding bovine brain lectin, with varying degree of affinity. Though many of these gangliosides have no terminal galactose groups, except in GM1 and GD1b (230) they were recognized by BBL sugar specifically. It may be recalled that terminal β -galactosyl residues are not necessary for high affinity binding of long poly N-acetyl lactosamine containing oligosaccharides to L-14 (the S-type lactose binding lectin) (290) or to the galactose-binding Ricinus communis lectin (RCA) (322).

Lectins are largely recognized for their role in cell to cell interaction. This is mainly achieved by binding of lectins to sugar moieties on glycolipids, glycoproteins or glycosaminoglycans. The interaction of BBL with gangliosides may have biological relevance in the light of the findings that specific binding of the proteins to gangliosides on cell surface might trigger many physiological changes within the cell. Also ample evidence is now available to believe that endogenous lectins and their glycoconjugate ligands are involved in cell to cell interaction. In view of the presence of autoantibodies to gangliosides in many neurological disorders and alteration of ganglioside (273) and lectin (96,97) expression in malignant transformation and metastasis, the role of brain lectin ganglioside interaction in such pathological states deserve attention. The biological significance of the interaction of brain lectin with endogenous gangliosides is far from clear at present. More investigations in this direction will be necessary to arrive at the physiological relevance of this interaction and may be helpful in the diagnosis or treatment of disorders of the nervous system.

PART II

**Sugar specific interaction of bovine brain lectin with
endogenous glycoprotein ligands from brain stem**

The β -galactoside-binding proteins have a widespread distribution in vertebrate cells (2). The preparation and characterization of the bovine brain 14 KDa β -galactoside-binding protein from bovine and rat brain have been reported (5). A similar galactose-binding lectin was reported from human brain grey matter and white matter though the specific activity in the latter was only one third of that in the former (6). We isolated a 14 KDa β -galactose-binding lectin from bovine brain stem and compared it to the lectin from cerebral cortex.

The β -galactose-binding lectins were isolated by lactose-Sepharose chromatography from bovine cerebral cortex and bovine brain stem. On SDS PAGE, the lectins from both the sources were resolved into a single subunit of identical mobility (Fig.12). Alkaline PAGE also gave similar mobility for both the proteins (Fig.13). In hemagglutination-inhibition study they exhibited similar sugar specificity, lactose being the potent inhibitor of lectin from both the sources. The antigenic crossreactivity of the antibody raised (in rabbit) against cerebral cortex lectin with that

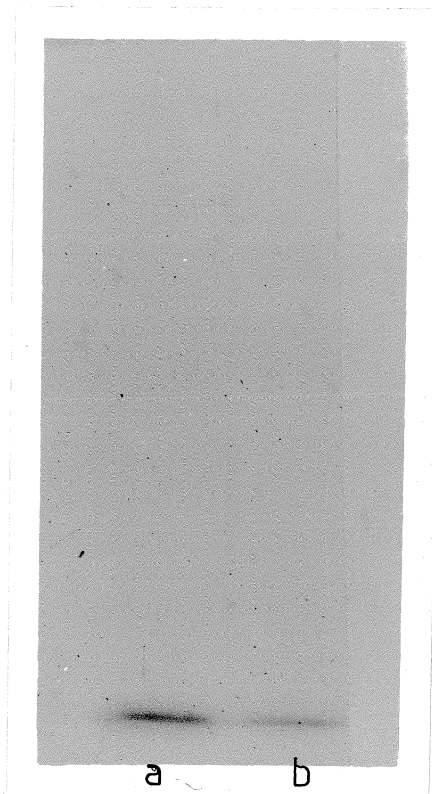


Fig.12. 10% SDS-PAGE of BBL ($5 \mu\text{g}$) isolated from (a) grey matter (b) white matter. Coomassie blue stain. See text for details.

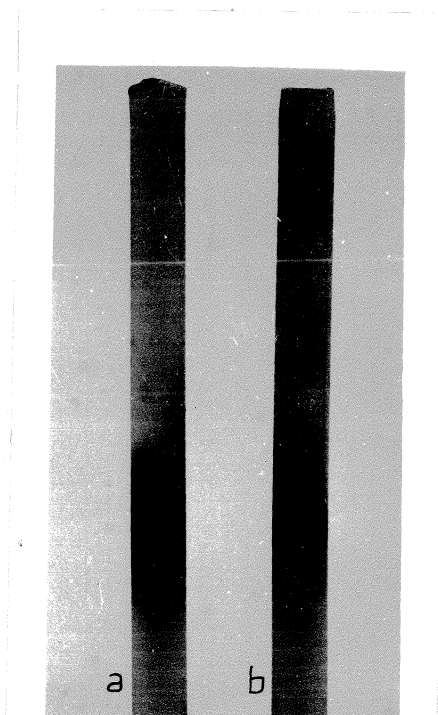


Fig.13. 7% Alkaline PAGE of BBL (10 μ g) isolated from (a) grey matter (b) white matter. Coomassie blue stain. Details are given in text.

from brain stem shows the presence of common antigenic epitopes in both the proteins (Fig.14). Also on carbohydrate estimation both the lectins were found to be non-glycosylated proteins. These different lines of evidences suggest that the lectin from brain stem is similar, if not identical, to the lectin from cerebral cortex. The wide distribution of this 14 KDa β -galactoside binding lectin with a highly conserved primary structure and carbohydrate binding activity also supports this contention (3).

Another mannose-specific lectin (Cerebellar soluble lectin, CSL) has been reported from cerebellum (45). In rats it is shown that this lectin is involved in myelination and is absent from non-myelinating Schwann cells (291). Further more, anti- CSL antibodies are present in the CSF of more than 93% of the multiple sclerosis patients (291). In combination with minor myelin associated glycoprotein, CSL is believed to play a crucial role in myelin compaction (51). It is not known whether 14 KDa β -galactose binding lectin has got a similar role in brain stem.

Bovine brain lectin in its amino acid composition and sugar specificity is similar to porcine heart lectin (Mr 14,700) (292). Also the lectin purified from bovine and rat brain are similar to the soluble β -galactoside binding lectin isolated from other mammalian organs (12).

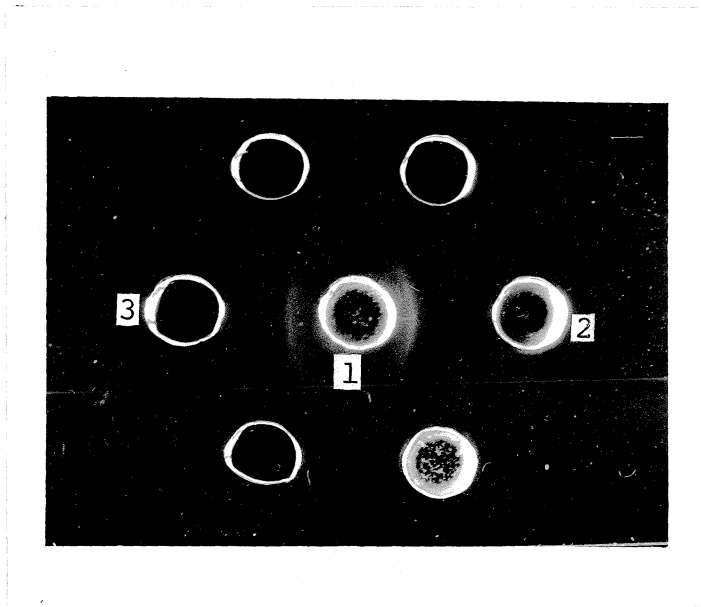


Fig.14. Ouchterlony immunodiffusion in 1% agarose gel showing the cross reactivity of antibody raised against BBL from grey matter with that from white matter. Samples loaded in wells were 1, antiserum against BBL isolated from grey matter; 2, BBL isolated from grey matter; 3, BBL isolated from white matter. See text for details.

In rat brain, a study of cerebral cortex and corpus callosum during brain development (27) showed the lectin to be predominantly neuronal and to increase from post natal day 1 to 10, decreasing thereafter. Studies also show that the lectin plays a role in intracellular traffic of molecules in nerves but is not generally externalised by them. During brain development however, lectins in neuroblast and axonal membrane could be involved in transient neuroblast adhesion, contact guidance, migration and fasciculation (3). In spite of lack of a signal sequence, the co-distribution of muscle lactose-binding lectin (14.5 KDa) with laminin in the myotube extracellular matrix (ECM) points to the export of this lectin from cytosol to ECM (293).

The cDNA cloning of soluble bovine galactose-binding lectin reveals the complete amino acid sequence and an antigenic relationship with the encephalitogenic domain of myelin basic protein (9). The 14 KDa lectin has been reported to have interesting immunoregulatory properties, suppressing experimental autoimmune myasthenia gravis (294), and experimental autoimmune encephalomyelitis (295) inducing the release of tumor necrosis factor from macrophages (296) and possibly also playing a part in prevention of maternal recognition of paternal antigens expressed on foetal tissue (295).

Allen et al. found tumor concentrations of the 14 KDa lectin to be variable, but generally elevated over control levels (297). Treatment of tumor cells with anti-lectin antibody in vitro, before injection into the tail vein of syngeneic mice, reduced upto 90%, the subsequent appearance of tumor colonies in the lung. It has been reported that the over expression of rat β -galactoside-binding protein in mouse BALB 3T3 fibroblast cells lead to acquisition of transformed phenotype (298). Transformation and growth-related changes in levels of nuclear and cytoplasmic proteins antigenically related to mammalian- β -galactoside binding lectin is also documented (64). Similarly the tumor microvessels lacked the variety of galactose-specific proteins found in those isolated from tumor-free cerebral tissues (299).

In short, the variety of roles played by the lectin in various normal and pathological states necessitates an in depth analysis of properties and regional localization of these proteins.

BBL-binding glycoproteins are present in the brain stem

Brain stem is mainly white matter and in view of the reduced content of gangliosides in white matter in comparison to grey matter, we searched for the soluble

glycoprotein ligands of BBL from brain stem. Streit et al. had shown the histochemical localization of galactose containing glycoconjugates in sensory neurons and their processes in the central and peripheral nervous system of rat using lectin peroxidase conjugates (300).

The glycoproteins obtained after RCA-Sepharose chromatography of bovine brain stem extract, on dot-blotting was recognized by BBL-HRP sugar specifically (Fig.15). This indicates the presence of BBL-interacting soluble glycoproteins in brain stem.

An 84 KDa protein from brain stem strongly interacts with BBL

Among the RCA-binding glycoproteins from brain stem, BBL-interacting proteins were identified by western blotting after SDS-PAGE followed by probing with BBL-HRP. An 84 KDa subunit was found to be the most prominent among glycoproteins recognized sugar-specifically by BBL. A few minor bands were also recognized sugar-specifically by the lectin (Fig.16). The intensity of the band obtained due to recognition of a glycoprotein by BBL-HRP is a function of both the affinity of the lectin to the glycoprotein and the amount of the glycoprotein in the band. From the Coomassie blue and amidoblack stain after SDS-PAGE, it appears that 84 KDa protein is relatively abundant in the mixture and

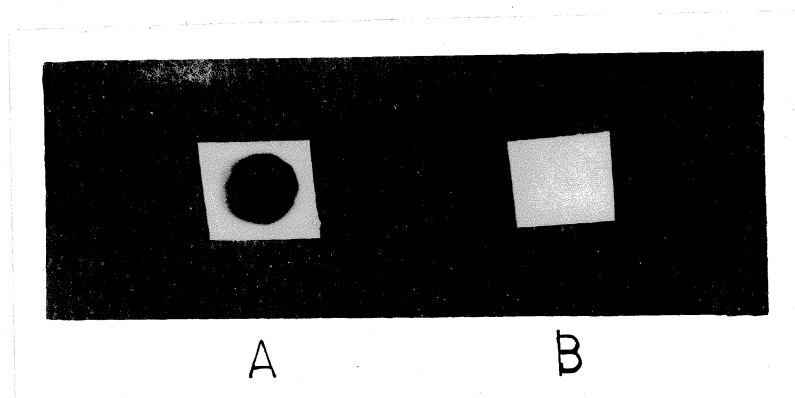


Fig.15. Binding of BBL-HRP conjugate to dot blot of RCA-binding bovine brain stem glycoproteins ($2\ \mu\text{g}$) in the absence (A) and presence (B) of $0.1\ \text{M}$ lactose. (Details are given in text).

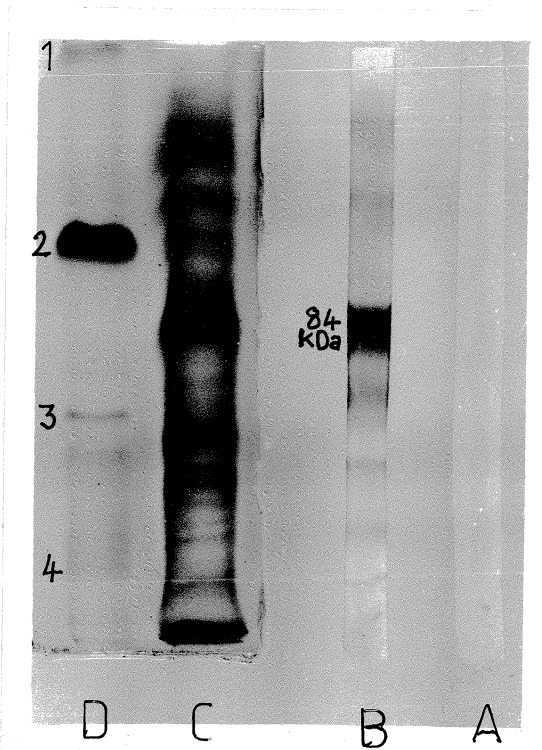


Fig.16. Recognition by BBL-HRP of nitrocellulose Western blot of RCA-binding bovine brain stem glycoproteins in the presence (A) and absence (B) of 0.1 M lactose [C, SDS-PAGE-separated RCA-binding glycoproteins stained by Coomassie blue before transfer; D, Standards run along with glycoproteins (1-myosin, 2- β -galactosidase, 3-BSA and 4-ovalbumin from hen egg)]. (Details are given in text).

that this may be one of the reasons for the observed major band due to this protein. Another important factor in deciding the affinity is the presence of a large number of lectin-interacting moieties (β -galactosyl groups) on the glycoprotein. Decreased transfer of the weakly interacting proteins to nitrocellulose may not be the reason for the less intense binding to these proteins because the weakly interacting proteins are present above and below the strongly interacting 84 KDa band. Amidoblack staining of the transferred proteins confirmed the proportionate transfer from SDS-PAGE to nitrocellulose sheets. The interaction between BBL and soluble glycoprotein from brain stem was entirely sugar dependent as there was no binding in the presence of 0.1M lactose. Joubert et al. (18) reported that human brain lectin has got a non-sugar-dependent interaction with one of the brain proteins, which was identified as actin. However, no sugar-independent interaction between BBL and any brain stem RCA-binding glycoprotein was observed by us.

Separation and isolation of BBL-interacting proteins

For separation and isolation of BBL-interacting proteins from the total RCA-binding proteins of brain stem various techniques were used.

a. HPLC-gel filtration

HPLC-gel filtration of RCA-binding glycoproteins (G 4000 SW gel filtration column) resolved the proteins into three peaks (Fig.17). Each peak on SDS-PAGE (Fig.18) and alkaline PAGE (Fig.19) gave multiple bands. This shows that none of the peaks were pure single protein peaks. So this method failed to separate the proteins completely. Therefore we attempted other methods of separation.

b. DEAE-HPLC

RCA-binding brain stem soluble glycoproteins came out as a single peak from DEAE-ion exchange high performance liquid chromatography column when eluted with a continuous gradient of sodium acetate (0-0.1M). Further, RCA-binding brain stem glycoproteins after alkylation using iodoacetic acid on DEAE-HPLC also gave only a single peak.

c. Electrophoresis

For the separation of native RCA-binding soluble glycoproteins, acid PAGE as well as alkaline PAGE was performed. No good separation was obtained in acid PAGE.

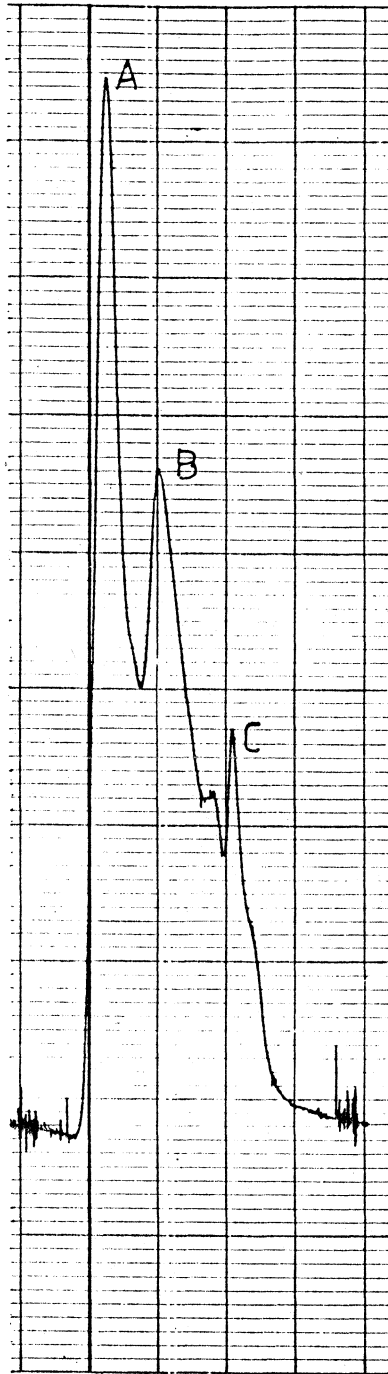


Fig.17. Gel filtration of RCA-binding glycoprotein from bovine brain stem using an HPLC column, G 4000 SW. Profiles A,B and C correspond to peak I, Peak II and Peak III. Eluant: PBS. Flow rate 1ml/min.)

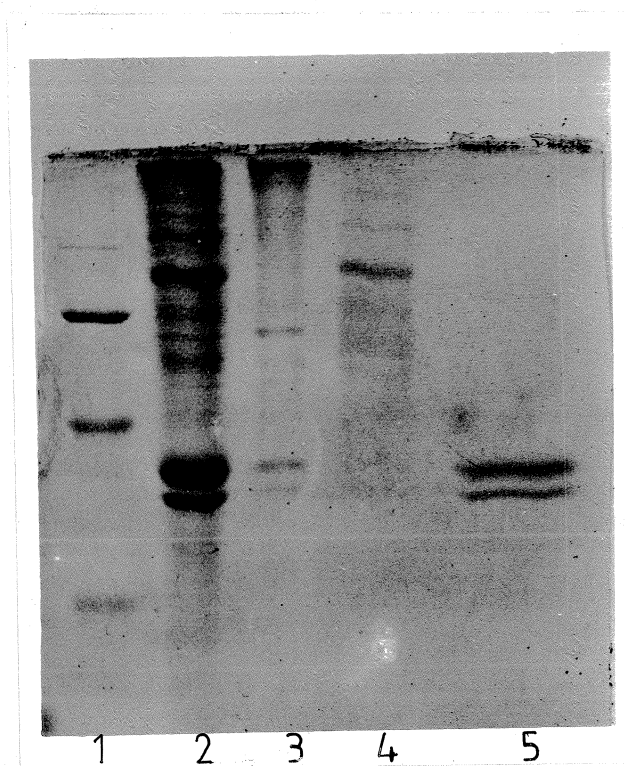
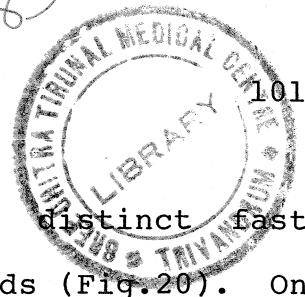


Fig.18. 10% SDS-PAGE of HPLC-gel filtration peaks of RCA-binding bovine brain stem glycoproteins. Lane 1 Molecular weight standards (from top to bottom; β -galactosidase, BSA and ovalbumin) Lane 2 -total RCA- binding glycoprotein. Lane 3, Lane 4 and Lane 5 are peak I, Peak II, and peak III respectively obtained on HPLC-gel filtration. Coomassie blue stain. Details are given in text.



Fig.19. 10% Alkaline PAGE of HPLC- gel filtration peaks of RCA-binding bovine brain stem glycoproteins. Lane 1 - total RCA-binding glycoprotein. Lane, 2, Lane 3 and Lane 4 are peak I, peak II and peak III respectively obtained on HPLC- gel filtration.

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Alkaline PAGE resolved the proteins into a distinct fast moving band and a cluster of slow moving bands (Fig.20). On alkaline PAGE of 7% and 10% acrylamide, the fast moving band remained as a single band itself without any splitting (Fig.20) suggesting that it may be a single protein. On dot blotting on nitrocellulose membrane and detection with BBL-HRP, the electroeluted alkaline PAGE fast moving protein was found to interact with BBL sugar specifically (Fig.21).

Isolation of BBL-interacting proteins from the total RCA-binding proteins of brain stem

Electroelution was used to isolate the BBL-interacting proteins. The 84 KDa protein in SDS-PAGE (10% gel) of RCA-binding glycoproteins is one of the proteins to which BBL binds strongly. This protein was isolated from SDS-PAGE gel by cutting out the band from unfixed gel after comparing with a stained portion of the gel and extracting out the proteins from the gel by electroelution. The alkaline PAGE fast moving protein which is also known to interact with BBL was isolated from alkaline PAGE tube gel (7% acrylamide) by cutting out the fast moving band and recovering the protein from the gel by electroelution.

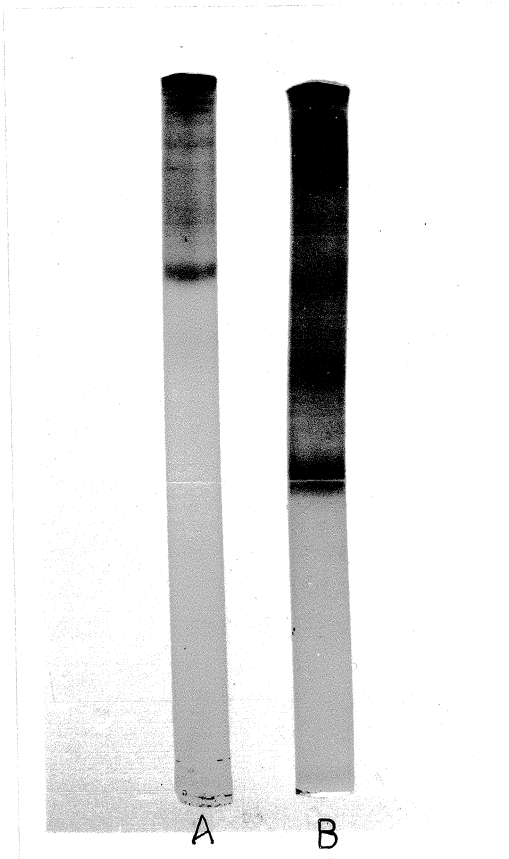


Fig.20. Alkaline pH tube gel electrophoresis of RCA-binding glycoproteins (50 μ g) from bovine brain stem stained with Coomassie blue [A, in 10% acrylamide gel; B, in 7% acrylamide gel]. (Details are given in text).

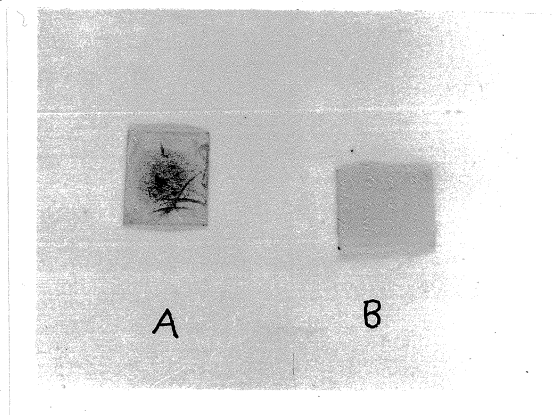


Fig.21. Binding of BBL-HRP conjugate to dot blot of electroeluted alkaline fast moving proteins from RCA-binding bovine brain stem glycoproteins (0.5 μ g) in the absence (A) and in the presence (B) of 0.1 M lactose. (Details are given in text).

Characterization of BBL-interacting proteins

a. Subunit molecular weight

Among total RCA-binding glycoproteins, the most prominent BBL-interacting subunit was of Mr 84 KDa as already mentioned. On SDS-PAGE, the alkaline PAGE fast moving protein was split into two subunit of Mr 55 KDa and 58 KDa (Fig.22).

b. Immunological characterization

The 84 KDa protein and alkaline PAGE fast moving protein are immunologically distinct. Antibodies were raised to 84 KDa protein and alkaline PAGE fast moving protein by injecting them in mice. The antiserum against the 84 KDa protein was found to interact with this glycoprotein as well as with the total RCA-binding protein on immunodiffusion as expected since the latter contains the 84 KDa protein (Fig.23). The antibody against the alkaline PAGE fast moving protein neither interacted with the 84 KDa protein nor with any other protein than the alkaline PAGE fast moving protein of the total RCA - binding glycoprotein (Fig.23). The RCA-binding glycoprotein devoid of the alkaline PAGE fast moving protein was prepared by electroeluting the proteins from alkaline PAGE tube gels after removing the alkaline PAGE

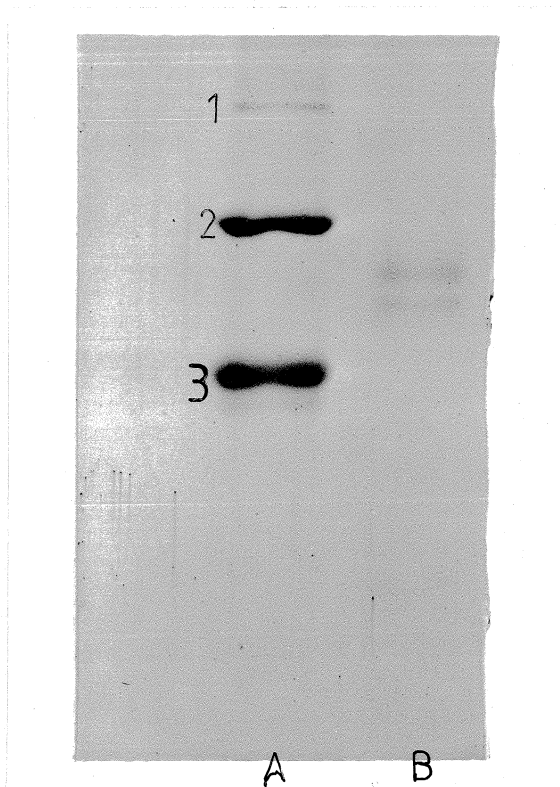


Fig.22. SDS-PAGE in 10% gel of electroeluted alkaline PAGE fast moving protein from RCA-binding bovine brain stem glycoproteins (B) and of molecular weight standards (A) [1, β -galactosidase; 2, BSA and 3, ovalbumin from hen egg]. (Details are given in text).

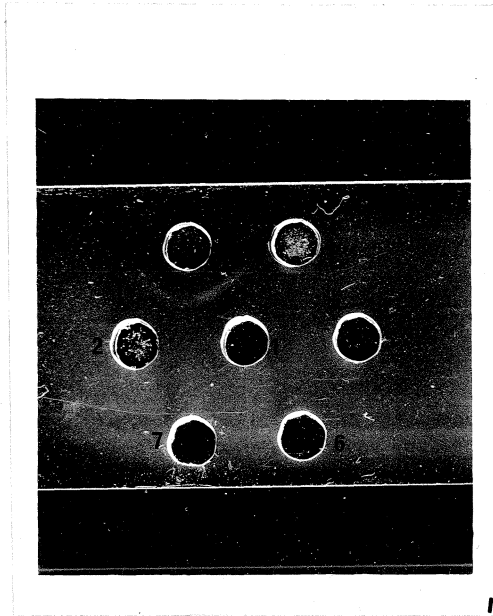


Fig.23. Ouchterlony immunodiffusion in 1% agarose gel to show the specificity of interaction of antibody raised against alkaline PAGE fast moving protein [samples loaded in wells were; 1, total RCA-binding glycoprotein; 2, electroeluted alkaline PAGE fast moving protein; 3, antiserum raised against electroeluted alkaline PAGE fast moving protein; 4, total RCA-binding proteins minus alkaline PAGE fast moving protein prepared by electroelution, 5 to 7, PBS]. (Details are given in text).

fast moving protein band. On electrophoresis as well as immunological properties the foregoing experiments suggested that two BBL-interacting proteins, the 84 KDa band and the alkaline PAGE fast moving band differ in their subunit size as well as antigenically.

c. Characterization of oligosaccharide part of the BBL-interacting brain stem glycoproteins

Plant lectins with defined specificities have been widely used to characterise the carbohydrate part of glycoproteins. For instance, carbohydrate chains of electrophoretically separated glycoproteins from murine B16 melanoma sublines of various metastatic properties were analysed by lectin binding to electrophoretically separated proteins (301). In another study, the microheterogeneity of oligosaccharide chains of HLA-DR, human major histocompatibility class II antigen, was followed using lectins (302). We used different carbohydrate binding proteins (lectins and carbohydrate specific antibodies) of known specificity to characterise the oligosaccharide part of BBL-interacting glycoproteins. This was performed by incubating the dot blot of glycoprotein with the HRP-conjugate of the carbohydrate binding proteins in presence or absence of the specific sugar. The binding of

different lectins/antibodies to the 84 KDa protein and alkaline PAGE fast moving protein is as shown in Table 3.

Concanavalin A binds to α -D-mannosyl and α -D-glucosyl residues. The strong binding of Con A to 84 KDa protein and alkaline PAGE fast moving protein indicated the presence of α -D-mannosyl and/or α -D-glucosyl residues in these glycoproteins.

Anti-Gal is a human serum antibody and it recognizes terminal α -galactosyl moieties. The observed positive interaction of this antibody with 84 KDa protein indicates the presence of terminal α -galactosyl residues in this protein. The binding of anti-Gal to alkaline PAGE fast moving protein was negative. This shows the absence of detectable terminal α -galactosyl residues in this protein.

Jacalin binds to terminal α -galactosyl residues and T-antigen (Gal β 1 --> 3 GalNAc α -) structure. Jacalin recognized sugar-specifically both 84 KDa protein and the alkaline PAGE fast moving protein. This result read along with the anti-Gal-binding data suggested that while the 84 KDa glycoprotein may contain terminal α -galactosyl group and/or T-antigen structure in its oligosaccharide sequence, the alkaline PAGE fast moving glycoprotein may contain T-antigen structure.

Alkaline PAGE fast moving protein has no terminal α -galactose but has got T-antigen structure because it is

TABLE 3

Comparison of binding affinity of Plant lectins and
anti-gal to glycoproteins

Lectin (50 µg/ml) or antibody (10 µg/ml)	Relative staining intensity on dot blots	
	84 KDa subunit	alkaline PAGE fast moving glycoprotein
Con A	+ + +	+ + +
Jacalin	+ + +	+ + +
WGA	+ + +	+ + +
PHA	+ +	--
UEA	--	--
anti-Gal	+	--

+, ++, +++ and - stand respectively for mildly positive, moderately positive, strongly positive and negative binding of HRP conjugate of lectin or antibody. Amount dotted was 2 µg of either glycoprotein. Sugar controls for all lectins and anti-Gal showed negative staining.

not interacting with anti-Gal but at the same time interacts with jacalin. The Thomsen-Friendenreich antigenic determinant (T-antigen) is revealed on human erythrocytes by neuraminidase treatment and is described as part of the MN blood group antigens. It is attached to glycoporphine or other proteins through O-serine or O-threonine linkages (303). Tn, the precursor of T-antigen is α -GalNAc-O-Serine/threonine. T-antigen is normally cryptic due to the presence of a terminal sialic acid residue, while Tn is exposed only in individuals with a recessive genetic disorder (304). Springer has claimed the expression of T and Tn determinants on over 90% of cancers of the breast, lung, and pancreas, although the nature of the molecules that bear these determinants and their exact structure was not clear (303).

Wheat germ agglutinin (WGA) binds to N-acetylglucosamine as well as sialic acid. Both the BBL-interacting proteins were sugar specifically recognized by WGA revealing the presence of N-acetyl glucosamine and/or sialic acid in these proteins. The presence of sialic acid was ascertained as the neuraminidase treatment of both the proteins reduced WGA binding. Wheat germ agglutinin has been used to identify four high molecular weight sialoglycoproteins in HT-29 human colon carcinoma cells and the relative intensity of WGA binding to these proteins differ depending on their growth phase (305).

The binding of Phaseolus haemagglutinin (PHA) to 84 KDa protein shows the presence of Gal as part of a complex type oligosaccharide structure specific for this lectin (305a) and lack of binding of the lectin to alkaline PAGE fast moving protein shows the absence of this structure in this glycoprotein.

The fucose-binding lectin Ulex europaeus agglutinin (UEA) was not binding to 84 KDa protein and alkaline PAGE fast moving protein, showing the absence of detectable L-fucose residues in these proteins. Ulex europaeus agglutinin is known to react with fucosylated oligosaccharides on most, but not all, species of carcinoembryonic antigen (CEA) but generally did not react with normal colon or liver extracts (306).

Ricinus communis agglutinin is known to recognize β - as well as α -linked galactose groups (231). Also, BBL, though generally regarded so far as a lectin recognizing β -galactosides, has recently been shown to recognize α -linked terminal galactose as well in this laboratory (319). In view of these data, from the above experimental results, presence of α -linked terminal galactose groups in the 84 KDa subunit is established, while β -linked galactosyl groups may or may not be present in this glycoprotein.

In brief it can be concluded that the 84 KDa protein contains the following sugar groups: terminal α -galactosyl groups with or without β -galactosyl,

α -D-mannosyl and/or α -D-glucosyl, sialic acid and galactose residue as part of a complex type of oligosaccharide structure. L-Fucose is undetectable in this protein. Similarly, the alkaline PAGE fast moving protein contains β -galactose, α -D-mannosyl and/or α -D-glucosyl, sialic acid residues and T-antigen structure. L-fucose and terminal α -galactosyl residues were undetectable in this protein.

The role of cell surface glycoproteins in central nervous system cell sociology has been emphasized by many investigators. Neural cell adhesion molecule (N-CAM) is a surface sialoglycoprotein which interacts with the N-CAM of another cell to establish a cell to cell contact (307). It is believed that nerve-muscle association is established through this interaction (307). The cell adhesion molecule called P-CAM of D. purpureum is also a glycoprotein (308). Lectins interact with the sugar moieties on glycoproteins and this interaction is also important in many physiological phenomena. Tissue fibronectin may function as an endogenous ligand for 14 KDa lectin as well as a ligand for integrin. The 14 KDa lectin may play a role in cell adhesion by interacting with extracellular matrix molecules such as fibronectin through protein carbohydrate interaction (309). Lectin-affinity chromatography combined with SDS-PAGE has revealed the change in glycoprotein on cell surface in relation to hematopoietic differentiation (310). An

association of galaptins (galactose-binding lectins) with laminin in vitro has been described (311) and could be of obvious biological significance as laminin has multiple activities in cell adhesion, growth, morphogenesis, migration, differentiation, neurite out growth and metastasis (312, 313).

Change in levels of glycosyl transferases reflects quantitative alteration in specific glycoproteins. In Rous sarcoma transformed baby hamster kidney cells there is an increase in the levels of N-acetyl glucosaminyl transferase V (314). Increased sialylation and β 1-->6 linked branching of complex type asparagine (Asn)-linked oligosaccharides (ie GlcNAc β 1-6 Man α -1-6 Man β 1-) are among many phenotypic changes observed following oncogenic transformation of cells. Moreover the sialylated lactosamine antennae and in particular the β 1-6 linked antennae of complex type oligosaccharides are important features of the metastatic phenotype (315). The alteration in oligosaccharide part of glycoproteins during malignant transformation arises out of incomplete synthesis of carbohydrate chains or due to neosynthesis of embryonic type structures. For instance in cancer-associated serum glycoprotein antigens expressing type 1 and type 2 chain polylactosamines, type 1 chain antigens, such as 2-3 and 2-6 sialylated Le^a antigens are more frequently associated with the incomplete synthesis of

the carbohydrate chains during the course of a malignant transformation, whereas the appearance of the cancer associated type 2 chain antigens, such as sialylated or fucosylated SSEA-1 antigens (sialylated Le^x -i and Le^y), are closely related to the neosynthesis of the embryonic type antigens in cancer cells (316). It is also observed that oncogenes conferring metastatic potential induce increased branching of Asn-linked oligosaccharides in rat 2 fibroblasts (317). Certain lines of human colon adenocarcinoma cells produce two Mac-2 (a galactose-binding lectin secreted by activated macrophages) binding glycoproteins, M2 BP-1 (98 KDa) and M2 BP-2 (70 KDa) (318). For cancer associated mucins, alterations in glycosylation have been observed, of which incomplete glycosylation occurs most frequently (104).

Avellana-Adalid et al. reported a few brain lectin interacting proteins from human brain in the molecular weight range 41×10^3 to 67×10^3 (17). Later, Joubert et al. found that human brain lectin binds to actin and that this interaction was sugar independent (18).

In our study, we found that bovine brain stem also contains a lectin very similar to the lectin from cerebral cortex. Therefore we searched for the endogenous glycoprotein ligands of this lectin in brain stem. The preliminary concentration of glycoproteins by RCA-chromato-

graphy helped to get glycoproteins which are likely to interact with BBL, since RCA resembled BBL most in sugar specificity. Thus even less concentrated BBL interacting glycoproteins which otherwise go undetected if total brain proteins were used, were available for electrophoresis and western blotting in quantities that allowed a detectable interaction with the lectin. The results here establish that the glycoproteins giving rise to 84 KDa subunit that dominated the Western blot and the alkaline PAGE fast moving glycoprotein are the major BBL-interacting glycoproteins in the brain stem.

In view of the role played by cerebellar soluble lectin and its glycoprotein ligands in myelin compaction (47,48), it will be interesting to investigate the role of BBL and its glycoprotein ligands in brain stem. The presence of a similar lectin (12,6) and glycoprotein ligands (17) in the cerebral cortex emphasises the wide tissue distribution and possible similar role for these molecules. The presence of carbohydrate ligand offers protection and stability to the lectin. The 14 KDa bovine lectin when treated with staphylococcal V8 protease or trypsin was completely protected by the presence of the ligand (lactose) from degradation while maltose (not recognized by the lectin) offered no protection (59). It remains to be seen whether the endogenous glycoprotein or glycolipid ligands offer

protection to the lectin in vivo. The exact role played by brain lectin and its glycoconjugate ligands in brain is not known. Since alteration in the expression of lectin and glycoprotein was observed in malignant transformation and metastasis, elucidating the role of lectin-glycoprotein interaction in brain will be helpful in better understanding of these pathological states and this will pave way for the better management of these ailments.

CHAPTER IV

GENERAL DISCUSSION AND SUMMARY

GENERAL DISCUSSION AND SUMMARY

A 14 KDa β -galactoside-binding lectin present in human and bovine brain cortex had been reported. We detected a similar lectin in the bovine brain stem as confirmed by sugar-specificity, electrophoretic mobility and antigenic cross reactivity.

Bovine brain lectin (BBL) from grey matter was found to recognize endogenous gangliosides sugar-specifically. This was demonstrated by two different ways.

(a) **Affinity electrophoresis.** Gangliosides incorporated in the polyacrylamide gels retained BBL when the latter was subjected to electrophoresis through the gel. This retention was fully reversible with the BBL-specific sugar lactose, but not by the non-specific sugar sucrose.

(b) **Inhibition of hemagglutination** in which gangliosides inhibited the agglutination of trypsinised rabbit erythrocytes by BBL. Desialylated gangliosides also give similar results in affinity electrophoresis and hemagglutination inhibition.

The possibility of a decrease in porosity of the polyacrylamide gel consequent on ganglioside incorporation causing reduced mobility of BBL was ruled out by the observation that the electrophoretic mobility of ovalbumin was unaffected by the presence of ganglioside in the gel.

Retention of BBL in the gel by gangliosides was confirmed by dot blot of an extract of the gel after electrophoresis and probing with rabbit anti-BBL antibody followed by anti-rabbit IgG coupled to horse radish peroxidase (HRP).

Contribution of traces of glycoproteins possibly contaminating the ganglioside mixture used, towards BBL-binding was also ruled out using dot blot of ganglioside sample probed with BBL-HRP conjugate.

Gangliosides did not undergo any alteration in structure under conditions of affinity electrophoresis since thin layer chromatogram of glycolipids extracted from the gel after electrophoresis was identical with that of gangliosides used for incorporation in the gel.

Carbohydrate estimation showed that 83% of gangliosides incorporated was recoverable from the gel after electrophoresis.

The chance of BBL binding to gangliosides through a sugar-independent mechanism and getting detached from them by a change in conformation of the lectin induced by lactose was ruled out by the observation that in presence of Ricinus communis agglutinin (RCA), GM1 ganglioside could not bind BBL (It was known that RCA binds to GM1 sugar-specifically).

Of the five different gangliosides mixed with BBL and loaded on 9% alkaline PAGE to observe their relative

capacity to retain BBL, the most interacting species was found to be GTIb. Other BBL-interacting gangliosides in the decreasing order of affinity towards BBL were GD1b, GM2, GM1 and GD1a.

For the isolation and characterization of BBL-interacting endogenous soluble glycoproteins from brain stem, we prepared RCA-binding soluble glycoproteins (by RCA-Sepharose column chromatography) from brain stem as RCA resembled BBL most in sugar specificity. The interaction of BBL with RCA-binding glycoproteins was shown by dot blotting. Among the RCA-binding glycoproteins BBL-interacting glycoproteins were identified by Western blotting after SDS-PAGE of the glycoproteins followed by probing with BBL-HRP. BBL strongly interacted with a band of Mr 84 KDa and weakly with a few other bands.

On alkaline PAGE, RCA-binding glycoproteins were resolved into a fast moving band and a cluster of very slow moving bands. After electroelution, proteins in the fast moving band was sugar specifically recognized by BBL-HRP and on SDS-PAGE showed two closely spaced bands of Mr 55 KDa and 58 KDa.

Binding of lectins and sugar-specific antibody revealed the presence of different types of carbohydrate moieties on BBL-interacting glycoproteins. Enzymatic deglycosylation was also employed to ascertain this. The

84 KDa protein and alkaline PAGE fast moving protein differ in their carbohydrate composition and are immunologically distinct as shown by the lack of cross reactivity of the antibodies raised to these proteins.

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SYNOPSIS

**"STUDIES ON MEMBRANE-BOUND AND
SOLUBLE GLYCOCONJUGATES RECOGNIZED
BY BOVINE BRAIN β -GALACTOSIDE
BINDING LECTIN: GANGLIOSIDES FROM
GREY MATTER AND GLYCOPROTEINS
FROM BRAIN STEM"**

SYNOPSIS

by

KANNAN V.M.

for Ph.D Degree

of

BIOCHEMISTRY

**SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL
SCIENCES AND TECHNOLOGY
TRIVANDRUM**

STUDIES ON MEMBRANE - BOUND AND SOLUBLE GLYCOCONJUGATES
RECOGNIZED BY BOVINE BRAIN β -GALACTOSIDE BINDING LECTIN:
GANGLIOSIDES FROM GREY MATTER AND GLYCOPROTEINS FROM
BRAIN STEM

Lectin - glycoconjugate interactions in vivo that facilitate cell-cell and cell-extracellular matrix adhesion have been implicated in development, differentiation, malignant transformation and metastasis (1). Quantitative and qualitative changes in glycoconjugate or lectin patterns have been reported to correlate with the metastatic potency of tumour cells (2,3). A 14 KDa β -galactoside binding lectin (BBL) present in human and bovine brain cortex has been reported (4). We detected a similar lectin in the bovine brain stem as well. The capacity of β -galactosides to extract these lectins from brain tissue as well as to inhibit lectin - mediated brain cell aggregation confirmed the presence of specific endogenous glycoconjugates. In this study we show that purified endogenous membrane - bound ligands (gangliosides from grey matter) and soluble ligands (glycoproteins from brain stem) are sugar - specifically recognized by BBL in vitro.

Comparison of BBL from brain stem with that from cerebral cortex

BBL from brain cortex or from brain stem was isolated from tissue homogenate by affinity chromatography on lactose-Sepharose. Both samples had identical electrophoretic mobility and similar sugar specificity in agglutination inhibition study. Moreover their antibodies were mutually cross-reactive, and both were non glycosylated. Thus BBL from cerebral cortex and brain stem were similar if not identical.

Interaction of BBL with ganglioside:-

Combined bovine brain grey matter gangliosides were prepared essentially as described by Folch-Pi et al, (5), and further purified by base treatment, silica gel chromatography and ion exchange chromatography. Interaction of BBL with gangliosides was studied using the following two techniques:-

a. Affinity electrophoresis:-

Combined bovine brain gangliosides (2mg/ml) with or without inhibitory sugar for BBL were incorporated in

7% polyacrylamide gel during polymerization. Normally, on electrophoresis at pH 8.2, BBL moved as a distinct band. But this movement as a band was abolished when ganglioside was present in the gel (6). This retention of BBL by gel impregnated ganglioside was sugar-dependent since lactose, if present in the gel, restored the BBL band in a concentration dependent manner, while sucrose was ineffective. The retaining effect of ganglioside was proportional to its concentration. Desialylated gangliosides also gave similar results. The possibility of a decrease in porosity of the gel consequent on ganglioside incorporation causing reduced mobility of BBL was ruled out by the observation that the electrophoretic mobility of ovalbumin was unaffected by the presence of ganglioside in the gel. Retention of BBL in the gel by ganglioside was confirmed by dot blot of an extract of the gel after electrophoresis and probing with rabbit anti-BBL antibody followed by anti-rabbit IgG coupled to horse radish peroxidase (HRP). Contribution of traces of glycoproteins possibly contaminating the ganglioside mixture used, towards BBL binding was also ruled out using dot blot of ganglioside sample probed with BBL-HRP conjugate. Binding of this conjugate to mixed ganglioside used in affinity electrophoresis was negative even as presence of ganglioside did not affect recognition of asialofetuin by the

conjugate. Gangliosides in the mixture did not undergo any alteration in structure under conditions of affinity electrophoresis since thin layer chromatogram of glycolipids extracted from the gel after electrophoresis was identical with that of gangliosides used for incorporation in gel. Carbohydrate estimation showed that 83% of ganglioside incorporated was recoverable from the gel after electrophoresis. The chance of BBL binding to gangliosides through a sugar independent mechanism and getting detached from them by a change in conformation of the lectin induced by lactose was ruled out by the observation that in presence of Ricinus communis agglutinin (RCA), GM1 ganglioside could not bind BBL. (It is known that RCA binds to GM1 sugar-specifically.

b. Inhibition of BBL-mediated hemagglutination:-

Study of inhibition by gangliosides of BBL-mediated agglutination of trypsinized rabbit RBC in presence of bovine serum albumin (to prevent sugar-independent agglutination by ganglioside) showed that both native and desialylated gangliosides inhibited agglutination to the same extent (6). Gangliosides purified further by DEAE-Sephadex chromatography were nearly 5 times as effective as untreated gangliosides, indicating that non-ganglioside

glycolipids possibly present in the ganglioside sample hardly contributed towards inhibition. This inhibition was sugar-specific since native and desialylated ganglioside at concentrations respectively 23 and 42 times that was needed for inhibition of BBL could not inhibit hemagglutination by human serum anti- α - galactoside antibody (anti-Gal) which recognizes terminal α - linked galactose only (7).

BBL-binding to individual gangliosides:-

Each of five different gangliosides was mixed with BBL and loaded on 9% PAGE to observe its capacity to retain BBL. Individual gangliosides differed in the extent of retention of BBL. The most interacting species was GT1b. Other BBL-interacting gangliosides in the decreasing order of affinity towards BBL were GD1b, GM2, GM1 and GD1a.

Detection and isolation of BBL-binding soluble glycoproteins from brain stem:

Membrane glycoproteins from bovine brain stem, prepared from 100,000 g pellet of homogenate of the tissue was found to possess no BBL-binding activity as detected on their dot-blot. The 100,000 g supernatant in which BBL-binding glycoproteins could be detected was loaded to a RCA-

Sepharose column, since among common lectins, RCA resembled BBL most in sugar specificity. RCA-binding glycoproteins were expected to be enriched in BBL-binding sugars. This assumption was confirmed by probing dot blot of RCA-binding brain stem glycoproteins using BBL-HRP. Western blot after SDS-PAGE, probed with BBL-HRP showed one strongly interacting band of Mr 84 KDa and a few weakly interacting bands. On electrophoresis of the RCA-binding glycoproteins at pH 8.2, in gels of both 7% and 10% acrylamide, a single prominent and fast moving band and a cluster of very slow moving bands were observed. The fast moving band was electroeluted by keeping the gel slices, before fixing, in Tris-acetate buffer, pH 8.0 in a dialysis bag and applying voltage (100V) across it. The electroeluted protein, on dot blots was sugar-specifically recognized by BBL-HRP and on SDS-PAGE, showed two closely spaced bands of Mr 55 KDa and 58 KDa. On electroelution, the 84 KDa glycoprotein subunit from direct SDS-PAGE of RCA-binding glycoproteins was also demonstrated to be BBL-binding on dot blots.

Characterization of BBL-binding brain stem glycoproteins:-

Using dot blots of glycoproteins and HRP conjugates of various sugar-binding proteins (lectins and antibodies) of known specificity, presence of various

carbohydrate moieties were ascertained. Enzymatic deglycosylation was also employed. Using antibodies in mice against electroeluted samples, the 84 KDa glycoprotein and the fast moving protein from alkaline PAGE were shown to be immunologically distinct.

CONCLUSION

This is the first demonstration of a sugar-specific interaction of an animal lectin with endogenous gangliosides. Since gangliosides are mostly cell-surface located and their patterns change during differentiation and tumorigenesis, this interaction, may have a regulatory role in cell sociology. In view of reduced ganglioside concentration in brain stem compared to grey matter and the absence of BBL-binding membrane-bound glycoproteins, the soluble BBL-binding glycoproteins may have a significant role in the physiological function of BBL in the brain stem.

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2. Major bovine brain stem glycoproteins sugar-specifically recognized by endogenous 14 KDa galactose-binding lectin V.M. Kannan and P.S. Appukuttan (1994) Indian J. Biochem Biophys 31,160-164.