IDENTIFICATION AND CHARACTERISATION OF ENDOGENOUS HUMAN GLYCOCONJUGATES RECOGNISED BY PLASMA ANTI-CARBOHYDRATE ANTIBODIES

A THESIS PRESENTED BY

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TO

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

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DECLARATION

I, Karthi. S hereby certify that I had personally carried out the work depicted in the thesis entitled "**Identification and characterization of endogenous human glycoconjugates recognised by plasma anti-carbohydrate antibodies.**" No part of the thesis has been submitted for award of any other degree or diploma prior to the date.

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CERTIFICATE

This is to certify that **Ms. Karthi. S**, in the Department of Biochemistry of this institute, has fulfilled the requirements prescribed for the Ph.D degree of the Sree Chitra Tirunal Instiute for Medical Sciences and Technology, Trivandrum.

The thesis entitled **"Identification and characterization of endogenous human glycoconjugates recognised by plasma anti-carbohydrate antibodies"** was carried out under my direct supervision. No part of the thesis was submitted for the award of any other degree or diploma prior to this date.

Clearances were obtained from the Institutional Ethics Committee [SCT-IEC/674, SCT-IEC/1032, SCT-IEC/ 1072] for carrying out the study.

Date: Dr. P. S. Appukuttan

The thesis entitled

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for the degree of

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Karthi.S

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ABBREVIATIONS

Aβ	amyloid β
ABG	anti-β-glucan antibody
AD	Alzheimer's disease
ADP	adenosine diphosphate
Anti-Gal	anti-α-galactoside antibody
AOP	AOP1 plus AOP2
AOP1	albumin-associated antibody-binding O-glycosylated protein 1
AOP2	albumin-associated antibody-binding O-glycosylated protein 2
apo(a)	apolipoprotein (a)
apoB	apolipoprotein B
APAG	affinity-purified anti-Gal
APABG	affinity-purified ABG
APP	amyloid precursor protein
APS	ammonium persulphate
CLGG	cross-linked guar galactomannan
CNS	central nervous system
Con A	concanavalin A
DTT	dithiothreitol
ELISA	enzyme linked immunosorbent assay

F-Aβ	fluorescent-labeled amyloid β
FITC	fluoroisothiocyanate
GalNAc	N-acetyl galactosamine
GlcNAc	N-acetyl glucosamine
HBSS	Hank's balanced salt solution
HRP	horse radish peroxidase
HSA	human serum albumin
Ig	immunoglobulin
Jn	jacalin
LDL	low density lipoprotein
Lp(a)	lipoprotein(a)
LRP	LDL receptor-related protein
2-ME	2-mercaptoethanol
MαG	methyl α-galactoside
MαM	methyl α-mannoside
MUC 1	mucin peptide 1
OPD	ortho-phenylenediamine
PAGE	polyacrylamide gel electrophoresis
PBS	20 mM potassium phosphate buffer containing 150 mM NaCl pH 7.4
PBST	PBS containing 0.05% Tween 20
PD	Parkinson's disease
PNA	peanut agglutinin
SDS	sodium dodecyl sulphate

STPS	serine- and threonine- rich peptide sequence
TAG	terminal α-galactoside
TEMED	N, N, N’, N’-tetraethyl ethylene diamine
Tg	thyroglobulin
TI	trypsin inhibitor
TIM	trypsin inhibitor-melibiose
TIC	trypsin inhibitor-cellobiose

SYNOPSIS

Background

Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common neurodegenerative disorders with steadily increasing incidence worldwide and accompanied by gradual and progressive decline in cognitive function and motor and non-motor functions due to damage and death of neurons in brain. Aggregation and abnormal deposition of proteins inside and around neurons are common pathological features in AD and PD. AD is characterized by the extra neuronal deposition of aggregated amyloid β (Aβ) as plaques and intra-neuronal deposition of microtubule-associated protein tau forming neurofibrillary tangles. In PD aggregation of α-synuclein causes accumulation of Lewy bodies. Monomeric Aβ, a 4 kDa protein formed as a cleavage product of amyloid precursor protein (APP) play important roles in synaptic plasticity, memory, antioxidant activity and also in maintaining the integrity of blood brain barrier. Tau is involved in axonal transport of biomolecules necessary for neural functions and α-synuclein, a small protein about 14 kDa in size and 140 amino acid residues is involved in neurotransmitter release and synaptic plasticity. When misfolded and aggregated these proteins are non-functional and cause neurotoxicity. There are reports of monoclonal antibodies against Aβ, tau, and α-synuclein being used in *in vitro* and animal trials to reduce formation of aggregates of these proteins since most substances that interact with them prevent their aggregation *in vitro*. However success, especially in animal trials has been few and uncertain. This prompted us to explore autologous molecules possessing structural features complementary to those of Aβ, tau or α-synuclein so as to interact with and

prevent aggregation of the latter molecules. Recently anti-α-galactoside (anti-Gal) antibody in human plasma has been reported from this laboratory to bind plasma lipoprotein(a) [Lp(a)] recognizing the serine- and threonine-rich peptide sequences (STPS) underlying their O-glycan chains as surrogate ligands. Notably tau and α-synuclein possess STPS and another plasma antibody (ABG) against β-glucosides also recognizes STPS. In the present work we detected two new albumin-associated antibody-binding O-glycosylated proteins AOP1 and AOP2 that interact with Aβ.

Hypothesis

Naturally occurring anti-carbohydrate antibodies anti-Gal and ABG, being specific to STPS that is characteristic of tau and α-synuclein, are possible natural provisions for binding to the latter molecules in order to prevent their aggregation. In support of this hypothesis human tau contain significantly more STPS. Anti-Gal synthesis started in evolutionary history at the same time when primates endowed with advanced brain and superior cognitive functions arose. Further, anti-Gal and ABG prepared from human plasma using their specific affinity matrices always contained as copurified molecules the O-glycosylated proteins AOP1 and AOP2 along with albumin while plasma albumin purified by albumin-specific affinity chromatography also contained AOP1 and AOP2 and the above two antibodies. These results prompted the hypothesis that plasma contains three member protein complexes (triplets) consisting of anti-Gal/ABG linked to albumin via an intermediary protein AOP1 or AOP2.

Though many reports ascribe plasma Aβ-binding activity to albumin purified albumin was inert to Aβ leading to the speculation that AOP1 and AOP2, the main albumin-associated plasma proteins played this role, particularly because these O-

glycoproteins are rich in STPS which is the domain recognized by Aβ on its cell surface receptor namely LDL receptor-related proteins (LRP).

Anti-Gal and ABG immune complexes with large antigens mandatorily have unoccupied binding sites that may be used to home these complexes to other smaller ligands including those on host cells, as shown by us earlier with reconstituted anti-Gal-Lp(a) immune complexes. Triplets being abundant in plasma and brain cells being uniquely rich in STPS-bearing LRP on their surface, triplets are possibly the ideal vehicles to carry the antibodies and O-glycoproteins to brain cells where they could prevent aggregation of Aβ, tau and α-synuclein. Verification of this carrier function of triplets was made using a model LRP-bearing host cell *viz* macrophages.

Reports are that platelets possess adhering antibodies and albumin in 1:1 and hold most of the circulating Aβ thereby acting as plasma sink for the peptide so as to reduce its re-entry into brain. Molecular mehanisms of this function is hardly known. Triplets contain immunoglobulin and albumin in 1:1 ratio as also Aβ-binding O-glycoproteins. Platelet surface O-glycoproteins possess STPS to anchor triplets through their antibodies. Molecular mechanism of triplet adhesion to platelets and consequences of triplet removal from platelets (denudation) were studied to explain platelet functions at the molecular level.

Objectives

- Identification and characterisation of endogenous O-glycoproteins that bridge between anti-Gal/ABG and albumin to form antibody-O-glycoprotein-albumin triplet
- To study the function of albumin in triplet formation

- To determine the role of albumin-associated antibody-binding O-glycosylated proteins in amyloid β binding
- To study entry of triplets into macrophages mediated through LRP receptors using the free binding sites in antibody
- Demonstrating recognition of STPS-rich brain glycoproteins like α-synuclein and tau by anti-Gal and ABG
- Explore molecular mechanisms of triplet adhesion to platelet surface and its consequences

Materials and methods

The presence of O-glycoproteins and albumin co-purified with anti-Gal and ABG were separated by electrophoresis and electroelution. The molecular weights of these proteins were determined by SDS gel electrophoresis and gel permeation chromatography. To check the glycosylation pattern and sialic acid contents proteins were directly coated and probed with HRP-conjugated jacalin or concanavalin A or peanut lectin respectively. The binding of O-glycoproteins to anti-Gal or ABG was checked using them as inhibitors of antibody binding to standard ligands in ELISA. For anti-Gal and ABG their binding to bovine thyroglobulin and trypsin inhibitor-cellulose (TIC) respectively, coated on polystyrene microwells were inhibited. Alternatively fluorescently labeled antibody in which ligand binding increases florescence in proportion to ligand concentration and ligand affinity was also employed. Albumin-glycoprotein complex was assayed by capturing on microplate-coated jacalin and probing with HRP-conjugated anti-albumin. Antibody-glycoprotein-albumin triplet was assayed by capturing on microplate-coated antibody ligands given above followed by probing with anti-albumin-HRP. Aβ binding to

glycoproteins was demonstrated using fluorescent-labeled Aβ and measuring its increase in fluorescence on incubating with glycoproteins. Entry of antibodies into macrophages was studied using macrophages cultured on sterile dishes for incubation with *de novo* antibody-glycoprotein-albumin complex in which either O-glycoprotein or anti-Gal/ABG was FITC-labeled. The surface bound fluorescence was removed using antibody-specific sugars and the cell bound fluorescence was measured after washing. It was also confirmed by observing under fluorescence microscope. The binding of antibody-glycoprotein-albumin complex to platelet surface glycoproteins was demonstrated by releasing the bound triplet into supernatant using antibody–specific sugars. The binding of anti-Gal and triplets to O-glycosylated proteins like GPIIb/IIIa was studied by incubating denuded platelets with anti-Gal-FITC or *de novo* triplet-FITC and measuring the cell bound fluorescence. To demonstrate the contribution of triplets in protecting platelets ADP-mediated aggregation before and after denudation was measured in terms of decrease in absorption of platelet suspension at 405 nm. The role of triplets in preventing fibrinogen binding to GPIIb/IIIa was shown by estimating the decrease in added triplets in supernatant in denuded platelets in presence and absence of fibrinogen. Prior blocking of platelets with jacalin, the O-glycan-specific plant lectin was employed to ascertain the role of O-glycosylated STPS-bearing cell surface proteins in ABG/anti-Gal or triplet adhesion.

Major findings:

1. Two new O-glycosylated proteins each binding albumin at one end and anti-carbohydrate antibodies anti-Gal or ABG at the other to form anti-Gal/ABG-O-glycoprotein-albumin triplet were identified and purified.

2. AOP1 and AOP2 are single polypeptide proteins of molecular weights of 107 kDa and 98 kDa and neutral sugar contents of 54% and 51 % respectively.

3. Nearly 36% of plasma albumin is AOP- or AOP2-bound though only a minor fraction of such binary complexes forms triplets. Availability of anti-Gal and ABG may be the rate-limiting factor in triplet formation since free antibodies are undetectable in plasma.

4. AOP1 and AOP2 are cross-reactive with LRP and bind to Aβ more strongly than equal amount of α-synuclein or β-cyclodextrin, known medicinal and endogenous receptors respectively for Aβ, did. In commercial albumin samples reported to bind Aβ the binding is actually to contaminant AOP1and AOP2 molecules present; purified albumin does not bind Aβ.

5. Albumin complexed to AOP1/AOP2 is big enough to leave adjacent binding site of antibody free after binding. Using this free binding site triplets bind to macrophages through their surface LRP receptors and deliver triplet components to the cell interior.

6. Anti-Gal and ABG in free and triplet forms bind STPS-containing brain glycoproteins like tau and α-synuclein.

7. Circulating platelets have adhering triplets bound possibly to their STPS-rich O-glycoprotein receptor GPIIb/IIIa. Triplets protect platelets from aggregation and triplet AOP1 and AOP2, rather than GPIIb/IIIa are Aβ ligands in normal platelets. Triplet-free platelets are more aggregative and attach far more of aggregating molecules fibrinogen and ADP. More triplets are platelet-bound than free in blood. Sugar specific to either antibody can release triplets of both.

Significance

Besides identifying two new O-glycoproteins that occupy nearly 36% of albumin in plasma results point to the role of antibody-O-glycoproptein-albumin triplets in protection of platelets and possibly in delivering antibodies and O-glycoproteins to LRP-rich brain endothelial cells and neurons, as a physiological provision to stall aggregation of Aβ, tau and α-synuclein. Increased susceptibility of diabetic patients in whom high blood glucose dismantles triplets and denudes their platelets, to AD and thrombotic vascular damages support the above conclusion.

1. INTRODUCTION

Neurodegenerative disorders

Neurodegeneration is any pathological condition primarly effecting the structure and function of neurons and is often multifactorial in origin though aging, genetic and environmental factors are thought to play significant roles. Of hundreds of different neurodegenerative disorders, more attention has been given to Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). We were interested in Alzheimer's disease and Parkinson's disease which were the most common and pressing problems of societies with aging populations. Formation and accumulation of insoluble protein aggregates are the major pathological hallmark associated with AD and PD (Forloni et al., 2002). AD is characterized by formation of extracellular β amyloid aggregates called amyloid plaques and intracellular tau protein aggregates called tau tangles causing irreversible loss of intellectual abilities, like reasoning and memory, thereby detrementally affecting the normal social and occupational lifestyle of the individual. PD is accompanied by α-synuclein aggregates in brain and is associated with several motor and non-motor deficits along with impaired cognitive, autonomic as well as psychiatric symptoms including tremor, muscle rigidity, loss of muscle coordination and bradykinesia. These aggregates can interfere with the normal plasticity of synapses by interrupting nutrient channels inside the neurons, axonal transport of synaptic vesicles, and impairing mitochondrial functions. A conformational change is associated with proteins involved in these neurodegenerative disorders as the α-helical structure of the proteins is converted to β-sheets associated with change from physiological function to pathological form (Kirkitadze et al., 2001). The toxic protein formed will eventually impair cellular function.

Role of amyloid beta in Alzheimer's disease

Amyloid β (Aβ) peptide of size about 4 kDa is derived from the larger Amyloid Precursor Protein (APP). Aβ extracted from senile plaques contains mainly $A\beta_{1-40}$ and $A\beta_{1-42}$ whereas vascular amyloid is predominantly $A\beta_{1-39}$ and $A\beta_{1-40.}$ A major soluble form of Aβ which is present in the blood, cerebrospinal fluid (CSF) and brain is $A\beta_{1-40}$. In normal physiology at lower concentrations, Aβ is involved in modulating synaptic activity, in memory and learning, has role in anti-oxidant activity, neurogenesis and maintaining blood brain barrier (Garcia-Osta & Alberini, 2009). Within the brain, catabolism of Aβ occurs but a significant amount of Aβ remains undegraded. Mechanism exists to transport Aβ across the blood brain barrier (BBB) and out into the circulation. If this mechanism is interfered it causes a large increase in the amount of Aβ that remains in the brain, leading to its ultimate accumulation. Soluble Aβ is exchanged across the BBB mainly by the low-density lipoprotein receptor-related protein (LRP) on the abluminal (brain) side (Shibata et al., 2000). The net efflux of Aβ across the BBB can predict the degree of cerebral amyloid burden (DeMattos et al., 2002) and the disruption of these mechanisms contributes significantly to development of amyloid pathology. The process of Aβ fibrillation involves a conformational shift which ultimately leads to the formation of extended β-sheets. In response to Aβ-induced neurotoxicity in AD, both humoral and cellular immunity are activated.

Role of tau protein in Alzheimer's disease

Tau is a microtubule-associated neuronal protein that binds to microtubules and assists their formation and stabilization and is most abundantly expressed in axons of

central nervous system neurons but can also be found in the somatodendritic compartment of neurons, oligodendrocytes, and non-neural tissues. When hyperphosphorylated tau is unable to bind to microtubules, becomes unstable and clumps to generate neurofibrillary tangles (NFT). Human tau exists in six major isoforms each showing different degree of phosphorylation and is capable of polymerising into fibrillar structures as seen in AD patients. There are about 79 serine and threonine residues in longest isoform of tau where more than 30 are susceptible for phosphorylation (Gong et al., 2005). Protein kinases and phosphatases which specifically recognize the serine and threonine rich regions of tau protein are responsible for the abnormal phosphorylation of protein (Goedert et al., 2012). These intracellular conformationally twisted tau tangles impair axonal transport between the cell body and numerous synapses, which is crucial to neuronal function and survival (Stefani & Dobson, 2003). Aggregated tau proteins can be involved in spread of AD pathology to neighboring tissues.

Role of α-synuclein in PD

Alpha-synuclein is a phospholipid-binding protein that is expressed highly in presynaptic terminals where it functions as a molecular chaperone to promote SNARE complex formation, to regulate synaptic vesicle formation and synaptic function and is involved in regulating the release of the neurotransmitter dopamine in controlling voluntary and involuntary movements. It is mainly expressed in the central nervous system (CNS), although the protein can also be detected in the peripheral nervous system and other tissues outside the CNS (Wakabayashi et al., 2010). It is normally localized to pre-synaptic terminals but in the diseased brain it

starts to accumulate in cell bodies and synapses. α-synuclein which exist normally as a soluble monomer attains a distinct secondary or tertiary structure that undergoes conformational changes to form intracellular fibrillar depositions called Lewy bodies which are insoluble neurotoxic forms capable of inducing membrane dysfunction and breakdown (Davidson et al., 1998).

Monoclonal antibodies against tau, amyloid β and α-synuclein

Immunotherapy has emerged as a promising tool to target and clear protein pathology in neurodegenerative diseases. There are monoclonal antibodies identified against amyloid β, tau and α-synuclein that can bind these proteins and prevent their aggregation. There are reports showing the presence of anti-Aβ antibodies in the blood and CSF of patients with AD and healthy subjects (Panza et al., 2010). On the other hand human anti-Aβ monoclonal antibodies have been used for the clearance of Aβ (Bohrmann et al., 2012). Blocking tau aggregates improved cognition *in vivo* (Yanamandra et al., 2013). Monoclonal antibodies that are highly specific for α-synuclein have been identified (Fagerqvist et al., 2013; Singh et al., 2017) and a human single-chain antibody fragment, specifically binding oligomeric α-synuclein, has also been developed and was reported to inhibit aggregation and prevent oligomer-induced toxicity (Emadi et al., 2004). Active immunization on transgenic mice showed decreased accumulation of α-synuclein aggregates (Masliah et al., 2011). But these monoclonal antibodies failed in Phase I or Phase II clinical trials and thus it was necessary to develop new therapeutic measures.

Anti-carbohydrate antibodies

They are naturally occuring antibodies forming the first line of defence against pathogens and are produced in all individuals without any deliberate immunization. Anti-α-galactoside antibody (anti-Gal) and anti-β-glucoside antibody (ABG) are the most abundant antibodies in humans. Anti-Gal is produced only in humans, apes and old world monkeys and is produced against α-Gal epitope (Galα1-3Galβ1-4GlcNAc-R). ABG present in other higher mammals as well is produced against β-glucan that are abundant in fungus and cell walls of plants like oats and barley. Humans get exposed to β-glucans by consuming diets rich in β-glucans and through fungal infections.

Anti-Gal and ABG are prepared from human plasma by capturing on respective affinity matrices and elution using antibody-spcific sugars (Geetha et al., 2007; Jaison & Appukuttan, 1992). On subjecting these affinity-purified anti-Gal and ABG to alkaline gel electrophoresis three proteins other than the antibody were detected. Reason for association of the copurified molecules with antibodies warranted investigation.

Tau and α-synuclein are proteins rich in serine- and threonine-rich peptide sequences (STPS) which are the sites for O-glycosylation and phosphorylation. Anti-Gal and ABG recognize STPS in proteins as their surrogate ligands and therefore recognize MUC-1 and apo(a) subunit of Lp(a) that are rich in STPS (Geetha et al., 2014; Sandrin et al., 1997). So it was imperative to check whether these natural antibodies could recognize tau and α-synuclein and be a natural mechanism preventing their aggregation and thereby protecting from neurodegenerative disorders.

HYPOTHESIS

Naturally occurring anti-carbohydrate antibodies anti-Gal and ABG, being specific to STPS that is characteristic of tau and α-synuclein, are possible natural provisions for binding to the latter molecules in order to prevent their aggregation. In support of this hypothesis human tau contain significantly more STPS. Anti-Gal synthesis started in evolutionary history at the same time when primates endowed with advanced brain and superior cognitive functions arose. Further, anti-Gal and ABG prepared from human plasma using their specific affinity matrices always contained as copurified molecules the O-glycosylated proteins AOP1 and AOP2 along with albumin while plasma albumin purified by albumin-specific affinity chromatography also contained AOP1 and AOP2 and the above two antibodies. These results prompted the hypothesis that plasma contains three member protein complexes (triplets) consisting of anti-Gal/ABG linked to albumin via an intermediary protein AOP1 or AOP2.

Though many reports ascribe plasma Aβ-binding activity to albumin, purified albumin was inert to Aβ leading to the speculation that AOP1 and AOP2, the main albumin-associated plasma proteins played this role, particularly because these O-glycoproteins are rich in STPS which is the domain recognized by Aβ on its cell surface receptor namely LDL receptor-related proteins (LRP).

Anti-Gal and ABG immune complexes with large antigens mandatorily have unoccupied binding sites that may be used to home these complexes to other smaller ligands including those on host cells, as shown in our laboratory earlier with reconstituted anti-Gal-Lp(a) immune complexes. Triplets being abundant in plasma and brain cells being uniquely rich in STPS-bearing LRP on their surface, triplets are

possibly the ideal vehicles to carry the antibodies and O-glycoproteins to brain cells where they could prevent aggregation of Aβ, tau and α-synuclein. Verification of this carrier function of triplets was made using a model LRP-bearing host cell *viz* macrophages.

Reports are that platelets possess adhering antibodies and albumin in 1:1 ratio. Based on the assumption that albumin was the chief Aβ-bearing molecule in circulation it was postulated that circulating platelets act as plasma sink for the peptide so as to reduce its re-entry into brain. Molecular mehanisms of this function is hardly known. Triplets contain immunoglobulin and albumin in 1:1 ratio as also Aβ-binding O-glycoproteins. Platelet surface O-glycoproteins possess STPS to anchor triplets through their antibodies. Molecular mechanism of triplet adhesion to platelets and consequences of triplet removal from platelets (denudation) were studied to explain platelet functions at the molecular level.

OBJECTIVES

1. Identification and characterisation of endogenous O-glycoproteins that bridge between anti-Gal/ABG and albumin to form antibody-O-glycoprotein-albumin triplet
2. To study the function of albumin in triplet formation
3. To determine the role of albumin-associated antibody-binding O-glycosylated proteins in amyloid β binding
4. To study entry of triplets into macrophages mediated through LRP receptors using the free binding site in antibody
5. Demonstrating recognition of STPS- rich brain glycoproteins like α-synuclein and tau by anti-Gal and ABG
6. Explore molecular mechanisms of triplet adhesion to platelet surface and its consequences

2. REVIEW OF LITERATURE

2.1. Glycoconjugates

A glycoconjugate is a compound in which one or more carbohydrate moieties (glycone) are covalently linked to a non-carbohydrate moiety (aglycone). Sugar chains may make a major portion in the mass of glycoconjugates. Carbohydrate moieties covalently attached to proteins or lipids are termed respectively as glycoproteins or glycolipids. The different monosaccharides attached to glycoconjugates in animals includes hexoses, sialic acids (most common is N-acetyl neuraminic acid), hexosamines, pentoses and uronic acids (Varki et al., 2009). Biosynthesis of glycans occurs both at the co-translational and post translational levels in the endoplasmic reticulum and Golgi apparatus. Most of the glycosylation reactions occur with the help of unique glycosyltranferase enzymes which belongs to a large family of enzymes.There are also certain types of glycoconjugates which are synthesized and reside within the nucleus and cytoplasm (Comer & Hart, 2000).

2.1.1. Glycoproteins

Glycosylation is the most common post translational modification in many of the proteins and they occur throughout the entire phylogenetic system ranging from algae to eukaryotes. More than 50% of human proteins are glycosylated and have crucial biological and physiological roles. Basic types of glycosylation include O-glycosylation and N-glycosylation. They are different in their location in protein chain, structure and biosynthesis.

N-linked glycosylation

N-Linked carbohydrate groups are attached to the aspargine residues through N-acetylglucosamine. N-Linked amino acid consensus sequence is Asn-any aminoacid-

Ser or Thr where the middle amino acid cannot be proline (Pro). Biosynthesis of N-glycans involves the attachment of a 14 residue high mannose precursor on dolichol which is a polyisoprenoid lipid in the membrane of endoplasmic reticulum (ER) acting as a carrier of the oligosaccharide. This oligosaccharide is then attached to asparagine residues of nascent polypeptide chain in the ER lumen and this precursor protein is conserved in plants, animals and eukaryotes [$Glc_3Man_9(GlcNAc)_2$] (Lodish et al., 2000). After this addition three glucose and one mannose residue are removed signaling that the protein is ready to get transferred to the polypeptide. There are differences in processing of different proteins and they also differ in different cell types and species producing N-linked proteins with a wide difference in structures. N-glycans attached to proteins are mainly of 3 different types: Oligomannose, complex and hybrid having the same core structure with three mannose residues and 2 N-acetylglucosamine residues.

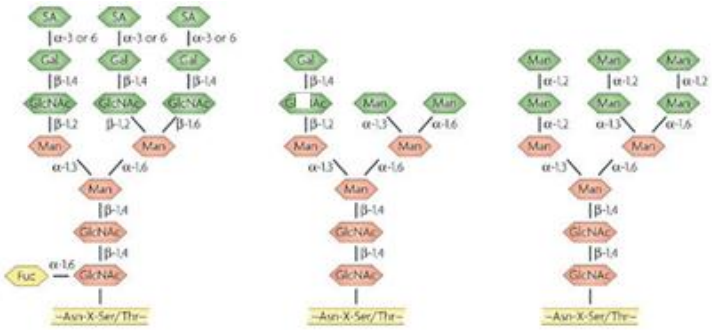


Figure 1. Structure of 3 main N-linked oligosaccharides

O-linked glycosylation

O-Linked glycans in proteins are linked through the hydroxyl groups of amino acids and are either mucin type or non-mucin tpe. Mucin type O-linked sugars are located in clusters in short regions of peptide chain containing repeating units of Ser, Thr,

and Pro. Most eukaryotic O-glycans are of the mucin-type which is linked through GalNAc attached to the hydroxyl groups of serine-or threonine residues of fully folded proteins. In non-mucin type α-linked fucose, β-linked xylose, α-linked mannose, β-linked N-acetyl glucosamine, α or β linked galactose, α or β linked glucose are attached to the hydroxyl groups of amino acids serine, threonine, hydroxylysine, hydroxyproline or tyrosine (Varki et al., 2009). O-Glycosylation is important for providing stability to glycoproteins by conferring resistance to proteases and heat as shown in mucins (Kramerov et al., 1996).

Mucin type O-glycosylation

Mucins are major glycoprotein components which line the surface of respiratory, digestive and urogenital tracts. All mucin polypeptide chains have domains rich in serine and threonine residues occurring as variable number of tandem repeats so that hydroxyl groups in these amino acids will contain O-glycans. The presence of GalNAcα-Ser/Thr is a hallmark of mucins (Spiro, 2002). There are also several proteins which are not mucins but are rich in 'mucin like domains', examples being fetuin, gonadotropins, glycophorins etc. The mucins or mucin type glycoproteins are rich in galactose, N-acetyl galactosamine, N-acetyl glucosamine and sialic acids with the carbohydrate groups closely clustered giving it a bottle-brush appearance projecting around an extended peptide backbone. There are mainly four common subtypes based on the different monosaccharide linkage attached to GalNAcα-Ser/Thr which includes Core 1 to Core 4.

Table 1. Mucin type O-glycans

Core types	Structure
Tn antigen	GalNAcαSer/Thr
Core 1 (T antigen/TF antigen)	Galβ1-3GalNAcαSer/Thr
Core 2	GlcNAcβ1-6(Galβ1-3)GalNAcαSer/Thr
Core 3	GlcNAcβ1-3GalNAcαSer/Thr
Core 4	GlcNAcβ1-6(GlcNAcβ1-3)GalNAcαSer/Thr

A frequently occurring O-linked glycosylation is the attachment of O-GlcNAc to nuclear and cytosolic proteins. O-Galactosyl glycans are also commonly found associated with collagens. O-Mannosyl, O-fucosyl and O-glucosyl are rare type of protein glycosylations. O-Glycan biosynthesis is simpler than N-glycan synthesis and is achieved by sequential addition of O-linked oligosaccharides in the ER and Golgi catalysed by several glycosyl transferases. The initiating event is the attachment of GalNAc to serine and threonine residues catalysed by GalNAc transferase enzyme. O-Glycans are also less branched compared to N-glycans and are commonly biantennary structures. Thomson Friedenreich antigen (T/TF antigen) is Core 1 structure, Thomson nouvelle (Tn antigen) is a precursor to Core 1 structure and its sialylated form is sialylated Tn (sTn). T, Tn and sTn antigens are cancer-sssociated and metastasis (Fu et al., 2016).

2.1.2. Role of glycans in biological systems

Glycans are a major and important part of all biological systems and are structurally diverse and rapidly evolving class of molecules (Varki, 2011). Glycosylation is a dynamic process with multiple mechanisms that alter glycosyltransferase and glycosidase expression and structure, as well as their accessibility to substrates. Glycans of non-vertebrate organisms can modulate the development and activation of the mammalian immune system (Cobb & Kasper, 2005). Mammalian glycans are well conserved, but species-specific variations exist which may be involved in the emergenc of distinct traits including susceptibilities to infectious pathogens (Gagneux & Varki, 1999). They have important role in cell-cell and cell-matrix interactions (Varki, 2017) including interactions of platelets, leucocytes and endothelial cells. Glycan-binding proteins and glycans bound on cell surface can interact with matrices or even with glycans on the same cell surface. A family of cell adhesion molecules called selectins are involved in rolling of leucocytes on endothelium. Glycoproteins have protective, stabilizing, organizational, barrier functions and possess distinct structural elements that govern interactions with other molecules. Glycans can promote or inhibit intra- and intermolecular binding including both homotypic and heterotypic interactions. Mammalian glycans can be substantial in size and frequency that they contribute the majority of mass and charge as in neural cell adhesion molecule (NCAM) having a uniquely large negative charge due to the presence of polysialic acids that inhibit homotypic NCAM protein-protein binding (Hoffman & Edelman, 1983). It has been shown that polysialic acid on NCAM must be regulated for selective axonal trafficking, emotional and cognitive memory. Glycans help in regulating protein folding, cell

adhesion, molecular trafficking and clearance, receptor activation, signal transduction, and endocytosis. Proteoglycans contain glycans which play important role in structure, porosity and integrity. Glycosylation has important roles in biological activity and function since human cells are more glycosylated compared to non-human cells and species more distant to humans in evolution are least glycosylated compared to humans (Brooks, 2004). In most animals N-linked glycans terminate in sialic acids which are commonly a mixture of N-acetylneuraminic acid or N-glycolylneuraminic acid but in humans they are mostly of N- acetylneuraminic acid form (Martinez-Duncker et al., 2014). O-GlcNAc modification associated with nucleocytoplasmic proteins mediates modulatory function by interplay with protein phosphorylation as they are attached to the same or adjacent serine or threonine residues (Hart et al., 1989). O-GlcNAc is a highly regulated post-translational modification required for the viability of many mammalian cell types by preventing protein phosphorylation. A thick layer of glycans on tissues provides tissue strength and heavily O-glycosylated proteins are resistant to a wide variety of proteinases.

2.1.3. Serine- and threonine-rich sequences as sites of glycosylation

Serine and threonine rich regions are motifs for glycosylation and phosphorylation. N-Linked glycosylation which occurs in all domains of life is attached to Asn-X-Ser/Thr consensus sequence (Wacker et al., 2006). O-Linked glycosylation is mainly mucin-type glycosylation where the acceptor sites contains domains rich in serine and threonine that exists in tandem repeats (Julenius et al., 2005).

2.1.4. Glycoproteins used in experiments for molecular recognition

Bovine Thyroglobulin

Thyroglobulin (Tg) is a 660-690 kDa dimeric glycoprotein produced by the follicular cells of the thyroid gland. They are rich in N-glycans which can be high mannose type rich in mannose and N-acetyl glucosamine and complex or hybrid type with mannose, galactose, N-acetyl glucosamine, N-acetyl neuraminic acid and fucose. Spiro and Bhoyroo in 1984 made a comparison of the glycosylation pattern of different species (calf, sheep, pig, dog, rat, rabbit, guinea pig and man) and they reported presence of Galα1-3Galβ1-4GlcNAc terminal structures (TAG group) which ranged from 11 mol/mol of protein (23% of total galactose) in calf to a complete absence in man. The presence of alpha-D-galactosyl groups resulted in a partial binding of the thyroglobulins (greater than 70% in calf and sheep) to *Grifonia simplicifolia* lectin (GSIB4) which specifically recognizes TAG group (Spiro & Bhoyroo, 1984). This was again confirmed by NMR spectroscopy (Dorland et al., 1984) and Thall et al 1990 again reported that thyroglobulin from nonprimate mammals and from New World monkeys express varying amounts of Gal-α1-3Galβ-1-4GlcNAc residues ranging between 0.01 and 11 residues per molecule, whereas no such residues were present on humans or Old World monkeys (Thall & Galili, 1990). The latter animals alone therefore synthesized anti-α-galactoside antibody (anti-Gal) which recognized thyroglobulin from non-primate animal and possessed similar binding specificity as GSIB4. TAG groups are absent in human thyroglobulin due to suppression of enzyme α-1,3 galactosyl transferase that occurred about 20-30 million years ago and are present in lower primates as the enzymes are expressed in them.

Guar galactomannan

Guar galactomannan is a hydrocolloid polysaccharide obtained from the endosperm of the leguminous plant *Cyamopsis tetragonolobus*. The endosperm contains a complex polysaccharide called galactomannan, which is a polymer of D-galactose and D-mannose with a linear backbone of α-1,4-linked β-D-mannopyranosyl units which are linked to 1,6-linked α-D-galactopyranosyl branches. All naturally occurring galactomannans possess this general structure but they differ in their mannose/galactose (M/G) ratio which in guar galactomannan is 2:1.

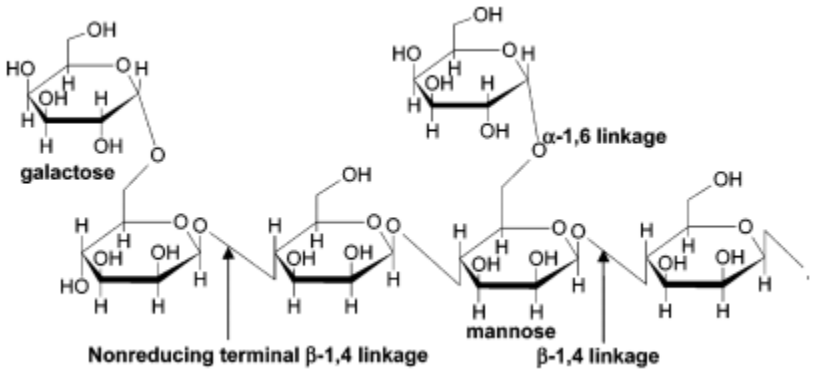


Figure 2. Structure of Guar galactomannan

Guar gum is rich in hydroxyl groups which in aquous media form hydrogen bonding making the solution viscous. They are easily soluble even in cold water and emulsify forming a gel and are stable over wide pH range (Thombare et al., 2016).

Fetuin

Fetuin is a glycoprotein of 48 kDa that was first isolated by Pedersen (1944) from fetal bovine serum which contain fetuin as a major protein component. It is an α-globulin structurally and biologically related to human α2HS. Fetuin has six carbohydrate side chains of which three are O-glycosylated and account for 20% of

the fetuin-bound carbohydrates and three are N-glycosylated and account for 80% of the fetuin-bound carbohydrates (Spiro & Bhoyroo, 1974). The N-linked oligosaccharides are tribranched and a minor population is dibranched and the sialic acid composition varies as majority are trisialylated (54%) and disialylated (35%) with minor populations tetrasialylated (8%) and monosialylated (3%) (Green et al., 1988). O-glycans in fetuin are mono or disialylated core-1 type O-glycans (Spiro & Bhoyroo, 1974). The large carbohydrate components in fetuin include galactose, mannose, glucosamine, galactosamine, and sialic acids where the hexosamines account for 5.5% of the weight of the protein or 6.8% when expressed as the N-acetyl derivatives (Spmot, 1960).

2.2. Lectins to characterize glycoconjugates

Lectins are proteins of non-immune origin that bind carbohydrates specifically and reversibly. They were first called as "haemagglutinins" or more commonly as phytohaemagglutinins as they were found exclusively in plants and also they were detected by erythrocyte agglutination and the first lectin to be identified was ricin from castor beans (Ricinus communis) by Hermann Stillmark in 1988. Since then, lectins were extracted from plants, including fungus and lichens, as well as in animals. The term lectin originated from the Latin "lectus", meaning "selected" which means the class of proteins that shows common characteristics of selectivity in the interaction with carbohydrates (Boyd & Shapleigh, 1954). The carbohydrate specificity of lectins was studied by hapten inhibition assay where different monosaccharides, oligosaccharides or glycopeptides were used to inhibit the haemagglutination produced with lectins (Rüdiger, 1993). As the interaction of

lectins with sugar is not through covalent bonding it is reversible and stability of its native structure is by the hydrophobic interactions.

Lectins can bind simple or complex carbohydrate conjugates which are either free in solution inside cells, in physiological fluids or on cell surfaces. The carbohydrate-binding activity of most of the lectins resides in a limited polypeptide segment which was termed as the carbohydrate-recognition domain (CRD) (Drickamer, 1988). All lectin molecules posses two or more carbohydrate binding sites that help them to agglutinate cells and to react with complex carbohydrates and some lectins can even bind up to five or more sugar residues. Since lectins differ in the types of carbohydrate structures they recognize they are usefull in the characterization of glycoconjugates. Many of these lectins are highly stable, can resist heat upto 70°C for more than 30 min and are not completely degraded with cooking. Some of them are even resistant to digestive enzymes and acids and are re-absorbed into the blood stream in animals and can cause an immune response.

Depending on their properties and their distribution in tissues, lectins can play important physiological roles. They exhibit different biological activities such as mitogenic and antiproliferative activities on cell lines of human cancer, inhibit bacterial and fungal growth, can cause cell aggregation and are involved in immunomodulatory activities and toxic effects.

2.2.1. Plant lectins

Plant lectins are used to study protein-carbohydrate recognition. They may exist in various tissues of the same plant and have different cellular localizations and molecular properties. The major sources of lectins in plants are seeds, legumes,

barks, heartwood, leaves and fruits. Other lectin sources are roots, tubers, bulbs, rhizomes and cotyledons. Plant lectins differ in amino acid sequence, in their number of subunits and in the nature of the polypeptides and they are relatively soluble and can be easily extracted. The activity of lectins can be usually measured by agglutination assay using erythrocytes. But some lectins like ricin are not agglutinins since they have only a single carbohydrate subunit and therefore some other methods like toxicity measurements have to be used for measuring their activity. Plant lectins are used to characterize the structures of animal cell glycoconjugates. Since they are highly specific the immobilized forms of these lectins are used in affinity purification of glycoproteins. The terminal modifications like sialylation or fucosylation in glycoproteins can be specifically recognized by plant lectins.

Jacalin

Jacalin is a plant lectin isolated from jackfruit (*Artocarpus heterophyllus*) seeds and was first reported by Ahmed and Chatterjee (1989). It is a tetrameric two-chain lectin with molecular mass of 65 kDa with a heavy α chain of 133 amino acid residues and a light β chain of 20–21 amino acid residues. It binds to O-linked glycoproteins and is highly specific for the O-glycoside present in the disaccharide Thomsen–Friedenreich antigen which is the core1 structure (Galβ1–3GalNAc), even in its sialylated form (Sastry et al., 1986). The GalNAcα- or Galα-structure at the reducing end is essential for the binding of jacalin. The presence of sialic acid (STn) at the C6 position of αGalNAc reduces its binding and the presence of an additional carbohydrate, such as βGlcNAc (in the cases of Core 2 and Core 6) abolishes their binding to jacalin. Jacalin was found to recognize the GalNAc moiety more extensively than the Gal moiety (Tachibana et al., 2005).

Jacalin binds specifically to the heavy chain of IgA and thus used as a diagnostic tool for IgA subclassing as it could distinguish between two subclasses of IgA in human serum and as a preparative tool for removing IgA from IgD and for removing IgA from biological samples and preparations (Aucouturier et al., 1989). It can completely block human immunodeficiency virus type 1 (HIV-1) by specifically inducing the proliferation of CD4+ T lymphocytes in human. Thus it can be used as a tool for the evaluation of the immune status of patients infected with human immunodeficiency virus (Corbeau et al., 1994).

Concanavalin A

Concanavalin A (Con A) was prepared from the common jackbeans (*Canavalia ensiformis*) in 1919 by Sumner and Howell (1936) and belongs to the family Leguminasae which is the most characterized family of plant lectins. They are metalloproteins with a tightly bound Ca2+ and a transition metal ion, usually Mn2+. Con A has a molecular weight of 104 kDa with 238 amino acid residues where about 20% of the amino acid residues are invariant in all legume lectins and another 20% are similar. The amino acid residues that are involved in interaction with sugars and those that coordinate the metal ions are conserved and include aspartic acid, asparagine, glycine which are conserved in all legume lectins and an aromatic amino acid or leucine. At physiological pH conA exists as a homotetramer with identical subunits each with a single saccharide-binding site.

ConA binds very tightly to high-mannose-type N-glycans and with weaker affinity to hybrid-type and biantennary complex-type N-glycans. Con A assembles into a compact β-barrel configuration devoid of α-helices. The binding site of

carbohydrates involves H bonds and hydrophobic interactions and the metals do not make direct contact with the sugars but help in stabilizing the amino acid chains required for binding (Reeke et al., 1975). When the specific sugar for Con A methyl α-D-glucopyranoside was used it stabilizes the lectin acting as a protector preventing conformational alterations at varying pH conditions. Con A acts as a mitogen that can activate the immune system, recruit lymphocytes and elicit cytokine production. They are mitogenic to peripheral blood lymphocytes and are cytotoxic to cells at higher concentrations (Krauss et al., 1999).

Peanut agglutinin (PNA)

PNA is a non-glycosylated lectin prepared from the fruits of peanut (*Arachis hypogaea*). It belongs to the family of legume lectins and therefore show similarity to Con A being metalloprotein containing Ca2+ and Mn2+ stabilizing its structure for ligand binding and shows similarity in tertiary structure with a β barreled structure and no α chains but with different quaternary structure. PNA is a homotetramer with an unusual open quaternary structure stabilised mainly by hydrophobic interactions and hydrogen-bonding. PNA has molecular weight 110 kDa (Natchiar et al., 2007).

PNA binds the carbohydrate sequence Galβ (1-3)-GalNAc that gets accessible to the lectin after neuramidase treatment of cell surface O-glycoproteins particularly those containing core 1 type glycans. PNA was used to study the surface carbohydrate expression of Galβ (1-3)-GalNAc by normal and malignant hemopoietic cells (Erber et al., 1992).

2.3. Neurodegenerative disorders: Alzheimer's disease and Parkinson's disease

Neurodegeneration is the progressive loss of structure and function of neurons even leading to death of neurons. Though a large number of neurodegenerative disorders are known Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common (Guttmacher et al., 2003). Increasing age is the consistent risk factor for developing these disorders. These disorders are generally called proteinopathies as a major hallmark of these disorders is the formation of misfolded protein aggregates. In normal brain these proteins are present in normal levels, and if these proteins are misfolded they will be either degraded or refolded correctly by chaperons which help in protein folding and stabilization. However in conditions of neurodegeneration these misfolded proteins form insoluble protein aggregates which are not degradable and become toxic (Sweeney et al., 2017).

2.3.1. Proteins involved in neuropathology of AD

Alzheimer's disease is the most common progressive irreversible neurodegenerative disorder which mainly affects the cortex and hippocampus. It results in loss of memory, ability to judge, decision making and language. AD is associated with high levels of amyloid-β and tau aggregates widely distributed throughout the brain forming extracellular amyloid plaques and intraneuronal tau tangles. In the last decades, researchers and pharmaceutical industries are focusing to develop drugs to cure AD where they target on amyloid β aggregates and neurofibrillary tangles. But clinical trials have largely failed, one possible reason cited for failure being a lack of biomarkers which could reliably identify AD in early stages for the therapy to be

successful. Therefore, new diagnostic tools capable of early detection are primarily needed.

(a) Amyloid β (Aβ)

Amyloid β, a 4 kDa protein is a cleavage form of large transmembrane protein Amyloid Precursor Protein (APP) (110-130 kDa). APP exists in different isoforms APP751, APP770, APP714, APP639, APP695 and LAPP ranging from 365-770 amino acids in length. APP695 that is rich in serine and threonine are present abundantly in humans (Tang & Gershon, 2003). In AD patients, there is a decrease in APP695 and increase in APP770 (Griffith et al., 1995). APP is involved in neuronal development and is processed through two pathways: amyloidogenic and non-amyloidogenic constitutive pathway. In non-amyloidogenic pathway, APP can be cleaved by α-secretase releasing soluble extracellular fragment called sAPP-α which have neuroprotective functions. In amyloidogenic pathway, it is cleaved by β-secretase producing soluble sAPP-β or by γ-secretase acts that produce Aβ which can exist as monomer, dimer or oligomers and can aggregate itself forming fibrils with β-sheet structure that can get deposited forming neuritic plaques (Selkoe & Hardy, 2016). These neuritic plaques are a hallmark of Alzheimer's disease. There are different Aβ species where those ending at position 40 (Aβ40) are the most abundant (~80-90%), followed by Aβ42 (~5-10%). Aβ42 are more hydrophobic and fibrillogenic, and are the principal form deposited in the brain.

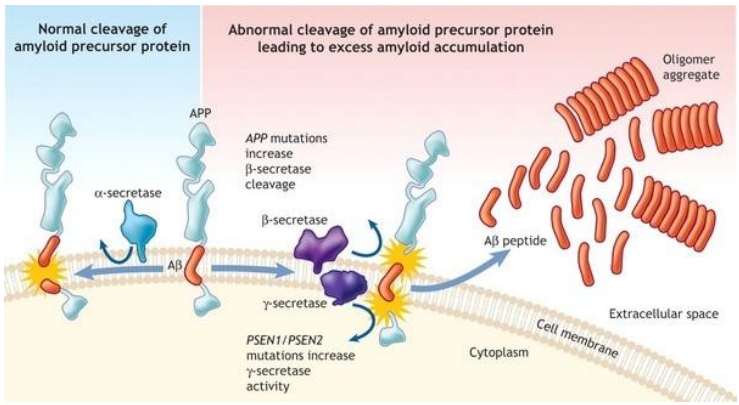


Figure 3. Processing of APP

Higher levels of Aβ have pathological potential whereas lower levels are physiologically necessary as it is important in synaptic activity, learning and memory (Plant et al., 2003). It has the ability to act as antioxidant by removing redox metals like copper, iron and zinc and has role in maintaining cellular homeostasis as they help to maintain blood brain barrier integrity (Atwood et al., 2003). There are reports that increased cholesterol levels enhance β and γ secretase activity promoting APP cleavage by the amyloidogenic pathway as amyloidogenic processing of APP occurs in the lipid rafts whereas when cholesterol levels are low it enhance α secretase activity leading to non-amyloidogenic pathway of APP metabolism. Amyloid β initially forms small clusters that are soluble but later form larger insoluble clusters which can bind to receptors on nerve cells blocking their synaptic contact with other cells and thus resulting in memory loss and impaired learning ability.

There are no effective treatments to prevent progression of AD. Inhibitors of β and γ secretases that generate amyloid β are known but it creates many side effects as both β and γ secretases has other substrates than APP and so its inhibition affects the

function of those substrates that are important in myelination, synaptic plasticity and cellular communication (Folch et al., 2016). Compounds that promote degradation of amyloid aggregates have been used which includes certain proteases but due to lack of specificity they have not reached advanced clinical development. In anti-amyloid immunotherapy, antibodies are used that reduce the levels of Aβ and thereby prevent formation of amyloid aggregates. These antibodies can also bind toxic amyloid deposits and neutralize them. But phase II clinical trial was not successful as some of the patient's immunized developed meningoencephalitis (Irwin et al., 2013). So further improved therapies have to be developed to clear amyloid deposits and thus protect from AD.

(b) Tau protein

Tau is a microtubule associated protein which is natively unfolded with a very low content of secondary structure, resistant to heat and acid and is predominantly present in the axons and at lower levels in dendrites. In normal brain, they stabilize microtubules that are essential for axonal transport. In mammalian brain, in adults it exists in 6 different isoforms varying in molecular weight from 48-67 kDa (352-441 amino acids in length) and in fetal brain only one isoform is present which is the smallest isoform containing 352 amino acids. These isoforms are coded by a single gene on chromosome 17 and are generated by alternative splicing of its pre-mRNA. They have the microtubule binding domain in the C terminal with an 18 residue repeating domain (R domain) ranging from R1-R4 and a projection domain in the N terminal which is projecting out from the microtubule surface and interact with other cytoskeletal elements and neuronal membranes. These isoforms differ in the

microtubule binding repeats (R) as they contain either 3R or 4R and in the N terminal domain which can be 0N, 1N or 2N.

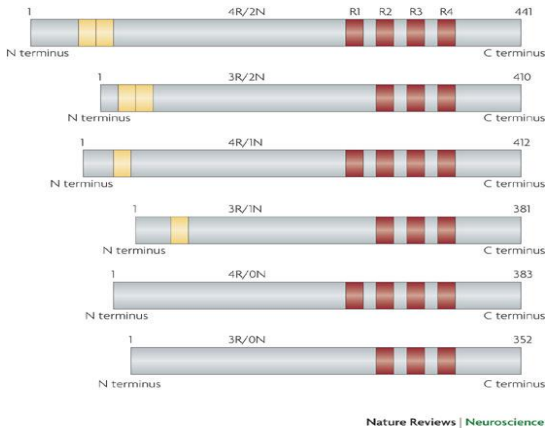


Figure 4. Tau isoforms

Tau protein is rich in serine- and threonine- rich sequences (STPS) so that in the longest tau isoform about 79 serine or threonine phosphorylation sites have been identified (Pevalova et al., 2006). Tau protein is phosphorylated more in embryonic period than in adults.

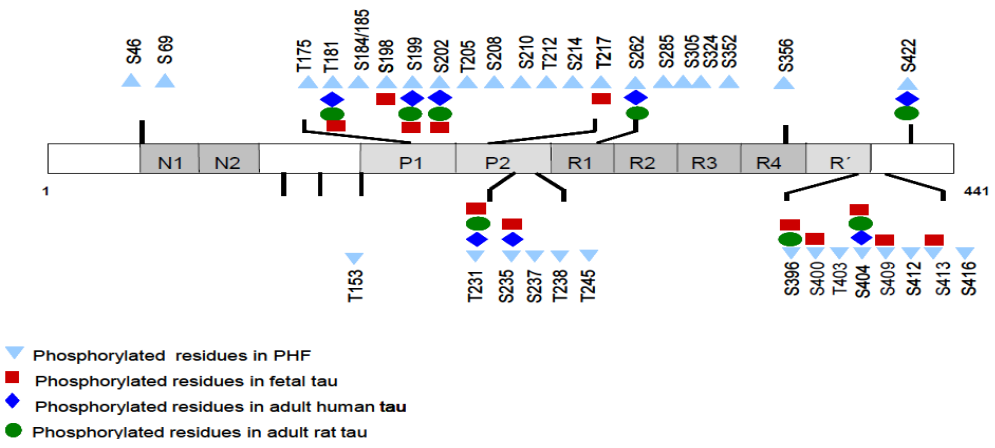


Figure 5. Tau protein phosphorylation

In AD, tau forms fibrillary tangles which have lost the capacity to bind microtubules. These aggregates are mostly intracellular but are also found in cerebrospinal fluids and as extracellular aggregates. Fibrillar tau which is detached from microtubules can mislocalise into presynaptic membranes and cause synaptic dysfunction. These tau aggregates can be taken up by other neurons and spread tau pathology. They form misfolded aggregates mainly due to abnormal post-translational modifications like hyperphosphorylation, acetylation or truncation where aberrant phosphorylation is the key feature associated with tau pathology. While site-specific tau phosphorylation, where 2-3 moles of phosphates will be attached per mole of protein, is important for normal function of tau, that is to bind microtubules, abnormal phosphorylation with about 5-9 moles of phosphates results in development of tau pathology (Köpke et al., 1993). They form neurofibrillary tangles (NFTs) that are composed of paired helical filaments (PHFs) with a β helical structure, which are the pathological filamentous aggregations of abnormally hyperphosphorylated tau. All six isoforms of adult brain tau are hyperphosphorylated and aggregated into tangles in AD. Human brain tau is modified with O-GlcNAcylation where β-N-acetylglucosamine (GlcNAc) attaches to serine/threonine residues (Liu et al., 2004). O-GlcNAcylation regulated phosphorylation of tau in a site-specific manner as abnormal hyperphosphorylation of tau could result from decreased tau O-GlcNAcylation, which probably is induced by deficient brain glucose uptake or metabolism in AD patients.

Therapeutic strategies targeting pathological tau protein has shown great potential in recent years with the use of antibody-mediated removal of tau aggregates. These tau

antibodies can bind to tau protein both extra and intracellularly but the importance of each site for tau clearance is not well defined. Anti-tau monoclonal antibodies on intra-cerebroventricular administration in human tau transgenic mice blocked tau uptake into neurons, its propagation between cells and thereby decreased the overall tau pathology (Yanamandra et al., 2015; Yanamandra et al., 2013). Only phase I clinical trials had been performed since toxicity like neuroautoimmune disorders has been identified on using full length recombinant tau administered in mice in combination with strong adjuvants (Sigurdsson, 2016).

2.3.2. Proteins involved in neuropathology of PD

Parkinson's disease (PD) is the second most common neurodegenerative disorder after AD which is characterized by the progressive death of dopaminergic neuronal cells in the substantia nigra of the brain and presence of lewy bodies in affected neurons. Substantia nigra is responsible for the production of dopamine which is a chemical messenger that transmits signals between two regions of the brain to coordinate activity ie, it connects the substantia nigra and the corpus striatum to regulate muscle activity.

(c) apha-synuclein

Alpha-synuclein is a small highly conserved 14 kDa presynaptic neuronal protein with 140 amino acid residues encoded by a single gene in chromosome 4. It is localized to presynaptic terminals, nucleus, cytosol and in some cellular membranes such as the mitochondria-associated membrane in the endoplasmic reticulum. Its main functions are vesicle trafficking during neurotransmitter release by binding to membrane phospholipids in vesicles. There are reports that α-synuclein is not an

intracellular protein and they can be secreted as their presence was detected in human plasma and CSF (El-Agnaf et al., 2003). They have an N-terminal domain (1–65), a non-amyloid-β component of plaques (NAC) domain (66–95), and a C-terminal domain (96–140) where the NAC domain is involved in aggregation.

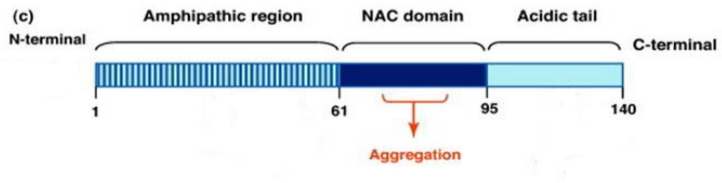


Figure 6. α-synuclein protein domain structure

There are mainly two hypotheses relating to its native structure: earlier studies indicated that they are monomeric with limited secondary structure and Bartels et al. showed that they are tetrameric protein which on getting destabilized into monomers get aggregated (Bartels et al., 2011). But there are recent reports showing that they are unstructured monomers which adopt a tetrameric structure only on membrane binding (Xu & Pu, 2016). In pathological form, they attain β sheeted secondary structure and form aggregates called lewy bodies which are proteinaceous aggregates that are hallmarks of PD. α-Synuclein may be modified by phosphorylation, oxidation, nitrosylation, and glycosylation. Of all such modifications, phosphorylation and glycosylation are the key modifications where S129 and S87 phosphorylation which occurs minimally in normal conditions rises in synuclein pathology resulting in synaptic and neuritic degeneration. O-Glycosylation is also an important modification in α-synuclein where O-GlcNAc is added to N-acetylglucosamine and this modification can prevent excessive phosphorylation as

both these modifications can occur on the same serine and threonine residues on protein. Thus O-GlcNAc addition protects from neurodegeneration (Marotta et al., 2015). The aggregated form induces toxicity by disrupting the normal function of α-synuclein in neurotransmitter release, impairing mitochondrial structure, disrupting ER-Golgi vesicular transport and eventually leading to cell injury and death. These proteins also adopt a self propagating property as they can spread from unaffected to affected regions and can induce neurodegeneration with motor disturbances, and also affect the cognitive and neuropsychological systems by inducing chronic neuroinflammation (Olanow & Brundin, 2013).

Early diagnosis is important for effective treatment and therapeutic methods are targeted to decrease α-synuclein aggregation, control its propagation and increase its clearance. There are studies showing the use of monoclonal antibodies (mAb) that can prevent synapse and neuron loss in primary neuronal cultures by preventing both uptake of misfolded proteins and subsequent cell-to cell transmission of pathology. An intraperitoneal administration of mAb specific for misfolded α-syn into nontransgenic mice with lewy bodies reduces pathology, reduces dopaminergic neuron loss and improves motor impairments (Tran et al., 2014).

2.3.3. Low density lipoprotein receptor-related proteins (LRP)

LRP is a member of the LDL receptor family (LDLR) which bind and endocytose numerous structurally diverse ligands with a variety of biological functions. There are seven type-I membrane proteins in this family, all sharing the ability to endocytose ligands. All members share five structural elements in common: 1) LDLR ligand-binding domains 2) epidermal growth factor (EGF)-like cysteine rich

repeats 3) YWTD domains 4) a single membrane spanning region and 5) cytoplasmic tails.

LRP is initially synthesized as a 600-kDa precursor protein that matures into a protein containing an extracellular ligand-binding subunit of 515 kDa and a transmembrane 85 kDa subunit that binds several adaptor proteins for efficient endocytic trafficking and signaling. Thus LRP is a type I integral membrane protein with a 515-kDa extracellular α-chain with multiple Ca2+ dependent ligand binding domains that are non-convalently bound to the 85 kDa membrane-spanning β-chain.

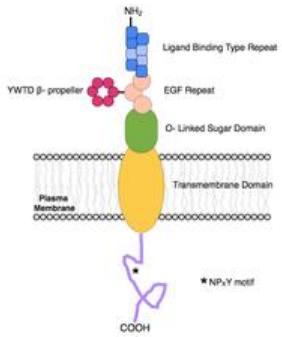


Figure 7. Structure of LRP

LRP is expressed in numerous cell types including fibroblasts, hepatocytes, adipocytes, macrophages and central nervous system (CNS) cells. In the normal human brain, LRP is abundantly expressed in the cell body and in proximal processes of cortical and hippocampal neurons and are expressed in microglia and astrocytes under some pathological conditions. In AD patients, the level of LRP in brain is significantly decreased suggesting that a decrease in LRP1 function might contribute to the cognitive decline (Sagare et al., 2011).

LRP can recognize about 30 different ligands which include lipoproteins, proteases, protease inhibitors, matrix proteins, intracellular proteins, bacterial toxins, viruses and growth factors. There are reports showing its role in regulating cell migration and modulating integrity of blood brain barrier (Lillis et al., 2005).

Impact of LRP on APP processing and Aβ clearance

The cytoplasmic tail in LRP is involved in APP processing. It was reported by Pietrzic et al. that in the absence of LRP Aβ production, APP secretion and internalization are affected and this effect is independent of APP isoforms and a neuronal adaptor protein Fe65 is responsible for linking APP and LRP (Pietrzik et al., 2004). LRP can mediate the export of Aβ from the brain. It has been demonstrated in vitro that LRP clears Aβ40 and Aβ42 (Fuentealba et al., 2010) and they also mediate neuronal degradation of Aβ. On neurons, LRP may endocytose Aβ, and degrade Aβ through the endosomal-lysosomal system. Experiments in animal models demonstrated increased Aβ toxicity when LRP is downregulated.

The 85 kDa transmembrane cytoplasmic β chain of LRP is responsible for cell signaling regulating endocytosis and for interacting with several intracellular adaptor proteins which includes Aβ. LRP was not only an endocytotic cargo transporter; it can transport several ligands transcellularly across the blood-brain barrier (BBB) including Aβ.

LRP is a protein rich in serine and threonine residues, its third domain is called the O-glycan domain that contain about 58 amino acid stretch highly enriched in serine and threonine residues. The association of various adaptor proteins with the LRP cytoplasmic domain is modulated by its phosphorylation state and serine and

threonine phosphorylation reduces the association of LRP with adaptor molecules of the endocytic machinery (Ranganathan et al., 2004).

2.4. Anti-carbohydrate antibodies

Polysaccharides are present in all living cells where they perform a myriad of functions. Eukaryotic cells have glycoproteins and glycolipids and bacteria have capsular polysaccharides and lipopolysaccharides. These antigens are the most common classes studied in carbohydrate–antibody complexes. The eukaryotic carbohydrates also include tumor antigens which includes Lewis x and y antigens and glycoprotein structures that viruses acquire from their host cell's biosynthetic machinery. It was hypothesized by Springer and Horton in 1969 that one of the major source stimulating antigen for the production of natural anti-carbohydrate antibodies are the bacteria that naturally colonize the GI tract (Springer & Horton, 1969). The multiple polysaccharides and oligosaccharides released from these bacteria are important sources of carbohydrate antigens stimulating the human immune system to produce antibodies. One class of natural antibodies is directed against blood group antigens where the A and B trisaccharide antigens are respectively: GalNAcα1,3 (Fucα1,2) Galβ-O-R and Galα1,3(Fucα1,2) Gal β-O-R, where R is a carbohydrate moiety of a glycolipid or glycoprotein. There are reports showing that natural antibodies in human sera interact with over 50 normal human glycomotifs using a microchip format glycan array to characterize individual carbohydrate recognition patterns by antibodies in sera of healthy individuals (Huflejt et al., 2009).

Anti-carbohydrate antibodies are the first line of defense against pathogens, dysfunctional cells and particles and are called natural antibodies (NAb) since they

are present in the sera of all individuals in the absence of deliberate immunization. They belong to IgG, IgM, IgA subtypes and are part of the innate immune system. Majority of these antibodies are polyclonal binding to different antigens and are predominantly of IgM isotype though IgA and IgG also exist. The affinities of anti-carbohydrate antibodies are lower by factors of 10^3–10^5 than antibodies specific for protein or peptide antigens. This is compensated by their initial expression as decavalent IgM and then class switching towards IgG3 in mice and IgG2 in humans by which they can self-associate through their constant regions to form multivalent networks (Cooper et al., 1991). The multivalent nature of these antibody clusters results in a marked increase in avidity and reflects an evolved mechanism for the recognition of multivalent or densely displayed carbohydrate antigens (Haji-Ghassemi et al., 2015).

According to N.Bovin, natural anti-carbohydrate antibodies can be divided into three groups: 1) conservative nAbs that are same in all healthy donors with respect to their epitope specificity and level in blood 2) allo-antibodies are antibodies produced against blood group antigens, for example as the anti-A antibodies in blood group A individuals where the level varies even within individuals of the same blood group 3) plastic antibodies where their level changes considerably during diseases and some temporary conditions, in particular inflammation and pregnancy (Bovin, 2013).

Diversity of anti-carbohydrate antibodies

The anti-carbohydrate antibodies that interact with carbohydrate antigens have a number of unusual characteristics. The modifications and relative degree of flexibility of many carbohydrates requires antibodies to utilize a variety of strategies

in their recognition. Carbohydrates are generally classified as T-cell independent antigens where B-lymphocytes are activated without the presentation of antigen fragments via MHC molecules to T-lymphocytes. However, the inability of most carbohydrate antigens to recruit T-cells results in a B-cell response lacking affinity maturation and therefore it required time for the production of IgM and IgG2 in human and IgM and IgG3 in mouse. The anti-carbohydrate immune response usually produces antibodies with "V-region restriction" where a relatively limited set of germ-line gene segments will generate antibodies against a broad range of epitopes (Brooks et al., 2010) and to overcome this restricted response glycoconjugate antigens have been developed in which carbohydrate antigens are coupled to proteins, and the protein moieties can then recruit T-cells. This makes these antibodies and the respective producer cells involved in transporting and presenting antigens for T cell stimulation. These antibodies are not produced upto 18-24 months of age and several antibodies like anti-B antibody and anti-phosphorylcholine antibody decrease in titer with age (Nordenstam et al., 1989).

2.4.1. Anti-α-galactoside antibody (anti-Gal)

Anti-Gal is a human natural antibody which interacts specifically with the alpha-galactosyl epitope Gal α-1-3Galβ1-4GlcNAc-R (Galili et al., 1987; Galili et al., 1984). It is a polyclonal antibody present in every individual and constitutes 1% of the immunoglobulins, and is predominantlyIgG, with traces of IgM and IgA. It is the only IgG antibody present abundantly in all individuals and are constantly produced in circulation in response to the alpha-galactosyl-like epitopes found on many bacteria present in the gastrointestinal tract, including *Klebsiella pneumonia, Escherichia coli and Serratia marcescens*. These antibodies are present abundantly

in humans, apes and Old World monkeys, but absent in New World monkeys, prosimians and nonprimate mammals possibly due to the presence of large amounts of alpha-galactosyl epitopes (more than 106 epitopes per cell) in the latter group. Anti-Gal appeared in ancestral Old World primates about 20-30 million years ago, possibly as a result of an evolutionary event which exerted a selective pressure for the suppression of alpha-galactosyl epitope expression by inactivation of the gene for the enzyme alpha 1,3 galactosyltransferase that are present in the Golgi of the cells where this enzyme catalyses the reaction Gal β1-4GlcNAc-R + UDP-Gal (α1-3-galactosyltransferase) →Gal α1-3Galβ1-4GlcNAc-R + UDP (Galili et al., 1988). In rhesus monkeys, orangutans and humans a variety of full-length transcripts were determined by sensitive polymerase chain reaction and on sequencing human genome partial sequences of the α1,3GT gene were observed but were corresponding pseudogenes (Panepistēmio tēs Krētēs et al., 2016).

The initial discovery of anti-Gal was on red blood cells (RBC) of patients with β-thallassaemia, on human normal senescent RBC and on sickle cell anemia RBC (Galili et al., 1984). In human RBC that are about 120 days old, or on thalassaemia and sickle cell anaemia RBC a cryptic antigen capable of binding anti-Gal is exposed. Anti-Gal isolated from AB individuals bind glycosphingolipids with Galα1-3Gal glycosidic epitope but not the closely related glycosphingolipid which is the blood group B determinant with a Galα1-3(Fucα1-2) Gal epitope. This inability of anti-Gal from AB individuals to interact with the B antigen is due to an effective immune tolerance mechanism.

Aberrant expression of the galactosyltransferase enzyme and formation of α-Gal epitope may result in the initiation of autoimmune disorders as in Grave's disease where α-Gal epitopes are present on thyroid cells. These epitopes produced by *Trypanosoma cruzi* interact with anti-Gal and induce inflammatory reactions in Chaga's disease (Galili, 2013). An increase in anti-Gal titre is present in autoimmune diseases including Henoch–Schonlein purpura, where the increase is in anti-Gal IgA antibody and in Crohn's disease where the increase is mainly of anti-Gal IgG antibody. On analyzing the anti-Gal IgG subclasses in human sera, all the four subtypes were present with IgG2 being the dominant subtype which is followed by IgG1, then IgG4 and least dominant is IgG3 [IgG2>IgG1>IgG4>IgG3]. Whereas in sera of infected patients as in malaria that is caused by *Plasmoduim falciparum* IgG3 level is higher. Anti-Gal acts as an immunological barrier that prevent the transplantation of pig organs into humans by binding to the millions of α-gal epitopes expressed on pig cell surface and thus causing hyperacute rejection of a pig xenograft (Galili, 2005). A xenograft with α-gal epitope introduced into human recipients increases anti-Gal titer to about 30-100 folds in 2-3 weeks. Anti-Gal IgM, which accounts for approximately 1–8% of total IgM Abs, is the predominant immunoglobulin involved in this phenomenon, although anti-Gal IgG, which accounts for 1–2.4% of total IgG are also involved. Anti-Gal can be exploited for clinical use in cancer immunotherapy by modifying cancer cells to express α-galactosyl epitopes for use as vaccines. These vaccines attract anti-Gal and the resulting activated Fc helps recognition of the vaccine by antigen-presenting cells to the lymph nodes to increase their immunogenicity (Tanemura et al., 2013).

It was reported in 1993 from our laboratory that anti-Gal recognizes epitopes on brain glycoproteins in man (Jaison et al., 1993). Anti-Gal can react with self MUC1 on mucin peptides expressed in large amounts on the surface of tumor cells but not on normal cells. It recognizes the serine- and threonine- rich peptide sequences (STPS) in MUC1 (Sandrin et al., 1997). This supports the earlier report that anti-Gal is expressed in humans as a natural antitumor defense system in humans (Castronovo et al., 1989). Our laboratory reported that anti-Gal binds to STPS present in the O-glycosylated protein apolipoprotein (a) [apo (a)] of lipoprotein (a) [Lp (a)] (Geetha et al., 2014).

2.4.2. Anti β-glucan antibody (ABG)

ABG is produced against β-glucans which are naturally occurring polysaccharides present in cell walls of plants such as oats, barley and seaweed, in fungi *Saccharomyces cerevisiae, Pneumocystis carinii, Cryptococcus neoformans, Aspergillus fumigatus, Histoplasma capsulatum, Candida albicans* and in pathogenic bacteria. β-Glucan is composed of β-1,3 and β-1,6- glycosyl linkages where β-1,3 linked β-D-glucopyranosyl units form a rigid skeleton providing physical strength to the cell wall whereas β-1,6 glucan acts as a connector linking β-1,3 glucans that varies in distribution and length. β-glucans derived from different sources have some differences in their structure as in oats and barleys they contain large regions of β-(1, 4) linkages separating shorter stretches of β-(1, 3) structures but in mushrooms they contain the backbone β-1, 3 linkage with short β-1, 6 branching and in yeast cell walls with long β-1,6 branching attached to backbone β-1,3. This difference in length of polysaccharide chain and extend of branching affects their solubility as β-glucans

from mushrooms are extractable in soluble form with hot water whereas those from yeast are only sparingly soluble.

Beta-glucan is an immunomodulator involved in boosting the immune system where the immunostimulating properties of mushrooms which contain polysaccharides that belong to the group of β-glucans have been known for thousands of years. β-glucans are isolated from variety of mushrooms and extracts of mushrooms were used for treatment purpose (Akramiene et al., 2007). They increase host immune defense by activating complement system, enhancing macrophages and natural killer cell function. The induction of cellular responses of β-glucans is by interacting specifically with several cell surface receptors on macrophages, neutrophils and NK cells. The immunological properties of β-glucans also vary based on their molecular weight. Large molecular weight β-glucans like zymosan which are obtained from yeast can easily activate leukocytes, stimulate phagocytic, cytotoxic and antimicrobial activities and production of reactive oxygen and nitrogen intermediates. Very low molecular weight β-glucans like laminarin are generally low in their immunological reactivity (Ishibashi et al., 2004).

Beta-glucans for oral consumption are widely available as oat bran and other bran-based products such as cereals with cholesterol-lowering properties and have beneficial anti-inflammatory and pro-apoptotic effects in inflammatory bowel disease and colitis-associated colon cancer (Barton et al., 2016). Chan et al in 2009 have shown in animal models that on oral administration of β-glucans, the specific backbone β1-3 linear beta-glycosidic chains cannot be digested and so they enter the proximal small intestine and some are captured by the macrophages. They are

internalized and fragmented within the cells, then transported by the macrophages to the endoplasmic reticular system where the smaller fragments will be released by the macrophages and taken up by other immune cells leading to various immune responses (Chan et al., 2009).

Beta-glucan shows anticarcinogenic activity, prevent oncogenesis and prevent metastasis by triggering complement-dependent antitumor cytotoxicity (Vannucci et al., 2013). Laminarin can also be used as an adjuvant to remove cancer antigens from melanomas by inducing dendritic cells to mature (Song et al., 2017). There were reports that use of anti-MUC-1 monoclonal antibodies in combination with β-glucan increased the rate of tumor regression to more than 20% in mice models (Hong et al., 2003).

Anti-β-glucan produced in response to β-glucan forms an antigen-antibody complex providing host defense against pathogenic organisms. Our laboratory reported that anti-β-glucoside antibody from normal plasma purified by affinity chromatography on cellulose had three times more IgA, largely as polymeric version, than IgG whereas about 90% of total serum IgA is monomer. Cellobiose and other β-glucosides were used to inhibit anti-β-glucan (Geetha et al., 2007). They produced all the four IgG isotypes though dominated by IgG2 which is the main IgG subclass elicited against β-glucan. In humans they recognize β-(1, 6)-linked glucans and more strongly β-(1-3) with branched β-(1,6)-glucans. But β-(1, 3)-linked glucan was recognized to a lesser extend (Chiani et al., 2009; Noss et al., 2015).

The first reported study on the production of anti-β-glucan was in rodents where β(1-3)- D-glucan with β(1-6) branching was conjugated to bovine serum albumin and

was used as the immunogen to raise anti-β-glucan (Adachi et al., 1994). There are reports showing the use of anti-glucan antibodies as fungicides against *Candida albicans*, Aspergillus species and *Cryptococcus neoformans* infections.

2.5. Albumin

Albumin is the most abundant multifunctional non-glycosylated plasma protein in human blood (35–50 g/L human serum) accounting for about 60% of the total protein in blood serum with a molecular weight of 66.5 kDa containing 585 amino acid residues. Albumin was precipitated from urine as early as 1500 AD and its basic properties were recognized as early as 1837 by Ancell who noted that "albumen" was needed for transport functions and for maintaining fluidity of the vascular system. An albumin molecule circulates in blood for about 19 days and is degraded if it is denatured or structurally altered and this longer half-life is mainly due to neonatal Fc receptor (FcRn)-mediated recycling.

Albumin is highly soluble in water or dilute salt solutions and its solubility is related to its high total electric charge. It is unusual among proteins in its solubility in polar organic solvents. Albumin is tolerant to high temperature but above a temperature of 60°C, it loses α-helical structure and gains β-form.

In dye-binding methods, only those dyes that bind very tightly to the albumin molecule were used so that practically 100% of the albumin present was bound to dye. The binding was unaffected by small changes in ionic strength and pH. The total albumin concentration was determined to be 3.5 mg/ml in average by Biuret and Kjeldahl's method (Kingsley, 2017). Later it was determined to be in range of 3.5-5 mg/ml by Bromocresol green method (McPherson & Everard, 1972).

2.5.1. Biosynthesis of albumin

Albumin is synthesized in the liver in a precursor form 'proalbumin' which is converted to albumin as it leaves the cell into serum (Campbell, 1975). As albumin is secreted from hepatocytes, it enters the circulation and translocates to the extracellular space through the pores of sinusoidal or fenestrated endothelium in certain organs, such as the liver, pancreas, small intestine and bone marrow. But in organs where a continuous endothelium predominates, albumin can traverse the endothelium through active transcytotic mechanisms using receptor-mediated mechanisms. Albumin synthesis is altered by environmental changes as its levels are higher in cold climates and as temperature increases albumin synthesis is depressed. Albumin degradation is also rather constant in man and in many lower animal species.

2.5.2. Structure of albumin

The entire amino acid sequence of both human and bovine albumin was determined by Meloun and his colleagues (Meloun et al., 1975). The secondary structure of albumin was determined by circular dichroism which determines that there are about 50-55% of α-helices and 15-18% β-sheets for human albumin and 46 % α-helices and 16% β sheets for bovine albumin. The covalent structure of albumin is shown to be a single peptide chain grouped into a series of nine disulfide-bonded loops where the loops appear to associate into three similar domains. The three-dimensional structure of human serum albumin was determined by X-ray crystallography by Xiao He and Daniel Carter in 1992 and was found to be heart shaped, but in solution they are ellipsoid (He & Carter, 1992). About 67% of the tertiary structure is composed of α helices. It consists of three homologous domains I, II, and III with each domain

containing two sub-domains (A and B) composed of 4 and 6 α helices respectively where each of these domain has two long loops with one shorter loop. These sub-domains are connected by flexible loops associated with proline residues which enable the sub-domains to move freely (Quinlan et al., 2005). These sub-domains are stabilized by 17 intra sub-domain disulphide bridges and mediate binding of endogenous and exogenous ligands. The domains are similar in structure but the N-terminal region is more compact than the C-terminal region and they have different hydrophobicity, net charge and ligand binding affinities. The crystal structure of albumin from various mammals shows similarities. It contains about 35 cysteine residues forming disulphide bridges contributing to its tertiary structure.

Figure 8. Structure of albumin

2.5.3. Functions of albumin

Albumin is a monomeric multi-domain macromolecule involved in transporting proteins, scavenging free radicals and regulating osmotic pressure and the main modulator of fluid distribution between body compartments. It is essential for binding and solubilising fatty acids, metabolism of lipids and in removal of toxins and it is used as a reference standard in the assay of proteins since it is free of carbohydrates. HSA displays an extraordinary ligand binding capacity for a wide range of endogenous and exogenous ligands. Several low and high affinity ligand

binding sites have been identified in albumin. N-terminal portion binds Cu, Ni and Co with high affinity. Interacting with serum albumin enabled small molecules to be present at a much higher concentration in blood plasma. HSA is important for passive permeability and penetration across the blood-brain barrier.

2.5.4. Albumin-binding proteins

Albumin-binding proteins were identified in vascular endothelial cells which have contributed to the transport of albumin through endocytosis. About seven membrane-associated albumin-binding proteins were discovered which includes: albondin/glycoprotein 60 (GP0), glycoprotein 18 (GP18), glycoprotein 30 (GP30), the neonatal Fc receptor (FcRn), heterogeneous nuclear ribonucleoproteins (hnRNPs), calreticulin, cubilin and megalin and a secreted protein acidic and rich in cysteine (SPARC).

Albondin/GP60

GP60 is a 60 kDa glycoprotein selectively expressed on the plasma membrane of continuous endothelium except in brain and operates to increase capillary permeability (Schnitzer et al., 1988). It is an endothelial sialoglycoprotein, apparently with O-linked oligosaccharides and its internalization is through the caveolin-dependent endocytic process. GP60 binds albumin and facilitates its internalization and subsequent transcytosis. It is proposed that about 50% of albumin leaves the capillary lumen through GP60.

GP18 and GP30

Glycoproteins GP30 and GP18 are both expressed in a variety of cells like macrophages and fibroblasts, smooth muscle cells, and endothelial cells and are

present in all tissues examined (heart, lung, skeletal muscle, diaphragm, duodenum, kidney, fat, brain, adrenal, pancreas, and liver). They bind conformationally-modified albumin but do not interact with native albumin. They are involved in endocytosis and degradation of various modified albumins may be as a protective pathway to remove altered, old, damaged or potentially deleterious albumins (Schnitzer & Bravo, 1993). Albumin may be modified through oxidation and non-enzymatic glycation as a result of normal aging or as a protective or pathological response. GP18 is also expressed in human breast cancer cells (Wang et al., 1994).

SPARC

SPARC also known as osteonectin is a 43 kDa glycoprotein expressed by many different types of cells and is associated with development and remodeling (Sage et al., 1984). It is a major non-collagenous protein of bone matrix and they are secreted by several cell types and also highly expressed in malignant cells and stromal cells. SPARC specifically interacts with native albumin in a similar way to albondin and also studies shows that SPARC and albondin share a native albumin-binding domain. It is a protein with 286 residues containing three distinct domains.

FcRn

FcRn is expressed in multiple cell-types and tissues which includes antigen-presenting cells, vascular endothelium, gut, lungs, kidneys and the blood-brain barrier (BBB) (i.e., endothelium and choroid plexus). This receptor protects albumin and IgG from degradation by binding them with high affinity only at a low pH (pH < 6.5) in acidic endosomes where it is protected from degradation and then

returning them to the extracellular space (pH 7.4) which also helps in extending the half-life of serum albumin (Anderson et al., 2006).

Cubilin and megalin

Cubilin is a multi-ligand receptor that is involved in the endocytosis and transcellular transport of numerous ligands, including albumin (Birn et al., 2000). It is localized to absorptive intestinal cells, placenta, visceral yolk-sac cells and proximal tubules of kidneys.

Megalin is a large trans-membrane protein that bind albumin (Cui et al., 1996). It is more widely expressed than cubilin and are also present in the choroid plexus, kidney proximal tubule cells and thyrocytes. Megalin binds to cubilin with high affinity and studies shows that cubilin associated with megalin is involved in the uptake of albumin (Amsellem et al., 2010). Cubilin- or megalin-deficient mice show a decrease in the uptake of albumin in the proximal tubules of kidney which results in albuminuria (Amsellem et al., 2010; Birn et al., 2000).

2.6. Platelets

Platelets were discovered by Giulio Bizzozero in 1882 (Ribatti & Crivellato, 2007). They are the smallest of the blood cells produced in bone marrow by fragmentation of megakaryocyte cytoplasm and circulate throughout blood vessels and survey the integrity of the vascular system. Megakaryocytes mature with nuclear duplication without cell division forming giant cells which fragment producing multiple platelets by the shear forces of circulating blood. Platelets circulate as discoid cellular fragments with internal actin and cytoskeletal filaments and they contain all the organelles as in other mammalian cells except nucleus. They have a diameter of

2-3 μm. The normal platelet count is 150,000-350,000 per microliter of blood, but since platelets are so small, they make up only a smaller fraction of the blood volume.

The primary function of platelets is to stop blood loss after tissue trauma and exposure of the subendothelial matrix. Formation of platelet plugs at sites of vascular damage requires a series of events such as i) initiation phase: platelets get arrested at the exposed subendothelium creating a monolayer of activated cells ii) extension phase: it involves recruitment and activation of additional platelets and iii) stabilization phase: stabilization of the platelet plug preventing premature disaggregation until wound healing occurs. Platelets are not only important in hemostasis; they also play roles in inflammation, antimicrobial activity, angiogenesis, tumor growth and metastasis.

2.6.1. Platelet receptors

Platelet receptors are important for normal functioning of the platelets. These receptors activate platelets or they act as adhesion molecules that interact with the damaged endothelium, other platelets and leukocytes. Some of the important platelet receptors are: integrins, leucine-rich repeat receptors, selectins, tetraspanins, transmembrane receptors, prostaglandin receptors, lipid receptors, immunoglobulin superfamily receptors and tyrosine kinase receptors.

Integrins: They are adhesion and signaling molecules which are non-covalently bound heterodimers of α and β subunits. Platelets have three types of integrins: β1, β2 and β3.

β1 family has three members : α2β1, α5β1 and α6β1. Receptor α2β1 or GPIb/IIa is an important platelet receptor for collagen which promotes platelet adhesion to collagen, stabilizing thrombus growth. Receptor α5β1 facilitates platelet adhesion to fibronectin and α6β1 integrin mediates platelet adhesion to laminin which is found in the basement membranes and extracellular matrix.

β2 family has only one member αLβ2 (CD102) which is also known as intercellular adhesion molecule 2 (ICAM-2). It has approximately 3,000 copies on the surface of the platelets and are important for platelet adhesion to neutrophils and for platelet-leukocyte interaction.

β3 family includes αIIbβ3 (CD41/CD61) also known as GPIIb/IIIa complex which is the most abundant platelet adhesion receptor (Quinn et al., 2003). Resting platelets express 80,000-100,000 molecules of αIIbβ3 on their surface. In addition to the surface receptors 20,000-40,000 molecules of αIIbβ3 are also present in the α-granules and dense granules. On platelet activation, αIIbβ3 is transformed from low affinity to high affinity state for attachment with its extra cellular ligands. This facilitates binding of αIIbβ3 to fibrinogen, von Willebrand Factor (vWF), fibronectin, vitronectin and thrombospondin and promotes platelet aggregation.

The αIIb subunit consists of 1008 amino acids, which is composed of a heavy and a light chain. The light chain contains a 20 amino acid cytoplasmic tail, a trans-membrane helix, and an extracellular segment that is disulfide linked to the heavy chain, which is entirely extracellular. The β3 subunit is a single polypeptide chain of 762 amino acids. The 2 subunits assemble into the divalent, cation-dependent

heterodimer during biosynthesis in megakaryocytes. It is rich in core 1 type O-glycans (T antigen) Galβ1-3GalNAcα1-Ser/T.

GPIIb/IIIa recognizes Arg-Gly-Asp (RGD) sequence of the ligands. The most important characteristic of this protein is the affinity modulation after its activation. In resting platelets, the affinity of GPIIb/IIIa for fibrinogen which is a main ligand of platelet aggregation is low and only minimal binding occurs despite the high levels of fibrinogen in blood. Activation of GPIIb/IIIa depends primarily on the conformational change of the receptors which is induced by platelet adhesion to extracellular matrix or mediated by agonists such as ADP, thrombin, or arachidonic acid. This requires transmission of information from within the cell to the extracellular domain of this receptor, a process referred to as "inside-out" signaling that changes receptors from a low- to high-affinity state. Integrin activation can involve changes not only in affinity for ligand, but also in avidity for ligand. Ligand-occupied integrin GPIIb/IIIa triggers various cellular processes, such as reorganization of the cytoskeleton within platelets.

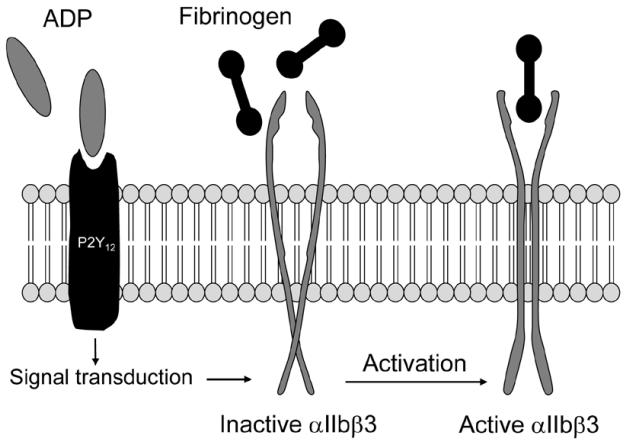


Figure 9. Signaling of GPIIb/IIIa

Leucine-rich repeats receptors (LRR): It is a protein structural motif composed of repeating 20-30 amino acids which are rich in the hydrophobic amino acid leucine. LRR motifs are the most important receptors of platelets that include GPIb-IX-V complex, toll-like receptors (TLR) and matrix metalloproteinases (MMP).

GPIb-IX-Vcomplex is the second most common platelet receptor after integrin GPIIb-IIIa, with approximately 50,000 copies per platelet (Romo et al., 1999). GPIb-IX-V complex is important in the initiation and propagation of both hemostasis and thrombosis. .

Transmembrane receptors: These receptors include ADP and thrombin receptors. ADP is secreted from the dense granules of platelets after activation. ADP performs a variety of functions in platelet activation that includes Ca2+ mobilization, shape changes, release reaction, production of thromboxane A2 (TXA2), αIIbβ3 activation and platelet aggregation by attachment with its receptor

Adenosine diphosphate (ADP) receptors include a guanosine triphosphate coupled protein receptor known as P2Y and an ion channel receptor called P2X1. These receptors play a pivotal role in platelet activation and aggregation (Gachet, 2001). P2X1 binds adenosine triphosphate and mediates extracellular calcium influx leading to alterations in platelet shape without platelet activation. P2Y receptors include P2Y1 and P2Y12 where ADP-stimulation of P2Y1 receptor activates phospholipase C (PLC) and induces a transient increase in intracellular calcium concentration resulting in platelet shape change and also cause a weak and transient platelet aggregation. Stable and firm platelet aggregation requires ADP binding to P2Y12

receptor and activation of GPIIb/IIIa receptors in the platelets membrane (Dorsam & Kunapuli, 2004). Activation of P2Y12 receptor liberates the G protein subunits, αGi and βγ. The subunit αGi decreases the platelet cyclic adenosine monophosphate (cAMP) level through the inhibition of adenylyl cyclase. This decrease in cAMP production leads to a reduction in the activation of protein kinase C (PKC) and after complex signal transduction processes cause activation of GPIIb/IIIa receptors. The subunit βγ activates the phosphatidylinositol 3-kinase (PI-3K), which regulates protein kinase Akt and contributes to the activation of GPIIb/IIIa receptors.

Immunoglobulin superfamily receptors: Immunoglobulin superfamily (IgSF) is a large group of cell surface proteins involved in the recognition, binding and adhesion of platelets. They include: FcRγ, FcγRIIA (CD32), FcεRI receptors (CD23), Junctional adhesion molecules (JAM), Platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD31).

2.6.2. Platelet aggregation

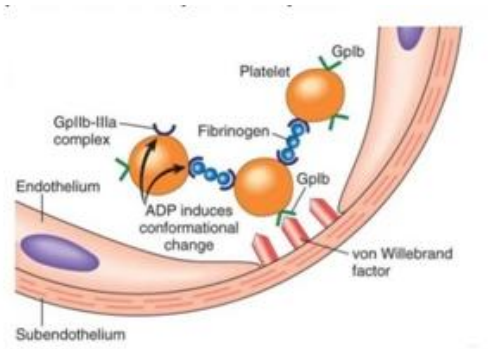
Platelet aggregation was first recognized in the late 1800s. Aggregation involves platelet-to-platelet adhesion and it is necessary for effective hemostasis following the initial adhesion of platelets to the site of injury. Platelets are activated by a number of agonists such as ADP and collagen present at the sites of vascular injury. These agonists activate platelets by binding to specific receptors present on the platelet surface. As the agonists bind these receptors, it leads to a series of downstream events that ultimately increases the intracytoplasmic concentration of calcium ions (Varga-Szabo et al., 2009). The increase in platelet intracellular calcium occurs through release from intracellular stores and calcium influx through the plasma

membrane. Receptors coupled to G-proteins including the ADP receptors and thromboxane A2 receptors activate phospholipase Cβ (PLCβ) and other receptors such as collagen receptor GpVI activates phospholipase Cγ (PLCγ). Activation of PLCβ or PLCγ results in the production of two second messengers diacylglycerol (DAG) and inositol trisphosphate (IP3). DAG mediates calcium influx while IP3 liberates calcium from intracellular stores. Calcium influx can also be induced directly by certain agonists, such as ATP binding to the ligand-gated ion channel receptor, P2X1.

Increased concentration of calcium in platelets results in a number of structural and functional changes of the platelet. Platelet changes its shape from a disc to a spiny sphere by the platelet cytoskeleton, composed by an organized network of microtubules and actin filaments and a number of associated proteins linked to a variety of platelet signaling molecules. Platelet shape change results in extensive reorganization of the cytoskeleton network, polymerization of actin, and myosin light chain phosphorylation. After changing shape, the granules in the platelet are centralized and their contents are discharged into the lumen from which they are then released to the exterior. The long membrane projections brought about by change in shape allows the platelets to interact with one another to form aggregates.

A main adhesion molecule involved in platelet aggregation is the membrane protein, GPIIb/IIIa complex. It exists as an inactive form in resting platelets and platelet activation by agonists induces conformational changes of GPIIb/IIIa and makes the receptor competent to bind soluble plasma fibrinogen.

The agonists that are involved in platelet aggregation are mainly: Fibrinogen, VWF, and fibronectin. All these proteins have similar binding affinities to the activated form of GPIIb/IIIa. In low shear conditions fibrinogen is the dominant ligand supporting platelet aggregation due to its high molar ratio in plasma relative to other GPIIb/IIIa ligands. As the shear increases (> 1000 s−1), the initiation of aggregation becomes progressively more dependent on VWF and fibronectin.



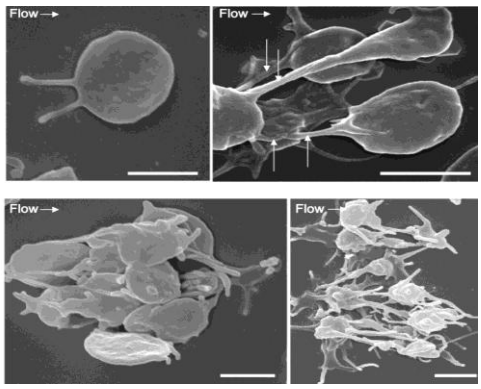


Figure 10.Platelet plug formation ***Figure 11.Platelet-platelet adhesive interactions***

2.6.3. Platelet activation enhanced by high glucose level

Diabetes mellitus (DM) and hyperglycaemia are associated with platelet activation with larger platelets and expressing more adhesion receptors on the cell surface. Acute hyperglycaemia may also alter platelet function making platelets hyperactive by increasing platelet membrane-bound protein kinase C (PKC) and enhancing collagen-induced platelet aggregation (Ferroni et al., 2004). There are reports that hyperglycaemia following a carbohydrate-rich meal (Yngen et al., 2006) causes platelet activation in vivo. Platelets from patients with diabetes mellitus have

dysregulated signaling pathways that lead to an increased tendency to activate and aggregate in response to a given stimulus. Platelets from patients with DM, unlike those from healthy individuals, are also associated with short-term activation of the calcium-sensitive PKCβ isoenzyme. Due to this impaired calcium homeostasis, activation of PKC, decreased production of platelet-derived NO, and increased formation of superoxide all add up causing platelet aggregation (Kakouros et al., 2011).

3. MATERIALS AND METHODS

3.1. Materials

Soybean tripsin inhibitor, sodium metaperiodate, potassium borohydride and 1-fluoro-dinitrobenzene (FDNB) were purchased from Fluka, Buchs, Switzerland. Bovine thyroglobulin, galactose, methyl α-D-galactopyranoside, methyl α-D-mannoside, cellobiose, melibiose, soluble guar gum, Coomassie brilliant blue G-250, Coomassie brilliant blue R-250, Cibacron blue F3GA, epichlorohydrin, sodium borohydride, Tween 20, Bovine serum albumin, β-galactosidase, phosphorylase B, myosin, polyvinylidene difluoride (PVDF), neuraminidase from *chlostridium perfringes*, 4-chloro-1-naphthol (4-CN), tetramethyl ethylenediamine (TEMED), ammonium persulfate (APS), agarose, riboflavin, bromophenol blue, horse radish peroxidase, orthophenylene diamine, N,N´-methylene bisacrylamide, acrylamide, Tris, glycine, dithiothreitol (DTT), fluoroisothiocyanate, α-synuclein, tau protein, sodium dodecyl sulphate, Histopaque 1077, RPMI-1640, penicillin, streptomycin, Hanks balanced salt solution (HBSS), dimethyl sulphoxide (DMSO), Sephadex G-100 and Hoest nuclear stain were purchased from Sigma aldrich, Bangalore. Polystyrenene 96 well microplate (MAXISORB) and sterile 35 mM cell culture dish were purchased from Nunc, Denmark. Anti human IgA, IgG, IgM, apo(a) and apoB antibodies raised in rabbit were purchased from Dako, Denmark. MitoSox for mitochondrial staining was purchased from Life technologies, California. Antibodies against LRP and anti-goat IgG HRP were from Santa Cruz biotechnologies, Texas. Fluorescent-labeled amyloid β (F-Aβ; HiLyte Fluor-488) was purchased from Anaspec, California. Other chemicals used in solvents and buffers were of analytical grade.

Seeds of *Canavalia ensiformis* (jackbeans) for concanavalin A preparation, *Arachis hypogaea* (peanut) for PNA and *Artocarpus integrifolia* (jackfruit seed) for jacalin preparation were obtained from local sources. Outdated plasma samples were collected from Department of Blood Transfusion Service of the institute with Institutional Ethics Committee (IEC) approval (SCT-IEC/674). Blood samples for isolating macrophages were collected from healthy volunteers outside SCTIMST with IEC approval (SCT-IEC/1032) and blood samples for isolating platelets were collected from healthy volunteers outside SCTIMST with IEC pproval (SCT-IEC/ 1072).

3.2. Methods

3.2.1. Protein estimation by Bradford's method

Coomassie brilliant blue G-250 dye solution was prepared as 0.06% solution using 3% perchloric acid and before use it was filtered through Whatman No.1 filter paper. This reagent and protein solution was mixed in the ratio 1:1 and absorbance measured immediately at 630 nm (Bradford, 1976).

3.2.2. Protein estimation by Lowry's method

a. Alkaline Copper Reagent: prepared fresh by mixing 1 ml of 2% sodium potassium tartrate solution and 1 ml of 1% copper sulphate solution and the mixture was made up to 100 ml with 2% sodium carbonate containing 0.1 N sodium hydroxide.

b. 1 N Folin Ciocalteu reagent: 0.5 ml of protein solution was mixed with 2.5 ml of alkaline copper reagent and incubated at 25°C for 10 minutes. This was followed by the addition of 0.25 ml of 1 N Folin Ciocalteu reagent and incubated at 25°C for 30

minutes. Absorbance was measured at 660 nm using bovine serum albumin as protein standard (Lowry et al., 1951).

3.2.3. Carbohydrate estimation by phenol sulphuric acid method

The total neutral sugar was estimated by phenol sulphuric acid method (DuBois et al., 1956). Samples (0.5 ml) were mixed with 1 ml of 5% phenol (prepared using distilled phenol mixed in the ratio 1:20 using water). To this mixture 4 ml of cold concentrated sulphuric acid was added while mixing. After incubating for 15 minutes at room temperature absorbance was measured at 485 nm.

3.2.4. Preparation of lectins

Jacalin, the agglutinin lectin from seeds of *Artocarpus integrifolia* (jack fruit) was isolated by the procedure described by Suresh Kumar et al. (1982). Briefly, 65% ammonium sulfate-precipitated fraction of seed (30 g) homogenate in PBS 6.5 was passed through cross-linked guar galactomannan (CLGG) column, followed by washing and eluting with galactose (150 mM in PBS 6.5). Peanut agglutinin was isolated from peanuts (*Arachis hypogea*) as described by Chacko and Appukuttan (2000) by passing peanut (50 g) homogenate in PBS pH 6.5 through CLGG column equilibrated in the same buffer. After washing out unbound proteins with PBS 6.5, bound proteins were eluted using 0.15 M lactose in the same buffer and concentrated using AMICON PM10 ultra-filtration membrane. Concanavalin A was isolated from the seeds of *Canavalia ensiformis* (jack beans) as described by Surolia et al. (1973) essentially by affinity chromatography on Sephadex G-50 column using 0.1 M dextrose in 1 M NaCl-0.1 M Tris HCl pH 7.4 for elution.

3.2.5. Preparation of crosslinked guar galactomannan (CLGG)

Soluble guar galactomannan was cross-linked to form an insoluble gel by a modification in the procedure described by Appukuttan et al. (1977). Guar gum powder (10 g) was mixed thoroughly with a finely dispersed emulsion of 2ml epicholorohydrin and 25 ml 3 N NaOH until the mixture became a solid cake. It was kept at 40°C in a water bath for 24 h and then at 70°C for 10 h. The resulting gel was soaked in distilled water and repeatedly washed with water and was then equilibrated with PBS 7.4. It was homogenized in a blender to obtain particles of about 300 μm size and fine particles were discarded by repeated decantation.

3.2.6. Preparation of yeast antigen

Yeast antigen was prepared from baker's yeast as described by Paul et al. (2011). Baker's yeast (5 g) suspended in 25 ml PBS was subjected to three subsequent freezing and thawing and homogenized in a POLYTRON homogenizer. The suspension was then sonicated for six 30s bouts. Then the sample was stirred for 30 min at 4°C, centrifuged at 12,000 g and the supernatant was collected and dialyzed against PBS 7.4.

3.2.7. Preparation of Blue Sephadex

Crosslinking of Sephadex: Sephadex G-100 was crosslinked using epichlorohydrin and coupled to the dye Cibacron F3 GA as in protocol by Böhme et al. (1972) to make blue Sephadex. Sephadex G-100 (50 g) was mixed with 45 ml of 1 M NaOH and 5 ml of epichlorohydrin at room temperature. Hundred mg of sodium borohydride was added to the above mixture and the suspension kept at 60°C for 2 h

with stirring. The gel was then washed till it was free of alkali and to this 1750 ml of distilled water was added.

Coupling of the dye: 40 mg dye (Cibacron Blue F3 GA) was used for 1 g of gel. Two gram of dye was dissolved in 60 ml water and the dye was added dropwise to the gel suspension at 60°C with stirring for 30 minutes. To this 225 g NaCl was added and stirring continued for 1 h. The temperature was raised to 80°C for 2 h. The gel was then cooled to room temperature and was washed by suction with water to make it free from excess dye. It was again washed successively with 500 ml of 20 mM citrate acetate buffer, pH 5.0 containing 2 M NaCl and 20 mM NaHCO3 buffer pH 8.5 containing 2 M NaCl and finally distilled water.

3.2.8. Preparation of affinity-purified albumin containing associated O-glycoproteins

Plasma (2 ml) dialyzed against 20 mM Tris HCl buffer pH 6.5 was loaded to 10 ml blue Sephadex in a glass chromatographic column equilibrated with the same buffer. Column was washed to remove unbound proteins and O-glycoprotein-bound albumin was eluted using 250 mM NaCl in the same buffer. From eluted proteins O-glycoprotein-free human serum albumin, O-glycoproteins and antibody were prepared by separation in 6% alkaline gel electrophoresis (pH 8.3), electroelution of protein bands and concentration by membrane filtration using AMICON ULTRA (MW cut-off 10 kDa).

3.2.9. Preparation of affinity-purified anti-α-galactoside antibody (APAG)

Anti-Gal was prepared from human plasma as described by Jaison and Appukuttan (1992). All steps were carried out at 4°C. Outdated human plasma (50 ml) from

healthy donors (25-40 years) collected from the blood bank of this institute was dialyzed extensively against 20 mM potassium phosphate buffer containing 150 mM NaCl, pH 7.4 (PBS). It was centrifuged at 12,000 g for 30 minutes and supernatant was passed through a CLGG column. After washing out unbound proteins using PBS 7.4, bound protein (anti-Gal) was eluted using 150 mM dialyzable galactose in the same buffer in 2 ml fractions. Protein-containing fractions were pooled, concentrated by ultra filtration (10,000 MW cut-off) and dialyzed against PBS to remove galactose and was stored at 2-4°C. Dialyzable galactose was obtained in the outer solution when appropriate concentration of galactose in PBS taken in 10,000 MW cut-off dialysis bag was dialyzed against three times its volume of PBS overnight.

3.2.10. Preparation of affinity purified anti-β-glucoside antibody (APABG)

ABG was prepared from human plasma as described by Geetha et al. (2007). All steps were carried out at 4°C. Outdated human plasma from healthy donors of age group 25-40 were collected from the blood bank of this institute and were dialysed thoroughly with a change in PBS 7.4. It was centrifuged at 15,000 g for 15 minutes and supernatant was passed through a column consisting of cellulose (microcrystalline) and celite (type 545, E.Merk, Germany) in 1:1 ratio (v/v). The column was washed in PBS 7.4 to make it protein free and bound protein was eluted using 0.2 M dialyzable dextrose in 2 ml fractions. Protein-containing fractions were pooled, concentrated by ultra filtration (10,000 MW cut-off) and dialyzed against PBS to remove dextrose and was stored at 2-4°C.

3.2.11. Isolation of glycoproteins AOP1 and AOP2 from affinity purified anti-Gal, ABG, blue Sephadex eluate and middle layer

Affinity purified anti-Gal or ABG (APAG and APABG; method 3.2.9 and 3.2.10), 250 mM NaCl eluate from blue Sephadex (method 3.2.8) or middle 400 µl obtained on subjecting plasma to ultracentrifugation at 535000 g for 4 h at 4°C was dialyzed and subjected to 6% alkaline gel electrophoresis at pH 8.3 and the glycoprotein bands that moved between immunoglobulin and albumin (AOP1 and AOP2) were cut, electroeluted and concentrated.

3.2.12. Gel permeation chromatography

Gel permeation chromatography of glycoproteins AOP1 and AOP2 was done in a 2 cm diameter x 43 cm height column of Sephadex G-100 in PBS 7.4 using phosphorylase B, β galactosidase and BSA as standards and 2 ml fractions collected in 10 minutes interval using a fraction collector. Molecular weight was determined from a plot of the ratio of $(V_t - V_e)$ and $(V_t - V_0)$ against molecular weight in logarithmic term, where V_t, V_e and V_0 are total gel volume, elution volume of protein standards, and void volume of the column respectively (Reiland, 1971).

3.2.13. Preparation of neoconjugates

Neoconjugates were prepared as described by Baues and Grays (1977). Melibiose or cellobiose was conjugated to the protein soybean trypsin inhibitor by reductive amination using sodium cyanoborohydride. Tripsin inhibitor, mellibiose or cellobiose and sodium cyanoborohydride were taken in the ratio 1:2:4 by weight in 2 ml 0.2 M trisodium phosphate-phosphoric acid buffer pH 9.0. The mixture was incubated at 25°C for 14 days and dialysed in PBS 7.4 and stored at -20°C.

3.2.14. Conjugation of HRP to lectins/antibodies

The HRP labeling of proteins was done as described by Heyderman et al. (1986) using periodate-activated horse radish peroxidase (HRP). HRP (2 mg) was dissolved in 0.2 ml freshly prepared 0.3 M NaHCO3 and to this 10 µl fluorodinitrobenzene (1% FDNB in absolute ethanol) was added, mixed and incubated for 1 h at room temperature (to prevent self coupling). The solution was then treated with 0.2 ml sodium metaperiodate (0.06 M in water) and incubated for 30 min at room temperature (to create aldehyde groups in HRP). To this 0.2 ml of ethylene glycol was added (0.32 M in water), mixed and incubated for 1 h at room temperature (to remove excess periodic acid). Finally the solution was made upto 1 ml with 0.01 M carbonate buffer pH 9.5 to make 2 mg/ml concentration and dialyzed in 0.01 M carbonate buffer pH 9.5. To conjugate activated HRP to proteins 1 mg protein (antibodies/lectin) in 1 ml 10 mM sodium bicarbonate buffer pH 9.5 was mixed with 0.67 mg periodate-activated HRP and incubated at 25°C for 2 h in dark. To this mixture 1% potassium borohydride solution in distilled water was added to make a final concentration of 0.1% and was incubated in dark again for 30 min at room temperature and the mixture was dialyzed thoroughly against PBS 7.4 with one change. The HRP labeled antibody/lectin was stored in 30% glycerol at -20°C.

3.2.15. Preparation of purified FITC conjugated antibodies and glycoproteins

FITC coupling to anti-Gal, ABG or glycoproteins was done by the protocol described by Hudson and Hay (1980). Antibodies and glycoproteins were concentrated to 1 mg/ml and dialyzed against 0.25 M carbonate-bicarbonate buffer (pH 9.0) and in case of antibodies they were preincubated with specific sugars (25 mM methyl α-D-galactoside for anti-Gal and 25 mM cellobiose for ABG) to protect antigen binding

sites from labeling. Antibodies and glycoproteins were then incubated with FITC (0.15 per mg protein) and kept overnight at 4ºC. For preparation of purified FITC-labeled antibodies, AOP1 and AOP2 FITC-labeled APAG or APABG was loaded to 6% alkaline gel electrophoresis, antibody and O-glycoprotein bands cut, electroeluted and concentrated by membrane filtration to prepare purified FITC-labeled proteins. Samples were dialyzed overnight in PBS 7.4 with two changes to remove reagents.

3.2.16. Fluorescence measurement

Purified anti-Gal/ABG-FITC (1 µg) or O-glycoprotein-FITC was incubated with defined quantities of ligands in 100 µl PBS overnight at 4°C. F-Aβ (50 ng) was incubated with defined amounts of ligands in 100 µl PBS for 3 h at 4°C. Incubated samples were brought to 300 µl in PBS and fluorescence was measured using excitation at 485 nm and emission at 528 nm in a BIOTEK fluorescence ELISA reader.

3.2.17. Density gradient ultracentrifugation (DGUC) of protein solutions and plasma

Plasma samples were centrifuged at 5000 g at 4°C to remove cells if any. After incubation with or without sugar molecules for 2 h at 4°C cell-free plasma or other protein solutions were adjusted to density 1.24 g/cc by addition of solid KBr and the resulting solution (1.1 ml) centrifuged in a himac CS 150 GXII microcentrifuge (HITACHI) at 535000 g for 4 h at 4°C. Tube contents were separated into top (400 µl), middle (400 µl) and bottom (300 µl) layers and dialyzed against PBS to remove KBr.

3.2.18. Enzyme linked immuno and lectin assays

Glycoproteins (AOP1 and AOP2), lectins, or other proteins were coated on polystyrene microtiter plates (NUNC Break apart-Maxisorb) by incubating defined amount in 200 µl PBS in the wells at 37°C for 3 h. Wells were washed with PBS containing 0.05% Tween-20 (PBS-T) and blocked by further incubation with 200 µl PBS containing 0.5 % Tween-20 for 30 min at 37°C. After another wash, wells were treated at 4°C with the primary reactant molecule with or without HRP label in 200 µl PBST for 2 h. After three washings in PBST if secondary reactants are involved its HRP conjugate in PBST is added and incubation continued for 2 h at 4°C. Finally plates washed thrice in PBST were incubated at 25°C with 200 µl OPD (0.5 mg/ml) in 0.1 M citrate-phosphate buffer, pH 5.0 containing 0.03% H_2O_2 for 15 min, followed by addition of 50 µl 12.5 % H_2SO_4 and absorbance measured at 490 nm in BIOTEK ELx800 ELISA reader.

3.2.19. Enzyme-linked inhibition assay

Polystyrene wells were coated with thyroglobulin (Tg), TIM, TIC or glycoproteins and blocked as explained above. Specified concentration of anti-Gal or ABG or HRP-conjugated lectin with or without inhibitors, was preincubated for 2 h at 4°C in 200 µl PBST before adding to the above wells and incubation at 4°C for 2 h. In the case of non-HRP-conjugated probes like antibodies washed wells were further incubated with HRP labeled anti-immunoglobulin mixture in 200 µl for 2 h at 4°C. Final washing, OPD treatment and reading were done as described above.

3.2.20. Determination of attenuation of anti-albumin binding to albumin by other molecules bound to albumin

Albumin (40 ng; purified) preincubated at 4°C overnight with or without 120 ng mixture of AOP1 and AOP2 was coated on NUNC microplates by 3 h incubation at 37°C. Wells washed and blocked as described above were then probed with anti-albumin-HRP conjugate (3.75 µg albumin per ml) in PBST for 2 h at 4°C and bound conjugate determined as given above. To determine anti-albumin response to albumin bound to AOP1/AOP2 and captured through the latter on plate-coated jacalin, microplates coated with jacalin (1µg per well) was treated with a mixture of 120 ng AOP1/AOP2 from APAG and 40 ng purified albumin in 200 µl PBST for 2 h at 4°C. After washing wells were probed with anti-albumin-HRP as above.

3.2.21. Testing identity of peptide sequences with those of known proteins by nano LC-MS/MS

Polyacrylamide gel containing AOP1 or AOP2 separated from APAG by alkaline electrophoresis was subjected to in-gel digestion and peptides subjected to nano LC-MS/MS analysis according to Shevchenko et al. (2007) in the Centre for Cellular and Molecular Platform at the National Centre for Biological Sciences, Bangalore, India. Data was generated on LTQ-Orbitrap-MS and the generated data was searched using MASSCOT 2.4 as search engine on proteome discoverer 1.4, against Uniprot Swiss-Prot data base.

3.2.22. Assaying anti-Gal and ABG triplet from plasma or triplet of anti-Gal and ABG prepared de novo

Outdated plasma was diluted 100 times in PBS 7.4 and was incubated for 2 h with 25 mM methyl α-D-galactoside (anti-Gal specific sugar), 25 mM cellobiose (ABG-specific sugar) or 25 mM methyl α-D-mannoside (nonspecific sugar). De novo anti-Gal and ABG triplets were prepared by incubating antibody overnight with AOP1/AOP2 which was pre-incubated with HSA for 3 h in PBST. Sugar-treated plasma or de novo triplet in mixture (200 µl) was added to guar galactomannan coating (1 µg per well) for anti-Gal triplet assay and to yeast antigen (1µg per well) coating for ABG triplet assay. Bound triplet was assayed using anti-albumin HRP.

3.2.23. Plasma anti-Gal assay

Melibiose conjugated trypsin inhibitor (TIM) prepared as described (5 µg/ml) was coated to polystyrene wells by incubating overnight at 4ºC or 3 h at 37ºC. The wells were blocked and treated with plasma (50 times diluted in PBST) and incubated at 4ºC for 2 h. The wells were then washed with 0.05 % cold PBST and followed with a mixture of HRP conjugates of anti-human IgG, IgA and IgM (1.5 µg/ml) and incubated for 2 h at 4ºC. The bound HRP was assayed using OPD as substrate as above. Known quantity of purified anti-Gal was used as standard. A well with TIM coating treated directly with HRP conjugate without adding plasma dilution was included as control and the OD obtained in this well was subtracted from the OD of the sample well to get the actual anti-Gal response of the sample.

3.2.24. Preparation of apo(a) and LDL

Plasma (1 ml) was adjusted to density 1.24 g/cc with potassium bromide and was subjected to ultracentrifugation at 535000 g for 4 h at 4°C. Top 20% layer containing lipoproteins (L1) was collected and dialyzed in PBS 7.4. To prepare apo (a) L1 was subjected to reduction using 4 mM DTT at 37°C for 15 minutes. The reduced sample was subjected to ultracentrifugation at 535000 g for 4 h at 4°C after adjusting the density to 1.24 g/cc using potassium bromide. Bottom 20% was collected as apo(a) and was dialyzed in PBS 7.4. To prepare LDL, L1 was subjected to 3.75% native PAGE in Tris-borate EDTA buffer (TBE gel electrophoresis as in methods). The fastest moving LDL band was cut, electroeluted, concentrated and dialyzed against PBS 7.4.

3.2.25. De-O-glycosylation of glycoproteins (AOP1 and AOP2)

AOP1 and AOP2 prepared from affinity purified anti-Gal was treated with 1 M Sodium borohydride in 0.05 N NaOH for 24 h at 37ºC and dialyzed against PBS 7.4.

3.2.26. Tris-Borate EDTA electrophoresis (TBE)

Lipoproteins were subjected to electrophoresis in 3.75% disc gels as described by Kalaivani and Appukuttan (2014).

Reagents:

Solution A: TBE buffer (should be prepared fresh in deionized distilled water).

0.05 M Tris, 0.025 M boric acid, 0.003 M disodium salt of EDTA.

Solution B: TBE buffer with TEMED (should be prepared fresh in deionized distilled water).

1 ml Solution A was mixed with 2 ml deionized distilled water containing 14 µl TEMED.

Solution C: 15% acrylamide/bisacrylamide (19:1, w/w)

Acrylamide: 14.25 g; Bis acrylamide: 0.75 g

Dissolved in 100 ml deionised distilled water, filtered through Whatman no1 filter paper and stored in dark bottles at 4ºC.

Solution D: 25% acrylamide/ bisacrylamide (20:5, w/w)

Acrylamide: 20 g; Bis acrylamide: 5 g. Dissolved in 100 ml deionised distilled water, filtered through Whatman no1 filter paper and stored in dark bottles at 4 ºC.

Solution E: Riboflavin 0.004% solution

Solution F: Ammonuim persulfate (0.2%) in deionised distilled water

Solution G: 0.005% Bromophenol blue as tracking dye

Solution H: 12.5% trichloroacetic acid in distilled water

Solution I: Tube gel stain-120 mg Coommassie brilliant blue (CBB R-250) in methanol: acetic acid: water (11:3:11, v/v)

Gel preparation:

The gels were casted in BROVIGA gel electrophoresis apparatus

A. Separating gel (3.75%): Solution A: 2 ml; Solution C: 2.5 ml; Solution F: 5.5 ml; TEMED: 10 µl

B. Spacer gel (polymerized on exposing to fluorescent light)

Solution B: 1 ml; Solution D: 1 ml; Solution E: 1 ml; Distilled water: 5 ml

Reservoir buffer: TBE buffer pH 8.7

Sample preparation: Samples were dialyzed overnight against TBE buffer (10 X diluted). Sample (50 µg) mixed with 10% glycerol was loaded. Inorder to track the

protein tracking dye bromophenol blue dissolved in TBE buffer was added to sample and loaded in one tube.

Electrophoresis: Run was at 25ºC at 3 mA current per tube till bromophenol blue ran out, plus 10% more time. After run one (marker) gel was fixed, stained and destained to identify position of bands.

3.2.27. Alkaline gel electrophoresis

Alkaline gel electrophoresis at pH 8.3 was done on 6% tube gel as described by Davis (1964).

Reagents:

Solution A: 1 N HCl-24 ml; Tris–18.1 g; TEMED-0.12 ml. Made upto 100 ml with water, pH 8-9.

Solution B:

1 N HCl-48 ml; Tris–5.98 g; TEMED-0.46 ml. Made upto 100 ml with water, pH 6.8.

Solution C: Acrylamide-28 g; Bisacrylamide-0.735 g. Dissolve in 100 ml distilled water.

Solution D: Acrylamide-20 g; Bisacrylamide-5 g. Dissolve in 100 ml water.

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

Solution G: 0.14 % Ammonium Persulphate (APS) in distilled water

Tracking dye: bromophenol blue

Chamber buffer: Tris-6 g; Glycine-28.8 g; Distilled water: 1 l, pH 8.3

Gel preparation: The gels were cast in BROVIGA gel electrophoresis apparatus.

Separating gel (6%): Solution A-5 ml; Solution G-10 ml; Distilled water-0.72 ml

Spacer gel (polymerized on exposing to fluorescent light): Solution B-1 ml; Solution D-1 ml; Solution E-1 ml; Distilled water-5 ml.

Sample preparation: Samples were dialyzed overnight against chamber buffer. Sample (50 µg) mixed with 10% glycerol was loaded. Inorder to track the protein tracking dye bromophenol blue dissolved in chamber buffer was added to sample and loaded in one tube.

Electrophoresis: It was run at 4ºC for isolation of purified anti-Gal and ABG, glycoproteins (AOP1 and AOP2) and albumin at 3 mA current per tube till bromophenol blue ran. After run one gel was fixed, stained and destained.

3.2.28. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was done as described by Laemmli (1970).

Reagents:

30% acrylamide: Acrylamide-30 g; Bisacrylamide-0.8 g

Dissolve in 100 ml distilled water and stored in amber bottle at 4 º C.

Buffer 1 (pH 8.8): Tris-18.58 g; SDS-410 mg; Distilled water-250 ml

Buffer 2 (pH 6.8): Tris-4.45 g; SDS-270 mg; Distilled water-250 ml

Chamber buffer (pH 8.3): Tris-0.756 g; Glycine-3.6 g; SDS-0.250 g; Distilled water-250 ml

Tracking dye: bromophenol blue

Fixative: 50% methanol-100 ml; Formaldehyde-50 µl

Stain: (a) Slab gel stain: 60 mg % Coommassie brilliant blue R-250 in methanol: acetic acid: water mixture (11:3:11, v/v)

(b) Tube gel stain: 120 mg % Coommassie brilliant blue R-250 in methanol: acetic acid: water mixture (11:3:11, v/v)

Destaining solution: methanol: acetic acid: water mixture (1:1.5:17.5, v/v)

Gel preparation:

The gels were cast in BROVIGA gel electrophoresis apparatus.

A. Separating gel (7 % was used for molecular weight determination of AOP1 and AOP2 and for western blotting of commercial BSA and blue Sephadex eluted HSA).

30% acrylamide: 3.375 ml; Distilled water: 1.125 ml; Buffer 1: 8.25 ml; Ammonuim Persulphate (APS) [15 mg/ml]: 0.625 ml; TEMED: 0.015 ml

B. Spacer gel

30% acrylamide: 0.5 ml; Buffer 2: 4.25 ml ;

Ammonuim Persulphate (APS) [15 mg/ml]: 0.25 ml; TEMED: 0.005 ml

Sample preparation: Samples were dialyzed overnight at 4ºC against chamber buffer without SDS.

For AOP1 and AOP2 size determination 50 µg of AOP1 and AOP2 mixed with 1% SDS and 10 % glycerol were added and kept in boiling water bath for 2 min and after cooling loaded on 7% slab gel together with markers. Markers used were TI (20 kDa), albumin (67 kDa), Phosphorylase B (97 kDa), β galactosidase (116 kDa).

Commercial fatty acid free BSA and blue Sephadex eluted HSA prior to western blotting, 100 µg each were mixed with 1% SDS and 10% glycerol and kept in boiling water bath for 2 min and after cooling loaded on 7% slab gel.

Electrophoresis: For tube gel electrophoresis, it was run at room temperature at 3 mA current per tube till bromophenol blue dye ran out. For slab gel

electrophoresis, it was run at room temperature at 18 mA current till bromophenol blue dye ran out. After run gel was fixed, stained and destained.

3.2.29. Electroelution of proteins

Electoelution of proteins from gels were done according to the method described by Ogden and Adams (1987). After polyacrylamide gel electrophoresis, one of the gels was stained with CBB R-250 and destained. Using this as marker, corresponding bands were sliced from the nonstained gels which had been kept at 4°C after the run. Gel slices containing the required protein band were minced with a scalpel blade, collected in about 2 ml of 0.01 M Tris acetate buffer, pH 8.0 and transferred to dialysis bags. Bags were immersed between opposite electrodes in a rectangular reservoir with 2L of cold Tris acetate buffer and a constant voltage of 100 V was applied across the electrodes for 2 h at 4°C and current was reversed for 5 min to detach proteins adhered to the dialysis bag surface facing the positive electrode. Supernatant containing protein was then extracted from the bag and was dialysed against PBS and concentrated by AMICON PM10 ultra filtration membrane.

3.2.30. Western blotting of commercial fatty acid free BSA and HSA

Western blotting was done as described by Towbin et al. (1979). After SDS PAGE in slab gel, proteins were transferred on PVDF membrane. The gel was transferred to transfer buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS and 5% methanol, pH 8.3 for 2 minutes. The PVDF membrane was also soaked in the same buffer. Into the anode plate, Whatman No.1 filter paper soaked in the same buffer was layed without air bubbles, over which PVDF membrane was placed and followed by the gel and again stacked with filter paper, cathode plate placed and a

constant current of 0.8 mA per cm^2 of gel was applied for 3 h at 25ºC. After transfer PVDF membrane was dried and preserved at 4ºC.

3.2.31. Enzyme linked immunoassay using Western blotted samples

Western blotted strips of BSA and HSA were cut into 22 equal fragments each about 3 mm X 3mm. Fragments were blocked in 0.5% PBST for 3 h at 25°C and transferred to microplates containing 375 ng jacalin-HRP in 200 µl PBST and incubated for 2 h at 4°C followed by washing with PBST thrice. Strips were again treated with 200 µl OPD solution in microplates for 2 h at 25°C, removed and well contents treated with H_2SO_4 and absorbance measured at 490 nm as described. Fragments 16 and 19 which gave maximum response for jacalin-HRP were cut from another strip and checked for anti-LRP response by treatment with 10 µg /ml anti-LRP-1 overnight following blocking as above and binding quantitated by treatment with anti goat IgG-HRP (1.5 µg /ml) for 3 h. As negative control for anti-LRP-1, fragments were probed for 3 h with 10 µg/ml anti apo B.

3.2.32. Dot blotting of α-synuclein

PVDF membrane cut into small square pieces (3 mm X 3 mm) were wetted in methanol followed by distilled water and PBS 7.4. A drop of protein α-synuclein was spotted at the centre of the blot and kept for drying in room temperature. Blots were washed in methanol, followed by water and PBS and blocked in 0.5% PBST for 2 h before addition to anti-Gal (5 µg/ml in PBST) which had been preincubated for 2 h at 4ºC with 25 mM specific sugar (1-O-methyl α-D-galactoside) or nonspecific sugar (1-O-methyl α-D-mannoside). After incubation for 2 h at 4ºC and washing, blots were further incubated with HRP conjugated anti-immunoglobulin for 2 h at

4 ºC. The blots were then transferred to substrate solution (1 mg 4-chloro naphthol dissolved in 0.4 ml methanol and mixed with 1.6 ml PBS containing 0.05% H_2O_2 where PBS was kept for 1 h in 37 °C water bath prior to addition) and kept in dark without shaking till colour of required intensity appears.The excess substrate was washed off and the blots were kept at 4 ºC.

3.2.33. Enzyme linked immunoassay using dot blotted samples

Tau protein was dot blotted on PVDF membrane as described and was incubated with anti-Gal or ABG which was treated in advance with specific sugars 25 mM in PBST of 1-O-methyl α-D-Gal or cellobiose or nonspecific sugar methyl α-D-Man. Blots were then treated with HRP-labeled anti-human Ig and washed. Bound antibody along with HRP-labeled anti-human Ig was extracted with specific sugar of either antibody. Extracted HRP was assayed using OPD as the substrate and absorbance measured at 490 nm using BIOTECK ELISA reader.

3.2.34. Preparation of total immunoglobulins (Ig) from plasma

From 10 ml plasma immune complexes were precipitated by 20% ammonium sulphate and were removed by centrifugation at 17400 g for 30 minutes. Ammonium sulphate concentration was then raised to 45% and the precipitate containing mostly immunoglobulins was collected by centrifuging again at 17400 g for 30 minutes. Precipitate collected was dissolved in 2 ml PBS and dialysed against PBS.

3.2.35. Isolation of human peripheral mononuclear cells and activation to macrophages

Peripheral blood mononuclear cells were isolated from healthy volunteers (IEC/SCT 1032) by a density gradient centrifugation method using Ficoll Histopaque 1077 in 1:1 ratio (v/v) as described by Panda et al. (2012).

Reagents:

A. RPMI 1640 meduim (containing L-glutamine and 25 mM HEPES, without sodium bicarbonate)

The entire powder in each pack was dissolved in 900 ml sterile distilled water with stirring under sterile condition. The pH of media was lowered to 4.0 with 1 N HCl to completely dissolve the media. After raising the pH to 7.2 with 1 N NaOH, 2 g sodium bicarbonate was added and stirred until dissolved. While stirring the pH of the medium was adjusted to 0.1-0.3 pH units below the desired pH since it may rise slightly on filtration. The solution was made to 1000 ml volume with sterile distilled water. To this 100 IU per ml penicillin, 100 µg /ml streptomycin and 100 µg/ml gentamycin was added and the media was then sterilized immediately by filtration using a membrane with 0.22 micron porosity and transferred into sterile containers and stored at 4ºC.

B. Heat inactivated donor specific (autologous) human serum

Serum was heat inactivated by placing in a 55ºC water bath for 30 min. It was further kept at 37 ºC for 1 h, and then filtered using a 25 mm Whatman syringe filter and stored at -20ºC as aliquots till use.

C. Sterile PBS;

D. Histopaque 1077

Isolation protocol:

Human venous blood was collected in a heparinised tube and mixed well by gently inverting the tube several times. In a 15 ml centrifuge tube 3 ml Ficoll Histopaque was taken and to this 3ml blood was gently layered on the top in such a way that blood and Ficoll Histopaque stayed as two different layers. The tubes were centrifuged for 20 min at 100 g in a swing-out bucket and the whitish buffy coat (PBMCs) formed at the inter-phase between histopaque and medium was aspirated and subjected to washing sequentially with 10 ml each of sterile PBS and sterile RPMI recovering the cells after each washing by centrifugation at 100 g for 10 min. The approximate yield of cells from 3 ml blood varied from 10^5 to 10^6. The cells were resuspended in 1.5 ml RPMI 1640 medium (without adding serum), cell viability was determined by trypan blue dye exclusion test was found to be greater than 95%. The mononuclear cells were then seeded onto sterile 35 mm plates and kept at 37°C in 5% CO_2 for 4 h allowing monocytes to adhere and lymphocytes to remain in suspension. Non adherent cells were removed by washing plates three times with sterile PBS and monocytes adhered to dishes were maintained in RPMI 1640 medium supplemented with 10% serum for 14 days for the cells to completely differentiate from monocytes to macrophages.

3.2.36. Fluorescence measurement in macrophages

Glycoproteins AOP1 and AOP2 mixture was incubated for 5 h at 4°C with albumin and further incubated overnight at 4°C with FITC labeled antibodies anti-Gal/ABG which was preincubated with nonspecific sugar methyl α-D-mannoside or with specific sugar methyl α-D-galactoside or cellobiose for 2 h at 4°C. Macrophages (after 14 days of culture) were again incubated in 4°C for 5 h with this labeled

mixture. After further washing with 100 mM specific sugar for 1 h cells were scraped and subjected to lysis in 350 µl distilled water overnight and fluorescence was measured at 485/528 nm in BIOTECK fluorescence ELISA reader. FITC-labeled glycoproteins incubated with unlabeled antibodies anti-Gal/ABG were also studied similarly.

3.2.37. Fluorescence imaging

For nuclear staining, macrophages were incubated for 5 min with 10 µM bis-benzimide H 33324 (Hoeschst 33342), washed with PBS and observed at 350/460 nm under inverted fluorescent microscope (Olympus 1X51-Singapore) in Regional Cancer Center (RCC), Trivandrum. For mitochondrial staining, macrophages were incubated for 10 min at 37°C with 5 µm MitoSOX diluted in Hanks balanced salt solution (HBSS) and observed at 510/580 nm. FITC labeling in macrophages were done by incubating macrophages with FITC labeled mixtures for 5 h, followed by washing thrice with PBS and observed at 485/528 nm.

3.2.38. Preparation of native and denuded (triplet-free) platelets

Platelets were isolated from fresh blood samples collected from healthy volunteers in accordance with Institutional Ethics Committee (IEC) clearance (IEC/1072) by the procedure described by Jennings and Phillips (1982). Anticoagulant-treated blood samples (10 ml) were centrifuged at 150 g for 5 min at 25°C to collect plasma to which one-tenth volume of 100 mM EDTA was added to prevent platelet activation. It was centrifuged at 230 g for 10 min at 25°C to remove erythrocytes and again centrifuged at 4530 g for 15 min whereby the supernatant was removed and the pellet containing platelets was collected in 1 ml PBS and counted using Horiba ABX

Pentra 60 C+ Hematology analyzer. This platelet suspension (300 µl) was incubated for 2 h at 25°C with a mixture of specific sugars for anti-Gal and ABG (15 mM each of MαG and cellobiose) and the supernatant containing platelet-extracted triplet was removed by centrifugation at 4530 g for 15 min. Pellet was washed twice with PBS to get denuded (triplet-free) platelets. Platelets treated as above, but with non-specific sugar MαM were used as native (non-denuded platelets).

3.2.39. Resolution of triplet components by electrophoresis

Following alkaline gel electrophoresis (methods 3.2.27) of platelet-extracted triplets in 6% polyacrylamide gel tubes in Tris-glycine pH 8.3, one gel was stained with Coomassie Brilliant blue G-250 and the bands corresponding to glycoproteins, albumin and antibody were cut and electroeluted separately. The two glycoprotein bands were also eluted together where required.

3.2.40. Platelet aggregation assay

Denuded and non-denuded platelets (methods 3.2.38) 250 µl were treated with 20 µM ADP and absorbance was measured after 2 minutes at 405 nm in a BIOTEK ELx800 ELISA reader.

3.2.41. Assay of amyloid β-binding activity of proteins

Denuded or native platelet suspension (method 3.2.38) (0.25 million cells/ µl) where platelets were counted using Horiba ABX Pentra 60 C+ Hematology analyzer was incubated for 3 h with fluorescent labeled-amyloid β at 25^0C and fluorescence of supernatant measured in polystyrene microwells at 485/528 nm as described earlier. The pellet was washed twice with PBS and incubated for 1 h at 25^0C with a mixture of anti-Gal- and ABG-specific sugars (MαG and cellobiose ; 15 mM each) and the

fluorescence of platelet-bound amyloid β released with specific sugars was measured as above.

3.2.42. Statistical analysis

Student's t test was done for statistical analysis using Microsoft excel 2010 and Graphpad prism 5. $P<0.05$ was considered statistically significant.

4. RESULTS AND DISCUSSION

PART- I

Identification and characterisation of endogenous O-glycoproteins that bridge between anti-Gal/ABG and albumin to form antibody-O-glycoprotein-albumin triplet

Introduction

Anti-carbohydrate antibodies being the first line of defense against pathogens constitute a major part of circulating antibodies in humans and are called natural antibodies as they are present in circulation without any deliberate immunization (Shoenfeld & Isenberg, 1989). The synthesis of anti-α-galactoside antibody (anti-Gal) was an evolutionary milestone observed only in humans, apes and old world monkeys (Galili et al., 1988). Galα1→3Gal epitopes are present on cell surface of other animals due to absence of anti-Gal and they act as a major effector of xenograft rejection in humans (Galili, 2005). A very minor fraction of anti-Gal in human plasma is known to form immune complex with lipoprotein(a) where it recognizes the serine- and threonine-rich peptide sequences (STPS) of the apo(a) subunit of lipoprotein(a) as surrogate ligand (Geetha et al., 2014). Anti-β-glucan antibodies (ABG) are produced against β-glucan present in fungal cell walls and in diet sources.

Plasma anti-Gal and ABG eluted from their respective affinity matrices using their specific sugar always contained as co-purified contaminants albumin and two other proteins. These glycoproteins and the same two antibodies were also co-purified with albumin captured from plasma on its affinity matrix and eluted using 250 mM NaCl. Present study was done to identify and characterize the proteins which were getting co-purified with anti-Gal /ABG and albumin and to examine their interaction with each other.

RESULTS

(A) Albumin and two other proteins co-purify with plasma anti-Gal and ABG.

Alkaline pH electrophoresis of affinity-purified anti-Gal (APAG) or affinity-purified ABG (APABG) showed a sluggish immunoglobulin band, two other protein bands with intermediate mobility and a fast moving band which was identified as human serum albumin (HSA) due to identical mobility with that of genuine HSA in alkaline and denaturing electrophoresis (Figure 12). Alkaline pH electrophoresis of affinity purified albumin captured from plasma by blue Sephadex and eluted using 250 mM NaCl also showed an albumin band, immunoglobulin band and the same two protein bands (Figure 12). These proteins do not bind to anti-Gal- or ABG-specific matrices or to blue Sephadex. Also anti-Gal or ABG does not bind to albumin specific matrix and albumin does not bind to antibody-specific matrices (Figure 13). Results suggest that anti-Gal or ABG, one or both of the new proteins and albumin formed a complex in plasma. As these proteins are albumin associated, O-glycosylated and antibody-binding they were named albumin-associated antibody-binding O-glycosylated proteins 1 and 2 (AOP1 and AOP2).

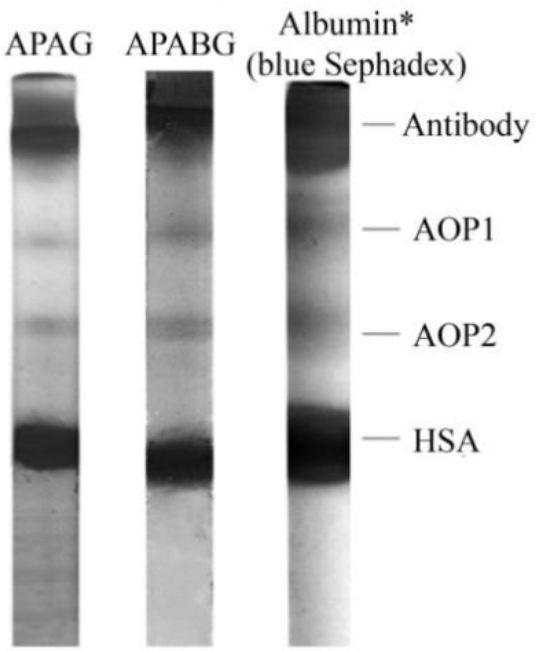


Figure 12. Albumin and two other proteins are associated with APAG or APABG from human plasma. *Electrophoresis of protein samples (50 µg) in 6% polyacrylamide gel in Tris-glycine pH 8.3 buffer. Gels were stained with Coomassie Brilliant blue G-250.*Plasma albumin loosely bound to blue Sephadex (eluted with 250 mM NaCl).*

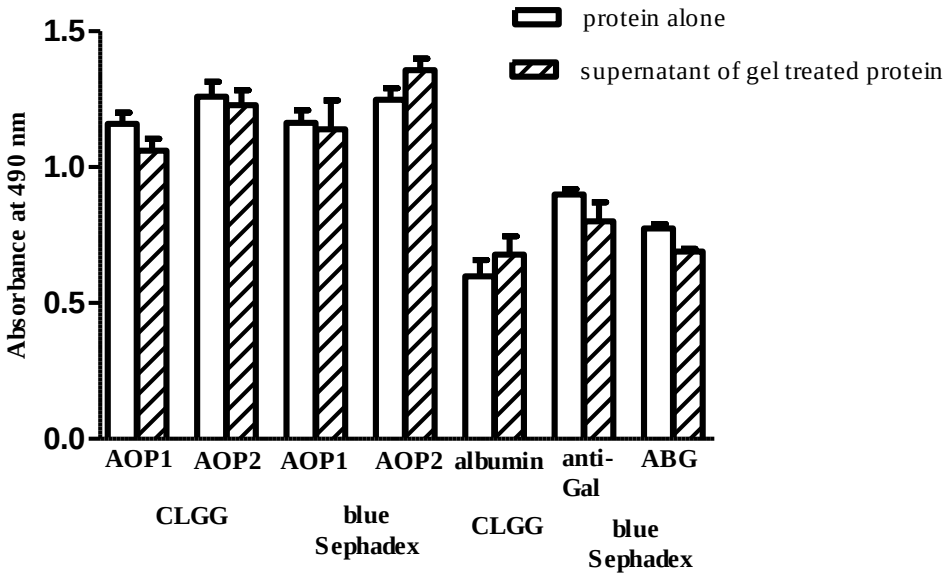


Figure 13. AOP1 and AOP2 does not bind to antibody specific or albumin specific matrices and anti-Gal or ABG does not bind to albumin specific matrix. *AOP1 or AOP2 (500 ng) in 200 µl was incubated overnight at 4°C with 0.4 ml CLGG or blue Sephadex and the total protein added and the superanatant after gel treatment was directly coated, washed and probed with jacalin-HRP (75 ng lectin/ml) as described under 'methods'. Similarly 100 ng albumin in 200 µl was incubated overnight at 4°C with 0.4 ml CLGG. Supernatant and the original protein added were directly coated on microplates, and probed with 3.75 µg/ml anti-albumin-HRP. Anti-Gal or ABG (100 ng) in 200 µl PBS was incubated overnight at 4°C with 0.4 ml blue Sephadex and the protein added and the superanatant after gel treatment was directly coated and probed with 1.5 µg/ml anti-immunoglobulin-HRP. Mean ± SD of 3 different samples.*

(B) Determining molecular weight of AOP1 and AOP2

Molecular weights of proteins were determined by SDS gel electrophoresis and gel permeation chromatography. Denaturing electrophoresis in 7% polyacrylamide gel containing 1% SDS in presence of 2-mercaptoethanol and using the molecular weight standards phosphorylase B (97 kDa), β-galactosidase (116 kDa), BSA (67 kDa) and soybean trypsin inhibitor (20 kDa) showed only one polypeptide of size 107 kDa and 98 kDa in AOP1 and AOP2 respectively (Figure 14). Molecular

weights of AOP1 and AOP2 in native state were determined by gel permeation chromatography as 110 kDa and 90 kDa respectively which was almost same as that determined by SDS gel electrophoresis suggesting that AOP1 and AOP2 are single monomeric proteins.

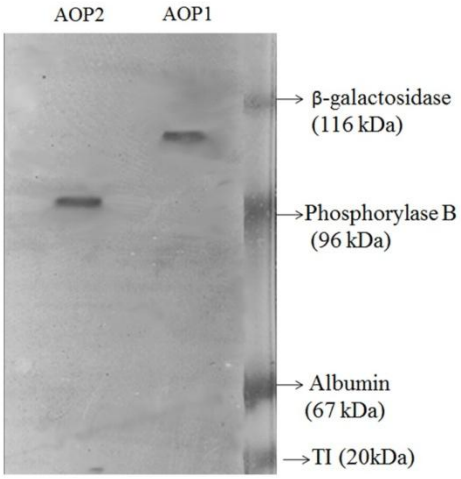


Figure14. Molecular weight determination of AOP1 and AOP2. *SDS-polyacrylamide gel (7%) electrophoresis of AOP1 and AOP2 (50 µg treated with 1% SDS and 0.05% v/v 2-mercaptoethanol at 95ºC for 5 min) along with molecular weight standards.*

(C) Proteins associated with anti-Gal/ABG and albumin (AOP1 and AOP2) are heavily O-glycosylated and poorly N-glycosylated

AOP1 and AOP2 electroeluted from the gel (Figure 12) was coated on polystyrene micro wells and probed with HRP-conjugated lectins for analyzing glycosylation. They showed strong binding of jacalin that specifically recognize O-glycans and poor binding of concanavalin A that recognize N-glycans (Goldstein et al., 1973; Sastry et al., 1986) indicating the high O-glycosylation and poor N-glycosylation in AOP1 and AOP2 (Figure 15). Neuraminidase-treated AOP1 and AOP2 were recognized by PNA which binds core-1 type O-glycans (Galβ1-3GalNAcαSer/Thr)

underlining presence of core -1 type O-glycans in AOP1 and AOP2. Apo(a) is a heavily glysosylated plasma lipoprotein with molecular weight ranging from 300-500 kDa and containing 28.1% carbohydrate by weight (Fless et al., 1986) comprised of 80% O-glycans and about 17% N-glycans (Garner et al., 2001). Ratio of responses to jacalin and concanavalin A was many fold higher for AOP1 and AOP2 than that for apo(a) (Figure 15 inset). Carbohydrate content of AOP1 or AOP2 was determined by phenol sulphuric acid method and the results (Figure 15 inset) show that it is nearly twice as that of apo(a).

	Response ratio (Jn/ConA)	Neutral sugar* (w/w)	MW[a] (kDa)
AOP1	5.12	54.25	107
AOP2	4.495	51.26	98
apo(a)	1.83	28	300-800

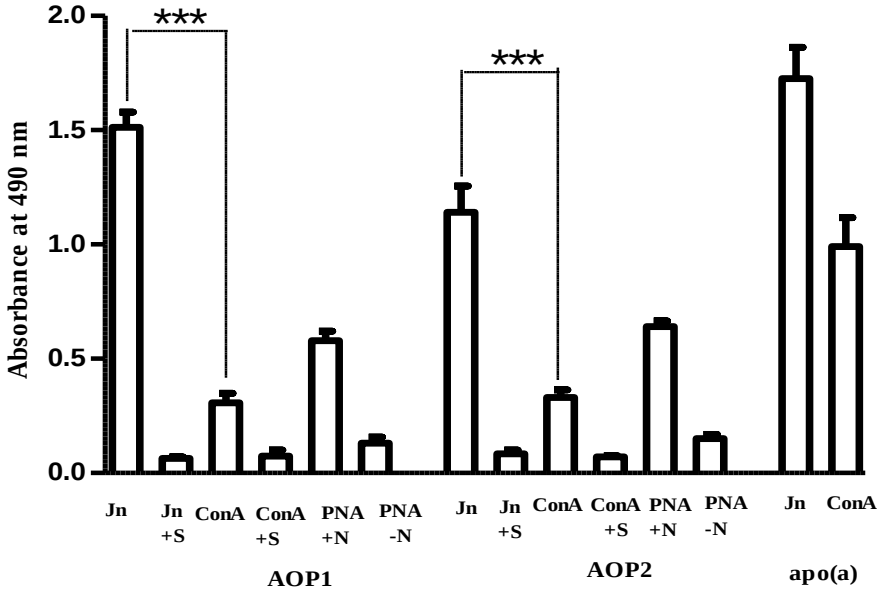


Figure 15. Lectin reactivity and glycan composition of AOP1 and AOP2. *AOP1, AOP2 or apo(a) (200 ng in 200 µl PBS) was coated on polystyrene (NUNC-Maxisorb) wells and blocked. Wells for PNA-HRP probing were treated with active*

*(+N) or heat inactivated (-N) neuraminidase in 200 µl PBST for 1 h. HRP-conjugated lectins [75 ng lectin/ml for jacalin (Jn), 10 µg lectin/ml for concanavalin A (Con A) and 7.5 µg lectin/ml for peanut aggutinin (PNA)] treated in advance with (+S) or without (-S) specific sugars (25 mM methyl α-D-Gal for jacalin and methyl α-D-Man for concanavalin A) were added in 200 µl PBST to washed wells. After incubation for 2 h at 4ºC and washing, bound conjugate was assayed by OPD treatment. Coating on wells, washing and conjugate assay are described under 'methods'. ***: P value <0.0001 for difference in response between jacalin and concanavalin A. Mean ± SD of 6 consecutive AOP1/AOP2 samples.* ***Inset:*** *Response ratio derived from data in main figure. *: Neutral sugar was estimated as percentage carbohydrate content estimated by phenol-sulphuric acid method in weighed mass of lyophilized AOP1 or AOP2. Mean ± SD of 3 samples. a: MW of AOP1 and AOP2 from Figure 14 and of apo(a) from literature.*

(D) AOP1 and AOP2 bind to O-glycoprotein-free anti-Gal and ABG

Anti-Gal recognizes epitopes with terminal α linked galactose (Galili et al., 1984) whereas β-glucosides including cellobiose are the best ligands for ABG (Geetha et al., 2007). Binding of O-glycoprotein-free anti-Gal and ABG to microplate-coated thyroglobulin (Tg, rich in terminal α-Gal moieties) and TIC respectively was inhibited significantly by pretreatment of either antibody with AOP1 or AOP2, showing that the latter interact with both anti-Gal and ABG (Figure 16).

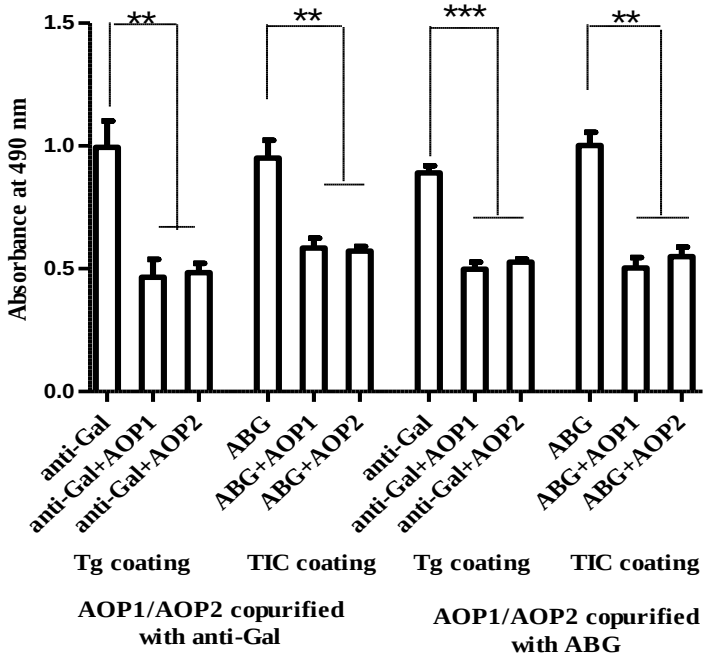


Figure16. AOP1 and AOP2 isolated from affinity-purified anti-Gal and ABG bind to glycoprotein-free anti-Gal and ABG. *Anti-Gal, ABG, AOP1 and AOP2 were isolated in pure form by electroelution from gel in Figure 12. Anti-Gal or ABG (100 ng) alone or with either AOP1 or AOP2 (1µg) co-purified with anti-Gal was incubated for 2 h at 4ºC before adding to microplate-coated Tg (for anti-Gal) or TIC (for ABG) and bound antibody quantitated using HRP-conjugated anti-immunoglobulin as described (methods 3.2.19). Alternatively AOP1 and AOP2 co-purified with ABG were also used. ***: P value <0.0007; **:P value <0.001. Mean ± SD of 6 consecutive AOP1/AOP2 samples.*

(E) AOP1 and AOP2 occupy sugar binding sites of antibodies

Fluorescence of FITC-labeled antibody increases on antigen binding due to antigen induced conformational change and this increase is proportional to affinity of macromolecular antigenic ligand interacting with the antibody (George et al., 2015). AOP1 and AOP2 produced an increase in fluorescence of anti-Gal-FITC and ABG-FITC greater than that by same amount of Tg or TIC respectively and this increase was totally abolished in presence of respective disaccharide inhibitors (Figure 17)

establishing that these glycoproteins occupied the sugar-binding site of respective antibodies.

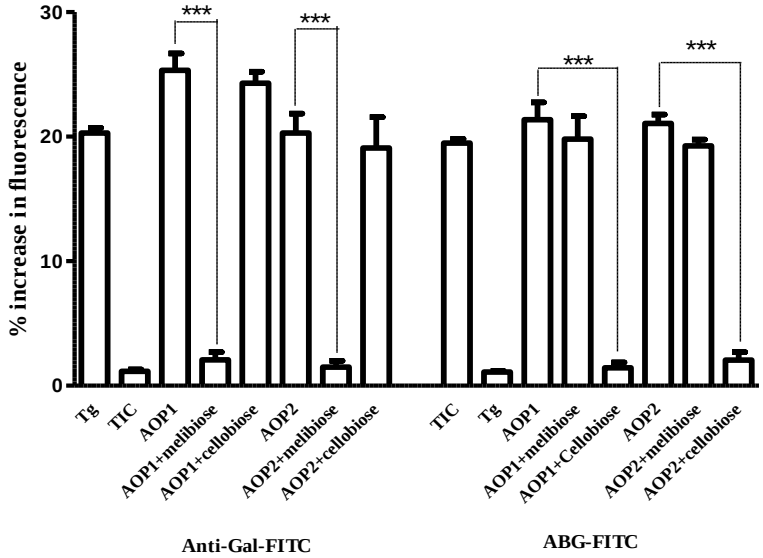


Figure 17. AOP1 and AOP2 occupy sugar-binding sites of glycoprotein-free anti-Gal and ABG. *Purified anti-Gal-FITC (separated from APAG-FITC by electrophoresis and elution; 1µg in 25 µl PBS) was incubated at 4ºC for 2 h with specific sugar (melibiose) or non-specific sugar (cellobiose) (each 25 mM). The mixture was added to 2 µg AOP1 or AOP2 purified from APAG in 75 µl PBS and incubated overnight at 4ºC before dilution to 300 µl and measurement of fluorescence as described in methods. Alternatively ABG-FITC (from APABG-FITC) treated with its specific sugar (cellobiose) or non-specific sugar (melibiose) was also tried. Tg and TIC were used instead of AOP1/AOP2 as glycoproteins bearing standard ligands for anti-Gal and ABG respectively. ***: P value <0.0003 for inhibition by specific sugar. Mean ± SD of 3 consecutive plasma samples.*

(F) Anti-Gal recognizes STPS in O-glycosylated regions of AOP1 and AOP2

Anti-Gal binds to MUC-1 and Lp(a) using STPS in latter (Geetha et al., 2014; Sandrin et al., 1997). Being human proteins AOP1 and AOP2 lack terminal α-galactoside residues, the classical anti-Gal ligand, but being heavily O-glycosylated with core-1 type O-glycans (as shown in Figure 15), were expected to be rich in STPS. The increase in fluorescence of anti-Gal-FITC produced by specific binding

of AOP1 or AOP2 was fully abolished by pretreatment of these glycoproteins with either jacalin or LDL (Figure 18). Jacalin binds to the Gal β1-3GalNAc core of O-glycans and apoB in LDL binds to negatively charged sialic acid moieties of O-glycans (Becker et al., 2004). Therefore the results shown in Figure 18 suggest that anti-Gal recognizes O-glycosylated region of the glycoproteins AOP1 and AOP2. But removal of O-glycan chains from AOP1 and AOP2 by de-O-glycosylation to convert them to DAOP1 and DAOP2 respectively did not affect their affinity towards anti-Gal unlike that towards jacalin (Figure 18 inset), showing that it could be the STPS at the O-glycosylated region rather than O-glycans per se that are ligands for anti-Gal. Effectiveness of de-O-glycosylation was verified by the loss of jacalin binding to DAOP1 and DAOP2 (Figure 18 inset).

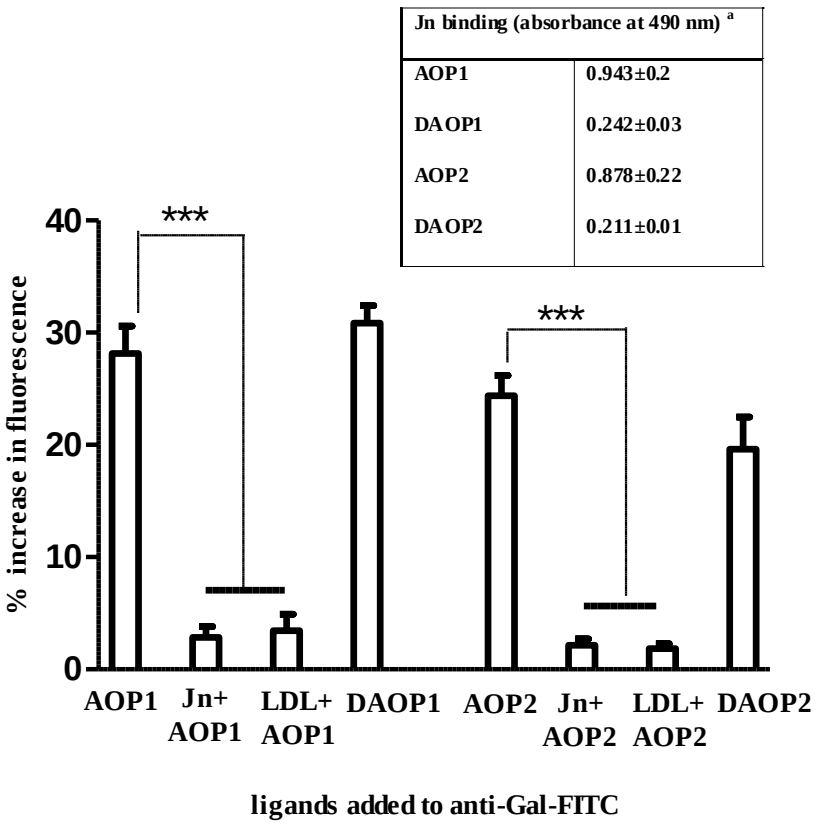


Figure 18. Anti-Gal recognizes STPS in O-glycosylated regions of AOP1 and AOP2. *Electroeluted AOP1 and AOP2 (2 µg in 50 µl PBS) was incubated with jacalin or LDL (4 µg each) in 25 µl PBS or with 25 µl PBS only for 2 h at 4ºC. These samples or de-O-glycosylated AOP1/AOP2 (DAOP1 and DAOP2) in 75 µl PBS was further incubated with electroeluted anti-Gal-FITC (1 µg in 25 µl PBS) overnight at 4ºC. All samples were diluted to 300 µl for fluorescence measurement as described. ***: P value <0.0001 for blocking of AOP1/AOP2 by jacalin or LDL. Mean ± SD of 6 consecutive plasma samples as AOP1/AOP2 source.* ***Inset:*** *Drop in jacalin-HRP binding to microplate-coated samples confirms de-O-glycosylation of AOP1 and AOP2. a: jacalin binds exclusively to O-glycans.*

(G) AOP1 and AOP2 are co-purified with plasma albumin

The two proteins other than immunoglobulins associated with albumin eluted from blue Sephadex using 250 mM NaCl were electroeluted from gel (Figure 12). These proteins as shown in Figure 12 were identical in mobility to AOP1 and AOP2

copurified with APAG and APABG and also were reactive towards anti-Gal and ABG as both increased the fluorescence of anti-Gal-FITC and ABG-FITC to the same extend as AOP1 and AOP2 co-purified from APAG and APABG did (Figure 19). The results confirm that AOP1 and AOP2 are associated with plasma albumin.

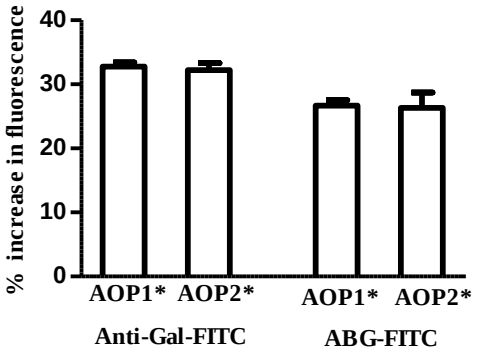


Figure 19. AOP1 and AOP2 are co-purified with plasma albumin. *2 µg AOP1 and AOP2 in 75 µl PBS was incubated with electroeluted anti-Gal-FITC or ABG-FITC (1 µg in 25 µl PBS) overnight at 4ºC. All the samples were diluted to 300 µl for fluorescence measurement as described. *Bands identical in mobility with AOP1 or AOP2 in alkaline electrophoresis. Mean ± SD of 6 samples.*

(H) Anti-Gal and ABG are the only plasma antibodies that are co-purified with albumin.

Immunoglobulins were electroeluted from gels of Figure12. From this sample those immunoglobulins that bound to Tg which were inhibitable by anti-Gal specific sugar melibiose and those that bound to TIC were inhibitable by ABG-specific sugar cellobiose suggesting that anti-Gal and ABG are the antibodies that are involved (Figure 20). Further, immunoglobulins co-purifying with albumin consisted exclusively of anti-Gal and ABG as all of them got absorbed to a mixture of cross-linked guar galactomannan and cellulose (Figure 20 inset).

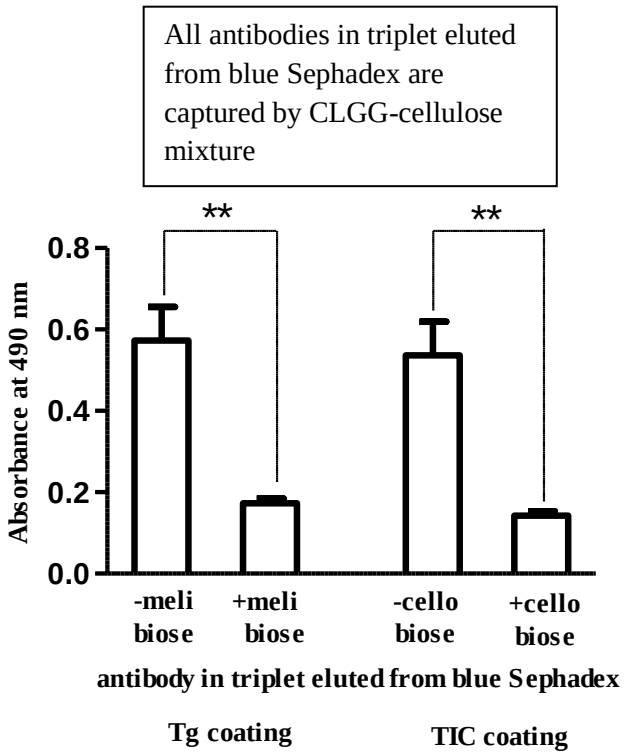


Figure 20. Anti-Gal and ABG are the only antibodies that bind along with albumin to blue-Sephadex. *Antibodies were separated by alkaline electrophoresis and electroelution from albumin bound to blue Sephadex and eluted by 250 mM NaCl. PBST (200 µl) containing 100 ng antibody was incubated with or without anti-Gal–specific sugar (melibiose; 25 mM) for 2 h before adding to 1 µg Tg coated on microplates and bound antibody probed using HRP-conjugated anti-immunoglobulins. Alternatively antibody incubated with or without 25 mM ABG-specific sugar (cellobiose) was added to 1 µg TIC coated on wells.**: P value<0.008 for inhibition by specific sugar. Mean ± SD of 3 antibody samples.* ***Inset****: From 200 ng antibody treated with a mixture of 0.2 ml CLGG and 0.2 ml cellulose in 1 ml PBS for 4 h at 4ºC unbound antibody in supernatant was determined by ELISA after coating 200 µl on microplates and assaying bound antibody using anti-immunoglobulin-HRP. Total antibody added was assayed similarly. Mean ± SD of 3 antibody samples.*

(I) All circulating anti-Gal and ABG molecules are involved in a protein complex containing albumin

Total plasma anti-Gal prepared by affinity chromatography on anti-Gal-specific matrix (APAG) could consist of complexed as well as free anti-Gal whereas anti-Gal molecules associated with albumin only would bind to blue Sephadex. To check the

presence of any free anti-Gal present in APAG the latter (500 ng) was treated with excess (0.4 ml) blue Sephadex and unbound protein was determined. While only 14.36±0.12% protein remained unbound to blue Sephadex among APAG samples (n=4) unbound protein had the same relative contents of immunoglobulins, albumin and glycoprotein as original APAG added (Figure 21a). Had there been free anti-Gal without being associated with albumin the supernatant would have been enriched in immunoglobulins. Results therefore suggest that there was no detectable amount of free anti-Gal in APAG though the affinity matrix used for APAG preparation could have captured free anti-Gal as well, if present in plasma. Again to confirm this result immunoglobulin and albumin contents of total anti-Gal population in plasma and of anti-Gal complexed with albumin were compared. Total albumin from plasma dilution and anti-Gal in albumin-containing complex (eluted from blue Sephadex) were incubated with microplate-coated guar galactomannan to capture all forms of anti-Gal and probed with HRP conjugates of anti-human immunoglobulins and anti-albumin separately. If there was any free anti-Gal in plasma the immunoglobulin: albumin ratio would have been higher in total anti-Gal from plasma, but the results shows that ratio was slightly higher in anti-Gal complexed with albumin (Figure 21b). Same was the result when plate-coated yeast antigen was used for capturing ABG and its complexes. Results in figures 21a and 21b together shows that all circulating anti-Gal and ABG are in complex with AOP1/AOP2 and albumin and no free anti-Gal or ABG are present in circulation.

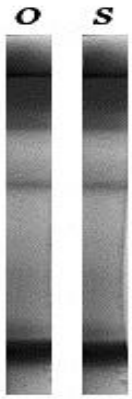


Figure 21a. No free anti-Gal is present in circulation*. More than 90% of affinity purified anti-Gal bind to blue Sephadex, unbound has same composition as original. APAG (500 ng) was treated with 0.4 ml blue Sephadex in 1.2 ml PBS for 3 h at 4ºC with stirring at 20 min intervals and the supernatant was collected and protein was assayed by Bradford method. Unbound fraction from several batches were combined, concentrated by filtration and compared with original in alkaline electrophoresis. Mean ± SD of 3 samples.* ***O****: original antibody sample,* ***S****: supernatant after gel treatment of* ***O****.*

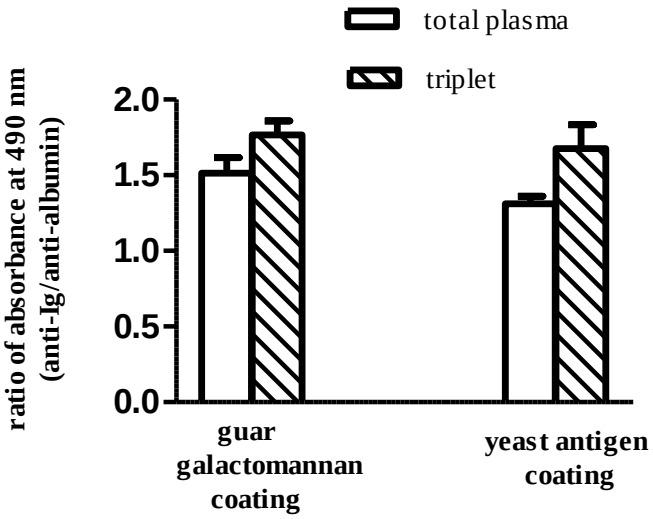


Figure 21b. All plasma anti-Gal and ABG are complexed with albumin. *Immunoglobulin: albumin ratio of total plasma anti-Gal or ABG is not greater than that of albumin-associated antibodies****.*** *Plasma dilution (50 X in PBST; 200 µl) or anti-Gal in complex with albumin (250 mM NaCl eluate from blue Sephadex; (Figure 12) (1 µg in 200 µl PBST) was added to microplate-coated (1 µg per well) soluble guar galactomannan or yeast antigen and were incubated at 4ºC for 2 h, washed and probed separately with anti-immunoglobulin-HRP and anti-albumin-HRP. Mean ± SD of 6 consecutive samples.*

(J) Plasma anti-Gal and ABG exist as triplets in which antibody and albumin are linked by AOP1 or AOP2

It is evident from the above results that both antibody-binding as well as albumin-binding matrices capture anti-Gal and ABG antibodies, O-glycoproteins AOP1 and AOP2 and albumin. Results also show that AOP1 and AOP2 can independently bind to anti-Gal or ABG. Since albumin which is devoid of either glycans or STPS is not a ligand for anti-Gal or ABG these results lead us to the conclusion that it is AOP1 or AOP2 that binds to albumin and antibody simultaneously and acts as a bridge between these two proteins and form the antibody-O-glycoprotein-albumin triplet. This assumption was confirmed by results in Figure 22. Capture of triplets from plasma dilution to microplate-coated guar galactomannan and assay of bound triplet by determining the bound albumin using anti-albumin-HRP was fully inhibited by anti-Gal-specific sugar. Similar was the case with plasma triplet binding to yeast antigen coating through ABG. Triplets due to anti-Gal and ABG were independent as sugar that dismantled one did not affect the other (Figure 22).

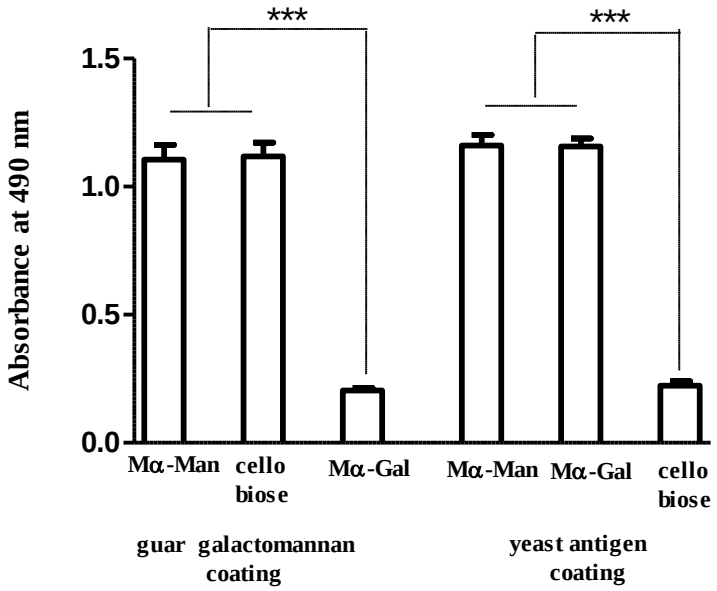


Figure 22. Triplets can be captured though antibodies and assayed using anti-albumin. *Cell-free plasma obtained by centrifuging plasma at 5000 g for 15 min at 25ºC was diluted 100 X in PBST and 200 µl pre-incubated with 25 mM specific sugar (methyl α-D-Gal for anti-Gal and cellobiose for ABG) or non-specific sugar (methyl α-D-mannoside) for 2 h before addition to microwell-coated guar galactomannan or yeast antigen (1 µg per well). After incubation for 2 h and washing with PBST bound albumin was detected by anti-albumin-HRP (3.75 µg antibody per ml) as described earlier .***: P value <0.0001 for inhibition by specific versus non-specific sugar for anti-Gal- and ABG-containing triplets. Mean ± SD of 6 plasma samples.*

(K) Novelty of AOP1 and AOP2

Nano LC-MS/MS analysis showed that while AOP1 and AOP2 were not identical with any other human protein for which sequences have been documented, a section of unique peptides detected in AOP1 and AOP2 have also been ascribed in the databases to human serum albumin. However identity of AOP1 or AOP2 with serum albumin was ruled out by differences in molecular size, electrophoretic mobility in alkaline gel, O-glycosylation and reactivity towards anti-Gal and ABG. AOP1 and

AOP2 were not recognized by anti-albumin antibody, but are ligands for albumin. Overlap of unique peptides between AOP1/AOP2 and albumin probably arose due to albumin samples used earlier for preparing its peptide sequence data having co-purified AOP1 and AOP2 adhering to albumin. These considerations and results listed above establish the novelty of AOP1 and AOP2.

DISCUSSION

Macromolecular antigens occupy only partial binding sites in the antibody enabling it to bind to smaller antigenic ligands using the free binding site(s) (George et al., 2017). This can be due to steric hindrance preventing the approach of a second macromolecule to a binding site adjacent to one already occupied by a macromolecule. The molecular mechanism of binding of antibody-macromolecule immune complexes in turn to other ligands, including those on host cells, has been reported (George et al., 2017). Present results show that all circulating anti-Gal and ABG antibodies are associated with two novel heavily O-glycosylated and poorly N-glycosylated glycoproteins which are inturn non-covalently bound to albumin.The chromatographic purification of anti-Gal and ABG despite these antibodies being associated with endogenous macromolecules (glycoprotein-albumin complex) was possible due to availability of unoccupied binding sites on these antibodies even after the above engagement. These albumin-associated antibody-binding O-glycosylated proteins 1 and 2 (AOP1 and AOP2) were monomeric proteins with molecular weights of 107 kDa and 98 kDa respectively and neutral sugar content of 54% and 51% respectively.

There are reports showing the binding of MUC-1 type peptides to anti-Gal using the serine- and threonine- rich peptide sequences (STPS) in MUC-1 (Sandrin et al., 1997). It has also been reported from this laboratory that lipoprotein (a) binds to anti-Gal using STPS in apo(a) subunit of Lp(a) (Geetha et al., 2014). The present results show that in AOP1 and AOP2 the remarkably more O-glycans than apo(a) and a larger number of serine-and threonine- rich sequences is responsible for its interaction with anti-Gal and ABG.

PART-II

Determining the role of albumin in triplet formation

Introduction

Human serum albumin is the most abundant plasma protein with reference range 3.5-6 g/dL. HSA is a single polypeptide protein with 585 aminoacids and is non-glycosylated. Ligands bind to hydrophobic pockets of albumin and can alter its hydrophobicity. As part 1 of this thesis established the presence in plasma of protein triplets consisting of anti-Gal or ABG bridged by O-glycoproteins AOP1 or AOP2 to albumin and delineated the interaction of either antibody with AOP1 and AOP2, present part examines the association of AOP1/AOP2 with albumin and how this association affects antibody-O-glycoprotein recognition and decide the physiological fate of the resulting triplet.

RESULTS

(A) Binding of AOP1 and AOP2 to albumin

AOP1/AOP2-FITC alone or preteated with HSA in 1 ml PBS was adjusted to density 1.24 g/cc by addition of solid KBr, where upon the volume increased to 1.1 ml. Following density gradient ultracentrifugation (DGUC) at 535000 g tube contents were separated into top (400 µl), middle (400 µl) and bottom (300 µl) layers and fluorescence was measured in middle and bottom layers. AOP1-FITC and AOP2-FITC remained mostly in the bottom layer (Figure 23). Upon DGUC of pure albumin in PBS (6.3 mg) majority of it (~4.3 mg) was recovered in middle layer while the rest (~1.9 mg) remained in the bottom layer (Figure 23 inset). However in the case of mixtures of HSA with AOP1 or AOP2 presence of albumin helped drag most of the

AOP1 and AOP2 to the middle layer (Figure 23) indicating that these O-glycosylated proteins bind to pure albumin.

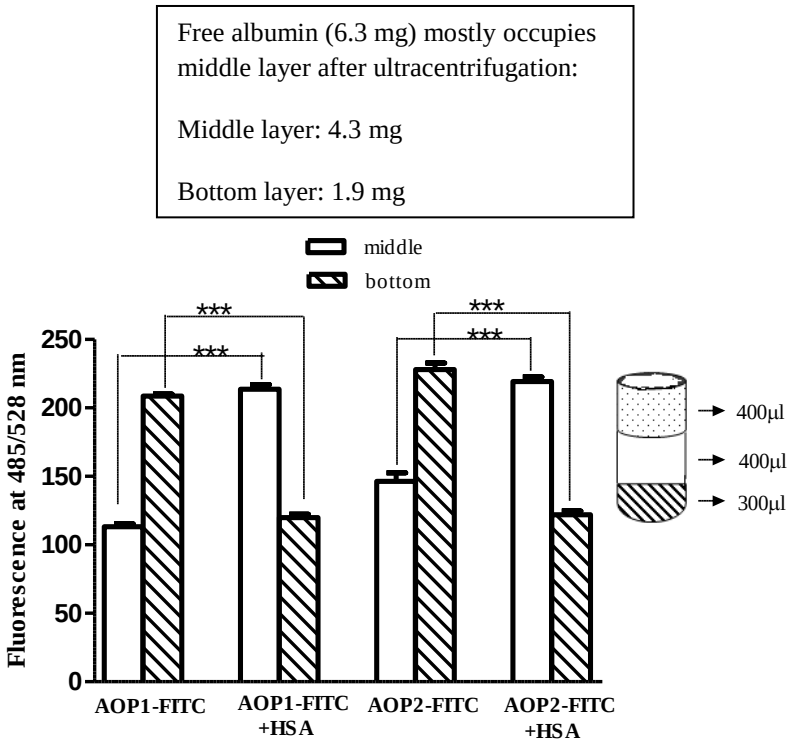


Figure 23. AOP1 and AOP2 bind to albumin. *Albumin, AOP1 and AOP2 were purified from affinity-purified anti-Gal (methods). PBS (1 ml) containing 100 µg AOP1-FITC or AOP2-FITC with or without 50 µg albumin was incubated overnight at 4^0C and subjected to DGUC (535000 g). Total fluorescence in middle (400 µl) and bottom (300 µl) layers were measured as described (methods). ***: P value <0.0001 for increase (in middle layer) and decrease (in bottom layer) of FITC-labeled AOP1 or AOP2 brought about by albumin. Mean ± SD of 6 samples.* ***Inset****: Albumin (6 mg in 1ml) was subjected to DGUC, middle (400 µl) and bottom (300 µl) layers as shown were collected and albumin assayed by coating dilutions (200 µl) in PBS on Nunc Maxisorb microplates and probing with anti-albumin -HRP (3.75 µg albumin per ml) (n=3).*

(B) Albumin binding restricts AOP1/AOP2 occupancy of anti-Gal and ABG; resulting triplet binds to other ligands

Binding of purified anti-Gal or ABG to microplate-coated thyroglobulin (Tg) or TIC respectively was partially inhibited by limited amount of AOP1 or AOP2. However

the same amount of AOP1 and AOP2 preincubated with albumin was non-inhibitory (Figure 24) showing that albumin-associated AOP1 or AOP2 already bound to the antibody was no hindrance to binding of the resulting triplet to smaller ligands including immobilized ones. It was reported that binding sites on anti-Gal and ABG were partially retained free even after occupation of other binding sites by macromolecular antigens enabling binding of the antibody along with macromolecular antigen to small ligands coated on polystyrene or exposed on cell surface (George et al., 2017). It follows therefore that free AOP1 or AOP2 unlike their albumin complex could occupy all binding sites of antibody molecules depending on O-glycoprotein concentration resulting in fully occupied antibody molecules which are unable to bind to plate-coated ligand in ELISA. On the other hand occupation of one binding of antibody by ligand-bearing macromolecules or their complexes exceeding a critical mass may leave adjacent binding site on the same antibody molecule free possibly because a second large molecule does not gain access to this site for steric reasons. Thus results in Figure 24 justifies the conclusion that binding of albumin to AOP1 or AOP2 ensures their restricted occupation on anti-Gal and ABG so that the triplet formed can bind in turn to smaller ligands utilizing the unoccupied binding site on antibody.

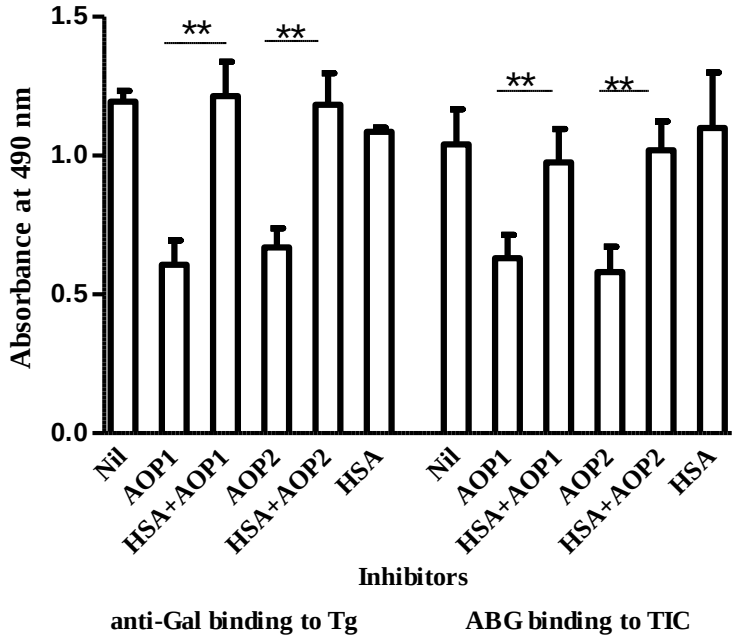


Figure 24. Albumin binding restricts AOP1/AOP2 occupancy of anti-Gal and ABG: resulting triplet binds to other ligands. *AOP1 or AOP2 (5 µg/ ml) alone or in presence of purified albumin (10 µg /ml) was incubated overnight at 4 °C. These samples or PBST (100 µl) were mixed with anti-Gal or ABG in PBST (100 ng in 100 µl), incubated for 2 h at 4^0C and added to microwell-coated with Tg or TIC respectively (1 µg). After 2 h incubation at 4^0C and washing of wells bound antibody was determined by probing with mixture of HRP-conjugated anti-human immunoglobulins (methods). **: P value < 0.005 for effect of albumin on inhibition of anti-Gal or ABG by AOP1 or AOP2. Mean ± SD of 6 plasma samples.*

(C) AOP1/AOP2 binding attenuates the hydrophobicity of albumin

Plasma albumin captured on cibacron blue F3GA immobilized on Sephadex G-100 (blue Sephadex) was eluted partially with 250 mM NaCl while the rest of the bound albumin required 1 M NaCl for elution. AOP1- and AOP2-bound albumin was eluted with 250 mM NaCl and it inhibited binding of jacalin-HRP to fetuin coated on microwells while same amount of protein eluted by 1 M NaCl after elution by 250 mM NaCl was ineffective (Figure 25). While primary event in binding of pure albumin to Cibacron blue F3GA is expected to be occupation of the hydrophobic

sites of the protein by the dye molecule, reversal of binding by 1M NaCl indicated that recognition of hydrophobic sites may be fortified by secondary ionic interactions. Therefore the above results indicate that O-glycoprotein binding had reduced the hydrophobicity of albumin enabling its elution from blue Sephadex by a lower (250 mM) NaCl concentration. This effect was also manifest in the response of albumin in Bradford's protein assay (Figure 25 inset). Assay response of 10 µg lyophilized powder of a mixture of AOP1 and AOP2 was less than 50 % of response by 10 µg standard BSA and the assay response of a mixture of 15 µg of AOP1/AOP2 mixture and 10 µg albumin was only about 80% of the sum of responses of AOP1/AOP2 and albumin measured separately suggesting that AOP1 and AOP2 which themselves reacted sluggishly with the dye in Bradford reagent could reduce albumin response to the dye as AOP1/AOP2 binds at its hydrophobic region where the dye in Bradford reagent mostly binds. But when anti-Gal was used instead of AOP1/AOP2 it did not produce this effect on albumin.

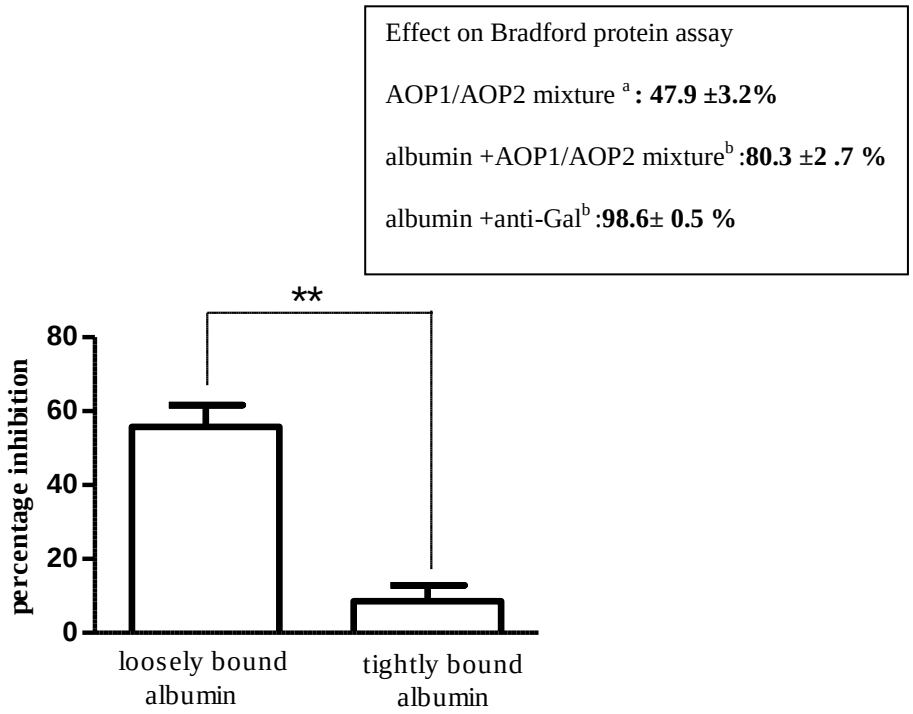


Figure 25. AOP1- or AOP2-bound albumin is less hydrophobic and eluted at lower NaCl concentration from blue Sephadex. *Albumin loosely bound (eluted at 250 mM NaCl) or tightly bound (eluted by 1M NaCl after 250 mM NaCl elution) to blue Sephadex (each 53 µg protein) was treated with jacalin-HRP (15 ng lectin) in 200 µl PBST for 1 h before adding the mixture to microplate-coated fetuin (1 µg per well). After incubation for 2 h at 4^0C and washing, bound HRP was assayed as described earlier. **: P value: 0.0028. Mean ± SD of 6 samples.* ***Inset****: Bradford protein assay response of purified albumin (10 µg), purified AOP1/AOP2 mixture (15 µg) and purified anti-Gal (15 µg), all prepared from weighed lyophilized powder, was measured individually or after incubation of given mixture for 1 h at 25^0C. a: Response as percentage of response of same weight of standard (HSA). b: Response of mixture as percentage of sum of responses of components. Mean ± SD of 3 samples.*

(D) De novo triplet formation requires anti-Gal or ABG, AOP1 or AOP2 and albumin

De novo triplets were formed by treating anti-Gal or ABG with AOP1 or AOP2 and albumin, all purified from APAG or APABG by electrophoresis and electroelution. Triplets formed were captured on microplate-coated anti-Gal- or ABG-specific ligands and assayed in terms of associated albumin using HRP-labeled anti-albumin

(Figure 26). Results confirm the linker role of AOP1 and AOP2 in triplet formation as antibody/albumin mixture or albumin alone was non-responsive in the assay.

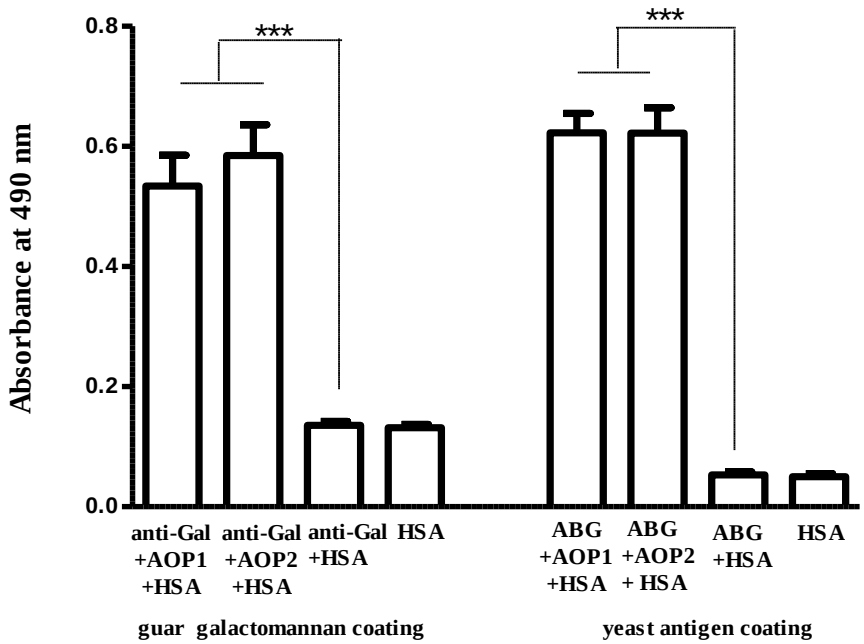


Figure 26. De novo triplet formation requires anti-Gal or ABG, AOP1 or AOP2 and albumin. *PBST (200 µl) containing purified anti-Gal or ABG (1 µg/ml) preincubated for 4 h with AOP1 or AOP2 (0.75 µg per ml) and albumin (2 µg/ml) was added to ELISA microplates coated with guar galactomannan or yeast antigen (1 µg per well), incubated for 2 h at 4^0C, and after washing, wells were probed with anti-albumin-HRP (3.75 µg antibody per ml). Albumin incubated alone or with antibody served as control. ***: P value < 0.0001 for response of anti-Gal/ABG, AOP1/AOP2 and albumin compared to that of antibody and albumin only. Mean ± SD of 6 samples.*

(E) Release of O-glycoproteins and albumin from immunoglobulin-containing complexes on treatment with anti-Gal- and ABG-specific sugars.

Addition of 25 mM methyl α-D-mannoside to plasma (1 ml) previously dialyzed against PBS to remove low molecular weight sugars did not alter the distribution of proteins in middle and bottom layers following DGUC (Table 2) proving that 25 mM

sugar exerted no colligative effect on protein distribution. On the other hand addition of methyl α-D-galactoside or cellobiose (25 mM) resulted in substantial increase (27% and 30% respectively) in protein in middle layer and a concomitant decrease of 26% and 25% respectively in protein in bottom layer. Corresponding rise in albumin content in middle layers were about 45% and 47% respectively while bottom layers registered loss of 43% and 47% albumin. O-Glycoproteins in middle layers increased about 46% and 45% respectively by anti-Gal- and ABG-specific sugars causing about 45% and 47% reductions in respective bottom layers. Results in table 2 suggest that a substantial part of albumin and O-glycoproteins of plasma remain associated with anti-Gal or ABG utilizing the sugar-binding site of these antibodies and that occupation of binding sites by competing sugar moieties causes displacement of O-glycoproteins along with albumin from antibodies.

Table 2.Treatment of cell-free plasma with anti-Gal- or ABG-specific sugar releases proteins including O-glycoproteins and albumin from bottom to middle layer on DGUC.

Sugar added (25 mM)	Components in middle and bottom layers after DGUC					
	Protein (mg)		O-glycoprotein [a]		Albumin [a]	
	Middle	bottom	Middle	bottom	Middle	bottom
no sugar	4.48±0.22	11.5±0.53	_	_	_	_
methyl α-D-Man	4.54±0.18	11.6±0.45	_	_	_	_
methyl α-D-Gal	5.78±0.12(↑27%*)	9.2±0.33(↓26%*)	↑ 46 % *	↓45%*	↑45% *	↓43%*
cellobiose	5.88±0.23 (↑30%*)	9.3 ±0.26(↓25%*)	↑ 45% *	↓47%*	↑47% *	↓47%*

Cell free plasma (1 ml) from supernatant centrifuged at 5000 g for 30 min and made normoglycemic by dialysis against PBS containing 4.5 mM glucose was further treated with anti-Gal-specific sugar methyl α-D-Gal, ABG-specific sugar cellobiose or non-specific sugar methyl α-D-Man (each 25 mM), incubated overnight, subjected to DGUC (535000 g, 4 hours at 4ºC) and middle (400 µl) and bottom (300 µl) layers (Figure 23) collected. Protein was estimated by Bradford assay. Both layers of methyl α-D-Man- treated samples diluted to make 1 µg protein per ml and others diluted to the same extent were directly coated (200 µl/well) on polystyrene wells and probed with jacalin-HRP (75 ng lectin /ml) for O-glycoprotein assay and with anti-albumin-HRP (3.75 µg antibody/ml) for albumin assay. n=6. % increase (↑) or decrease (↓) over corresponding values using methyl α-D-Man shown. [a] *:response of microplate-coated samples probed with jacalin-HRP or anti-albumin-HRP compared.*

(F) Anti-Gal or ABG binding to AOP1 or AOP2 enhances the stability of the latter's complex with albumin

DGUC (in 1 ml tubes) of plasma or PBS after addition of AOP1-FITC or AOP2-FITC and measuring the fluorescence in middle and bottom layers showed that in both plasma and PBS added AOP1- or AOP2-FITC sequestered more to the bottom

layer compared to middle (Figure 27). However when plasma treated with anti-Gal- or ABG- specific sugar was used as medium the distribution of FITC-labeled O-glycoproteins was reversed, with middle layer containing more label than the bottom layer. A likely reason for this reshuffle is that removal of antibody from plasma triplets by treatment with specific sugar temporarily destabilizes the AOP1/AOP2-albumin bondage and now the newly liberated albumin could bind to any AOP1/AOP2 molecule including FITC-labeled ones. But how the freshly liberated albumin differs from AOP1-and AOP2-free albumin likely to be present in plasma is unclear.

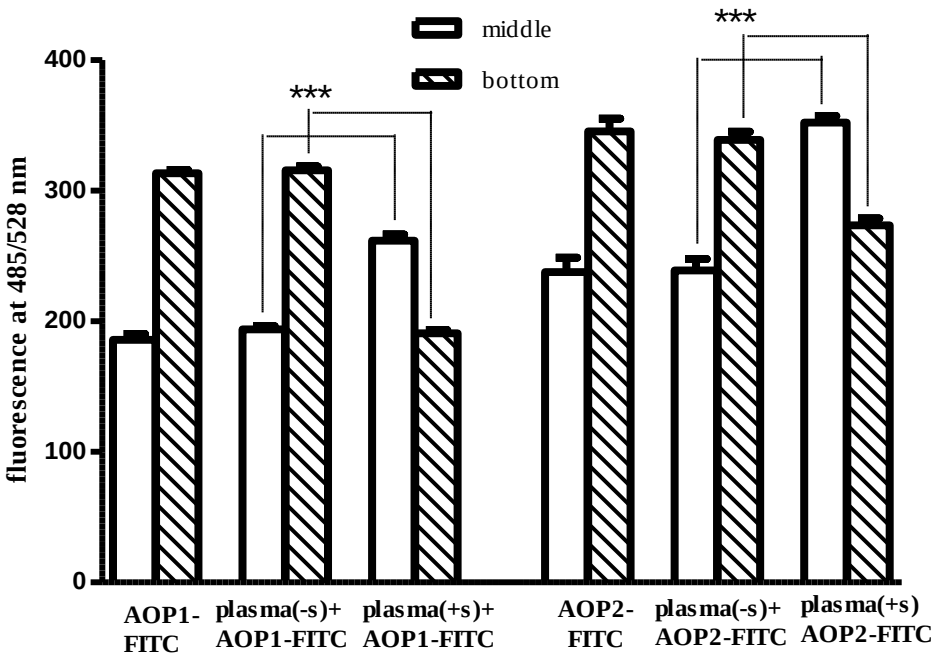


Figure 27. Anti-Gal or ABG binding to AOP1 or AOP2 increases the stability of the latter's complex with albumin. *Plasma diluted with PBS to 1 mg protein/ml was treated with cellobiose and methyl α-D-galactoside (25 mM each), incubated for 3 h at 4^0C and again for 3 h after addition of AOP1-FITC or AOP2–FITC to final concentration of 1 mg/ml. After DGUC as described total fluorescence in middle (400 µl) and bottom (300 µl) layers were measured. ***: P value < 0.0001 for increase in middle layer and decrease in bottom layer of added AOP1-FITC or AOP2-FITC brought about by treatment of plasma with antibody-specific sugars. Mean ± SD of 6 samples.*

(G) Antibody-bound or not, O-glycoproteins are associated with albumin

Anticoagulant mixture used for blood collection raises glucose level in plasma. Any effect of this on triplet structure was reversed by dialysis of cell-free plasma against PBS containing normoglycemic glucose level (4.5 mM). Lipoprotein- and immunoglobulin-free middle layer (Figure 23 and table 2) obtained in DGUC following treatment of plasma with anti-Gal-specific (methyl α-D-galactoside) or ABG-specific (cellobiose) sugar was dialyzed against PBS, diluted in PBST and added to jacalin-coated microplates to capture O-glycoproteins and associated macromolecules. Probing of washed plates with HRP-labeled anti-albumin revealed that O-glycoproteins released from triplets were associated with albumin and migrated to the middle layer (Figure 28). Pre-existing albumin-O-glycoprotein binary complexes that sequester in middle layer from plasma treated with the inert sugar methyl α-D-mannoside, were also considerable though much lower than in same layer obtained after treatment with antibody-specific sugars. This proved that normal plasma contained, in addition to antibody-O-glycoprotein-albumin complexes, free albumin-O-glycoprotein binary complexes ready for antibody binding and is in agreement with our finding that no free anti-Gal or ABG exists in circulation.

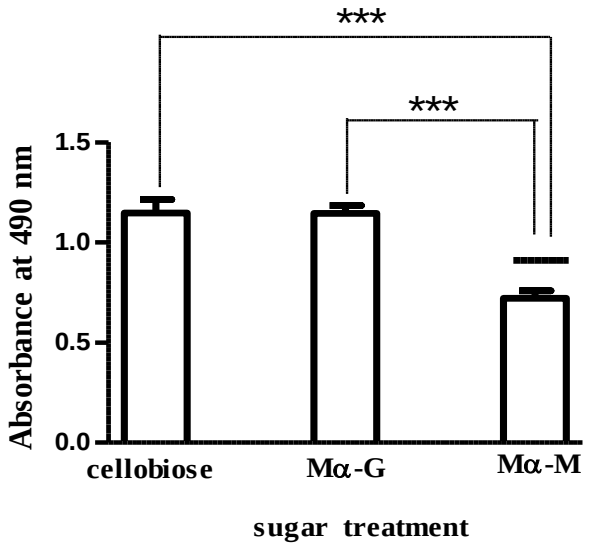


Figure 28. O-Glycoproteins remain associated with albumin following DGUC after dissociation of antibody from triplets. *Plasma middle layers collected after DGUC of cell-free plasma treated with anti-Gal/ABG-specific or non-specific sugar (table 2) were dialyzed against PBS and 8000 X dilutions in PBST added to microplate-coated jacalin (1 µg). After 2 h incubation at 4^0C and washing with PBST albumin associated with O-glycoproteins bound to jacalin was assayed by probing with anti-albumin-HRP (3.75 µg antibody per ml) as decsribed under 'methods'. ***: P value < 0.0003 for increase of albumin-glycoprotein combine in middle layer following removal of antibody from triplet by treatment with cellobiose or methyl α-D-Gal over corresponding values using α-D-Man. Mean ± SD of 6 plasma samples.*

(H) O-Glycoproteins in plasma middle layer after DGUC are AOP1 and AOP2

Middle layer obtained after DGUC of plasma which was dialyzed against 4.5 mM glucose in PBS to make it normoglycemic was resolved by alkaline pH electrophoresis. Of three proteins obtained (Figure 29 inset) the fastest was identified as albumin using anti-albumin antibody and the other two were ligands for anti-Gal and ABG since they inhibited binding of these antibodies to thyroglobulin and TIC respectively (Figure 29). The same electrophoretic bands were obtained for middle layer proteins obtained after cellobiose and methyl α-D-galactoside treatment of plasma eventhough this middle layer consisted substantially of AOP1 and AOP2

released from antibodies. These results established that the slow and fast moving O-glycoproteins in middle layer after DGUC of untreated plasma were also AOP1 and AOP2 mostly, if not fully, albumin-bound (Figure 23).

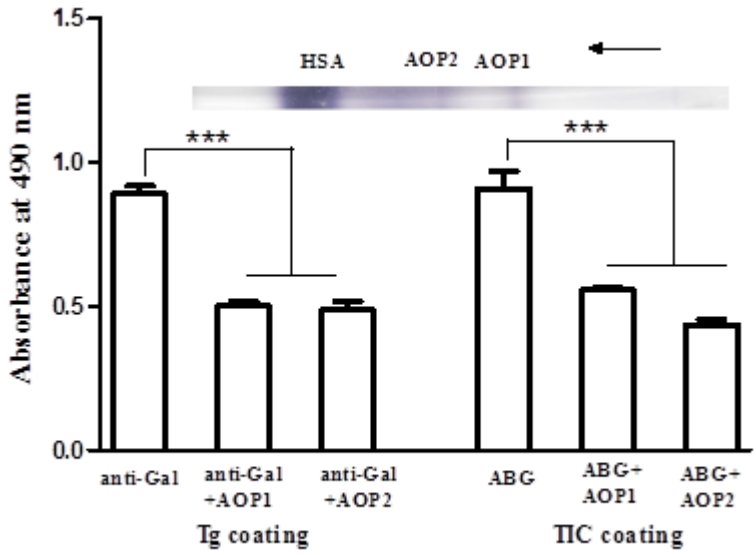


Figure 29. Plasma middle layer O-glycoproteins are identical with AOP1 or AOP2 co-purifying with anti-Gal or ABG and inhibit antibody binding to ligands. *Proteins in middle layer (400 µl) obtained upon DGUC of plasma (Figure 23) when resolved on 6% alkaline polyacrylamide gel electrophoresis contained albumin and two other proteins (inset) identical in mobility with AOP1 and AOP2. The latter separately electroeluted (1 µg) were incubated with anti-Gal or ABG (100 ng) in 200 µl PBST for 2h at 4^0C before addition to antibody ligands (Tg or TIC; 1 µg per well) coated on ELISA microwells. After 2h incubattion at at 4^0C and washing bound antibody was assayed by probing with 200 µl HRP-conjugated anti-immunoglobulin (1.5 µg antibody per ml in PBST). *: initially assumed to be AOP1 or AOP2 and confirmed by results. ***: P value <0.0003 for inhibition of anti-Gal by AOP1 or AOP2; **: P value <0.004 for inhibition of ABG by AOP1 or AOP2. Mean ± SD of 3 samples.*

(I) Albumin binding protects triplet formation from getting blocked by LDL

AOP1 or AOP2 produces a large increase on fluorescence of anti-Gal-FITC and ABG-FITC which was significantly reduced when albumin-bound AOP1 or AOP2 was used instead, though albumin per se was inert towards either antibody (Figure 30). More importantly while pure AOP1 or AOP2 is blocked fully by LDL from

binding to anti-Gal, binding of albumin-associated AOP1 and AOP2 from middle layer of plasma after DGUC is unaffected by LDL (Figure 30) indicating another physiological advantage accrued by the association of AOP1 and AOP2 with albumin. Thus though albumin treatment reduces conformational shift produced by AOP1 or AOP2 on anti-Gal and ABG, it protects triplet formation from getting blocked by LDL.

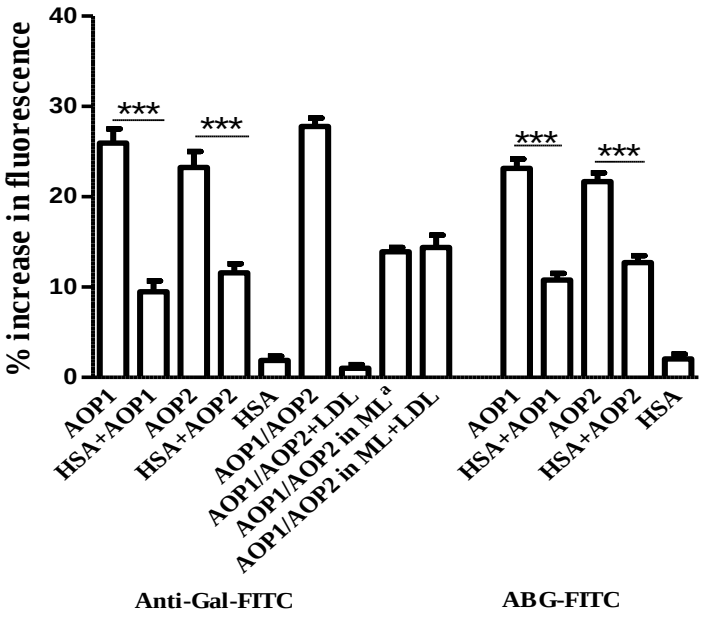


Figure 30. Albumin binding protects triplet from getting blocked by LDL*. AOP1/AOP2 mixture (electroeluted together) and purified samples of AOP1 and AOP2 (2 µg of each in PBS) were incubated separately with 4 µg albumin (HSA) in 50 µl PBS for 2 h. This mixture or albumin/AOP1/AOP2 mixture from middle layer after DGUC of normal plasma with O-glycoprotein content equal to 2 µg AOP1/AOP2 mixture (determined by jacalin binding) in 50 µl was mixed with 25 µl PBS or PBS containing 4 µg LDL and incubated for 2 h at 4^0C. Samples were mixed with 1 µg purified anti-Gal-FITC in 25 µl PBS and incubation continued overnight. Mixtures were diluted to 300 µl and fluorescence measured as described. Alternatively effect of AOP1 or AOP2 incubated with or without albumin on fluorescence of 1 µg purified ABG–FITC was also measured under same conditions. a: middle layer albumin-O-glycoprotein mixture. ***: P value <0.0005 for the reduction brought about by presence of albumin in fluorescence enhancement caused in anti-Gal-FITC and ABG-FITC by AOP1/AOP2 or mixture. Mean ± SD of 6 antibody samples.*

(J) Binding of AOP1 /AOP2 reduces the avidity of anti-albumin for albumin

The anti-albumin-HRP response of 40 ng microplate-coated purified albumin is shown in Figure 31. When same amount of albumin was bound to AOP1/AOP2 mixture (120 ng) and the latter captured on microplate-coated jacalin the response of anti-albumin-HRP was significantly reduced. Engagement of all molecules of 40 ng albumin by 120 ng AOP1/AOP2 mixture used as well as complete capture of the resulting mixture on plate-coated jacalin (1 µg per well) was ensured.

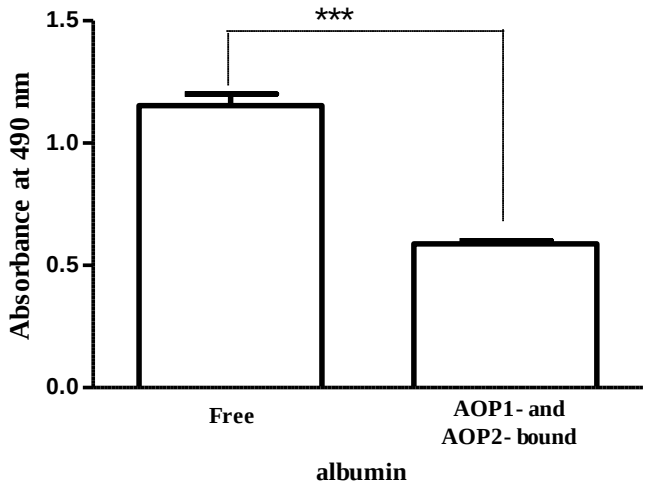


Figure 31. Attachment of AOP1 and AOP2 to albumin reduces its binding to anti-albumin. *Pure albumin (40 ng) was directly coated to ELISA microwells or bound to 120 ng AOP1/AOP2 mixture and captured through the latter on microwell-coated jacalin (1 µg per well). Complete capture of albumin in the latter case was ensured. Anti-albumin-HRP binding to either type of wells was assayed by probing with anti albumin-HRP as above (Figure 28). *** : P value: 0.0003. Mean ± SD of 3 samples.* ***Inset:*** *extent of attenuation of anti-albumin response derived from values in figure.*

(K) Nearly 36% of all plasma albumin is bound to either AOP1 or AOP2

Plasma (1 ml) adjusted to density to 1.24 g/cc on addition of solid KBr and volume increased to 1.1 ml was subjected to DGUC. Albumin-free and lipoprotein-rich top 100 µl was discarded and bottom 1 ml was dialyzed against PBS. To determine O-

glycoprotein-bound albumin the latter was captured on microplate-coated jacalin (1µg per well) from 200 µl of 40,000 X dilution of the dialyzed sample above and assayed using anti-albumin-HRP conjugate as probe. Since this assay response (A^1, table 3) is a quenched response due to binding to AOP1/AOP2, and through the latter to jacalin, actual amount of O-glycoprotein-bound albumin (A) was derived using attenuation factor AF1(1.67) described in table 3. Total albumin in plasma was determined by coating 80,000 X dilution in PBS (200 µl per well) of the 1 ml albumin layer above (B) and probing as above. This response included both the unaffected response of free albumin and the attenuated response of O-glycoprotein-bound albumin. The latter part (A^2) could be calculated from actual amount of O-glycoprotein-bound albumin (A) using attenuation factor 2 (AF2; 1.51) (table 3). Free albumin alone therefore was B-A^2 and total albumin was B-A^2+A. Fraction of O-glycoprotein-bound albumin (A) in total albumin of normal plasma samples was 35.7 ± 2.7% (n=6). This result is in agreement with our observation that close to 50% of plasma albumin failed to bind to blue Sephadex in presence of 250 mM NaCl. Assays utilizing dye binding to peptide sequences have reported plasma albumin levels of 35-52 mg per ml (Dati et al., 1996). Since dyes bind to proteins in proportion to the hydrophobicity of the latter roughly 36% of albumin molecules in serum would have been underestimated in these assays, being O-glycoprotein-bound (table 3), so that actual albumin values would be in a higher range. This is in agreement with plasma albumin concentration arrived by present protocol (55.9±5.43 mg per ml) though an entirely different property of albumin was utilized to arrive at this value.

Table 3. Derivation of plasma albumin fraction bound to O-glycosylated proteins

Albumin sample in plasma	Anti-albumin response of albumin in unit plasma volume
O-Glycosylated protein-bound albumin captured on microplate-coated jacalin (attenuated by jacalin binding through O-glycosylated proteins)	A^1
Attenuation factor for response of albumin bound to plate coated jacalin through O-glycosylated proteins	$AF1^a$
Corrected response of O-glycosylated protein-bound albumin (A^1 multiplied by AF1)	A
Total albumin captured on plate from plasma dilution (free + O-glycosylated protein-bound in attenuated state)	B
Attenuation factor for response of albumin bound to O-glycosylated proteins only	$AF2^b$
O-Glycosylated protein-bound component in B (A divided by AF2)	A^2
Free albumin	$B-A^2$
Total albumin	$B-A^2+A$
O-Glycosylated protein-bound albumin as percentage of total	$(A \times 100) / (B-A^2+A)$
Experimental values	
Albumin in undiluted plasma* = $(B-A^2+A) \times 8 / 7$	**55.9 ±5.43 mg per ml**
Fraction of O-glycosylated protein-bound albumin as % of total (mean± SD, n=6)	**35.7±2.68%**

DISCUSSION

Data above lead to the conclusion that AOP1 and AOP2 are designed to bind simultaneously to albumin and anti-Gal/ABG antibody. During their interaction with these antibodies, unlike pure AOP1 or AOP2 their complexes with albumin leave unoccupied binding sites on antibodies enabling the triplet formed to bind to smaller ligands, free or cell bound. Albumin being a protein abundant in plasma may thus be helping to incease the plasma half life of the above antibodies by preventing the O-glycosylated proteins from occupying both binding sites of their immunoglobulin monomer units and getting easily removed from circulation due to their Fc component having got fully activated. An equally significant contribution of albumin towards triplet formation is complete protection of AOP1 and AOP2 from getting inaccessible to antibodies by LDL occupation of their O-glycosylated regions. A unique property of albumin is its high solubility (> 50 mg per ml) in plasma competing with scores of other proteins and ions that are also in need of water molecules for solubility. Its association with AOP1 and AOP2 could be partially the reason for its high solubility despite possessing a distinct hydrophobic domain and no glycosylation. Our results here show that nearly 36% of albumin is bound to either of the O-glycosylated proteins. AOP1 or AOP2 and albumin use their hydrophobic domains for binding to each other and the hydrophilic O-glycans in AOP1/AOP2-albumin complex render the resulting complex highly soluble in plasma.

PART-III

Role of albumin-associated antibody-binding O-glycosylated proteins in amyloid β binding

Introduction

Molecular pathology of Alzheimer's disease has been attributed largely to formation of amyloid plaques by aggregation of amyloid β (Aβ) peptides in brain intercellular regions and consequent loss of neuronal functions especially in the hippocampus (Musiek & Holtzman, 2015). At lower levels Aβ is physiologically important in synaptic activity, learning and memory and has the ability to act as antioxidants (Plant et al., 2003). They also play a role in maintaining cellular homeostasis by helping to maintain blood-brain barrier integrity (Atwood et al., 2003). LDL receptor-related protein (LRP) mediates Aβ transport from brain to blood (Liu et al., 2004). LRP acts mainly as an endocytotic transporter of ligands including Aβ across the blood-brain barrier. The extracellular α chain (550 kDa) of LRP binds Aβ. Defects in expression or function of molecules such as LRP that bind and sequester Aβ from brain to CSF and circulation have been shown to precipitate amyloidogenesis and cognitive dysfunction (Liu et al., 2004). There are reports that over 90% of Aβ in plasma are albumin-bound (Biere et al., 1996) though no Aβ binding domain had been demonstrated in albumin. Since the major pathway for clearance of Aβ from brain extracellular fluids involves its binding to LRP on neuronal surface we investigated whether AOP1 and AOP2 that share the STPS and O-glycan abundance of LRP is also a receptor for Aβ.

RESULTS

(A) Amyloid β bind to AOP1 and AOP2 and not albumin

Treatment with AOP1 or AOP2 alone, in presence of purified albumin or in presence of albumin along with anti-Gal or ABG produced equal and significant increase of nearly 45% in fluorescence of F-Aβ (Figure 32) whereas purified albumin or antibody did not produce any change in fluorescence of F-Aβ.

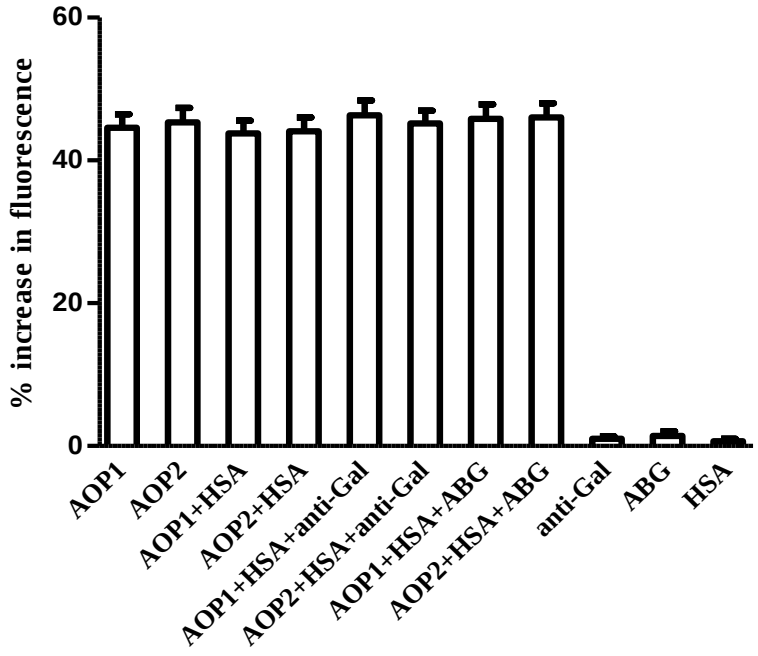


Figure 32. AOP1and AOP2 interact with amyloid β; albumin, anti-Gal or ABG does not. *AOP1 and AOP2 were obtained by electroelution after separation of APAG by alkaline pH electrophoresis in 6% polyacrylamide gel. AOP1 or AOP2 (0.5 µg in 75 µl PBS) alone or pre-incubated with 2.35 µg albumin (HSA) for 3 h or AOP1/AOP2-albumin mixture incubated overnight with 3.75 µg anti-Gal or ABG to form triplet was further incubated with 50 ng F-Aβ in 25 µl PBS for 3 h, mixture diluted to 300 µl and fluorescence measured at 528 nm after excitation at 485 nm. Anti-Gal, ABG (3.75 µg each) or albumin (2.35 µg) alone was also used for incubation with F-Aβ. P value < 0.0001 for response of AOP1/AOP2-containing samples vs antibody or albumin. Mean ± SD of proteins from 6 consecutive plasma samples.*

(B) AOP1 and AOP2 in free, albumin-bound or triplet form react equally with Aβ

After adjusting plasma (1 ml) to density 1.24 g per cc DGUC result in segregation of albumin and its immunoglobulin-free complexes to a middle layer of 400 µl, immunoglobulins and their conjugates to bottom 300 µl and lipoproteins to top 400 µl (Figure 33 inset). As shown in earlier results middle layer contains albumin-bound AOP1 and AOP2 that are not bound to anti-Gal or ABG. Albumin-bound form of AOP1 and AOP2 obtained in this middle layer was compared with the mixture of AOP1 and AOP2 eluted together after electrophoretic separation of APAG and with AOP1 and AOP2 in triplet form for their F-Aβ reactivity, using equal O-glycan content of all samples as determined by jacalin-HRP binding to their microplate-coated forms. Results (Figure 33) show that AOP1 and AOP2 in free, albumin-bound or triplet form with the same O-glycan content produced equal response in F-Aβ. Thyroglobulin, a strong anti-Gal ligand without O-glycans was non-reactive.

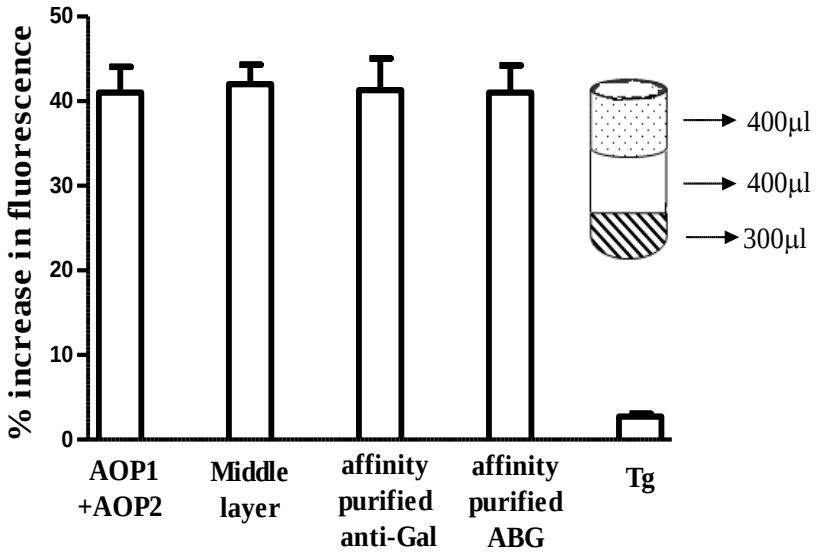


Figure 33. Free, albumin-bound and triplet AOP1 and AOP2 react equally with Aβ. *Albumin-AOP1/AOP2 complex from normal plasma was obtained in the middle 400 µl following DGUC of plasma samples. Following electrophoretic separation in 6% alkaline polyacrylamide gel a mixture of AOP1 and AOP2 in the same ratio as in plasma was obtained by electroelution of a mixture of gels containing corresponding bands. This AOP1/AOP2 mixture (0.5 µg protein) or total middle layer proteins, APAG or APABG (all of equal O-glycan content as AOP1/AOP2 mixture as judged by HRP-labeled jacalin binding to their microplate-coated versions) was incubated with 50 ng F-Aβ in 75 µl PBS for 3 h, diluted to 300 µl in PBS and fluorescence measured as for Figure 32. P value <0.0005 for AOP1/AOP2-containing samples vs Tg. Mean ± SD of proteins from 3 consecutive plasma samples.*

(C) Aβ bind and remain attached to AOP1 and AOP2

Upon DGUC of a mixture of albumin and other proteins in PBS, albumin along with its complexes with less dense proteins were more in the middle 500 µl volume below the top 200 µl while bottom 400 µl retained denser proteins. Result in table 4 shows that Aβ alone when subjected to DGUC gravitated more to the bottom layer than to middle layer. Presence of albumin along with Aβ makes no change in the above distribution of Aβ. However in presence of AOP1/AOP2 mixture along with albumin, Aβ level was significantly elevated in the albumin rich middle layer and

correspondingly reduced in the bottom layer. Since AOP1 and AOP2 bind albumin the result shows that it is the AOP1/ AOP2-bound Aβ that got attached to albumin.

Table 4. Binding of AOP1/AOP2 is responsible for fluorescence increase in F-Aβ

Protein in PBS medium	Fluorescence (increase or decrease as percentage change from value with F-Aβ only)		
	Top 200 µl	Middle 500 µl	Bottom 400 µl
F-Aβ	211(0%)	452(0%)	522(0%)
F-Aβ+HSA	200 (↓ 4.24%)	440 (↓ 4.24%)	537 (↑2.87%)
F-Aβ+HSA+AOP1/AOP2 mixture	220 (↑ 4.26%)	617(↑ 36.4%)	413(↓23%)

Middle 400 µl collected after DGUC of plasma at 535000 g was used as HSA+AOP1/AOP2 mixture. This sample or electroluted HSA alone (each 1 mg) was treated with 0.5 µg F-Aβ in 1 ml PBS for 3 h at 4^0C. Incubated mixtures or F-Aβ alone in PBS were further subjected to DGUC at 535000 g and top 200 µl, middle 500 µl, and bottom 400 µl. Top layer diluted to 300 µl with PBS and 300 µl from bottom layers were used for fluorescence measurement at 485/528 nm. Percentage change in fluorescence was calculated on considering the fluorescence of F-Aβ alone.

(D) Aβ bound to plasma triplets remain in the AOP1/AOP2-albumin complex following dissociation by sugar

Plasma was incubated with F-Aβ and further treated with anti-Gal-specific sugar (methyl α-D-Gal) or control sugar (methyl α-D-Man) and subjected to DGUC. Middle layer (400 µl) below the top 400 µl and bottom 300 µl were collected and

assayed for Aβ fluorescence. While distribution of fluorescence between layers was only marginally affected by non-specific sugar, specific sugar upset the above distribution and elevated total fluorescence in middle layer significantly (Figure 34). It has been shown in earlier results that AOP1 and AOP2 migrate along with albumin from bottom layer to middle layer upon DGUC following sugar-mediated dissociation of triplets in plasma (table 2). Since Aβ does not bind albumin the above observation again establishes that attachment of Aβ is to AOP1 and AOP2.

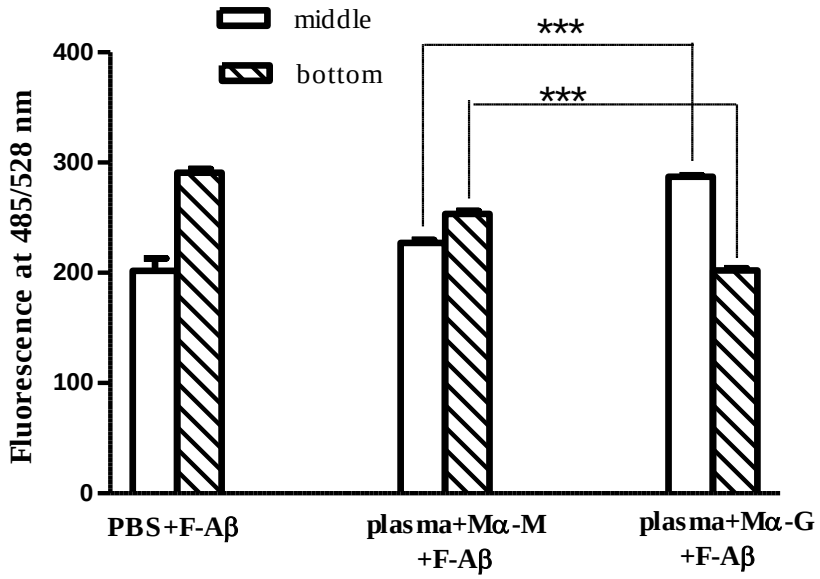


Figure 34. Aβ binds to AOP1/AOP2-albumin complex and not to antibodies of plasma triplets. *Plasma (950 µl) was incubated at 4^0C with anti-Gal-specific sugar methyl α-D-galactoside (Mα-G) or nonspecific sugar methyl α-D-mannoside (Mα-M) (25 mM each) overnight and then with 0.5µg F-Aβ for 3 h in 1 ml ultracentrifuge tubes. After adjusting density to 1.24 g/cc by addition of KBr followed by DGUC at 535000g middle 400 µl and bottom 300 µl were collected and total fluorescence in either layer measured as above. F-Aβ alone in PBS served as control. ***: P value < 0.0001 for increase of F-Aβ in middle layer and decrease of F-Aβ in bottom layer brought about by specific sugar. Mean ± SD of 6 plsma samples.*

(E) Aβ binds at the O-glycosylated region of AOP1 and AOP2

Jacalin recognizes core 1 type disaccharide group that is plenty in O-glycans of AOP1 and AOP2 (Figure 15) and LDL attaches to O-glycan rich region of O-glycosylated proteins due to ionic interaction between basic aminoacid residues in apoB chain of LDL and negatively charged terminal sialic acid moieties in O-glycans (Becker et al., 2004). AOP1 and AOP2 binding to Aβ was blocked completely by either jacalin or LDL (Figure 35) which confirms that Aβ binds to AOP1 and AOP2 at their O-glycan-rich region.

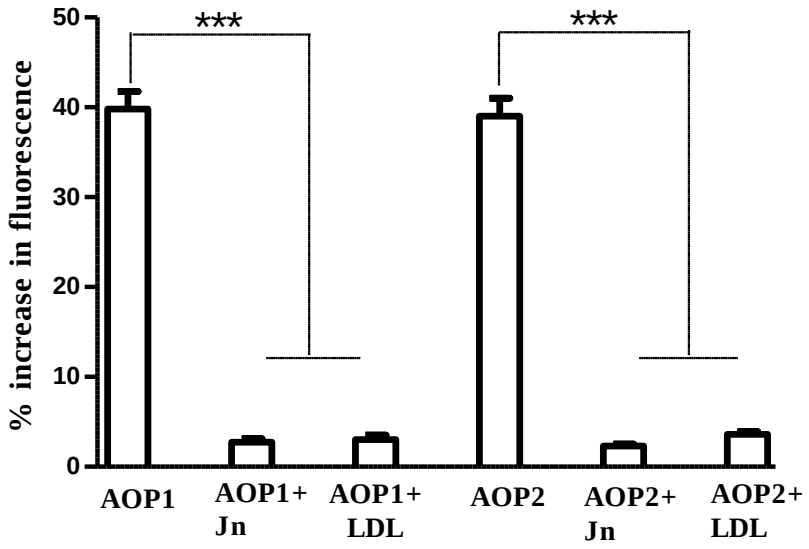


Figure 35. Aβ binds at the O-glycosylated region of AOP1 & AOP2. *AOP1 or AOP2 (0.5 µg) (prepared by electroelution after alkaline electrophoresis of APAG) alone or with 2 µg jacalin (Jn) or with 2 µg LDL was incubated overnight in 75 µl PBS at 4^0C. F-Aβ (50 ng in 25 µl PBS) was added and incubation continued for 3 h. After dilution to 300 µl with PBS fluorescence was measured as above. ***: P value < 0.0001 for decrease brought about by bound jacalin or LDL in AOP1/ AOP2-induced fluorescence in F-Aβ. Mean ± SD of 3 AOP1/AOP2 samples.*

(F) Aβ binding to AOP1 or AOP2 in albumin-associated or triplet form is not inhibited by LDL, unlike that to free AOP1 or AOP2

F-Aβ binding to a mixture of AOP1 and AOP2 electroeluted from APAG was inhibited by LDL. But F-Aβ binding to the same amount of AOP1 and AOP2 in middle layer obtained after DGUC of normal plasma and consisting mainly of albumin with and without bound AOP1or AOP2, or in triplet form as APAG was not affected by LDL (Figure 36) indicating that physiological forms of AOP1 and AOP2 that are always albumin-bound are unaffected by LDL in their Aβ-binding function. A possible reason is that presence of albumin bound to AOP1 or AOP2 precludes approach of the extraordinary large LDL molecule to their O-glycosylated domains.

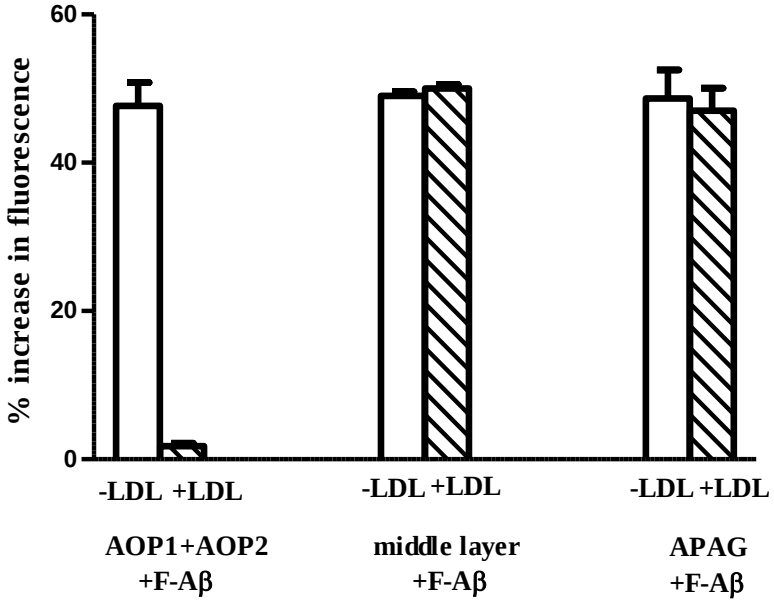


Figure 36. Unlike free AOP1 or AOP2 their albumin complex or triplet with anti-Gal/ABG is not inhibited by LDL in Aβ binding. *AOP1/AOP2 mixture, total middle layer proteins or APAG (all of same quantity and origin as in Figure 33) was pre-incubated with or without 2 µg LDL in 75 µl PBS overnight at 4^0C and then with F-Aβ (50 ng in 25 µl PBS) for 3 h. After dilution to 300 µl with PBS fluorescence was measured as above. Mean ± SD of 3 samples.*

(G) STPS confirmed as Aβ ligand in AOP1 and AOP2

De-O-glycosylation using alkaline sodium borohydride removes the O-glycans and exposes the underlying STPS sequence in O-glycoproteins (Geetha et al., 2014). Removal of O-glycans of AOP1/AOP2 by this procedure was confirmed by complete absence of jacalin binding to the de-O-glycosylated versions DAOP1 and DAOP2 (Figure 37 inset). De-O-glycosylation increased Aβ binding to AOP1/AOP2 and reduced the LDL-mediated inhibition of this binding (Figure 37). LDL binds to O-glycan-rich proteins due to negative charges on sialic acid moieties on the latter so that on removal of O-glycans the inhitory effect of LDL was also reduced. Result confirms that STPS rather than O-glycans per se mediated Aβ binding to AOP1 or AOP2.

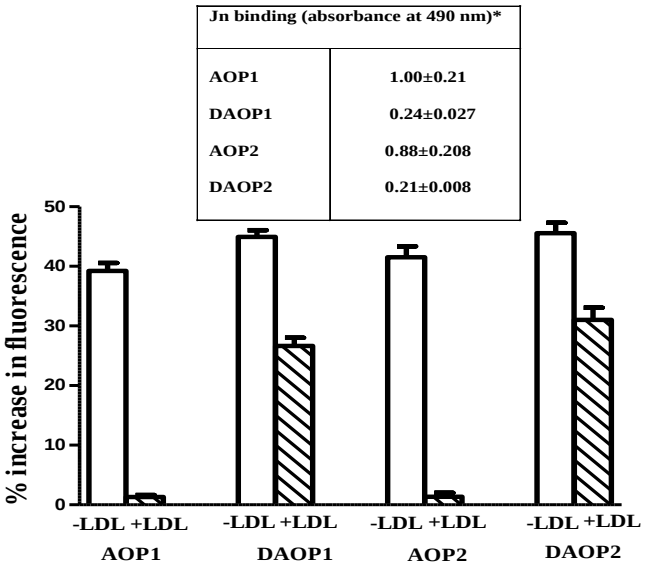


Figure 37. Serine- and threonine-rich peptide sequences of AOP1 and AOP2 mediate Aβ binding. *AOP1 and AOP2 (prepared by electroelution after alkaline electrophoresis of APAG) were de-O glycosylated to obtain de-O-glycosylated AOP1 (DAOP1) and de-O-glycosylated AOP2 (DAOP2). AOP1, AOP2, DAOP1 and DAOP2 (0.5µg each) were pre-incubated with or without 2 µg LDL in 75 µl PBS overnight at 4^0C and then with F-Aβ (50 ng in 25 µl PBS) for 3 h. After dilution to 300 µl fluorescence was measured as above.* **** Inset****: Protein samples coated on microplates were treated with jacalin-HRP (75 ng per ml) and bound HRP assayed as described under 'methods'. Mean ± SD of 6 AOP1/AOP2 samples.*

(H) AOP1 and AOP2 are immunologically cross reactive with LRP

Microplate-coated AOP1 and AOP2, but not albumin was recognized by anti-LRP (Figure 38). As negative controls, goat anti-apoB instead of anti-LRP as well as direct binding of HRP-conjugated anti-goat IgG to microplate-coated AOP1 were used.

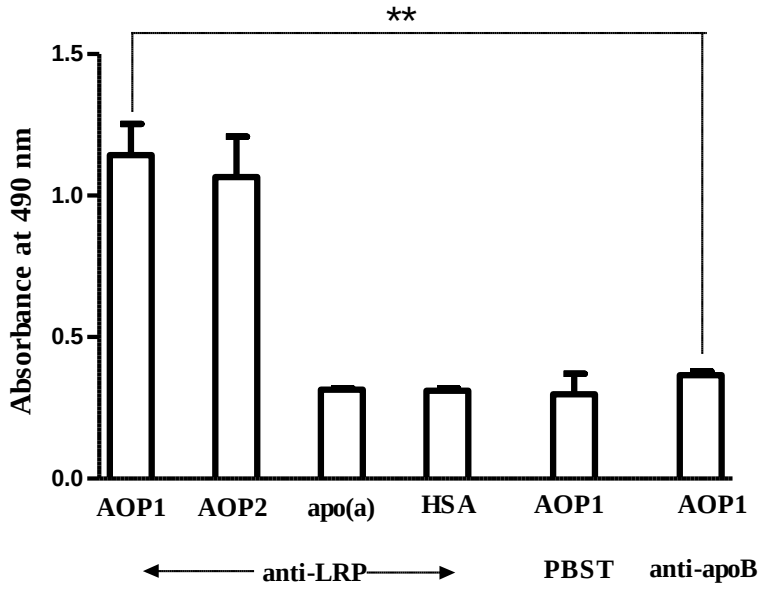


Figure 38. AOP1 and AOP2 are immunologically cross-reactive with LRP. *AOP1 and AOP2 (obtained by electro elution after electrophoresis of APAG) was coated on NUNC microplates (500 ng each in PBS), treated with 200 µl goat anti-LRP-1 in PBS-T (1µg/ml) for 2h at 4^0C, washed and then probed with 200 µl HRP-conjugated anti-goat IgG (0.3 µg /ml in PBS-T). Controls included apo(a) and albumin (HSA) coatings (500 ng per well), direct binding of HRP-conjugated anti-goat IgG to AOP1/AOP2 and goat anti-apoB antibody instead of anti-LRP-1. **: P value 0.009 for response of anti-LRP1vs anti-apoB towards AOP1. Mean ± SD of 4 AOP1/AOP2 samples.*

(I) Displacing AOP1/AOP2–albumin from bottom layer reduces LRP-like molecules

After DGUC of plasma pre-treated with anti-Gal- and ABG- specific sugars (methyl α-D-Gal and cellobiose, 25 mM each) bottom layer was collected as described for Figure 33 and was tested for presence of molecules cross-reactive with LRP. Molecules cross reactive with LRP were assayed by capturing on microplate-coated anti-LRP1 and probing with jacalin-HRP since known biomolecules cross-reactive with LRP are O-glycosylated. Results (Figure 39) showed that close to half of LRP-related proteins in bottom layer had escaped on treatment with specific sugars. From earlier results it is clear that proteins released from bottom layer by sugar treatment are mostly albumin and AOP1/AOP2. So the results confirm the immunological cross-reactivity of AOP1/AOP2 with LRP.

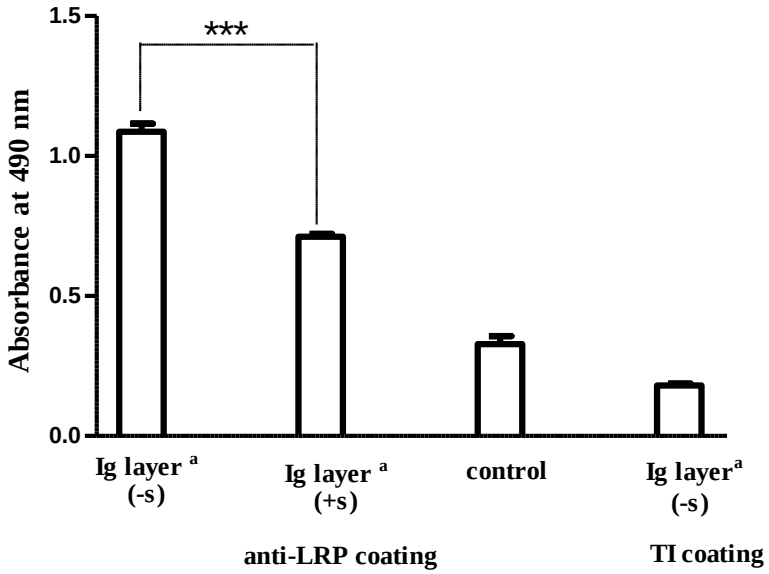


Figure 39. Displacement of AOP1/AOP2-albumin reduces LRP-like molecules in antibody-containing layer of plasma. *Bottom 300 µl containing antibodies was collected following DGUC of plasma treated with or without a mixture of 25 mM of cellobiose and 25 mM methyl α-D-galactoside. Its 50 X dilution in PBS-T was added to anti-LRP1 (400 ng in 200 µl PBS) coated on microplates. After 2 h incubation at 4^0C well-bound O-glycosylated proteins were assayed using HRP-conjugated jacalin (15 ng jacalin in 200 µl conjugate in PBST). As controls direct binding of jacalin conjugate to coated anti-LRP1 and binding of untreated bottom layer dilution to plate-coated non-specific protein (trypsin inhibitor [TI]; 400 ng per well) were checked. a: Immunoglobulin-containing layer after DGUC of plasma. ***: P value 0.0002 for sugar treated versus non-sugar treated antibody layer. Mean ± SD of 3 samples.*

(J) Proteins other than native BSA in commercial BSA react with Aβ

Alkaline pH electrophoresis of fatty acid- free BSA (Sigma) showed several high molecular weight bands (Figure 40 inset) other than the most prominent and fastest moving band that was identified as native BSA from its size and antigenicity. The non-BSA proteins electroeluted together interacted with Aβ increasing fluorescence of F-Aβ to about 28% whereas native BSA did not interact with Aβ (Figure 40) suggesting that either aggregated albumin or albumin contamination with

AOP1/AOP2 in commercial BSA accounted for its Aβ binding reported in earlier publications (Biere et al., 1996; Yamamoto et al., 2014).

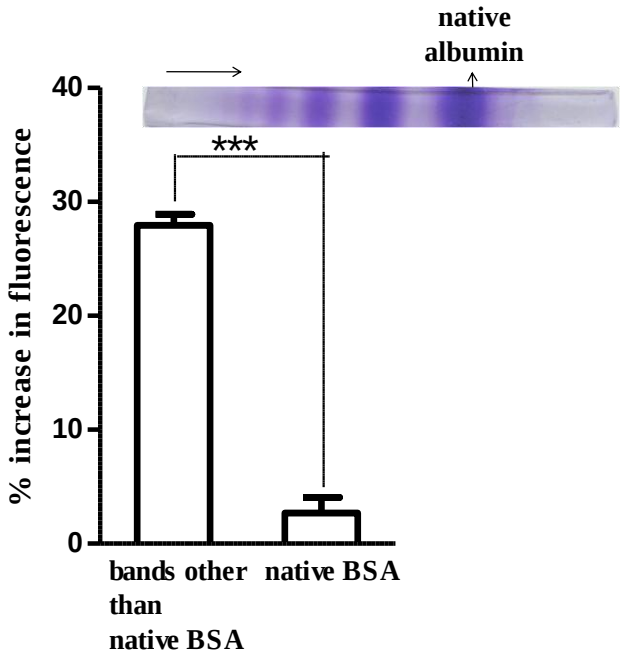


Figure 40. Proteins other than native BSA in commercial fatty acid-free BSA react with Aβ. *BSA (commercial, fatty acid-free) subjected to 6% alkaline polyacrylamide gel electrophoresis* ***(inset)****. Fastest moving band (native albumin) as well as all other bands pooled was separately electroeluted. Proteins in either group (1 µg) was incubated with 50 ng F-Aβ in 100 µl PBS for 5 h at 4^0C, mixture diluted to 300 µl and fluorescence measured at 485/528 nm. ***: P value <0.0001 for response of bands other than native BSA versus native BSA with F-Aβ. Mean ± SD of 3 samples.*

(K) Commercial BSA contains O-glycosylated proteins

In microplate-coated form native BSA separated from commercial BSA and native HSA was non-responsive to jacalin whereas total commercial BSA samples contained jacalin-binding proteins (Figure 41). Results indicate the possibility that the reported binding of Aβ to BSA (Biere et al., 1996; Yamamoto et al., 2014) was mediated by AOP1 and AOP2 contaminating the commercial BSA sample used in that report.

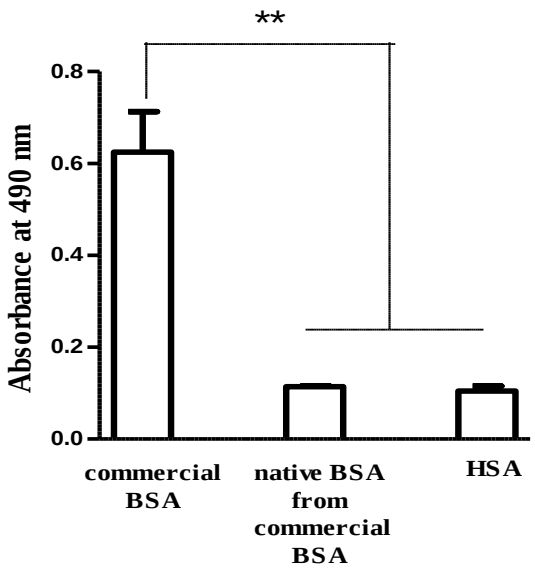
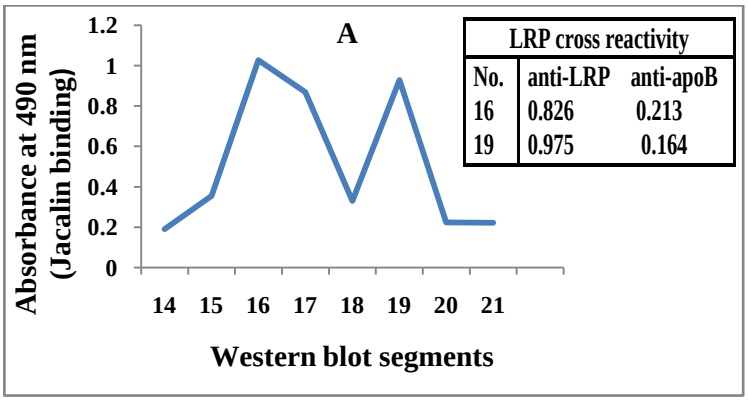


Figure 41.Commercial BSA contains O-glycosylated co-purified proteins whereas albumin is not O-glycosylated. *Commercial fatty acid-free BSA, lower band (native albumin) electro eluted after electrophoresis (Figure 40) and purified HSA (prepared similarly from blue Sephadex-binding human plasma albumin eluted by 250 mM NaCl were coated on microplates (1.25 µg per well) and probed with jacalin-HRP (75 ng jacalin per ml) in PBST. **: P value 0.004 for difference in jacalin binding to commercial BSA versus native BSA or HSA. Mean ± SD of 3 samples.*

(L) O-Glycosylated proteins in commercial BSA are cross-reactive with LRP and of same size as AOP1 or AOP2

Results in Figure 42 shows that two jacalin-binding glycoproteins of same mobility in SDS-polyacrylamide gel electrophoresis as AOP1 or AOP2 in human serum albumin prepared by affinity chromatography on blue Sephadex are present in commercial fatty acid-free BSA. Similar to AOP1 or AOP2 the O-glycosylated proteins in commercial BSA were cross-reactive with LRP (Figure 42 A&B insets).



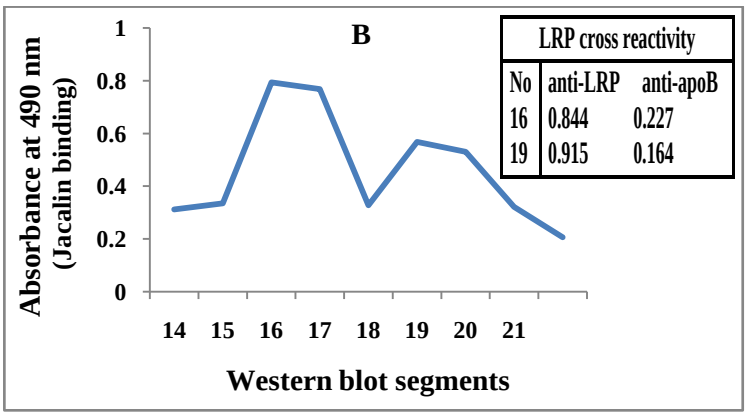


Figure 42. O-Glycosylated proteins in commercial BSA (fatty acid-free) are cross reactive with LRP and of same size as AOP1 and AOP2. *Western blotted strips of commercial BSA (A) and albumin from human plasma bound to blue Sephadex and eluted with 1M NaCl (B) were cut into fragments of length 3 mm. Fragments were blocked by treating with 0.5% Tween 20 PBS for 3 h at 37^0C, washed and treated with 200 µl jacalin-HRP (375 ng jacalin per ml) in PBST for 3 h at 4^0C. After washing with PBST thrice fragments were added to OPD solution (200 µl) in microplates. After 15 min strips were removed, 12.5% H_2SO_4 (50 µl) added to wells and color read at 490 nm.* ***Inset (A and B)****: Strips 16 and 19, which gave maximum response with jacalin-HRP in Western blot of both human and bovine albumin samples used, were cut from parallel strips and checked for anti-LRP1 response by treating with 10 µg per ml anti-LRP-1 overnight at 4^0C followed by incubation with HRP conjugate of anti-goat IgG (1.5 µg /ml) for 3 h, washing and assay of bound HRP as given above for jacain-binding. As negative control for anti-LRP1, fragments were treated for 3 h with 10 µg per ml anti apoB.*

(M) AOP1 and AOP2 are better receptors for amyloid β than α-synuclein or β-cyclodextrin

β-Cyclodextrin and α-synuclein are amphipathic molecules that bind amyloid β and can prevent its aggregation (Camilleri et al., 1994; Jensen et al., 1997). AOP1 or AOP2 alone produced an increase in fluorescence of 46% over that of untreated F-Aβ whereas β-cyclodextrin produced an increase of only 9.4 % (Figure 43) suggesting that AOP1 and AOP2 are more effective ligands for Aβ compared to β-cyclodextrin. Further AOP1/AOP2 act synergistically with β-cyclodextrin since together they produced a fluorescence increase of about 77% (Figure 43). In contrast α-synuclein brings about an increase of only 22 % in fluorescence of F-Aβ and does not act in synergy with AOP1 and AOP2 since together they produce only an additive effect of about 72% increase in fluorescence (Figure 44).

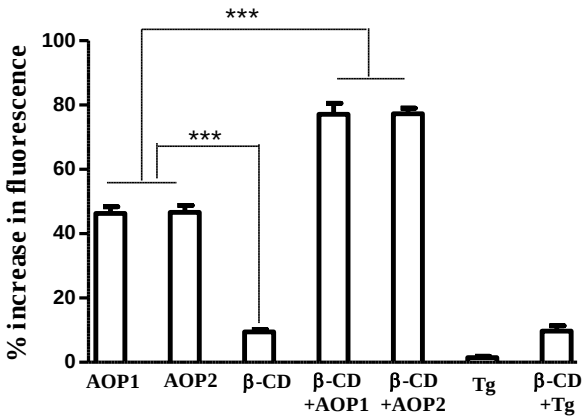


Figure 43. AOP1and AOP2 are better ligands for Aβ compared to β-cyclodextrin. *AOP1 and AOP2 electroeluted from APAG (0.5 µg in 50 µl PBS) or β-cyclodextrin (2 µg in 50 µl PBS) was incubated separately with F-Aβ (50 ng in 25 µl PBS) for 6 h at 4ºC. Alternatively 2 µg β-cyclodextrin and 50 ng Aβ in 50 µl PBS was preincubated for 6 h and further incubated overnight with 0.5 µg AOP1/AOP2 added in 25 µl PBS. As control 0.5 µg Tg was used instead of AOP1/AOP2. Samples were diluted to 300 µl with PBS and fluorescence was measured as described. ***: P value <0.0001 for response to AOP1/AOP2 versus that to β-cyclodextrin and for response to AOP1/AOP2 versus that to AOP1/AOP2 + β-cyclodextrin. Mean ± SD of 6 AOP1/AOP2 samples.*

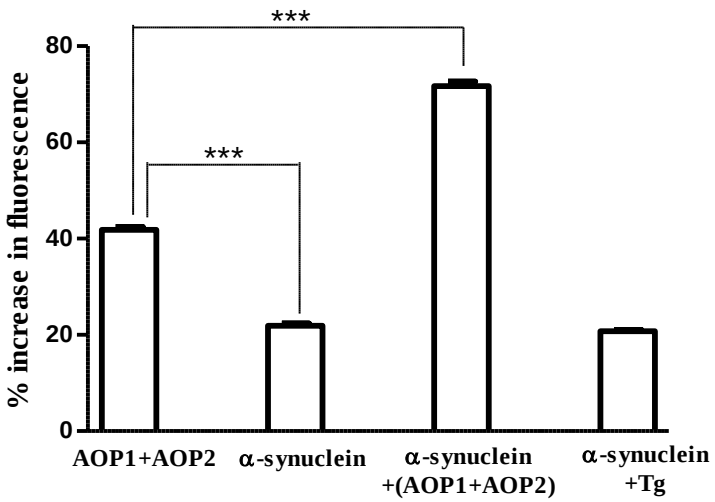


Figure 44. AOP1/AOP2 binds to Aβ better than α-synuclein. *AOP1 and AOP2 electroeluted together from APAG and α-synuclien (both 100 ng) in 50 µl PBS was incubated separately for 6 h at 4ºC with 50 ng F- Aβ in 25 µl PBS. Alternatively, α-synuclein (100 ng) was preincubated with F-Aβ (50 ng) in 50 µl PBS for 6 h and further incubated overnight with AOP1+AOP2 mixture (100 ng added in 25 µl PBS). Samples were diluted to 300 µl with PBS and fluorescence was measured as described. ***: P value <0.0001 for response of both AOP1+AOP2 mixture versus α-synuclein and AOP1+AOP2 mixture versus AOP-α-synuclein mixture. Mean ± SD of 6 samples.*

DISCUSSION

AOP1 and AOP2 in free form, in complex with albumin or as a bridge between albumin and anti-Gal/ABG in triplet, bind strongly with Aβ as evidenced by the remarkable rise in fluorescence of F-Aβ. There are reports in which binding of Aβ in plasma had been attributed to albumin and claimed that albumin bound 95% of serum Aβ (Yamamoto et al., 2014) and reversed Aβ fibril formation in animal brain (Stanyon & Viles, 2012). But those reports were based on experiments that used commercial fatty acid-free bovine and human albumin samples (Biere et al., 1996)

which, as we have shown here, contain O-glycosylated protein equalents of AOP1 and AOP2. We could confirm that AOP1 and AOP2 are chiefly the molecules that actually recognize Aβ since co-purified AOP1 and AOP2 in albumin samples were responsible for these results and electrophoretically purified and O-glycoprotein-free native albumin from the above albumin samples and the electroeluted albumin samples from human albumin samples were inert towards Aβ. AOP1 and AOP2 are immunologically cross-reactive with LRP and screening the Western blots of commercial BSA and affinity purified HSA using jacalin and anti-LRP indicated that the size of albumin-associated Aβ-binding glycoproteins is well preserved along the evolutionary distance from bovine to human species. Aβ binding to AOP1 and AOP2 in pure forms is blocked by LDL whereas when they are bound to albumin LDL ceases to be effective. AOP1 and AOP2 together occupy about 36% of albumin prompting the assumption that these O-glycoproteins, rather than albumin act as sink for plasma amyloid β particularly since pre-bound albumin protects their albumin complexes and triplets from getting blocked by LDL in this function.

There are several small and large molecules that interact with amyloid β and are found to retard its aggregation (Re et al., 2010; Wang et al., 2011). STPS-containing protein α-synuclein which are abundant in brain as well as the drug β-cyclodextrin, are both amphipathic molecules reported to interact with amyloid β and inhibit amyloid β aggregation and plaque formation (Bachhuber et al., 2015; Camilleri et al., 1994). AOP1/AOP2 are also amphipathic molecules since they bind to the hydrophobic region of albumin on one hand and are obviously hydrophilic too, due to their heavy O-glycan content. It was therefore natural that they were more

effective ligands for Aβ than both α-synuclein and β-cyclodextrin. Results indicate the possibility that besides being effective sinks for Aβ in plasma, AOP1and AOP2 could be natural blockers of aggregation of amyloid β in brain to prevent formation of Aβ plaques characteristic of Alzheimer's disease and other cognitive disorders since these amphipathic proteins are uniquely pevileged to be associated with the most abundant plasma protein that not only protects them from LDL-mediated blocking of their recognition by two prominent STPS-specific natural antibodies, but also ensures unoccupied binding sites on the resulting antibody-AOP1/AOP2-albumin triplet so that the latter can convey the O-glycoproteins as well as antibodies to cells to which they bind, especially of brain. The following chapter provides results supporting this speculation.

PART IV

Internalization of anti-Gal/ABG-AOP1/AOP2-albumin triplet by macrophages using cell surface LRP as ligand for free binding site in antibody

Introduction

There are reports showing that Lp(a)-anti-Gal-immune complex uses its free binding site to bind to macrophages (Sheela et al., 2016). So we investigated whether anti-Gal/ABG-AOP1/AOP2-albumin complex could use its free binding site to bind to LRP receptors abundant on macrophages and brain cells and thus mediate the entry of glycoproteins and antibody into these cells. We used macrophages as model cells.

RESULTS

(A) Anti-Gal and ABG triplets enter into macrophages using the free binding site in anti-Gal/ABG

Monocyte-derived macrophages were incubated with anti-Gal/ABG-AOP1/AOP2-albumin triplets prepared de novo using FITC labeled-O-glycoprotein or FITC labeled–antibodies (anti-Gal/ABG). Triplets were treated with sugars specific for respective antibody or with non-specific sugar (methyl α-D-mannoside) before incubation with the cells. Following incubation they were washed with solutions of antibody-specific sugar to remove triplets still on the cell surface. Results in Figure 45 and 46 shows that cells incubated with triplets treated with non-specific sugar internalized significantly more triplet components than those incubated with triplets treated with specific sugars. This was true regardless of whether antibody or AOP1/AOP2 was labeled with FITC. Anti-Gal and ABG triplets were similar in this respect.

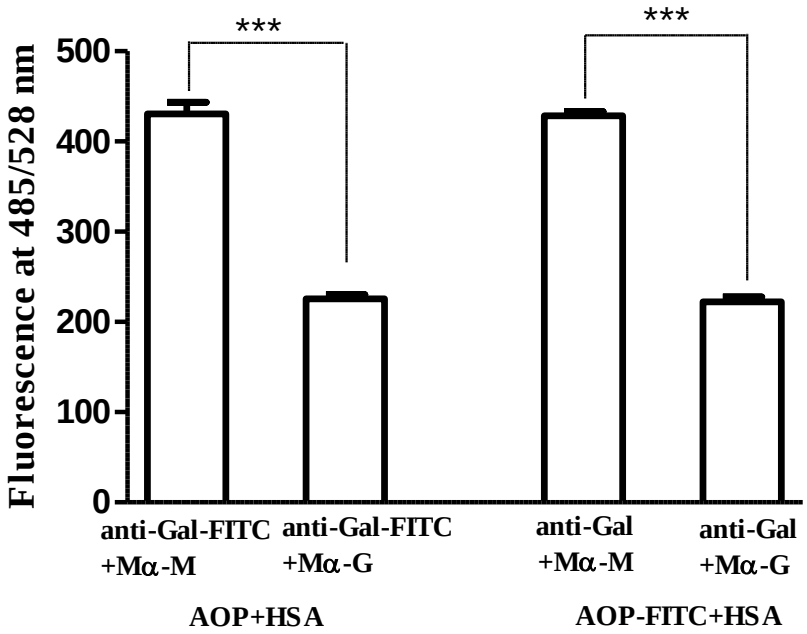


Figure 45. Anti-Gal triplet with its free binding site binds to macrophages. *AOP1+AOP2 electroeluted together from middle 400 µl (17 µg in 40 µl PBS) was incubated for 6 h at 4ºC with HSA (30 µg in 40 µl PBS) and further incubated overnight with electroeluted anti-Gal-FITC (5 µg in 50 µl PBS) which had been preincubated for 2 h with specific sugar methyl α-D-galactoside or non-specific sugar methyl α-D-mannoside (25 mM). Mixtures were made to 1 ml in RPMI-1640 medium. Monocyte derived-macrophages (after 14 days culture) in 35 mm culture dish were incubated with the above mixture for 5 h at 4ºC. The incubated dishes were then washed with 100 mM methyl α-D-galactoside in PBS for 1 h and the cells were scraped in 350 µl distilled water and kept overnight for lysis. Fluorescence was measured using 300 µl as described. A similar protocol was followed using (AOP1+AOP2)-FITC, HSA and anti-Gal. ***: P value <0.0001 for antibody treated with mannoside versus galactoside in both anti-Gal-FITC-(AOP1+AOP2)-HSA triplet and anti-Gal-(AOP1+AOP2)-FITC-HSA triplet. Mean ± SD of 5 samples.*

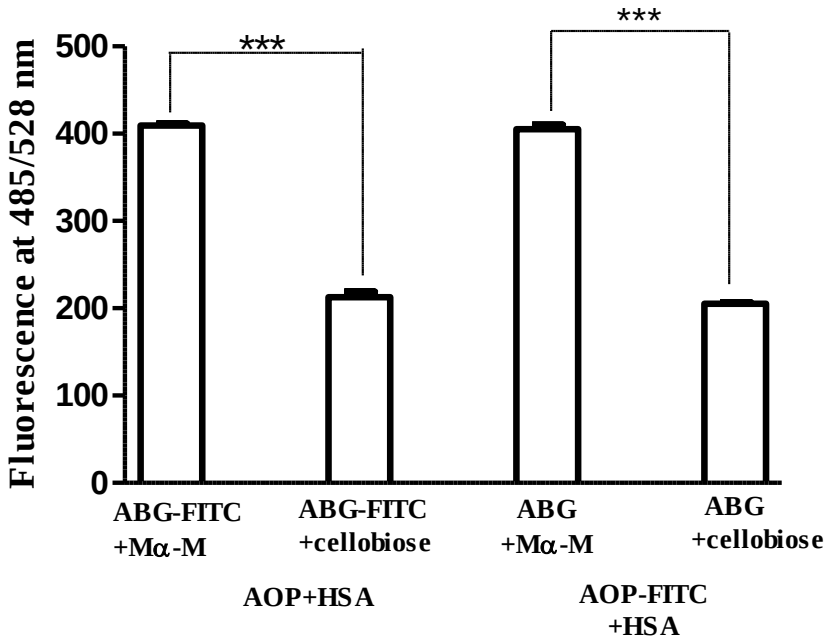


Figure 46. ABG triplet with its free binding site(s) binds to macrophages. *AOP1+AOP2 electroeluted together from middle 400 µl (17 µg in 40 µl PBS) was incubated for 6 h at 4ºC with HSA (30 µg in 40 µl PBS) and further incubated overnight with electroeluted ABG-FITC (5 µg in 50 µl PBS) which had been preincubated for 2 h with specific sugar cellobiose or non-specific sugar methyl α-D-mannoside (25 mM). Mixtures were made to 1 ml in RPMI-1640 medium. Monocyte derived-macrophages (after 14 days culture) in 35 mm culture dish were incubated with the above mixture for 5 h at 4ºC. The incubated dishes were then washed with 100 mM cellobiose in PBS for 1 h and the cells were scraped in 350 µl distilled water and kept overnight for lysis. Fluorescence was measured using 300 µl as described. A similar protocol was followed using (AOP1+AOP2)-FITC, HSA and ABG. ***: P value <0.0001 for antibody treated with mannoside versus cellobiose in both ABG-FITC-(AOP1+AOP2)-HSA triplet and ABG-(AOP1+AOP2)-FITC-HSA triplet. Mean ± SD of 5 samples.*

(B) Fluorescence imaging showing triplet entry into the cells using antibodies anti-Gal and ABG

Monocyte-derived macrophages were incubated with de novo prepared anti-Gal/ABG- FITC-labeled O-glycoprotein-albumin triplets or anti-Gal/ABG-FITC-O-glycoprotein-albumin triplets employing antibodies or their FITC conjugates

preincubated with specific sugars ie, anti-Gal/anti-Gal-FITC with methyl α-D-galactoside and ABG/ABG-FITC with cellobiose or with non-specific sugar methyl α-D-mannoside. Inorder to identify cell nucleus and mitochondria cells were treated with fluorescent dyes Hoeshst 33342 and mitoSOX respectively. In cells incubated with triplets where the antibody was preincubated with non-specific sugar, triplets entered into cells and cells appeared green at 485/528 nm and at the same position it appeared red at 510/580 nm indicating mitochondria and blue at 350/460 nm indicating nucleus (Figure 47 a,c,f,h). Notably fluorescent triplet component entered cells regardless of whether FITC was conjugated to antbody or O-glycoprotein. In cells treated with triplets where antibody was preincubated with specific sugars cells emitted red and blue florescence as in Figure 47 b, d, g, i but did not emit green fluorescence indicating that the triplets had not been formed due to presence of sugar inhibitory to the antibody so that labeled O-glycoproteins could not get into the cells. In cells incubated with FITC-labeled O-glycoprotein alone in absence of antibody, they did not emit green fluorescence (Figure 47e) indicating that entry of O-glycoprotein into the cells is always mediated through anti-Gal/ABG and in absence of antibody O-glycoprotein entry is restricted.

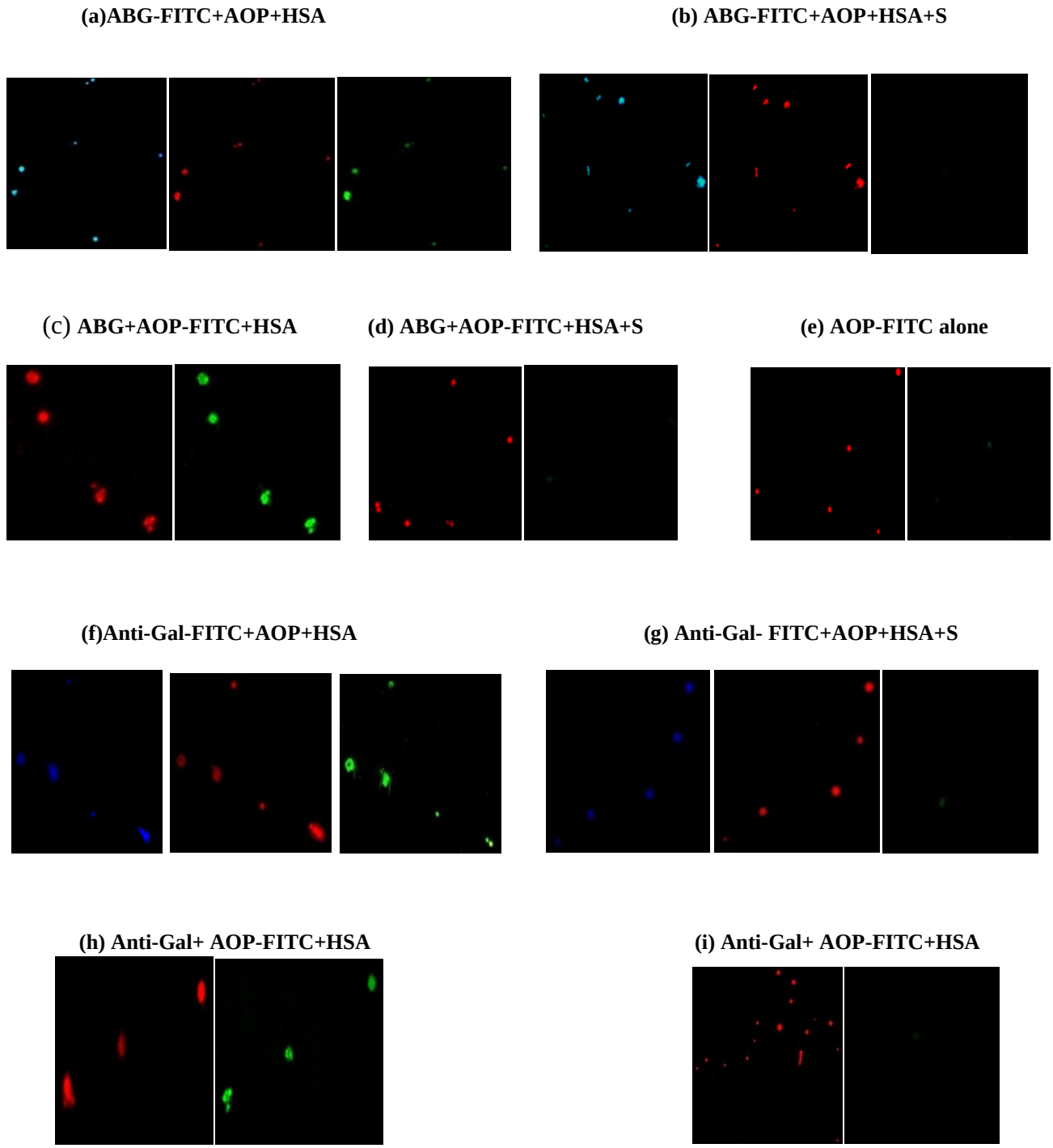


Figure 47. Fluorescent microscope images showing triplet entry into the cells using antibodies anti-Gal and ABG. *AOP1 +AOP2 electroeluted together from middle 400 µl (denoted AOP; labeled or unlabeled with FITC; 17 µg in 40 µl PBS) was incubated for 6 h at 4ºC with HSA (30 µg in 40 µl PBS) and further incubated overnight with electroeluted*

anti-Gal or anti-Gal-FITC respectively (5 µg in 50 µl PBS) which had been preincubated for 2 h with specific sugar methyl-α-D-galactoside or non-specific sugar methyl α-D-mannoside (25 mM). This yields anti-Gal-AOP-FITC-HSA and anti-Gal-FITC-AOP-HSA triplets having been incubated with specific or non-specific sugar. Equalent triplets were prepared from ABG as well. Monocyte-differentiated to macrophages (14 days culture) in 35 mm culture dish were incubated for 5 h with the above triplet samples diluted to 1 ml in PBS, washed thrice with PBS and observed under fluorescence microscope at 485/528 nm. AOP-FITC alone without antibodies or HSA was also used (Figure e). The same cells were again incubated for 5 min with 10 µM Bis-benzimide H 33324 (Hoeschst 33342), washed with PBS and observed under fluorescence microscope at 350/460 nm for staining nucleus (blue fluorescence). Cells were then incubated for 10 min at room temperature with 5 µM mitoSOX (dilutions in HBSS), washed thrice in PBS and observed under fluorescence microscope at 510/580 nm for staining mitochondria (red fluorescence). Presence of specific sugar only indicated (+S).

(C) Anti-Gal entry into macrophages is LRP mediated

Monocyte-derived macrophages were treated with either anti-LRP which can block LRP on cell surface or anti-apoB as a non-specific antibody instead of anti-LRP. On incubating the treated cells with FITC labeled O-glycoprotein-albumin-anti-Gal triplet and washing the cells with anti-Gal specific sugar methyl α-D-galactoside all the surface bound triplets were removed and those that entered inside the cells would be spared. Upon lysis the anti-LRP treated cells gave a total fluorescence response of only 220 whereas anti-apoB treated cells and untreated cells gave a fluorescence reading of more than 400 (Figure 48) which indicated that the entry of triplets into the cells is LRP mediated.

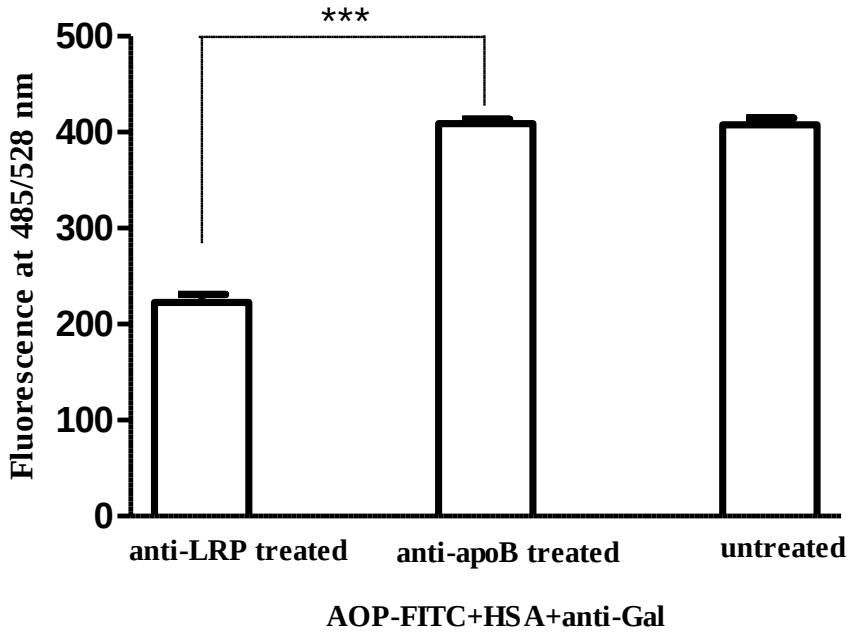


Figure 48. Anti-Gal entry into macrophages is LRP mediated. *AOP1 +AOP2 (AOP) electroeluted together from middle 400 µl following DGUC of plasma in 1 ml tubes was labeled with FITC. Seventeen µg of this sample in 40 µl PBS was incubated for 6 h at 4ºC with 30 µg HSA added in 40 µl PBS and further incubated overnight with electroeluted anti-Gal (5 µg in 50 µl PBS) which had been preincubated for 2 h with specific sugar methyl α-D-galactoside or non-specific sugar methyl α-D-mannoside (25 mM). Mixtures were made up to 1 ml using RPMI-1640 medium. Monocyte derived-macrophages (after 14 days culture) in 35 mm culture dish was incubated overnight at 4ºC with 12 µg anti-LRP-1 or anti-apoB and further treated with the AOP-FITC-HSA-anti-Gal mixture and incubated for 5 h in 4ºC. The incubated dishes were washed with 100 mM specific sugar methyl α-D-galactoside for 1 h and the cells were scraped in 350 µl distilled water and kept overnight at 4ºC. Fluorescence was measured using 300 µl as described above. ***: P value <0.0001 for anti-LRP versus anti-apoB treated macrophages. Mean ± SD of 5 samples.*

DISCUSSION

Treatment of human macrophages which expressed LRP receptors on their surface with anti-Gal or ABG triplets prepared de novo resulted in incorporation of both the antibody used and AOP1/AOP2 into cells by a binding event which was inhibitable with antibody-specific sugar or anti-LRP suggesting that LRP present on

macrophage cell surface mediated the capture of triplets. Though macrophages are among the few circulating LRP-bearing cells (Petersen et al., 1987; Quinn et al., 1997) LRP-like molecules that possess STPS domains are abundant in brain endothelial cells, choroid plexus and neurons (Herzog et al., 2014; Johanson et al., 2006; Kanekiyo & Bu, 2014). STPS present in these LRP receptors enable anti-Gal/ABG-AOP1/AOP2-albumin triplets with free binding site for attachment. AOP1 and AOP2 which on entering brain cells through LRP mediation, are potential candidates for preventing aggregation of amyloid β and formation of plaques since most amyloid β-binding molecules known prevent aggregation of this peptide. Anti-Gal/ABG-AOP1/AOP2-albumin triplets in which the O-glycoproteins play a pivotal role may thus be a natural protective mechanism against neurodegenerative disorders.

PART-V

Recognition of STPS-rich brain glycoproteins α-synuclein and tau by anti-Gal and ABG

Introduction

Tau and alpha-synuclein are brain proteins rich in serine-and threonine- rich peptide sequences (STPS). Under pathological conditions they form toxic oligomers and abnormal intracellular aggregates that can trigger synaptic damage, network dysfunction and eventually lead to loss of neuronal populations. The accumulations of tau and α-synuclein aggregates are hallmarks of Alzheimer's disease and Parkinson's disease respectively. Anti-tau and anti-α-synuclein monoclonal antibodies are presently on trial for preventing the aggregation and enhancing the clearance of aggregated tau, or α-syn as strategy for preventing their toxicity (Valera et al., 2016). These antibodies have been tried only in phase I clinical trials in mouse models (Sigurdsson, 2016). Since anti-Gal and ABG recognize STPS-rich proteins we examined recognition of tau and α-synuclein by anti-Gal or ABG since they appear to be natural provisions to prevent tau and α-synuclein aggregation.

RESULTS

(A) Tau protein interacts with anti-Gal and ABG

Tau protein was dot blotted on PVDF membrane and incubated with anti-Gal or ABG which had been treated in advance with specific sugar 25 mM (methyl α-D Gal)/ cellobiose or non specific sugar mannoside. Blots were then treated with HRP-labeled anti-human immunoglobulin and the bound HRP conjugate was extracted with 50 mM of specific sugar for anti-Gal (methyl α-D-Gal) or ABG (cellobiose) and assayed using OPD as the substrate as described in methods and absorbance was measured at 490 nm. Results show that tau protein interacted with both anti-Gal and

ABG and this binding was inhibitable using their specific sugars showing that tau and α-synuclein bind to the sugar binding site of anti-Gal and ABG (Figure 49).Total immunoglobulin (Ig fraction) was used as a negative control which did not interact with tau protein.

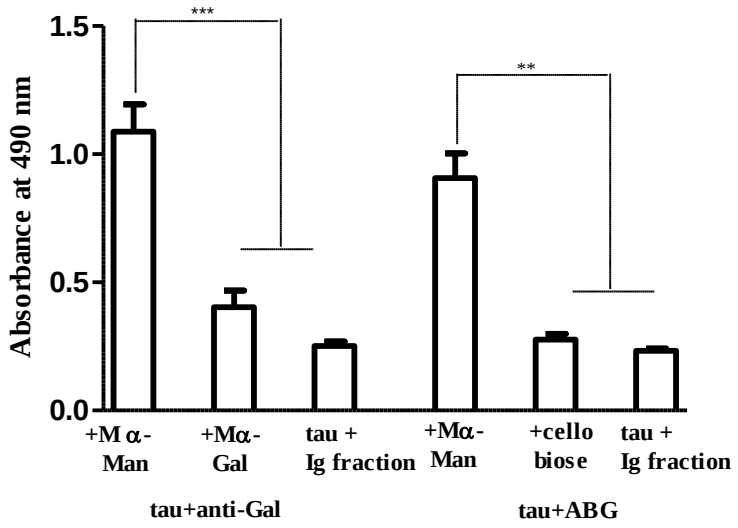


Figure 49. Tau interacts with anti-Gal and ABG. *Tau protein (2 µg in 3 µl) was dot blotted on 3 mm X 3 mm PVDF sheets and blocked overnight in 0.5% PBST. Anti-Gal or ABG (5 µg/ml in 200 µl 0.05% PBST) was preincubated for 2 h with 25 mM specific sugar (methyl α-D-Gal and cellobiose respectively) or non-specific sugar methyl α-D-Man. Separate blots were incubated in each of these solutions at 4°C for 2 h, washed in PBST thrice and added to anti-immunoglobulin-HRP (1.5 µg per ml; 200 µl) in PBST for 2 h at 4°C. As control for anti-Gal/ABG total human immunoglobulin (Ig ; 5 µg/ml) was used. After washing the blots anti-Gal or ABG bound to the blots was extracted along with anti-immunoglobulin-HRP bound to them by incubating with 50 mM specific sugars methyl α-D-Gal or cellobiose in 220 µl citrate phosphate buffer pH 5 for 1 h and 200 µl of the released antibody was assayed in microtiter wells by adding 25 µl OPD solution and reaction stopped with addition of 25 µl H_2SO_4 within 5 minutes and absorbance measured at 490 nm. Mean ± SD of 6 different anti-Gal, ABG and Ig fractions. ***: P value<0.0006 for decrease in tau binding with specific sugar or Ig fraction versus non-specific sugar in anti-Gal. **: P value <0.003 ABG for decrease in tau binding with specific sugar or Ig fraction versus non-specific sugar in ABG.*

(B) α-synuclein interacts with anti-Gal and ABG

Alpha-synuclein was incubated with glycoprotein-free anti-Gal-FITC and ABG-FITC preincubated with or without 25 mM specific sugars for anti-Gal (methyl α-D-Gal) and ABG (cellobiose) and fluorescence of mixture was measured. α-synuclein increased fluorescence of anti-Gal-FITC to about 26% and ABG-FITC to about 19.8% and this increase was inhitable using their specific sugars (Figure 50) suggesting that α-synuclein binds to the sugar binding site of anti-Gal and ABG. FITC-labeled total human immunoglobulin fraction (Ig-FITC) used as control instead of anti-Gal/ABG-FITC did not produce any response (Figure 50).

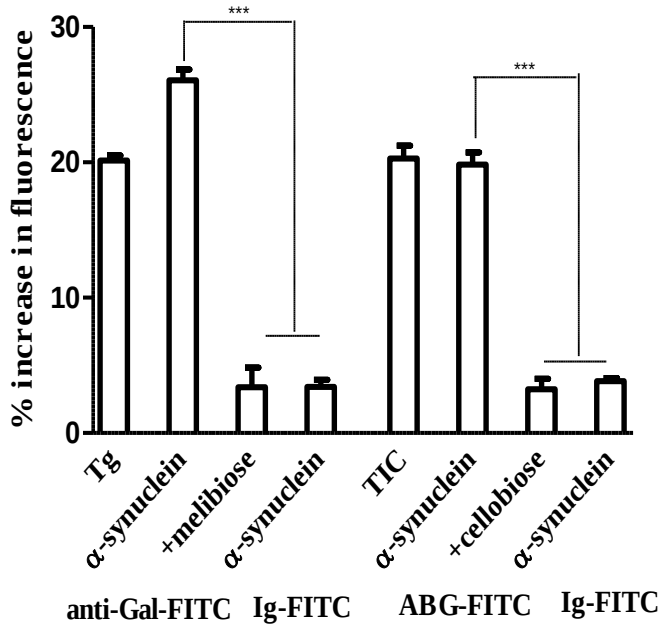


Figure 50. α-Synuclein interacts with anti-Gal and ABG. *α-synuclein (5 µg) in 50 µl was incubated overnight in 4°C with 1 µg glycoprotein-free anti-Gal-FITC or ABG-FITC in 25 µl PBS which was preincubated for 2 h with anti-Gal specific sugar 25 mM methyl α-D-Gal or ABG specific sugar cellobiose. The mixture was diluted to 300 µl and fluorescence measured at 485/528 nm. Total immunoglobulin fraction (Ig)-FITC labeled was used instead of anti-Gal or ABG. Mean ± SD of 6 different anti-Gal, ABG and Ig fractions. ***: P value <0.0001 for decrease in fluorescence of FITC-labeled anti-Gal or ABG using specific sugars.*

(C) Anti-Gal recognizes dot blotted α-synuclein sugar dependently

Dot blot of α-synuclein in a PVDF membrane (3 mm X 3 mm) was incubated overnight in anti-Gal which had been preincubated with or without specific sugar methyl α-D-Gal for 2 h and the bound antibody was probed using anti-immunoglobulin-HRP. Results shows that dot blotted α-synuclein was recognized by anti-Gal and it was inhibited in presence of anti-Gal specific sugar (Figure 51) confirming again that anti-Gal recognizes α-synuclein sugar dependently.

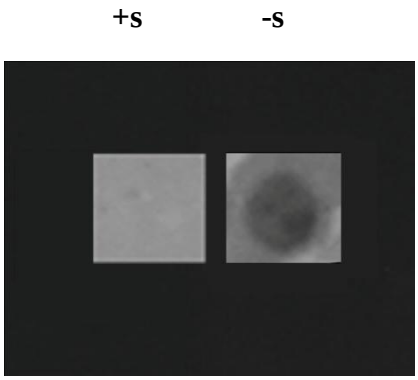


Figure 51. Anti-Gal recognizes dot blotted α-synuclein sugar dependently. *α-synuclein 2 µl was dot blotted in a PVDF membrane which had been wetted in advance in methanol followed by water and PBS. After the protein gets dried on the blot the latter was washed and blocked in 0.5% PBST for 2 h. The blot was washed and then incubated overnight at 4°C in 5 µg/ml anti-Gal in 200 µl PBST with or without pre-incubation for 2 h with specific sugar 25 mM methyl α-D-Gal and the bound antibody was probed using 15 µg/ml anti-immunoglobulin-HRP for 2 h. It was then transferred to substrate solution 4-chloronaphthol as described in methods and kept in dark without shaking till colour appears.*

(D) α-Synuclein interacts with triplet anti-Gal and ABG

Anti-Gal and ABG normaly exist in circulation in the form of triplets, viz. APAG and APABG respectively. On treating α-synuclein with APAG-FITC or APABG-FITC, fluorescence of the latter was increased significantly (Figure 52).

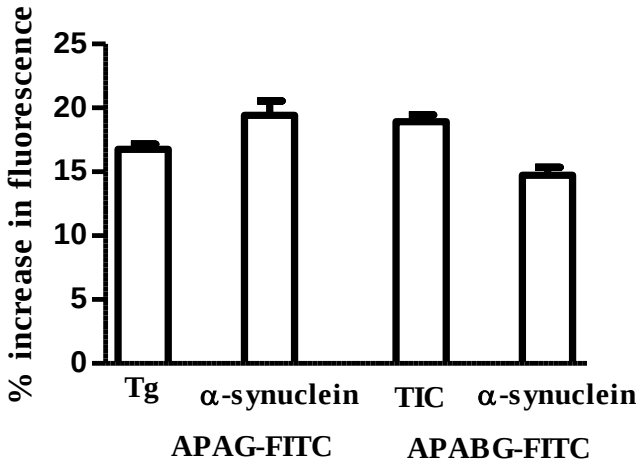


Figure 52. α-Synuclein interacts with triplet anti-Gal and ABG. *α-synuclein (5 µg) in 50 µl was incubated overnight at 4°C with 3 µg APAG-FITC or APABG-FITC in 25 µl PBS and fluorescence was measured as described for Figure 50. Mean ± SD of 4 samples.*

DISCUSSION

Tau and α synuclein forming aggregates are the primary event in the molecular pathology of Alzheimer's disease and Parkinson's disease respectively (Wang & Mandelkow, 2015; Wong & Krainc, 2017). Present results show that anti-Gal and ABG bind through their sugar binding sites to tau and α-synuclein which are two serine-and threonine-rich proteins particularly abundant in brain suggesting another possible physiological role for triplets. As discussed in the previous section anti-Gal/ABG-AOP1/AOP2-albumin triplets may enter the brain cells mediated by LRP receptors that are present abundantly in these cells. Since monoclonal antibodies are used to disassemble the aggregates (Castillo-Carranza et al., 2014; Congdon et al., 2014; Emadi et al., 2004) natural anti-carbohydrate antibodies, as part of triplets capable of binding to LRP-rich cells such as in brain emerge as the natural provision

to bind tau and α-synuclein so as to prevent/undo their aggregation and thereby protect from neurodegenerative disorders.

PART VI

Molecular mechanisms of triplet adhesion to platelet surface and its consequences

Introduction

Platelets are rich in STPS-containing O-glycosylated proteins like GPIIb/IIIa present in high density on their surface membrane (60,000-80,000 copies per platelet) (Coller & Shattil, 2008; Wagner et al., 1996). GPIIb/IIIa molecules act as integrins that enable platelets to bind to sub-endothelial tissues where under pathological conditions conformational changes occur on platelet surface receptors and induce platelet aggregation. Platelets have been reported to carry adhering immunoglobulins and albumins in 1:1 ratio and also amyloid β but the exact molecular mechanisms of these events is not known (George, 1991). Present part of the thesis was undertaken to explore whether anti-Gal and ABG triplets use their free binding sites to anchor on platelet surface utilizing the STPS-rich GPIIb/IIIa as receptor and to check if bound triplets affect platelet aggregation and, through their AOP1 and AOP2 components, mediate amyloid β binding by platelets.

RESULTS

(A) Either or both of two O-glycosylated proteins and albumin are attached to platelets through anti-Gal or ABG

Proteins eluted from platelets using a mixture of 15 mM each of MαG (anti-Gal-specific) and cellobiose (ABG-specific), run on 6% alkaline polyacrylamide gel electrophoresis showed protein bands with mobilities identical to those of a mixture of equal amounts of plasma triplets of anti-Gal (APAG) and of ABG (APABG) (Figure 53 inset). The fastest and slowest moving bands were identified as albumin (HSA) and immunoglobulins respectively using corresponding enzyme-labeled antibodies (Figure 53). Specificity of antibodies was clear from the absence of

recognition of albumin by antibody against immunoglobulin and vice versa. Bands identical in mobility with AOP1 or AOP2 were recognized by the O-glycan specific lectin jacalin showing that they are heavily O-glycosylated (Figure 53). Results confirm the presence of two O-glycosylated proteins with identical mobility and O-glycan content as AOP1 and AOP2, albumin and antibodies in protein released from platelets using anti-Gal and ABG specific sugars.

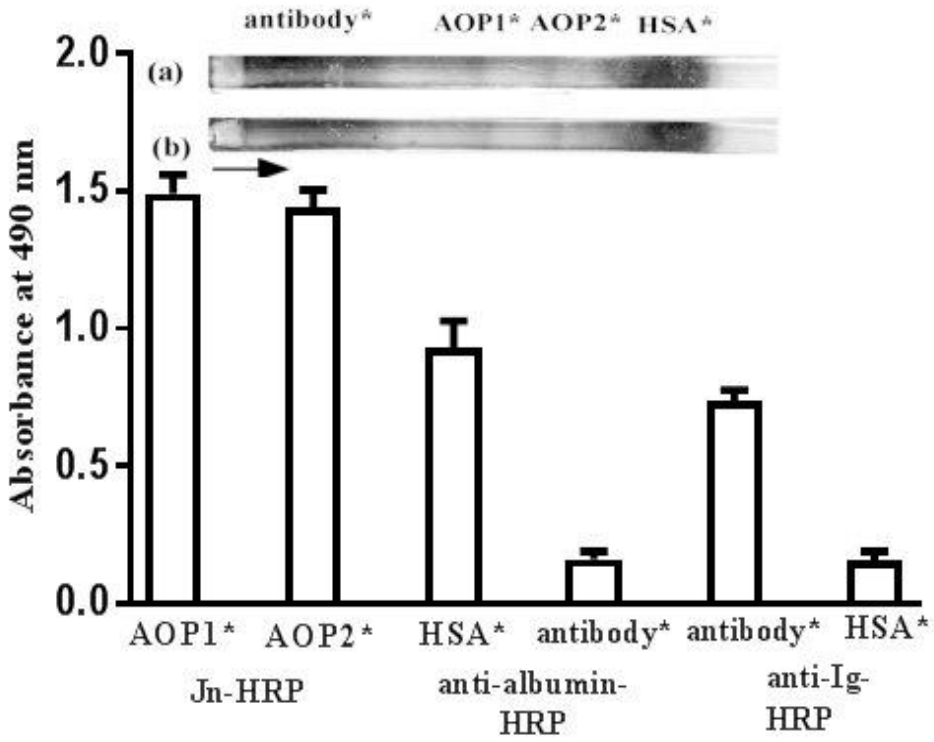


Figure 53. Two O-glycosylated proteins, albumin and antibodies are released from platelets treated with anti-Gal- and ABG-specific sugars. ***Inset:*** *(a) Electophoresis of platelet-bound triplet (50 µg) and (b) mixture of APAG and APABG (25 µg each) in 6% polyacrylamide gel in Tris-glycine pH 8.3 buffer. Gels were stained with Coomassie Brilliant blue G-250. Bands corresponding to two O-glycoproteins of plasma triplets, albumin and antibody were electroeluted from unstained gel of inset (a). Protein from each band (200 ng in 200 µl PBS) was directly coated in ELISA* microwells. *Proteins corresponding to O-glycoproteins, antibodies and albumin were probed with jacalin-HRP (75 ng per ml), anti-immunoglobulin-HRP (1.5 µg per ml), and anti-albumin-HRP (3.75 µg per ml) respectively in 200 µl PBST. * : suspected to be AOP1, AOP2 or albumin (HSA) in plasma triplets due to identical mobility in electrophoresis. Mean ± SD of 3 samples.*

(B) Anti-Gal and ABG are the antibodies released from platelets on treatment with methyl α-D-Gal or cellobiose

Antibodies released by a mixture of anti-Gal- and ABG-specific sugars from platelets and separated from other released molecules by electrophoresis and electroelution contained anti-Gal since part of them bound to microplate-coated thyroglobulin terminating in α-galactoside moieties and was inhibited by MαG but not by cellobiose. In addition the platelet-derived antibody above also contained ABG since part of them bound to microplate-coated TIC and was inhibited by cellobiose but not by MαG (Figure 54). Antibodies eluted from platelets by MαG +cellulose mixture contained only anti-Gal and ABG since an excess of a mixture of the affinity matrices for these antibodies, namely CLGG and cellulose, could capture nearly all of both the platelet-eluted immunoglobulin mixture and the electrophoretically purified plasma anti-Gal and ABG (1:1 by weight) out of the 200 ng mixture of each added to the gel mixture (Figure 54 inset).

% of antibodies bound to CLGG +cellulose mixture (n=3)	
Anti-Gal/ABG mixture	95.2±0.51
Platelet-bound antibodies	94.4±2.1

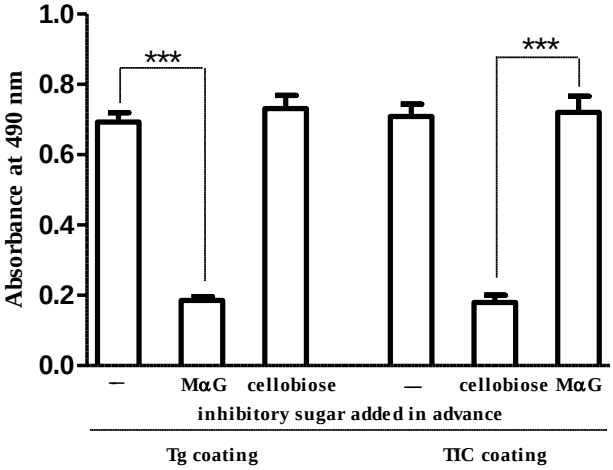


Figure 54. Anti-Gal and ABG are the antibodies released from platelets by methyl α-D-Gal or cellobiose treatment. *From triplets released from platelets, antibodies separated by 6% alkaline gel electrophoresis were electroeluted. Antibody (100 ng in 200 µl PBST) was added to 1 µg Tg coating after preincubation with 25 mM anti-Gal-specific-sugar methyl α-D-Gal, 25 mM ABG-specific sugar cellobiose or no sugar for 2 h in 4°C. Alternatively antibody was added to 1 µg TIC coating after preincubation with 25 mM ABG-specific sugar cellobiose, 25 mM anti-Gal-specific-sugar methyl α-D-Gal or no sugar. In both cases the bound antibody was probed using 1.5 µg/ml anti-immunoglobulin-HRP. ***: P value < 0.0001 for inhibition using specific sugars. Mean ± SD of 6 samples.* ***Inset:*** *A mixture of 0.2 ml CLGG and 0.2 ml cellulose in 1 ml PBS was treated with 200 ng antibody eluted from platelet-bound triplets and 100 ng of each anti-Gal and ABG mixture for 4 h at 4ºC and the unbound antibody in supernatant was determined by ELISA after coating 200 µl on microplates and assaying bound antibody using 1.5 µg/ml anti-immunoglobulin-HRP. Mean ± SD of 3 samples.*

(C) Anti-Gal/ABG-O-glycosylated protein-albumin triplet are released from platelets treated with antibody-specific sugars; either sugar releases triplets of both antibodies

Triplets in plasma formed by simultaneous binding of AOP1 or AOP2 to either anti-Gal or ABG on one side and albumin on the other were assayed by capturing on

plate-coated antibody ligand, washing and assaying the albumin on the other end of the bound triplet using HRP-labeled anti-albumin antibody as probe (Figure 22). Proteins released from platelets on treatment with anti-Gal- or ABG-specific sugar were dialysed and assayed by the above protocol. From proteins released from platelets by MαG or cellobiose significantly more albumin was bound through anti-Gal or ABG to plate-coated antibody ligand than from the same dilution of proteins released by the non-specific sugar MαM (Figure 55). Since albumin is not directly associated with either of these two antibodies (anti-Gal/ABG) as shown in earlier sections, and two O-glycoproteins identical in mobility and O-glycan content as AOP1 and AOP2 are present in the released proteins (Figure 53) results point to the presence of antibody-O-glycoprotein-albumin triplets in proteins released from platelets on treatment with antibody-specific sugars. Involvement of O-glycoproteins was confirmed by capturing the platelet proteins, released by a mixture of specific sugars of either antibody, through their O-glycoproteins to the O-glycan-specific lectin jacalin coated on microwells and probing bound proteins using HRP-labeled anti-albumin (Figure 55). These data indicated presence of anti-Gal or ABG at one side and albumin at the other of a bridging O-glycoprotein molecule resulting in triplet as detected in cell-free plasma.

Though anti-Gal and ABG share a common affinity for STPS their specificities towards small sugars are distinct and different whether isolated from plasma triplets (Figure 22) or platelet-bound triplets (Figure 54). In this context release of as much anti-Gal triplets as ABG triplets by ABG-specific sugars and vice versa (Figure 55) was noteworty. A possible reason for this behavior could be that antibody detachment from triplet of one antibody results in temporary destabilization of its O-

glycoprotein-albumin bondage as well, as observed earlier in the case of plasma triplets (Figure 27). The resulting newly generated free O-glycoproteins resemble small sugars in that they can occupy all binding sites of antibodies as small sugar moieties do and being common to both triplets, may liberate the triplet of the other antibody as well.

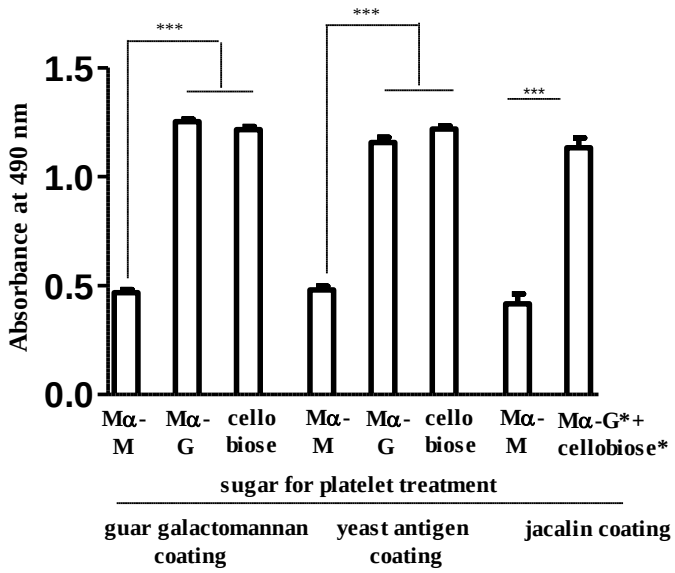


Figure 55. Anti-Gal or ABG triplets are released from platelets treated with anti-Gal-specific sugar methyl α-D-galactoside and ABG-specific sugar cellobiose: either sugar releases both triplets. *Platelet prepared as in methods (0.25 million cells/µl PBS; 300µl) was incubated for 2 h at room temperature with 25 mM non-specific sugar (methyl α-D-mannoside) or 25 mM ABG specific sugar (cellobiose) or 25 mM anti-Gal specific sugar (methyl α-D-Gal). It was centrifuged at 4500 g for 15 min at 25°C and 100 X dilution of the supernatant in 200 µl was added to microwell-coated guar galactomannan or yeast antigen (1 µg per well). After incubation for 2 h in 4°C wells were washed with PBST and probed with anti-albumin-HRP (3.75 µg antibody per ml). To verify the presence of O-glycoproteins associated with albumin in proteins bound to platelets through antibodies these proteins were released by treatment of platelets with a mixture of specific sugars for anti-Gal and ABG (methyl α-D-Gal and cellobiose, 15 mM each). The released protein diluted as above was added to 1µg jacalin coating on microplates and the glycoprotein-bound albumin was assayed using 3.75 µg/ml anti-albumin-HRP. ***: P value <0.0001 for release by specific sugar versus non-specific sugar of anti-Gal-containing triplets, ABG-containing triplets and O-glycoprotein-bound albumin. Mean ± SD of 7 blood samples.*

(D) Plasma and platelet-bound triplets are identical; anti-Gal and its triplet bind significantly more to denuded platelets

Platelets deprived of their attached triplets by treatment with a mixture of anti-Gal- and ABG-specific sugars (denuded platelets) were treated with anti-Gal-FITC or plasma triplets of anti-Gal reconstituted using albumin-FITC, anti-Gal and either AOP1 or AOP2. Both anti-Gal alone and reconstituted plasma triplet got attached to denuded platelets unless inhibited by specific sugars. In contrast native platelets in which naturally adhering triplets were intact attached significantly less of both (Figure 56). Since triplets from cell-free plasma could substitute for platelet-bound triplets and O-glycoprotein of triplets from plasma and platelet were identical in alkaline gel electrophoresis it seems reasonable to conclude that triplets bound to platelets or in free form in plasma are identical and consist of anti-Gal or ABG and albumin linked by AOP1 or AOP2, as demonstrated in plasma. Comparison of distribution of triplets between platelets and cell-free plasma shows that more of both anti-Gal and ABG triplets are born by platelets than in free form in same blood volume (Figure 56 inset).

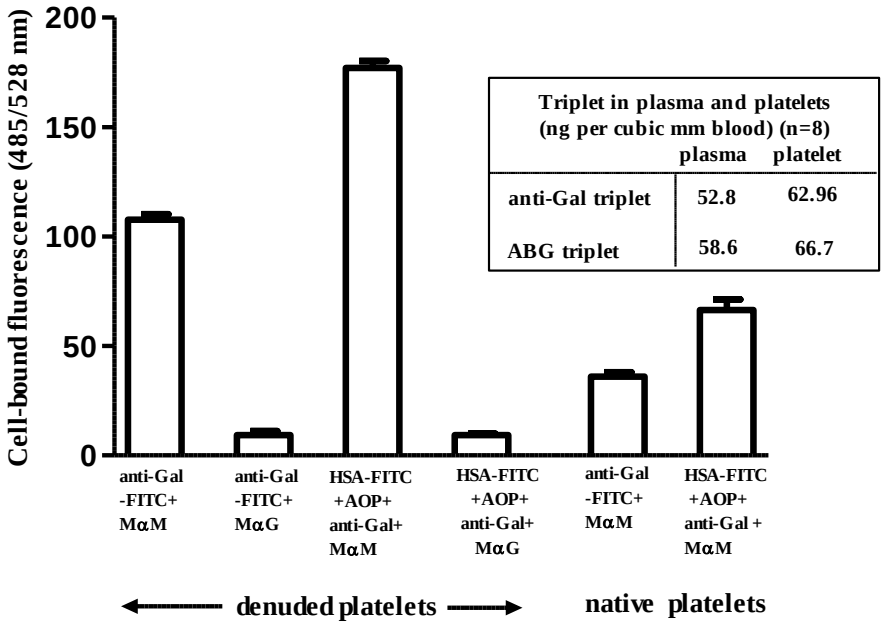


Figure 56. Anti-Gal and its triplet bind significantly more to denuded platelets. *Glycoprotein-free anti-Gal-FITC (1.2 µg) in 400 µl PBS was preincubated for 2 h at 4°C with 25 mM specific sugar methyl α-D-Gal or non-specific sugar methyl α-D-mannoside (25 mM each). De novo triplets reconstituted using its constituents from plasma was prepared by adding 600 ng anti-Gal in 200 µl, pretreated for 2 h with specific sugar methyl α-D-Gal or non-specific sugar methyl α-D-mannoside (both 25 mM), to a mixture of AOP1 and AOP2 electroeluted together (400 ng in 100µl) and HSA-FITC (200 ng in 100 µl) preincubated for 2 h at 4°C and incubating the mixture again for 4 h at 4°C. Denuded platelets were prepared by treating native platelets (0.25 million cells/µl; 300 µl) with MαG and cellobiose (15 mM each) for 1 h, removing supernatant containing platelet-extracted triplet by washing with PBS twice. Pellet containing denuded platelets was resuspended in anti-Gal-FITC or FITC-labeled triplets (above) and incubated for 2 h at 4°C. Supernatant was removed and pellet washed twice with PBS by centrifugation at 4530 g for 15 min. Pellet was resuspended in 320 µl PBS and fluorescence was measured using 300 µl as described. ***: P value < 0.0001 for difference between native and denuded platelets in binding of anti-Gal-FITC or FITC-labeled triplets. Mean ± SD 6 different blood samples.* ***Inset: Distribution of triplets in plasma and platelets.*** *Platelet-bound triplets (0.25 million cells per µl) released in 250 µl using mixture of cellobiose and methyl α-D-Gal (15 mM each) and cell free plasma prepared on removing the pellet after centrifugation at 5000 g for 15 min were both diluted 100 X in 200 µl PBST was added to microwell-coated TIM or TIC (1 µg per well) and probed using 3.75 µg/ml anti-albumin-HRP to estimate anti-Gal triplet and ABG-triplet respectively in both platelets and plasma. Anti-albumin response of APAG and APABG (0.5 µg per ml) added to microwell coated TIM or TIC (1µg per ml) was used as the standard. Mean ± SD of 8 different sets of samples.*

(E) Anti-Gal and its triplet recognize the O-glycosylated region of platelet surface glycoprotein receptor

Platelet surface are rich in O-glycoproteins like GPIIb/IIIa. To determine ligands on platelet surface recognized by antibodies in triplets O-glycosylated regions of proteins on platelet surface were blocked by O-glycan specific lectin jacalin. Glycoprotein-free anti-Gal-FITC from platelets or its reconstituted triplet (using AOP1, AOP2 and albumin-FITC from platelet) could not bind to denuded platelets treated in advance with jacalin whereas heat-inactive jacalin did not cause this blocking (Figure 57). Results show that STPS that underlie the O-glycosylated regions of O-glycoproteins on platelet surface which gets blocked upon jacalin occupation of the overlying O-glycans are the true ligands for anti-Gal and O-glycans per se are not ligands for anti-Gal since the classical antigenic ligand for anti-Gal terminal α-linked galactose is absent in humans. In fact, removal of O-glycans from O-glycoproteins enhances anti-Gal reactivity possibly due to greater access to the STPS [(Geetha et al., 2014); Figure 18].

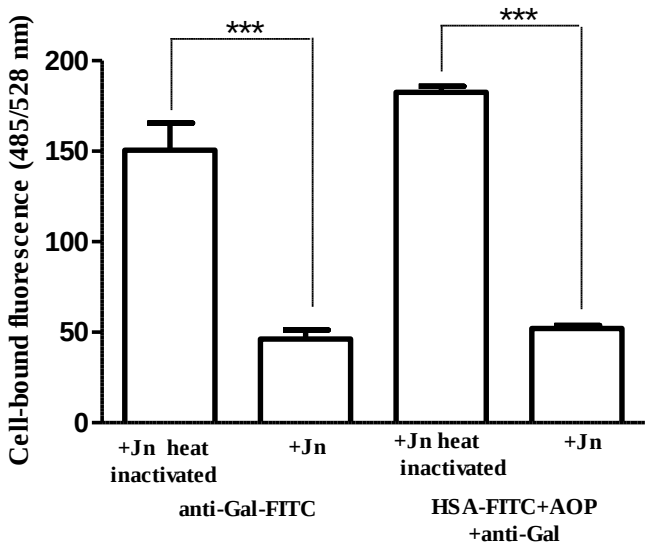


Figure 57. Antibody–O-glycoprotein–albumin triplet and anti-Gal bind to O-glycosylated membrane glycoprotein(s) on platelet surface. *Denuded platelets (0.25 million cells per µl; 250 µl) were incubated with 50 ng jacalin or heat-inactivated jacalin for1 h at 4°C followed by removal of supernatant by centrifugation at 4500 g for 15 min. Platelets in pellet were washed twice with PBS and incubated at 25°C for 3 h in 300 µl PBS with either 4 µg glycoprotein-free anti-Gal-FITC or de novo triplet with FITC-labeled albumin but prepared from platelet-derived APAG. After washing thrice in PBS by centrifugation at 4500 g the pellet was resuspended in 320 µl PBS and fluorescence measured as for Figure 56 protocol. ***: P value<0.0001 for difference between cells pretreated with jacalin and inactive jacalin in bound fluorescence due to FITC-labeled anti-Gal or triplet. Mean ± SD of 6 blood samples.*

(F) Triplets bind on denuded platelets at the same site where fibrinogen binds

Fibinogen is a known ligand for GPIIb/IIIa (Wang et al., 2012). Denuded platelets were treated with or without fibrinogen, washed and treated with platelet-derived triplets. Unbound triplet remaining in the supernatant was assayed by capturing on plate-coated antibody ligand and probing the associated albumin using HRP-labeled anti-albumin antibody (as in Figure 22 and Figure 55). Prior treatment of denuded platelets with fibrinogen resulted in complete blocking of triplet binding to them (Figure 58). Interference of fibrinogen during assay of unbound triplet was ruled out

(Figure 58 inset). Since fibrinogen recognizes the GPIIb/IIIa integrin present on platelet surface the present result further support the assumption that GPIIb/IIIa is the receptor for anti-Gal and ABG antibodies present in triplets.

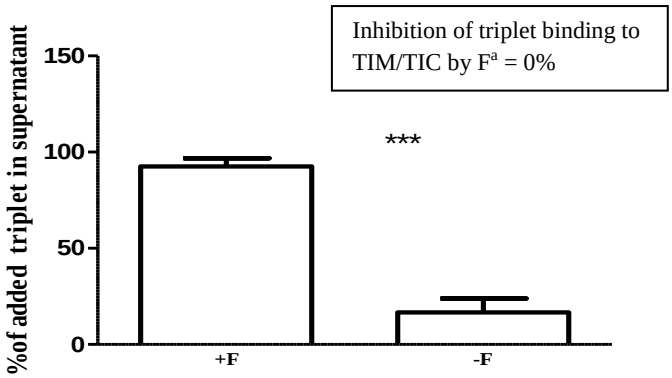


Figure 58. Triplets bind on denuded platelets at the same site where fibrinogen binds. *Denuded platelets (0.25 million cells /µl; 350 µl) were incubated with 1µM fibrinogen (F) at 25°C for 2h. After removing supernatant the pellet was washed twice with PBS and again incubated for 4 h 25°C with 2 µg of triplet released from platelets using sugars. From supernatant collected on centrifugation at 4500 g for 15 min 200 µl was added to wells coated with both TIM and TIC (0.5 µg each). After 2 h incubation at 4^0C and washing bound triplet was assayed using anti-albumin-HRP as probe as described earlier. Mean ± SD of 6 samples. F: Fibrinogen; a: 1 µM Fibrinogen.*

(G) Triplet-free platelets are more aggregative and remarkably so on adding ADP

ADP is released when platelets are prone to aggregate. In turn ADP binds to the two G-protein-coupled receptors P2Y1 and P2Y12 present on platelet surface (Dorsam & Kunapuli, 2004) thereby altering the conformation of receptors and making platelets aggregate. Aggregation of platelets in suspension was monitored in terms of reduction in the absorbance of the suspension at 405 nm. Triplet-free (denuded) platelets are more prone to aggregation than their native counterparts even before

ADP addition while addition of ADP caused a substantial increase in aggregation whereas native (non-denuded) platelets were little aggregated upon addition of ADP regardless of pre-treatment of platelets with jacalin (Figure 59). However, pre-treatment of denuded triplets with jacalin provides complete protection from ADP-mediated aggregation. There is no data available regarding the binding of ADP to O-glycosylated proteins on platelet membrane but involvement of GPIIb/IIIa in subsequent events in ADP-mediated aggregation has been reported explaining the jacalin-mediated inhibition of aggregation of denuded platelets by ADP.

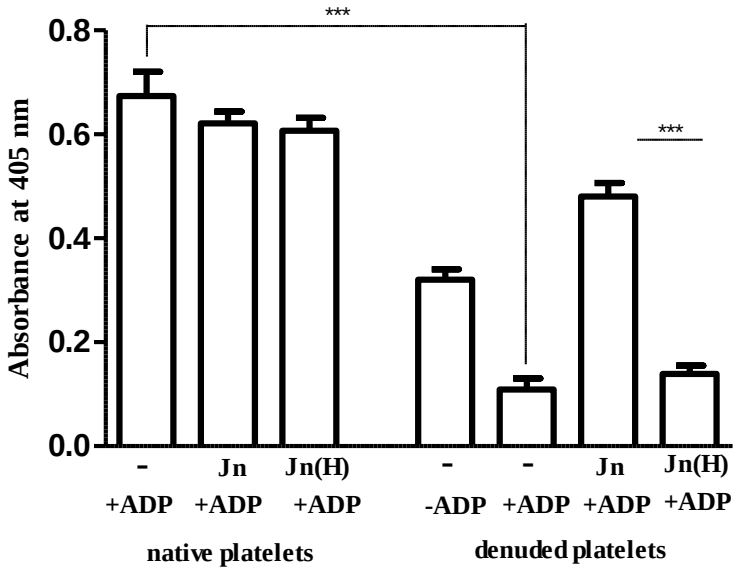


Figure 59. Triplet-free platelets are more aggregable and remarkably so on adding ADP; jacalin blocks ADP binding to platelets. *Suspensions in PBS of native and denuded platelets prepared as described under 'methods' (0.25 million cells per µl; 250 µl) were incubated with or without 50 ng of active or heat-inactivated jacalin for1 h at 4°C. After removing supernatant and three washings with PBS the pellet was dispersed in 220 µl PBS with or without 20 µM ADP and absorbance at 405 nm was measured after 2 min. Mean ± SD of 10 blood samples.*

(H) Amyloid β bind to the O-glycoproteins of adhering triplets rather than to GPIIb/IIIa on circulating platelets

There are several reports that amyloid β binds to GPIIb/IIIa present on platelet surface and also that aggregated platelets release more amyloid β. We have shown that AOP1 and AOP2 of triplets are efficient receptors for amyloid β binding. Results in Figures 58 and 59 indicated that triplets in native platelets are bound to GPIIb/IIIa. So we examined the relative contributions of GPIIb/IIIa which gets exposed in denuded platelets and AOP1/AOP2 in native platelets towards amyloid β capture. Results in Figure 60 show that denuded and native platelets captured nearly the same amount of a limited amount of amyloid β presented to them. But on treatment of resulting amyloid β-bearing platelets with sugars capable of releasing triplets the amount of amyloid β released to supernatant by denuded platelets was far less than by native platelets, suggesting that amyloid β binding on native platelets was to the O-glycoproteins AOP1 and AOP2 of triplets. Denuded platelets also captured amyloid β where the amyloid β was possibly bound to the GPIIb/IIIa which are exposed in these platelets. Our earlier finding that the ultimate receptor for amyloid β on proteins is the STPS at their O-glycosylated regions support this conclusion since like AOP1 and AOP2 GPIIb/IIIa is also O-glycosylated.

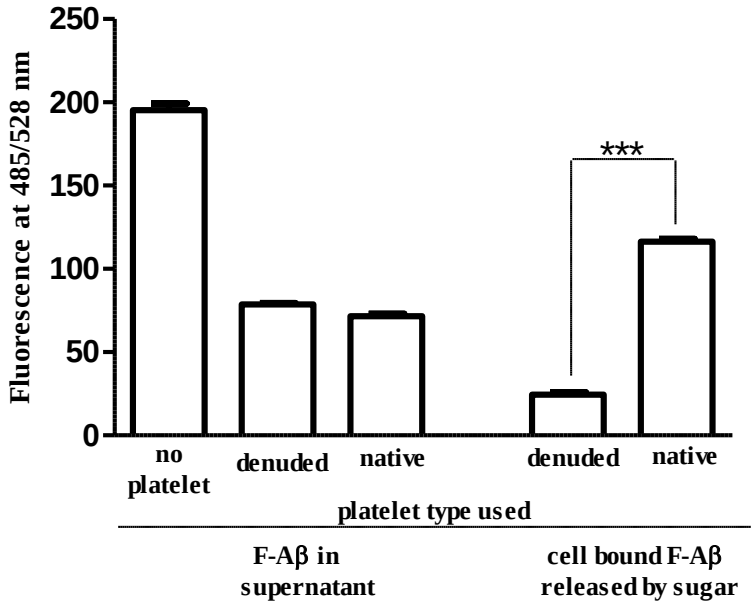


Figure 60. Amyloid-β binds to triplets bound to platelets. *Denuded and native platelets ('methods') in 320 µl PBS (0.25 million cells per µl) were incubated for 3 h with 75 ng fluorescent labeled amyloid β at 25°C. Following centrifugation at 4500 g for 15 min at 25°C fluorescence in supernatant was measured using 300 µl at 485/528 nm. The pellet was washed twice with PBS and incubated for 1 h at 25°C with a mixture of 15 mM each of anti-Gal- and ABG-specific sugars (Mα-G and cellobiose) in 350 µl PBS and the triplet-bound amyloid β now released with specific sugars was measured using 300 µl supernatant as above. ***: P value <0.0001 for the difference between native and denuded platelets in amyloid β released by antibody-specific sugars. Mean ± SD of 9 blood samples.*

DISCUSSION

Platelets play important roles in homeostasis by adhering and aggregating at sites of vascular injury to form an anti-hemorrhagic plug. But platelet aggregation is also a key event associated with pathogenesis of several disorders like immune thrombocytopenic purpura (ITP), hyperglycaemia, in cardiovascular disorders and Alzheimer's disease (Bhatt & Topol, 2003; Sevush et al., 1998). A phenomenon of great clinical importance but largely unexplained at molecular levels is the increased

susceptibility of platelets of diabetes patients to activation and aggregation causing widespread vascular damage observed in these patients.

Platelets surface is known to be rich in O-glycoproteins containing core-1 glycans Galβ1-3GalNAcα1-Ser/Thr (T antigen). Most prominent among them, GPIIb/IIIa, occurs at a density of nearly 80,000 molecules per platelet which is among the highest in any cell known (Wang et al., 2012). Several evidences in the present study indicate the possibility that GPIIb/IIIa is the chief receptor and anchor for anti-Gal/ABG-O-glycoprotein-albumin triplets on platelets. Evidences implicating GPIIb/IIIa in triplet attachment are a) the protection offered by triplet from ADP-mediated aggregation, and b) blocking by pre-bound jacalin of ADP-mediated aggregation of denuded platelets, since GPIIb/IIIa activation is essential for ADP-mediated activation and aggregation of platelets (Coller & Shattil, 2008; Dorsam & Kunapuli, 2004) and c) complete blocking of this adhesion by preincubation of platelets with fibrinogen which directly binds to GPIIb/IIIa. Data reveal that triplets protect platelets from haphazard aggregation apparently by covering its strategic receptors such as GPIIb/IIIa from being recognized by pro-aggregation factors including fibrinogen. Even more significantly, denudation of triplets under hyperglycemic conditions, leading to their enhanced susceptibility to aggregation and activation, explains several unanswered questions in diabetes-driven vascular pathology (Sudic et al., 2006).

Platelets play the role of carriers of amyloid β, thereby acting as a circulating protective sink to reduce its concentration in the brain (Chen et al., 1995). Reports shows that GPIIb/IIIa receptor bind amyloid β on platelets (Donner et al., 2016). Our

results show that this may be true only of denuded platelets and that in circulating normal platelets the AOP1 and AOP2 of triplets mediate amyloid β binding. Triplet binding to platelets is also of major significance to Alzheimer's disease which is accompanied by increased level of platelet activation (Sevush et al., 1998). A general drop in triplet generation that curtails triplet availability for platelets and increases their vulnerability for activation may also independenty cause or worsen Alzheimer's disease due to reduced triplet influx into brain. Alternatively platelets disabled by reduced triplet load may be poor fixers of circulating amyloid β and thus promote Alzheimer's disease. The proposition that triplet O-glycoproteins rather than platelet membrane proteins retain amyloid β is in line with the earlier report that amyloid β is released from activated platelets (Davies et al., 2000). Diabetes is identified as the most common predisposing factor for AD, possibly because high concentration in circulation of glucose that is specific for ABG, but can disassemble both triplets as seen above, gets platelets rid of both anti-Gal and ABG-containing triplets. Though the resulting denuded platelets are capable of amyloid β binding due to exposed GPIIb/IIIa they are more aggregative as shown here and hence less stable. As a result AD patient's blood showed 39.57% increase of platelet aggregates and 53.3 % increase in leukocyte-platelet complexes (Sevush et al., 1998).

Triplet-mediated protection of platelets is also evidenced by the myriad of platelet-mediated vascular injuries accompanying diabetes. Platelet-leukocyte adhesion facilitated by GPIIb/IIIa, which in the case of denuded platelets becomes more exposed, is trigger for synthesis and release of several inflammatory factors by leukocytes, reported in acute myocardial infarction (Neumann et al., 1999) and

stroke (Zeller et al., 2005), underlining the physiological role of the cover offered by triplets to platelets.

5. SUMMARY AND CONCLUSION

Neurodegenerative disorders are leading causes of death and disability in the world today with AD and PD, the most common among them imposing a prolonged burden on patients and caregivers. Extracellular deposition of amyloid β aggregates called amyloid plaques and intracellular deposition of hyperphosphorylated tau aggregates called neurofibrillary tangles are the pathological hallmarks of AD while mostly intracellular deposition of α-synuclein aggregates is the major pathological feature of PD. Though monoclonal antibodies against amyloid β, tau and α-synuclein have been tried as new immunotherapeutic strategy to prevent aggregation of these proteins and thereby slow the progression of these disorders many patients started developing autoimmune disorders even during phase I clinical trials. Search for natural mechanisms that exist in the body to prevent pathological aggregation of brain proteins also appeared relevant. There are previous reports from this laboratory showing recognition of serine- and threonine- rich peptide sequences (STPS) in proteins like lipoprotein(a) by anti-α-galactoside antibody (anti-Gal) which is a natural antibody present in every individual without any deliberate immunisation. Since tau and α-synuclein are STPS-rich human proteins we aimed to check the binding of these proteins by anti-carbohydrate antibodies.

During efforts to purify natural anti-carbohydrate antibodies like anti-Gal and ABG by affinity chromatography two new albumin-associated antibody-binding O-glycoproteins AOP1 and AOP2 of molecular weight 107 kDa and 97 kDa respectively and albumin were copurified showing that antibody, either or both of the O-glycoproteins and albumin were in association in such a way that at least a part of the binding sites in the antibody was still open. We showed that either AOP1 or

AOP2 bridged between anti-Gal or ABG on one side and albumin on the other by binding to both resulting in anti-Gal/ABG-AOP1/AOP2-albumin triplet. We demonstrated that STPS-containing regions of AOP1 and AOP2 that carry their uniquely large number of O-glycans are the ligands recognized by the antibodies. Albumin ensures free binding sites in antibodies even after the O-glycoprotein-albumin complex has bound to it, in contrast to free O-glycoprotein that saturates all binding sites in the antibody. Besides, albumin-bound O-glycoproteins, unlike their free counterparts, are not inhibited by LDL in being recognized by antibody. Almost all anti-Gal and ABG exist in circulation in the form of triplet. Mass spectrometry-based comparison of peptide sequences with those of known proteins showed partial overlap of sequences with those of serum albumin. Since this result arose most probably due to contamination of albumin samples, used earlier for its own sequencing, by AOP1 and AOP2 as shown and the O-glycoproteins possessed size and properties too different from those of albumin for them to be identical with the latter, AOP1 and AOP2 are new additions to the list of plasma proteins. They might have escaped detection so far due to their high glycan content (about 54%) obscuring detection by dye binding and their association with albumin.

In proof of the above structure of the complex involving antibody, O-glycoprotein and albumin in circulation, plasma proteins captured by an albumin-specific affinity matrix and eluted at low ionic strength contained antibodies and both AOP1 and AOP2, in adition to albumin. In further proof of presence of anti-Gal/ABG-O-glycoprotein-albumin triplets in plasma the latter could be captured from plasma either on antibody-specific ligands or the O-glycoprotein-specific lectin jacalin

coated on polystyrene microplates and assayed by tracking the albumin at the other end using enzyme-labeled anti-albumin antibody. Such assays revealed that nearly 36% of plasma albumin is O-glycoprotein-bound, with or without antibody bound to the O-glycoproteins since antibodies are much less in number than O-glycoproteins. As a result there is no detectable free anti-Gal or ABG in circulation.

We showed here that amyloid β-binding activity in plasma which was reported earlier to be carried out by albumin was in fact a function of O-glycoproteins associated with the commercial albumin samples used in the above reports since electrophoretically purified albumin from these samples was inert towards amyloid β while AOP1 and AOP2 were very efficient receptors for the peptide, increasing the fluorescence of fluorolabeled-amyloid β upto 50%. AOP1 and AOP2 were even more effective in binding amyloid β than was α-synuclein which has been reported to be a physiological amyloid β-binding molecule. Amyloid β recognized the STPS in O-glysosylated regions of AOP1 and AOP2. Since STPS is also present on LDL receptor-related proteins (LRP), the known receptors of amyloid β on brain cells, our finding that AOP1 and AOP2, and not albumin are immunologically cross-reactive with LRP confirmed the role of these O-glycoproteins as major amyloid β-binding molecules in plasma. Significantly, though not as much as free AOP1 and AOP2 were, albumin-bound AOP1 and AOP2 were also inhibited by LDL from binding amyloid β, accounting for the observed increased incidence of AD in people with high serum LDL.

Using ligand-induced increase in fluorescence of FITC-labeled antibody as an index of affinity of the ligand we showed that anti-Gal and ABG bind to both tau and α-

synuclein using the sugar-binding site of the antibody for accommodating the latter. This result suggests the possibility that these two antibodies are natural modulators of tau and α-synuclein aggregation given the fact that these are the only antibodies that form triplets with free binding sites mandatorily left free and that cell surface LRP family of receptors containing specific ligands (STPS) for these antibodies are especially abundant in brain. Experimental evidence supporting this possibility was obtained using as model cells macrophages that are the circulating cells richest in LRP. De novo triplets of both antibodies reconstituted from components containing FITC label on either antibody or O-glycoprotein entered macrophages unless antibody-specific sugar or anti-LRP was present, as detected by fluorescence microscopy. Results underline the potential of anti-Gal and ABG as therapeutic antibodies due to their tau- and α-synuclein-binding activity. Such a role of these antibodies in the biology of cognition is also supported by the fact that start of synthesis of anti-Gal, an even more efficient tau- and α-synuclein-binding antibody than ABG which is present in lower animals as well, almost coincided in evolutionary history with the origin of apes and man. Presumably higher cognitive functions demanded in primates by selection pressure necessitated acquisition of more effective modulators of key molecules in learning and memory.

More triplets were found to be associated with platelets than in cell-free plasma in the same blood volume and contained the three components in the same proportion as in plasma. Platelet-bound triplets, bound to the cell surface STPS-rich molecules using the unoccupied binding sites on the antibodies, were extractable with antibody-specific sugars, leaving the resulting denuded platelets vulnerable to aggregation,

more so on adding fibrinogen or ADP. Triplet adhesion therefore appears to be a natural provision to protect platelets from haphazard aggregation, especially when we consider the role of albumin in ensuring free binding sites on antibodies. A steady 1:1 ratio between albumin and immunoglobulins associated with platelets reported earlier is accounted for by the adhering triplet in which antibody, O-gycoprotein and albumin are in 1:1:1 ratio. Curiously sugar moiety specific to either anti-Gal or ABG could dislodge triplets of both antibodies from platelets, apparently due to transiently produced fee AOP1 and AOP2. Pysiological consequence of this effect is that during diabetic hyperglycemia the high glucose level in plasma results in large scale denudation of platelets, their increased aggregation and above all the increased platelet-leukocyte adhesion which is known to be the main cause of ischemia. Thus our results explain in molecular terms the origin of the most prominent but still unexplained contributing factor towards diabetes-driven vascular damage.

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7. ANNEXURES