IMMUNE COMPLEX FORMATION BETWEEN DIETARY AND MICROBIAL POLYSACCHARIDES AND ANTI-CARBOHYDRATE ANTIBODIES

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DECLARATION

I, **Genu George**, hereby declare that I had personally carried out the work depicted in the thesis entitled **"Immune complex formation between dietary and microbial polysaccharides and anti-carbohydrate antibodies"** under the direct supervision of **Dr. P.S Appukuttan**, Professor and Head, Department of Biochemistry, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. No part of the thesis has been submitted for award of any other degree or diploma prior to this date.

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Dr. P. S Appukuttan

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ABBREVIATIONS

ABG	anti-β-glucan
AGA	anti-glucan antibodies
AGE	advanced glycation end products
Anti-Gal	anti-α-galactoside antibody
APC	antigen presenting cells
APS	anti-phospholipid syndrome
BHL	bovine heart lectin
B-NSMAg	biotin labelled NSMAg
CIC	circulating immune complex
CLGG	cross-linked guar galactomannan
CNBr	cyanogen bromide
CPL	circular polarisation of luminescence
CRP	C-reactive protein
CSBG	Candida species β(1–3) glucan
CVD	cardiovascular diseases
DEAE	diethyl aminoethyl
DIg	dextran binding immunoglobulins
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
FDNB	1-fluoro 2-4-dinitrobenzene
FITC	fluorescein isothiocyanate
Gal-1	galectin-1

GalNAc	N-acetyl galactosamine
GCs	germinal centers
GlcNAc	N-acetyl glucosamine
HPL	human placental lectin
HRP	horse radish peroxidise
HSA	human serum albumin
HSPs	heat shock proteins
IC	immune complexes
IE	infective endocarditis
Ig	immunoglobulin
LacNAc	N-acetyl lactosamine
LIg	lactose-binding immunoglobulins
Lp(a)	lipoprotein (a)
LPS	lipopolysaccharides
2ME	2-mercaptoethanol
MHC	major histocompatibility complex
NAbs	natural antibodies
NDS	non-dialysable sugar
NSMAg	negatively charged *S mutans* antigens
OPD	ortho-phenylenediamine
Ox-LDL	oxidised-LDL
PAAP	platelet aggregation associated protein
PAGE	polyacrylamide gel electrophoresis
PAc	protein antigen c

PBS	20 mM potassium phosphate buffer containing 150 mM NaCl, pH 7.4
PBS-T	PBS containing 0.05 % Tween-20
PEG	polyethyleneglycol
PpiA	lipoprotein prolyl-cis/trans isomerase
PRRs	pattern recognition receptors
PSMAg	positively charged *S mutans* antigens
RA	rheumatoid arthritis
RES	reticuloendothelial system
RGP	rhamnose glucan polymer
SLE	systemic lupus erythematosus
SMBP	*Streptococcus mutans* binding proteins
STPS	serine- and threonine-rich peptide sequence
TAG	terminal α-galactoside
TEMED	N,N,N',N'- tetramethyl ethylene diamine
TD	T-lymphocyte-dependent
TF	thomson Freidenreich antigen
Tg	thyroglobulin
TGF-β	transforming growth factor-β
Th	T-helper cells
TI	trypsin inhibitor
TI	T-lymphocyte-independent
TIC	trypsin inhibitor-cellobiose
TIg	total immunoglobulin fraction

TIM	trypsin inhibitor-melibiose
TLRs	toll-like receptors
VBS	veronal-buffered saline
YBG	yeast-β-glucan

SYNOPSIS

Studies on cardiovascular disease (CVD) pathogenesis have focussed traditionally on dislipidemia. With increasing appreciation of inflammatory pathogenesis in CVD, infections, including those caused by oral bacteria are implicated in CVD progression. Dietary and microbial antigens are reported to enter circulation and get exposed to the immune system. Polysaccharide antigens are the most common and most abundant antigens expressed by commensal or pathogenic microbes. The antigenic potential of polysaccharide antigens accrue not only due to their abundance but also due to their cell surface location. As a result anti-glycan antibodies (AGA) constitute the major part of antibodies raised against these microbes. Recognition of these microbial or dietary antigens by natural or induced AGA can also lead to the formation of immune complexes (IC). High level of circulating IC per se has been correlated with vascular pathology and CVD. AGA immune complexes are relatively more potential pathological mediators since it is reported that AGA are enriched in IgA that has tendency for tissue deposition disorders such as IgA nephropathy, since investigations in this laboratory had shown that among immunoglobulins IgA is particularly strong ligand for tissue-derived lectin galectin-1.

Apart from gut and other epithelial microbiota and diet, polysaccharide and other glycoprotein infusion into circulation can take place through dental infections. It is reported that oral infections with bacteria such as *Streptococcus mutans* can increasingly predispose subjects to CVD. A notable feature of such bacteria is the production and secretion of α- and β-glucans of very high molecular size. Dental plaques are known to contain deposits of such glucans. *Streptococcus mutans* is one

of the major organisms reported in various vascular pathology including endocarditis, ulceritis, and several other inflammatory diseases. Another route of entry of high molecular weight glucan into the body is through edible sugar marketed in tropical regions, which has been found to contain high molecular weight polymers interacting with serum antibodies. These glucans are synthesised by bacteria that contaminate sugarcane harvest.

In this project we indent to examine the potential of dietary and microbial antigens to form IC with naturally occurring antibodies and to study their characteristics crucial for recognition by tissue lectins.

Objectives:

1. To resolve and characterize high molecular weight dextran or glycoproteins present in contaminated edible sugar.

2. To identify human serum antibodies that react with polysaccharides released by the major bacteria causing dental plaques, viz. *Streptococcus mutans*

3. Isolation and characterisation of constituent polysaccharides /glycoprotein antigens in *S mutans*

4. Examine formation of immune complexes on mixing polysaccharides from cariogenic bacteria with normal serum.

5. To study the stoichiometry and residual binding sites in immune complexes formed of polysaccharides described above and specific antibodies

6. To study the composition of microbial antigen ICs in terms of their susceptibility to tissue deposition mediated by galectin-1.

Methods:

Anti-glycan antibodies (dextran binding immunoglobulins or DIg, anti-β-glucan antibody or ABG and anti-α-Galactoside antibody or anti-Gal) and lectins (ConA, jacalin, bovine heart and human placental galectin-1 [BHL and HPL]) were prepared by affinity chromatography. High molecular weight polysaccharides from commercially available sugar samples were prepared by removing small sugars by dialysis or by precipitating using 50% alcohol. Characterisation of anti-carbohydrate antibody and lectin binding to these polysaccharide antigens were done by ELISA. *Streptococcus mutans* (MTCC 890) cells mixed with Biogel-P4 was used as an affinity matrix to isolate plasma antibodies recognising the cell surface antigens of *S mutans*. Specificity of *Streptococcus mutans* binding proteins (SMBP) were checked on dextran, β-glucan and TIM coated wells for capturing SMBP and probing with anti-Ig-HRP. The immunoglobulin distribution of SMBP was assayed by direct coating followed by probing with anti-Ig-HRP. Crude antigens from *S mutans* released by freeze thawing and ultrasonication was further resolved by chromatographic (gel filtration and ion exchange) and electrophoretic separation (acid PAGE). The binding specificity of naturally occurring anti-carbohydrate antibodies to different antigens from *S mutans* were done using ELISA and the subsequent conformational changes in antibodies upon antigen binding were checked using FITC-labelled antibodies using a new protocol developed for this purpose. Purified antibody of defined specificity was FITC-labeled in presence of specific low molecular weight antigen to protect binding site and purified by gel filtration. Binding of macromolecular but not low molecular weight antigens produced substantial fluorescence enhancement in the FITC-antibody due to Fc activation. *De novo* IC

with *S mutans* antigens were prepared by mixing with immunoglobulin rich (plasma Ig) fraction. The latter was obtained as bottom 30% fraction after ultracentrifugation at 202,000 g of plasma from which high molecular weight proteins had been removed earlier by 20% ammonium sulphate precipitation. PEG (2%) was used for the precipitation of IC. ICs were also prepared using biotin labelled *S mutans* (NSMAg) antigen. The incorporation of biotin labelled antigen in IC were detected by capturing the IC on streptavidin-coated wells followed by probing with anti-Ig-HRP. The immunoglobulin distribution in IC was also determined by a similar protocol. Participation of naturally occurring ABG and DIg in formation of IC with *S mutans* antigens was confirmed by the substantial reduction in IC formation by plasma Ig fraction from which ABG and DIg were removed. Presence of free binding sites on *S mutans* antigen ABG complex was detected using ELISA. The ability of these ICs to get recognised by tissue galectin-1 was checked by using immobilised galectin-1.

Results and Discussion:

High molecular weight polysaccharides obtained from commercially available sugar samples contained antigenic epitopes for naturally occurring DIg and ABG molecules, with DIg being slightly better than ABG in recognising them. DIg molecules are known to be multi-specific, with the antigen binding site capable of recognising either α-D-glucoside or α-D-galactoside group. There was a wide variation in the α-gal binding ability of different DIg molecules. The α-Gal binding DIg which is more abundant than anti-Gal often encounter dextran-like polysaccharides many times daily. These α-Gal specific DIg-IC may contain additional free binding sites (as has been shown in the case of Lp(a)-bound anti-Gal) which may enable them binding in turn to host cell surfaces.

Streptococcus mutans is a commonly occurring oral bacteria causing dental infection and plaques. There is a high possibility of these microorganisms gaining entry into circulation. There are several reports of the existence of these organisms in extirpated heart valves as well as in atherosclerotic plaques although the actual mechanism of their localisation at these sites is not yet clear. We here demonstrated that naturally occurring antibodies, mostly IgG recognizing surface antigens of *S mutans*. The cell surface antigen from *S mutans* was resolved into different components and the negatively charged protein antigens (NSMAg) were most reactive against anti-carbohydrate antibodies. ABG antibodies were particularly superior to other anti-carbohydrate antibodies in recognising *S mutans* antigens. The ABG binding to *S mutans* antigens were further confirmed by fluorescence enhancement of FITC-labelled ABG upon antigen binding. Results suggested that fluorescence enhancement upon antigen binding could be used as a tool to measure the antigen binding affinity of an antibody.

These antigens (NSMAg) when treated with plasma Ig fraction were capable of forming immune complexes. ABG and DIg were the major antibodies forming ICs with *S mutans* antigen since removal of ABG and DIg caused considerable reduction in IC formation.

ABG occupied by *S mutans* antigen (NSMAg) can as well bind to a β-glucan ligand on a macromolecule confirming the presence of free binding sites on ABG-NSMAg complex and availability of this unoccupied binding site for binding to a different antigen. The occurrence of free binding site could be due to the steric constraint in accommodating two large ligands simultaneously on both binding sites leaving the complex still capable of binding to other cell surface ligands.

Anti-glycan antibodies including ABG are reported to be particularly enriched in polymeric IgA which is a strong ligand for Galectin-1. There was a significantly higher IgA content per unit protein in NSMAg-IC compared to plasma IC in general. Galectin-1 which is the most ubiquitous carbohydrate binding lectin on mammalian surfaces and interior is a possible route through which ABG-NSMAg- IC could get deposited at the tissue surface. In summary polysaccharides and other glycoconjugates introduced into host through food and other environmental and microbial factors pose a challenge when the former are indigestible by the body and when antibodies recognizing these antigens are present in circulation. Since these antigens are recognized by naturally occurring antibodies in healthy individuals the immune complexes thus formed could cause vascular diseases and inflammatory reaction at the tissue surface.

SIGNIFICANCE:

The results from fluorescence studies suggest that conformational shift in Fc produced by occupation of binding sites by large antigens resulted in the enhancement of fluorescence of FITC tags on Fc. Data thus provides a tool for detection and measurement of specific ligands using fluorolabeled whole antibodies.

We demonstrate that ABG and DIg were among the major anti-carbohydrate antibodies recognising *Streptococcus mutans* antigen to form circulating ICs. This recognition and IC formation offers a possible mechanism for IC-mediated vascular inflammation mediated by complement and Fc receptors. Significantly higher content of the galectin-1 binding IgA and free antigen binding site remaining are other factors facilitating their tissue deposition.

INTRODUCTION

Cardiovascular diseases (CVD) now recognised as the leading cause of death worldwide and it is being expected that by 2020, it would prevail as the leading cause of death and disability over infectious diseases globally (Gupta et al., 2013). Advances in understanding pathogenesis of various cardiovascular diseases have stimulated interest in the identification of several novel risk factors for CVD. It has been now well established that inflammation of the endothelium plays an important role in the pathogenesis of atherosclerosis and this knowledge had lead to the identification of novel risk factors. These risk factors include lipid variables (small, dense, low density lipoproteins, oxidised low-density lipoproteins, apolipoprotein B), inflammatory marker like CRP level, immune complexes, bacterial and viral infections, thrombogenic/hemostatic factors (fibrinogen, homocysteine).

It is now widely recognised that the evolving atherosclerotic lesion has all the characteristic of an inflammatory reaction, including the presence of various immune cells in the atherosclerotic plaque. In addition to this non-cellular components of immunity including immunoglobulins, specific disease-related pathogens as well as CRP and complement were also found (Binder et al., 2005). Deposition of Ag-Ab complexes (immune complexes or ICs) in tissues underlies the pathogenesis of a range of human autoimmune diseases from glomerulonephritis, systemic lupus erythematosus (SLE), arthritis and transplantation rejection to rheumatic fever. Fc receptor signalling and complement activation may modulate the inflammation in atherosclerosis by IC's and antibody isotype may direct the role that IC's play in atherogenesis (Burut et al., 2010). Antibodies or B1 cells alone or in the form of IC may play an important role in the pathogenesis of atherosclerosis

(Hollander et al., 1979). Complement participates in the elimination of IC so that deposition of IC in various organs occurs in complement deficient and depleted states and also in non-complement fixing IC (IgA-IC) (Schifferli and Taylor, 1989).

Naturally occurring anti-carbohydrate antibodies:

A wide variety of natural antibodies or immunoglobulins generated by B1 cells are directed against glycans (Bovin, 2013) and they represent the first line of defense against invading pathogens which are involved in the removal of dysfunctional or malignant cells and particles (Huflejt et al., 2009). They are present in the sera of all individuals in the absence of deliberate immunisation (Shoenfeld and Isenberg, 1989) and are thus part of the innate immune system. There is both a bacterial paradigm theory (Springer and Horton, 1969) and germline theory (Coutinho et al., 1995) explaining the occurrence of natural antibodies. The naturally occurring anti-carbohydrate antibodies include, anti-blood group antibodies, anti-Gal, DIg, ABG, LIg, antibodies against Forssman glycolipid antigen etc. Glycan epitopes entering the human body through diet, environment and microbes are enormous and are capable of offering antigenic epitopes for naturally occurring anti-carbohydrate antibodies.

Most anti-carbohydrate antibodies are polyreactive and the ligand binding pocket are found to be more flexible and thereby are capable of binding to a wide variety of different antigens that too in different intensities (Notkins, 2004). Avrameas (1991) reported the role of polar amino acids in the complementarity determining regions to be responsible for the reaction of natural antibodies with

various antigenically unrelated antigens. It is estimated that around 20 % of the B-cells in the peripheral blood are involved in the production of polyreactive antibodies (Schwartz-Albiez, 2012) and most of them were found to belong to the IgM class.

Polysaccharide antigens:

Glycans being the predominant surface components of cells such as erythrocytes, immune cells and microorganisms, they can give rise to anti-carbohydrate antibodies of all classes. Anti-carbohydrate antibodies are now being considered to represent a new class of risk factor that is associated with advanced atherosclerosis (Mosedale et al., 2006). Two of the polysaccharide antigens which we come across daily from microbial and dietary sources are β-glucan and dextran. β-Glucan is found as a major component of the cell wall of plants and fungi. As traces of plant and fungal cell wall are ubiquitous in our environment, there is a high possibility of their entry into our body and thereby eliciting an immune inflammatory reaction. Plant β-glucan concentration in normal individual is found to be less than 10 pg/ml and is found to increase to as high as 1 ng/ml in systemic fungal infections (Obayashi et al., 1995). Eventhough β-glucan in small amount is a very poor immunogen, the abundance of these polysaccharides in the environment and the presence of natural antibodies recognising β-glucan (ABG) in healthy human plasma may contribute to its immune inflammatory potential (Masuzawa et al., 2003, and Geetha et al., 2007).

Dextran, another class of polysaccharide antigen found as bacterial extracellular polysaccharides. One of the major organism involved in dextran synthesis is *Leuconostoc mesentroides* which infect the cane sugar (Sidebotham, 1974). They are also synthesised by dental plaque forming bacteria *S mutans*. Most of these bacteria employ dextran as a protective coating to evade host phagocytes (Meddens et al., 1984). The antigenicity of dextran first reported by Kabat and Berg (1953) was found to increase with its molecular weight (Kabat and Bezer, 1958). Naturally occurring DIg were found in healthy human plasma (Kraft et al., 1982, Anastase et al., 1996 and Chacko and Appukuttan, 2003). Both DIg and ABG were found to be particularly enriched in polymeric IgA (Geetha et al., 2007 and Paul et al., 2011) and IgA is a potent ligand for tissue galectin-1 (Sangeetha and Appukuttan, 2005). The level of anti-dextran antibodies was elevated in several pathological conditions including IgA nephropathy (Kennel et al., 1995 and Palosuo and Milgrom, 1981).

Role of immune complexes in vascular pathology*:*

In support to the suggestion that atherosclerosis is a chronic inflammatory disease, studies have demonstrated the occurrence of antigens and their cognate antibodies in the serum of patients with CVD. The IC's being proinflammatory may be involved in the progression of atherosclerosis (Tsimikas et al., 2007). Several of the autoimmune diseases associated with immune complex diposition such as SLE, anti-phospholipid syndrome and rheumatic arthritis are now known to be established risk factors for CVD (Turesson et al., 2008). The formation of IC is a protective

mechanism but under situations of chronic inflammation, secondary events associated with IC formation such as complement activation and Fc receptor cross-linkage may mediate disease process leading to organ/tissue injury such as seen during atherosclerosis. Several complement activation products have been detected in atherosclerotic lesions. The IC-C1q complex is capable of binding to C1q receptors on endothelial cell surface and this binding will stimulate the expression of several cell adhesion molecules and down-regulate the expression of cholesterol-27-hydroxylase which is involved in the removal of cholesterol from the arterial wall (Galkina and Ley, 2007). Human atherosclerotic lesions were detected to contain all three antibody classes, IgG, IgM and IgA (Hollander et al., 1979).

Infection-associated risk for CVD:

The high concentration of WBC within the plaque is influenced by several factors including infection (Dunne, 2000). The role of infection in atherosclerosis was first suggested over hundred years ago with the finding that acute infection with *Bacillus typhosus* resulted in fatty sclerotic changes in the arterial wall (Nieto, 1998). The release of various pro-inflammatory mediators in response to bacterial LPS, HSP and IC's are capable of initiating an immune response in the arterial wall. Another route through which infection may result in the initiation or progression of an atherosclerotic lesion involves the dissemination of microorganisms from the site of infection directly to the endothelial cell surface (Kozarov et al., 2005). Among the viral pathogens associated with CVD include cytomegalovirus and Herps simplex virus and the bacterial pathogens involved include various dental organisms,

Helicobacter pylori, *Chlamydia pneumonia* and *Mycoplasma pneumonia* (Danesh, 1999).

Oral infections in CVD:

A vast number of studies have now recognised that oral inections may be a risk factor for CVD. Oral cavity can act as a site of dissemination of pathogenic organisms to distant body sites in immune-compromised hosts (Li et al., 2000). The anatomic proximity of these microfloras to the blood stream could facilitate bacteraemia and systemic spread of bacterial products and IC's.

Oral infections can affect systemic health by several possible mechanisms, by direct transfer of infection from the oral cavity into deeper adjacent tissues, by passage of inflammatory mediators from the periodontium into the circulation and thereby affecting distant sites (atherosclerosis), by penetration of oral bacteria into the systemic circulation to cause infections to distant sites (endocarditis, thrombosis and atherosclerosis) and by the spread of bacterial products or bacteria-induced host products to distant mucosal sites to promote or exacerbate disease (Scannapieco et al., 2003).

There are several studies suggesting the direct effects of oral bacteria on atheroma formation including the detection of oral bacteria like *P gingivalis* and *S mutans* in carotid and coronary atheroma (Nakano et al., 2008 and Nakano et al., 2006a) and studies related to platelet aggregation associated with thrombus formation by Streptococcus and Porphyromonas species (Herzberg and Meyer, 1996). Indirect effects include the elevation in the level of CRP and fibrinogens

which are recognised as independent risk factors for CVD (Shklair et al., 1968). Oral infections being so common, oral bacterial products could be a potential source of antigen for naturally occurring anti-carbohydrate antibodies in healthy human plasma.

Objectives of the work:

1. To resolve and characterize high molecular weight dextran or glycoproteins present in contaminated edible sugar.

2. To identify human serum antibodies that react with polysaccharides released by the major bacteria causing dental plaques, viz. *Streptococcus mutans*

3. Isolation and characterisation of constituent polysaccharides/glycoprotein antigens in *S mutans*

4. Examine formation of immune complexes on mixing polysaccharides from cariogenic bacteria with normal serum.

5. To examine residual binding sites in antibodies involved in forming immune complexes with polysaccharides described above.

6. To study the composition of microbial antigen ICs in terms of their susceptibility to tissue deposition mediated by galectin-1.

REVIEW OF LITERATURE

2.1.Glycoproteins:

Most eukaryotic proteins are glycoproteins in which the glycan (carbohydrate) parts are involved in a variety of biological processes such as intra- and extracellular signalling and in the mediation of cell-cell interaction. In proteoglycans saccharides is the dominant part while in glycoproteins they constitute only a small portion of the entire molecule (Roth et al., 2012). The attachment of sugar residues to proteins is one of the most complicated co- or post-translational modification that a protein undergoes (Spiro, 1973). Enzymatic glycosylation is controlled by factors that differ greatly among cell types and species. The formation of a sugar aminoacid bond is the most defining event in the biogenesis of peptide-linked oligosaccharides and in most of the cases it determines the nature of the carbohydrate units which in turn influences the protein's biological activity (Spiro, 2002).

Five distinct types of sugar peptide bonds have been identified viz. N-glycosyl, O-glycosyl, C-glycosyl, P-glycosyl and glypiation. N-glycosidic bond involves the β-glycosylamine linkage of GlcNAc to Asn and is one of the most widely distributed carbohydrate peptide bond. In O-glycosylation, sugar is attached to an aminoacid containing a hydroxyl group. The C-mannosyl or C-glycosyl bond is a newly detected carbohydrate-protein linkage involving the attachment of a α-mannosyl residue to the C-2 of Trp through a C-C bond. Another distinct type of glycopeptide linkage involves attachment of sugar to a protein by a phosphodiester bond and GlcNAc, Man, Xyl and Fuc have been found to be involved in this. GPI

anchor is an example for glypiated linkage in which Man is linked to phosphoethanolamine which in turn is attached to the terminal carboxyl group of the protein (Spiro, 2002).

All cells in nature are covered with a dense coating of glycan and are required for various biological processes as well as for the binding of pathogens to them. Hosts evade the more rapidly evolving pathogens that infect them by changing their glycan expression patterns without compromising their own survival and thus the cell surface glycans may be trapped in the never-ending cycles of evolutionary "red queen" effects (Varki, 2006).

The biosynthesis of glycans is primarily determined by glycosyltransferases that are capable of assembling monosaccharide moieties into linear and branched chain glycans. Glycosyltransferases constitute a very large family of enzymes. A few of the enzymes involved in the biosynthesis of glycans are glycosidases which can remove monosaccharides to form intermediates which are acted upon by glycosyltransferases (Varkey, 2009).

2.1.1. N-Glycans:

N-glycans are covalently attached to protein at Asn residues by an N-glycosidic bond. The N-glycans were initially described in ovalbumin and later were demonstrated on many secreted and membrane bound glycoproteins (Johansen et al., 1961). The addition of N-glycans occurs at the Asn residue at consensus sequences Asn-X-Ser/Thr where X can be any aminoacid other than proline. Five different n-

glycan linkages have been so far reported of which the most common one is the N-acetylglucosamine to Asn (GlcNAcβ1-Asn) (Varkey, 2009).

N-glycans are classified into 3 types and all of which share a common core sugar sequence, Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn-X-Ser/Thr. The three types of N-glycans are 1) oligomannose in which only mannose residues are attached to the core; 2) complex type in which "antennae" initiated by an N-acetylyglucosaminyltransferases (GlcNAcTs) are attached to the core; and 3) hybrid type in which only mannose residues are attached to Manα1-6 arm of the core and two or more antennae are on the Manα1-3 arm (Kornfeld and Kornfeld, 1985).

Although the presence of Asn-X-Ser/Thr sequon is necessary for the synthesis of N-glycans, conformational or other constraints during glycoprotein folding makes these sequences inaccessible to the glycosyltransferase enzyme and thus the transfer of N-glycan to this sequence does not always occur. The identity of X can also reduce the efficiency of glycosylation (Kelleher and Gilmore, 2006). These glycans are initially synthesised on a lipid like molecule called dolichol-phosphate followed by "en bloc" transfer of the entire glycan of 14 sugars to proteins (Varkey, 2009). Initial linkages involving the two core GlcNAc monosaccharides and the first five mannose residues occur on the cytosolic side of the ER membrane. The monosaccharide donors include UDP-GlcNAc and GDP-Man for the first seven linkage reactions and it occurs on the cytosolic side of the ER membrane. The dolichol-oligosaccharide precursor flips across the membrane

bilayer and become oriented in the lumen of the ER. Four mannose residues are then added in succession using dolichol-phosphate mannose as donor. The assembly is completed by the addition of three glucose residues donated by dolichol-phosphate-Glc and now the dolichol linked oligosaccharide precursor consisting of 14 saccharide units is ready for transfer to Asn residue on proteins. The terminal three glucose residues are associated with protein folding (Rosner et al., 1982).

2.1.2. O-glycans:

The modification of Ser/Thr residues on proteins by the addition of a GalNAc results in an O-linked oligosaccharide or O-glycan. Here the sugar is attached to an aminoacid with a hydroxyl group (Eg: Ser, Thr, Tyr, Hyp, Hyl) (Spiro, 2002). O-glycan biosynthesis is simpler than N-glycan in that a lipid-linked oligosaccharide precursor is not required for transfer to protein. It is initiated by the addition of the monosaccharide GalNAc (from UDP-GalNAc) to mainly Ser and Thr residues catalysed by a polypeptide GalNAc transferase.

The GalNAc-α-Ser/Thr linkage has been considered a hallmark of mucins. However a wide variety of glycoproteins are known to contain this linkage such as fetuin, human gonadotrophins, glycophorins and anti-freeze proteins (Spiro, 1973). The β-anomer of this linkage has been reported to occur in the archaebacterium *Aneurinibacillus thermoaerophilus* (Schäffer et al., 1999). O-glycans can thus be classified as mucin type and non-mucin type. Non-mucin type glycans include α-linked fucose, β-linked-O-xylose, α –linked-O-mannose, β-linked O-GlcNAc (N-

acetylglucosamine), α- or β-linked O-galactose and α- or β-linked O-glucose attached to the hydroxyl group of the aminoacids.

O-glycan structure formed by Gal-NAc transferase action include four common subtypes. Core 1 to core 4 O-glycans comprise the majority of O-glycan structures with most of them being of the core 2 subtype. Others are less common and further complexities in O-glycan diversification continue to arise with novel core subtypes being discovered.

O-glycan	**Structure**
Core	
Tn antigen	GalNAcαSer/Thr
Sialyl-Tn antige	Siaα2-6GalNAcαSer/Thr
Core 1 or T 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

Table:1. Structure of O-glycan core found in mucins (Varkey, 2009).

The biosynthesis of O-glycans can be modified and terminated with the addition of sialic acid residues. These sialic acid addition gives rise to a series of O-glycan structures that generally restricts further biosynthesis steps.

2.1.3. Biological role of glycans:

Glycans have many protective, stabilising, organisational and barrier functions. For example, glycans attached to proteoglycans are important for the maintenance of tissue structure, porosity and integrity. The externally located glycan on most glycoproteins is involved in protecting the underlying polypeptide from recognition by proteases or antibodies. Other functions include regulation of protein folding, cell adhesion, molecular trafficking and clearance, receptor activation, signal transduction and endocytosis. In all of evolution, there wouldn't be an example of cell that is not covered by glycans. The complexity and structural plasticity of glycans makes them suited to be at the frontlines of our constant evolutionary conflict with invading microbes (Hart, 2013). The diversification of glycans is most likely to be led by the heightened evolutionary pressure of being at the frontlines of cellular collaboration. The glycoconjugate coating covering a whole cell can present a 'glycocalyx' forming a protective barrier. The proteins that are incorrectly glycosylated fail to fold properly and are consequently degraded by the proteosome machinery. While supporting evidences exist for each of the theories regarding oligosaccharide function exception to each can also be observed (Varki, 1993a).

The involvement of lectins that are by definition glycan-binding proteins in cell-cell adhesion was best characterised for selectins and their glycan ligands (Lowe, 2003). This interaction is known to play an important role in leukocyte trafficking responses that are essential for immune system homeostasis, hematopoiesis and inflammation (Rosen, 2004). Glycosylation was also reported to modulate cell-cell adhesion in early mammalian embryos.

The role of glycosylation in self/non-self recognition is based on the ability of mammalian lectins to recognise glycans from divergent organisms such as bacteria, yeast and invertebrates. There are several receptors like Toll-like receptors which can activate immune system by binding to bacterial glycan ligands (Barton and Medzhitov, 2003)

The best studied example of the importance of glycans in trafficking is the role of mannose-6-phosphate residues in targeting newly synthesised lysosomal proteins to their final destination in lysosomes from the ER golgi compartment (Varki, 1993a).

Another structural/modulatory role of glycan is to act as a protective storage depot for biologically important molecules. They are also involved in endocytosis which plays an important role in providing access to material from extracellular compartments, directing molecular cargo to distinct organelles, and in inducing the turnover of cell surface molecules. Mammalian glycans produced in the Golgi can modulate the endocytosis of cell surface glycoproteins and thereby can control receptor expression (Ohtsubo and Marth, 2006). Just as certain oligosaccharides act

as traitorous signspots for microbial and immune attack, some can serve to abrogate these detrimental reactions. In these cases the addition of specific monosaccharides or modifications can mask the sequences recognised by microorganisms or autoimmune antibodies. For example the extension of the oligosaccharide chain of GM1 would prevent the binding of cholera toxin. Oligosaccharide sequences on soluble glycoconjugates such as mucins can act as 'decoys' for microorganisms and parasites. Thus pathogenic organisms attempting to gain access to mucosal membranes might first encounter their cognate oligosaccharide ligands attached to soluble mucins (Varki, 1993a).

Glycosylation can modulate interactions of receptors and ligands with themselves, co-regulatory molecules and with distinct membrane domains of intact cells and can thereby alter signal transduction. For example, fibroblast growth factor receptors bind to specific heparin sulphate glycosaminoglycans on some proteoglycans and thereby fascilitate the co-presentation of ligand monomers to achieve receptor dimerisation and activation (Ohtsubo and Marth, 2006).

Defects in catabolic steps like endocytosis and trafficking to lysosomes include glycosidase deficiency and it forms the basis for several cellular storage diseases such as Gaucherr's, Niemann Pick, Sandhoff's and Tay Sach's disease. Example for the defect in anabolic process of glycan formation include I-cell disease resulting from the failure to produce mannose-6-phosphate modification on N-glycans in Golgi which is required for trafficking of hydrolases to lysosomes. In addition to this several other diseases have also been recognised which include IgA

nephropathy, muscular dystrophies, paranoxisomal nocturnal hemoglobinuria etc (Ohtsubo and Marth, 2006 and Varki, 1993b).

2.2. Natural Antibodies (NAbs):

Natural antibodies (NAbs) are usually defined as antibodies that are found in normal individuals in the complete absence of stimulation by any exogenous antigen. The observation that the occurrence of NAbs to toxins, bacteria and erythrocytes are present in the sera of normal individuals goes back to the beginning of immunology with the observation of anti-B agglutinins in the sera of group A individuals and vice-versa (Schwartz, 2007). They form an important part of the house keeping function by facilitating the clearance of damaged cells and cellular debris. In addition to their capacity to bind microbial structures, they are known to be autoreactive with their ability to bind to self structures (Boes, 2000). Most of the NAbs were found to be polyreactive and this property is required for the rapid and immediate recognition and protection against invading pathogens (Binder et al., 2005). Analysis of these antibodies have revealed that they are predominantly of IgM type followed with IgG and IgA and are found to react with more than two antigens, although monospecific antibodies are also present (Avrameas, 1991).

2.2.1. Naturally occurring anti-carbohydrate antibodies:

Several research groups have reported the existence of antibodies against carbohydrate structures and they are classified as natural antibodies since they are present in healthy individuals without any immunisation (Shoenfeld and Isenberg, 1989) and are thus a part of the innate immune system. The recognition of

carbohydrate antigens by antibodies began with the observation of Dochez and Avery (1917) when a substance in the culture fluid of Pneumococci was precipitated specifically by anti-sera against Pneumococcus. Later the precipitating substance was identified to be polysaccharide derivatives from bacterial surface (Heidelberger and Avery, 1924). Although most of the anti-carbohydrate antibodies are classified as natural antibodies, some are produced after contact with an exogenous antigen. Most of these antibodies have been suggested to play an important role in house-keeping functions. There are reports of these anti-carbohydrate antibodies as part of an anti-tumour immune response while their presence in healthy individuals is still waiting for a plausible explanation (Schwartz-Albiez, 2012).

The best known and studied NAbs are directed to blood group antigen A and B. A variety of human anti-carbohydrate antibodies act against antigens which occur frequently in bacteria or non-human mammalians as are the anti-Gal antibodies specific to (Galα1-3Galβ1-4GlcNac) and comprises around 1% of immunoglobulins (Galili et al., 1984). Other naturally occurring anti-carbohydrate antibodies in normal healthy plasma include dextran binding immunoglobulins (DIg), anti-β-glucan (ABG) and lactose binding immunoglobulins (LIg). Others include antibodies to Forssman glycolipid antigen (GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc) (Huflejt et al., 2009) and to tumour associated antigens such as Galβ1-3GalNAcα (TF, Thomson-Friedenreich antigen) and GalNAcα1-OSer/Thr (Tn) (Springer, 1984). Bovin (2013) classified NAbs to glycans into three groups i) conservative antibodies that is practically the same in all healthy donors with respect to their epitope specificity and level in the blood, ii) alloantibodies to blood group

antigens and iii) plastic antibodies related to the first or second group changes considerably during diseases and some temporary conditions, in particular inflammation and pregnancy. Antibodies from the third group proved to be prospective markers of a number of diseases.

2.2.2. Origin of anti-carbohydrate antibodies and their polyreactive nature:

A majority of anti-carbohydrate antibodies seem to be derived from the pool of NAbs produced already during fetal life, which do not need a stimulus by an exogenous antigen. Evidence for this is based on the immunoglobulin structure of autoantibodies including those against carbohydrates. Nonmutated germline variable region configuration of genes encoding heavy and light chains is a feature of NAbs (Siminovitch et al., 1989).

Another hypothesis suggests the occurrence of these anti-carbohydrate antibodies in response to bacterial lipopolysaccharides colonised in the gastrointestinal tract. The presence of these anti-carbohydrate antibodies thus reflects the large variety of bacterial oligosaccharides to which the young organisms are confronted with (Springer and Horton, 1969).

Competition between invading pathogenic microorganisms and the host defence system to evade this attack might also play an important role in creating the diversity of surface oligosaccharides on both sides and for the pathogens to continuously invent new mechanisms of mimicry to escape recognition by the immune system. The complexity of carbohydrate structures of our body may have further arisen by our need to both evade pathogenic relationships and to co-evolve

symbiotic relationships with our non-pathogenic resident microbes. Apart from anti-carbohydrate antibodies, the host defence system has also developed glycan-binding proteins/lectins which are known to function in the control of innate and adaptive immune reponse (Gagneux and Varki, 1999).

B-lymphocytes producing these antibodies belong to the splenic marginal zone B-lymphocytes and B1 type of the peritoneal and pleural cavities. They are characterised by the expression of CD5 ($CD5^+$ B-cells). $CD5^+$ B-cells have been involved in the synthesis of natural antibodies although $CD5^-$ B-cells may also participate in their production (Avrameas, 1991). B1 cells are further classified into B1a and B1b cells depending on their function. While B1a cells produce NAbs, which are stimulated by the danger signals as part of the innate immune system, B1b cells and splenic marginal zone B cells are responsible for the production of the anti-carbohydrate antibodies. Although it is the B2 cells which undergo affinity maturation, peritoneal B1 cells mainly of IgA and much less of IgM antibodies of B1b cells may undergo somatic hypermutations (Foote and Kearney, 2009). Although anti-carbohydrate antibodies are predominantly of IgM class (Avrameas, 1991), one may also find IgG class of anti-carbohydrate antibodies with somatic mutation in the variable region which is consistent with an antigen driven process. Most of the anti-carbohydrate antibodies belong to the class of NAbs, one cannot exclude the presence of those generated by an antigen-driven process.

Most NAbs including the anti-carbohydrate antibodies are polyreactive and the antigen binding pocket are found to be more flexible and thereby capable of

binding to several different antigens that too in different intensities. The antigen antibody reaction of classic monoreative antibodies has been described as lock and key in which the antigen binding pocket of the antibody and an epitope on antigen were complementary and rigid in nature. In contrast, polyreactive antibodies are known to change conformation at the antigen binding site in order to accommodate several different antigens (Notkins, 2004). NAbs encoded by unmutated germ-line genes are characterised by the presence of several arginine and lysine residues in their complementarity determining regions and it has been suggested that these polar aminoacids might be responsible for the reaction of NAbs with various antigenically unrelated antigens (Avrameas, 1991). It has been reported that this type of interaction is followed by conformational changes that lead to a more intimate contact between the antigen and the antibody (Notkins, 2004). However the high specificity of anti-carbohydrate antibodies recognising even small anomeric differences in glycosidic bonds of sugar remains to be explained in more depth. It is estimated that about 20% of B lymphocytes in the peripheral blood are involved in the production of polyreactive antibodies and most of these belong to the IgM class (Schwartz-Albiez, 2012). Notably and in contrast to the majority of naturally occurring anti-carbohydrate antibodies, xenoreactive antibodies like anti-Gal are predominantly of IgG isotype.

2.3. Polysaccharide antigens and B-cell response to polysaccharide antigens:

Glycans are the predominant surface components of cells such as erythrocytes, immune cells and microorganisms and as such they can give rise to anti-glycan antibodies of all classes. Carbohydrates in the form of capsular polysaccharides or lipopolysaccharides form the major components on the surface of bacteria and other microorganisms. The polysaccharides on the surface of most bacteria are in the form of capsules, glycoproteins or glycolipids. It is now well established that the immune response against the surface polysaccharides of microorganisms confers protection against the same (Weintraub, 2003). Anti-polysaccharide immune response are characterised by lack of T-lymphocyte memory, isotype restriction and delayed ontogeny.

An antigen can be immunologically classified into T-lymphocyte dependent (TD) and T-lymphocyte independent (TI) antigens. Proteins and peptides are the TD antigens since they require stimulation from T-helper (Th) cells in order to elicit an immune response where as TI antigens can function without stimulation from Th cells. The TD protein antigens are presented to T-cells by the major histocompatibility complex (MHC) present on the antigen presenting cells (APC) like macrophages, B-cells and dendritic cells. TD antigens induce long lasting immune response due to the formation of memory B and T-cells. The antibodies against TD antigens are of high affinity and of multiple isotypes. In contrast to this TI antigens are incapable of giving rise to immunological memory. Memory

responses are characterised by high avidity antibodies i.e. antibodies strongly binding to antigens (Kuby et al., 2007).

The TI antigens are further classified into type I TI antigens and type II TI antigens based on their interaction with B-cells (Mosier et al., 1977). TI type I antigens are capable of inducing proliferation and differentiation of both mature and naive B-lymphocytes (Janeway C. A. et al., 2001). These antigens activate B-cells and may induce immune response in neonates, adults and in mice with X-linked B-cell defect (Xid) (Mosier et al., 1977). Common example for a type I TI antigens are the bacterial lipopolysaccharide that elicit polyclonal B-cell activation via Toll-like receptors. TI type II antigens are particularly high molecular weight repetitive polysaccharide structures that exhibit no intrinsic B-cell stimulating activity. They are also characterised by their poor in vivo degradability and their inability to stimulate MHC class II restricted T-cell help. They are capable of activating only the mature B-cells resulting in the production of antigen specific antibodies (Weintraub, 2003). Antibodies synthesised against carbohydrate antigens are produced by the CD5+ B1 cells and these B-cells constitute 5% of the total B-cell population. B1 cells are characterised by the lack of affinity maturation and little proclivity to class-switching or differentiation to memory cells (Kuby et al., 2007) so that IgM forms the major immunoglobulin type in most of the naturally occurring anti-carbohydrate antibodies. However, Obukhanych and Nussenzweig (2006) have reported that polysaccharide antigen can elicit memory B-cells that are phenotypically different from those elicited by protein antigens. While T-cell may not provide a direct help in inducing TI immune response, it can enhance the antibody production by these

antigens via cytokines such as IL-3, GMCSF and IFN-γ (Kuby et al., 2007). Two polysaccharide antigens which we come across daily are β-glucan and dextran.

2.3.1. β-Glucan:

β-Glucans, are long chain polymers of glucose in β(1→3) (1→6) linkages. They are known for long as plant constituents and as major components of the cell walls of plants and fungi. They are produced by a variety of plants like oats, barley and seaweeds. In addition to plants and fungi, β-glucans are present as a constituent of the cell wall of certain pathogenic bacteria including *Streptococcus mutans*. As traces of plant and microbial matter are ubiquitous in all environments β-glucan is present to some level almost everywhere. Human exposure can occur through breathing, fungal infections, systemic administration of β-glucan containing pharmaceuticals etc and recent studies have revealed that plasma (1→3) β-D glucan measurement is useful for screening for invasive fungal infections (Usami et al., 2002).

2.3.2. β-Glucans as immunomodulating agents:

Immune modulatory functions induced by β-glucans involve both innate and adaptive immune response. They possess both anti-infective and anti-tumorigenic properties that stem from their ability to activate leukotrienes thereby stimulating their phagocytic activity, production of reactive oxygen intermediates, inflammatory mediators and cytokines including TNF-α (Czop, 1986). Dectin-1 receptor binding by β-glucans and subsequent downstream signalling will eventually lead to the

release of cytokines including IL-2, IL-6, TNF-α and IL-10. Some of these cytokines may play important role in cancer therapy (Chan et al., 2009).

It was reported that TLR-4 signalling plays an important role in β-glucan induced dendritic cell maturation and these glucans were reported to be effective in inducing human peripheral blood mononuclear cell proliferation (Chan et al., 2007). It was also found to enhance phenotypic and functional maturation of monocyte derived dendritic cells with significant production of IL-12 and IL-10.

The immunomodulatory effects differ among β-glucans and it depends on the size and branching complexity. In addition to this β-glucan is capable of stimulating the antigen presenting cells (APC) involved in production of IL-4 which is required for the development of Th2 cells which play a key role in allergic immune response (Del Prete et al., 1988). β-Glucans have been widely used in anti-tumour applications although they themselves do not have any direct cytotoxic effects nor trigger any apoptotic pathway. In contrast they stimulate the proliferation of monocyte lineage leukemic cells and were effective in facilitating the maturation of dendritic cells derived from leukemic cells (Chan et al., 2008). Hence the application of β-glucans on leukemic patients remains controversial. Most of the studies on the use of β-glucans as anti-cancer agents were done with crude herbal extracts or a fraction of extracts instead of purified β-glucans and were based on the assessment of toxicity profile or underlying immune changes without addressing changes in cancer status (Chan et al., 2009). Therefore it is difficult to identify whether the anti-cancer effects were related to β-glucan or other confounding

chemicals found in the mixture. Although oral β-glucan were effective in stimulating proliferation and activation of peripheral blood monocytes in patients with advanced breast cancer (Demir et al., 2007). Other application of β-glucans included wound healing (Wei et al., 2002) and stimulation of general immunity and immunoprotection against infectious agents (Akramiene et al., 2007).

2.3.3. Anti-β-glucan:

It is generally accepted that β-glucans are not a good antigen for eliciting an immune response. However a β-glucan binding antibody was found in human sera (Masuzawa et al., 2003). When Candida species β(1→3) glucan (CSBG) was coated on polystyrene wells, sera from healthy human volunteers showed high reactivity towards coated material and this was neutralised by adding soluble CSBG as a competitor. The anti-β-glucan (ABG) antibody was found to be highly reactive to fungal β-glucan and was much less reactive to six branched β(1→3) glucan prepared from mushroom. The titre of ABG differed significantly in the sera from healthy human volunteers and showed fluctuations in patients with deep mycosis (Ishibashi et al., 2010).

While β-glucan in small amount is a very poor immunogen, the abundance of these polysaccharides in the environment makes it a potent immunogen. The presence of β-glucan as a moiety in complex microbial cell wall molecules particularly proteins makes it a natural glycoconjugate enhancing its immunogenicity (Chiani et al., 2009).

The ABG in normal human plasma purified by affinity chromatography on cellulose were found to contain three times higher IgA to IgG ratio and substantially higher polymeric IgA content than total serum immunoglobulins. Cellobiose and other β-glucosides were the best inhibitor of its binding to polystyrene well coated β-glucosides. Natural ligands recognised included β(1→3) glucan from *Saccharomyces cervisiae*, *Candida albicans* and barley in decreasing order of affinity. High IgA content along with the capacity to bind to commonly encountered microbial and dietary antigens shows the immune inflammatory potential of the antibody (Geetha et al., 2007)

Ishibashi et al. (2005) has reported the role of ABG in host defence against pathogenic fungi. The naturally occurring ABG ineracts with the fungal cell or extracellular glucan and plays an important role in modifying the host defense system. A remarkable decrease in antibody titre was observed when pathogenic fungal cell wall glucan from Candida species and Aspergillus species was administered suggesting the formation of an antigen-antibody complex. In patients with deep mycoses, whose sera was positive for the presence of β(1→3) glucan, the ABG titer was low and this decrease in titer correlated with other parameters such as CRP. It was suggested that this antibody function in collaboration with other recognition molecule such as β-glucan receptor or complement in human. ABG monoclonal antibody can reduce fungal burden following systemic infection with Candida species and Aspergillus species. Similarly ABG in normal sera could play a protective role against fungal infections (Torosantucci et al., 2009).

Schwarz et al. (2003) reported the presence of an anti-cellulose antibody that binds specifically to β(1→4) linked saccharides with a preference for glucopyranose over galactopyranose residues using a novel glycan array. Although anti-glycan antibodies are present in normal healthy plasma, anti-mannan antibody may exert competing, inhibitory effects on the protective capacity of anti-β-glucan antibodies (Bromuro et al., 2002).

2.3.4. Dextran:

Dextran make up the class of polysaccharides that are D-glucans with α(1→6) glycosidic linkages in the main chains and α(1→2), α(1→3) and α(1→4) branch glycosidic linkages depending on the specificity of the particular dextran sucrase. The enzyme responsible for the synthesis of dextran from sucrose are known as dextran sucrase, glucan sucrase and glycosyl transferases (Kim et al., 2003). Historically dextrans have been recognised as contaminants in sugar and the name dextran was given by Schiebler in 1874 who demonstrated dextran as a carbohydrate with molecular formula $(C_6H_{10}O_6)_n$. Dextrans are found as bacterial extracellular polysaccharides and are synthesised from sucrose by beneficial lactic acid bacteria such as *Leuconostoc mesentroides* and *Lactobacillus brevis* and also by dental plaque forming species *Streptococcus mutans*. Most of these bacteria employ dextran as a protective coating in biofilm formation to evade host phagocytes in the case of pathogenic bacteria (Meddens et al., 1984).

Dextran is produced by microorganisms which infect cane and feed on sucrose. The bacteria involved is Leuconostoc species and is ubiquitous in the soil.

They enter the cane at places of exposed tissue and any delay in kill to mill time allows the bacteria to proliferate and makes the dextran levels to soar. Thus dextran is present as a contaminant in commercially available sucrose (Cheetham et al., 1990). Another source of entry of dextran into our body is through dental infections and dental caries. Among the various oral microorganisms, *S mutans* has been identified as a plaque forming bacterium and is dependent on the presence of sucrose. Plaque formation is mediated by an initial reversible interaction between the organism and the saliva coated tooth surfaces and an irreversible stage mediated by water insoluble glucan (dextran) synthesised from sucrose by the action of glucosyltransferase (Hamada et al., 1984).

Leuconostoc and Streptococcus species are used for commercial production of dextran. Leuconostoc species require sucrose as an inducing agent where as Streptococcus species do not require glucose in the medium (Kim et al., 2003). The physical and chemical properties of purified dextrans vary depending on the microbial strains from which they are produced. Other species involved in dextran production include Acetobacter and Rhizopus species. Acetobacter is capable of producing dextran from dextrins and the enzyme involved is dextrin dextranase (Hehre, 1951).

The long history of safety of dextrans has allowed them to be used in food, chemicals, pharmaceuticals and in cosmetics industry. The various applications of dextrans include decreasing vascular thrombosis by its binding to erythrocytes, platelets and vascular endothelium, use in eye drops as a lubricant and in certain

intravenous fluids to solubilise other factors, and as an anticoagulant agent. Incorporation of dextran can improve softness and loaf volume in bakery products. In addition to these, dextrans are used as additives in candies and icecreams, as a moisturizer and thickner in cosmetics industry and in photographic industry to improve the quality of silver emulsion of photographs. Thus there are several sources through which dextran can gain entry into our body.

2.3.5. Dextran binding immunoglobulins (DIg):

Kabat and Berg (1953) were the first to report the antigenicity of dextran. The antigenicity of dextran in man, its occurrence in commercial sugar and its elaboration by microorganisms in the gastrointestinal tract provide an explanation for the occurrence of allergic reactions in man on infusion of dextran and for the occurrence of small quantities of anti-dextran in individuals not previously infected with dextran (Kabat and Berg, 1953). Native dextrans are large molecules with multiple repeats of α(1→6) linked glucose and is non-catabolisable in mammals (Jeanes, 1986).

Sporadic cases of occurrence of serum anti-dextran antibodies had been reported in some Western populations (Kraft et al., 1982 and Anastase et al., 1996). Dextran binding immunoglobulins (DIg) was found in all blood donors tested in province of Kerala who had no history of exposure to dextran (Chacko and Appukuttan, 2003). Kabat (1960) checked the capacity of a series of dextran fractions of different molecular weight to stimulate antibody formation in man by injecting 1 mg dextran and dextran of average molecular weight of 51,300 Da or

below was poor in eliciting antibody formation. Thus the antigenicity of dextran was found to increase with the molecular weight of the same (Kabat and Bezer, 1958).

Persisting sources of antigenic epitopes causing synthesis and maturation of DIg in man include microbes colonising the gut, dextran present in abundance in dental plaques and those from commercially available sucrose (Sidebotham, 1974). Under diabetic conditions, serum glucose level will be several fold higher than the normal and are capable of competitively inhibiting DIg binding to non-self antigens and thereby making the individual susceptible to infections (Chacko and Appukuttan, 2003). Paul et al. (2009) used an improved affinity chromatography using Sephadex-G200 for the isolation of DIg and the purified DIg contained mostly of IgM and IgG and was particularly enriched in polymeric IgA. DIg thus isolated was multispecific and recognised α(1→6) and β(1→3) linked glucose and α(1→3) linked galactose in natural glycoconjugates.

DIg has been reported in several pathological conditions including IgA nephropathy. Serum anti-dextran IgG or DIg were found at significantly higher levels in IgA nephropathy (IgAN) patients than in controls (Kennel et al., 1995). Cationic dextran was capable of inducing mesangioproliferative nephritis in rats independent of glomerular IgA deposition (Burg et al., 1997). Dextran or dextran like material was detected in sera of several patients with various gastrointestinal ulcers and also in the sera of aged people. The level of anti-dextran antibodies especially of IgG class was elevated in these pathological conditions (Palosuo and Milgrom, 1981).

In mice, dextran was reported to induce a classical TI(type II) pattern of antibody response with IgM and IgA predominating and a few IgG3 (Fernandez and Möller, 1990). Unlike protein antigens, dextran cannot induce class switching to IgG isotypes upon the second stimulation. However immune response to dextran may persist for a prolonged period (Vicari and Courtenay, 1977). In striking contrast to previous reports, Wang et al. (1994) reported that dextran can induce the formation of germinal centers (GCs). The involvement of GC in the dextran immune response may account for some of the variable region diversity characteristic of this response. Although the response of dextran has some of the characteristics common with TD response, the isotype profile of the antibody produced in response to dextran is characteristic of TI-type II response with IgM predominating and little IgG (Wang et al., 1994 and Paul et al., 2009). Thus the occurrence of GCs in itself is not sufficient to induce IgG class switching, which require Th cell derived cytokines also. Antibodies specific for TI-type II antigens are primarily produced by marginal zone and B1 subsets and they were reported to play an important role in boosted production of dextran specific antibody titers upon second challenge with the same (Foote and Kearney, 2009).

2.3.6. Anti-Gal:

Anti-Gal is one of the most abundant anti-carbohydrate antibodies found in humans. It constitutes around 1% of the circulating IgG and binds specifically with the α-gal epitope (Galα1→3Galβ1→4GlcNAc-R) (Galili et al., 1993). Its synthesis is proposed to be triggered by terminal α-linked galactose in gut bacterial antigens.

The occurrence of anti-Gal antibody was first suggested in a study on in vivo binding of antibodies to pathologic RBC of patients with β-thalassemia (Galili et al., 1983). Similar IgG molecules were demonstrated on human normal senescent red blood cells (Galili et al., 1986a) and on RBC of patients with sickle cell anemia (Galili et al., 1986b). Around 1% of human B-cells can produce anti-Gal but most of this are quiescent and only those along the gastrointestinal tract produce this antibody in response to continuous stimulation by the gastrointestinal bacteria (Galili, 2013).

α(1→3) galactosyl transferase (α1,3GT) gene is involved in the production of α-Gal epitope and this gene is completely inactive in old world primates (i.e. humans, apes and old world monkeys). Because of this, humans, apes and old world monkeys produce very large amounts of anti-Gal and this antibody is responsible for xenograft rejection because it readily binds to the α-Gal epitopes on such xenografts (Galili and Swanson, 1991). However Jaison et al. (1993) had reported the presence of anti-Gal reactive epitopes in human brain grey matter and later atherosclerotic plaque in humans were also found to contain antibodies reactive against TAG (Mosedale et al., 2006). Aberrant expression of α-Gal epitope or of antigens mimicking this epitope in humans may result in autoimmune processes as in Grave's disease. The expression of α-Gal epitopes on Trypanosoma cruzi can cause autoimmune like inflammatory reactions in Chaga's disease (Galili, 2013).

Glycolipid or glycoprotein antigens with carbohydrate chains other than the α-Gal epitope (eg. bovine fetuin, human thyroglobulin, human laminin etc) do not

interact with anti-Gal indicating that this antibody is highly specific and is not polyreactive like other naturally occurring anti-carbohydrate antibodies (Galili et al., 1985). However on analysis of anti-Gal interaction with peptide libraries, this antibody was reported to interact with peptide sequence that is also found in mucin molecules on malignant cells (Sandrin et al., 1997). This supports the previous reports by Castronovo et al. (1989) who observed binding of anti-Gal to mammary carcinoma cells. Although no reports of the role of anti-Gal in facilitating in vivo destruction of tumour cells is available so far. Recently it was reported that all samples of affinity purified anti-Gal from plasma contained co-purified lipoprotein (a) [Lp(a)] (Mandagini et al., 2013). The carbohydrate binding site of anti-Gal was utilised in forming IC with Lp(a). Since Lp(a) lacks TAG epitopes it was concluded that, STPS (serine-and threonine peptide sequence) or mucin type peptide at O-glycosylation sites of apo(a) subunit in Lp(a) acted as surrogate to TAG as a ligand for anti-Gal (Geetha et al., 2014).

Analysis of the isotypes of anti-Gal demonstrated the predominance of IgG (Galili et al., 1995) which was capable of binding to various cells that express α-Gal epitopes, and opsonise them for phagocytosis by macrophages (LaTemple et al., 1996). In Henoch Schonlein purpura there occurs an increase in the titer of anti-Gal IgA within the serum, though their pathological role is not clear (Davin et al., 1987). Anti-Gal IgG analysis in human sera demonstrated the presence of all four subclasses with anti-Gal IgG1 being the most prevalent (Ravindran et al., 1988). Unlike other anti-carbohydrate antibodies anti-Gal undergoes isotype switch according to the general pattern of antibody response. Since isotype switching is

considered as a T-cell dependent process, it is possible that anti-Gal IgG production is associated with T-cell activity (Galili et al., 1997).

2.4. Immune Complexes (ICs):

ICs are formed every time antibody meets antigen, and generally they are removed effectively by the mononuclear phagocyte system, but occasionally they persist and eventually deposit in a range of tissues and organs. The complement and effector cell mediated damage that follows is known as type III hypersensitivity reaction, or IC disease. The sites of IC deposition are partly determined by the localization of the antigen in the tissues and partly by how circulating ICs (CICs) become deposited (Roitt et al., 2001). Deposition of ICs in tissues underlies the pathogenesis of a range of diseases from glomerulonephritis, systemic lupus erythematosus (SLE), arthritis and transplantation rejection to rheumatic fever (Mayadas et al., 2009).

Pathogenic ICs are either locally formed or deposited from circulation. Local formation of IC is characterised by the antigens being a part of the organ or the unrelated antigens getting selectively being deposited in the organ and antibodies from circulation reacting with these antigens. In this case characteristically one organ is involved and ICs are not present in circulation. When ICs are present in circulation, more than one organ system becomes involved due to the deposition of these ICs from circulation (Mannik, 1980).

The number of antigenic determinants of a molecule profoundly influences the kind of antigen antibody complexes that may form with specific antibodies. The

lattice of ICs which reflects the number of antigen and number of antibody molecules in each complex influences its biological properties. The valence of antigen influences the lattice of IC that can be formed. A monovalent antigen molecule can form Ag_2Ab_1. Small latticed ICs are found to be soluble. The addition of excess antigen beyond the point of equivalence leads to the formation of soluble ICs. At relatively low degree of antigen excess soluble large latticed ICs are formed. But with increasing addition of antigen only small latticed ICs are obtained (Arend and Mannik, 1974). The lattice of ICs formed will influence the fate and tissue deposition potential of these complexes. The lattice formation is influenced by the association constant between the antigen and antibody also. A lower association constant results in the formation of small latticed ICs while higher association constant results in the formation of large latticed ICs (Mannik, 1980). The biological properties of ICs which are important in IC mediated diseases are their tissue deposition potential, complement activation and interaction with cell surface receptors.

2.4.1. The fate of ICs in circulation:

The concentration of ICs and their fate in circulation is determined by the rate of IC formation and the rate of their clearance from circulation. Wilson and Dixon (1971) has reported that very small proportion of antigen were deposited in renal glomeruli during disease process and yet these small amounts of ICs were sufficient to cause tissue damage and organ failure. Large latticed ICs (greater than Ag_2Ab_2) were removed relatively rapidly than small latticed ICs. With increasing

occurrence of large latticed complexes, the clearance of these complexes reached a plateau, indicating saturation of reticuloendothelial system involved in its removal. Kupffer cells are responsible for the uptake of ICs and with increasing doses of these complexes the Fc receptors get saturated (Haakenstad and Mannik, 1974). The small latticed ICs defined as containing one or two antibody molecules were removed more slowly from circulation than large latticed ICs. The nature of antigens and antibodies in the ICs can also alter the fate of these complexes in circulation independent of the lattice. Human IgA, IgG2 and IgG4 are ineffective in interacting with Fc receptors on phagocytic cells. Therefore ICs containing these antibodies might persist longer in circulation than complexes containing antibodies that react effectively with Fc receptors. Large latticed ICs with reduced and alkylated antibodies persisted in circulation due to decreased hepatic uptake and were removed at rates comparable to small latticed IC leading to increased glomerular deposition of these ICs (Spiegelberg, 1974).

2.4.2. The pathological role of ICs in atherogenesis:

Atherosclerosis is now considered as a chronic inflammatory disease predominantly affecting medium and large sized arteries and is considered as the major underlying cause of CVD. Several studies have demonstrated the occurrence of antigens and their cognate antibodies in the serum of patients with CVD. The antigens included oxidised low-density lipoproteins (ox-LDL), heat shock protein (HSPs) and bacterial antigens which have the potential to form IC with the corresponding antibodies (Tsimikas et al., 2007). The ICs being proinflammatory

may then be subsequently involved in progression of atherosclerosis. Infact several of the autoimmune diseases associated with the presence of IC's, such as SLE, anti-phospholipid syndrome (APS) and rheumatoid arthritis (RA) are also known to be established risk factors for CVD (Turesson et al., 2008). Although in normal circumstances, the formation of IC is a protective mechanism and is an essential part of the immune defence for the acute clearance of foreign antigens. But in situations of chronic inflammation secondary events, such as complement activation associated with IC formation may mediate disease process leading to organ/tissue injury such as that seen in atherosclerosis. The two principal mechanisms by which IC formation may lead to progression of atherosclerosis include immunoglobulin Fc receptor cross-linkage and activation and complement activation.

Invitro studies on the putative atherogenic properties of ICs were done with ICs consisting predominantly of IgG type. These ICs were capable of reducing monocyte apoptosis and thereby promoted their survival probably by inducing the generation of spingosene-1-phosphate, a positive modulator of cell growth. This would probably increase the macrophage derived foam cell content, a hallmark of early atherosclerotic lesion development. These ICs were also capable of inducing the release of various proinflammatory mediators like IL-1, IL-6, IL-12, TNF and the enzyme matrix metalloproteinase-1 (Burut et al., 2010).

In addition to Fcγ receptors, several complement activation products have also been detected in atherosclerotic lesions suggesting a possible role of these components in atherosclerosis. The IC-C1q complex in circulation is capable of

binding to C1q receptors expressed on endothelial cell surface whereby it can stimulate the expression of intercellular and vascular cell adhesion molecules on endothelial cells and downregulation of cholesterol-27-hydroxylase which is involved in the removal of cholesterol from the arterial wall (Galkina and Ley, 2007 and Babiker et al., 1997). The expression of adhesion receptors will in turn promotes the recruitment of mononuclear phagocytic cells to the arterial wall. Thus several studies suggest that Fc receptor cross-linking and complement activation by ICs may promote inflammatory processes eventually leading to atherosclerosis.

Human atherosclerotic plaque lesions have been detected to contain all three antibody classes IgG, IgM and IgA (Hollander et al., 1979) although IgA-ICs are known to play an important role in atherosclerosis progression in diabetic patients. IgA could not activate complement efficiently and their inflammatory action is mediated via the cross-linking of Fcα receptors found on macrophages while IgG-ICs are capable of inducing inflammatory reactions via Fcγ as well as by complement receptor activation on the endothelial cell surface (Burut et al., 2010). Studies on the levels of circulating IgM antibodies have led to the proposal that they are involved in a protective role in atherogenesis. Oksjoki et al. (2007) has reported the differential distribution of IgM antibodies in the atherosclerotic plaque and the IgM-ICs were present in the superficial layers of the intima rather than the deeper musculoelastic layer. These antibodies may be the polyreactive natural IgM antibodies that would react with multiple similar conserved motifs, thus producing an immune response. The higher level of circulating IC may result in their

deposition into the intima which is analogous to that observed in diseases such as serum sickness, glomerulonephritis and vasculitis.

Factors affecting complex deposition (Carter, 1973):

i) Hydrostatic pressure: Differences in the hydrostatic pressure are the most important factor in the deposition of ICs in the kidneys and skin. Acute glomerulonephritis is seen in glomeruli situated close to the medulla as the intraglomerular hydrostatic pressure in these glomeruli is higher compared to other regions.

ii) Vascular permeability: Increased vascular permeability is also known to fascilitate the deposition of complexes in the vessel walls and the release of vasoactive amines as part of a mechanism resulting from complex deposition.

iii) Complex concentarion: A low concentration of soluble complexes present for a prolonged time is sufficient to cause glomerulonephritis while higher concentration of complexes is required for the arterial deposition of complexes.

iv) Complex composition: This is one of the main factors responsible for complex deposition within the tissues. It is expected that highly soluble complexes with formulae of the order of Ag_2Ab might penetrate to greater distances within blood vessel walls than those with formulae of the order Ag_3Ab_2.

v) Time: if there is a continuous supply of antigenic material for a prolonged period of time it could result in the saturation of reticuloendothelial system and can result in the IC deposition.

vi) Host factors that influence the IC deposition depend on the age, weight and sex of the immunised animal and the magnitude of response often depend on genetic factors.

Other factors include antibody type and valency, affinity of the antibody for the Fc receptors and their ability to bind and activate complement

2.5. Atherosclerosis:

Atherosclerosis or hardening of arteries is considered as one of the underlying cause of most cardiovascular diseases including ischemic gangrene, abdominal aortic aneurisms and many cases of heart failure and stroke. This constitutes one of the main causes of death in the Western world today. World Health Organisation expects cardiovascular diseases to be the major cause of death globally by 2020 owing to both its rapidly increasing prevalence in developing countries and Eastern Europe and an accumulation of metabolic risk factors, including obesity and diabetes among the Western world (Hansson et al., 2006). Atherosclerotic lesions (atheromata) are asymmetric focal thickenings of the innermost layer of the artery and consist of cells, connective tissue elements, lipids and debris. Other components include blood borne inflammatory and immune cells as well as vascular endothelial and smooth muscle cells (Hansson, 2005a). Atheroma is preceded by fatty streak which are sites of accumulation of lipid

droplets and immune cells. Fatty streaks are dominated by the lipid laden macrophages termed foam cells in addition to T-cells, dendritic cells and macrophages. Foam cells and extracellular lipid droplets form a core region in the centre of the atheroma which is surrounded by a cap of smooth muscle cells and a collagen rich matrix. T-cells, mast cells and macrophages infiltrate the lesion and are abundant in the atheroma (Jonasson et al., 1986). Many of the immune cells within the atheroma exhibit signs of activation and produce inflammatory cytokines.

Prevention of blood flow through the coronary artery by the atheromatous process leads to myocardial infarction. Most cases of infarction are due to the formation of an occluding thrombus on the surface of the plaque and the two major causes of coronary thrombosis are plaque rupture and endothelial erosion (Libby, 2002). There are several theories explaining the reason for the occurrence of atherosclerotic plaque.

2.5.1. Inflammation in atherosclerosis:

This is the most widely accepted theory explaining the probable mechanism in the occurrence of atherosclerosis. Signs of inflammation in atherosclerotic plaques and their role in atherosclerosis have been observed for several centuries and were the basis of controversy in the 19th century between Carl von Roktansky and Rudolf Virchow who were the pioneers to suggest the role of inflammation in atherosclerosis. While the former suggested a secondary role to these inflammatory arterial changes, Virchow considered them to be of primary importance (Mayerl et al., 2006). Histopthological analysis of atherosclerotic lesions revealed the presence

of cells, connective tissue elements, lipids and debris. Recent experimental, clinical and epidemiological studies have confirmed the role of inflammation in atherosclerosis and revealed a cross talk between inflammation and lipid metabolism, identified inflammatory molecules as markers of disease activity and suggested inflammatory targets for possible future therapy. Most of the cells found were immune and inflammatory cells and the rest were found to be vascular endothelial and smooth muscle cells. Fatty streaks were also revealed to contain T-cells, mast cells and macrophages or foam cells. Most of the immune cells in atheroma exhibits signs of activation and can induce the production of pro-inflammatory cytokines (Hansson et al., 2006).

The chemokine/chemokine receptor network is essential for direction of leukocyte migration in homeostatic and inflammatory conditions. Macrophages were the first inflammatory cells to be associated with atherosclerosis and they express pattern recognition receptors (PRRs) including scavenger receptors and Toll-like receptors that connect the innate and adaptive immune response during atherosclerosis. The activated macrophages produces proteases, oxygen and nitrogen radical molecules in addition to inflammatory cytokines. Similar effects are observed with dendritic cells, mast cells and endothelial cells which also express Toll-like receptors and plaque activation may partly depend on this pathway (Edfeldt et al., 2002).

T-cell infiltrate present in atherosclerotic lesion is predominantly CD4+ T-cells and CD4+ T-cells reactive to the oxidised LDL, disease related antigen, hsp-60

and Chlamedia proteins have been cloned from human lesions (Galkina and Ley, 2009). Natural killer T-cells are prevalent during the early lesion development. In addition to these two subpopulations CD8+ T-cells are also present in atherosclerotic lesions and are involved in the recognition of viral antigens at the lesion site (Hansson, 2005a). The activation of T-cells results in the expression of a set of cytokines, cell surface molecules and enzymes. Th1 cells are capable initiating an inflammatory disease similar to delayed hypersensitivity and Th2 cells are capable of eliciting an allergic inflammation. The atherosclerotic lesion contains cytokines that promote a Th1 response rather than a Th2 response. Th1 activation results in the production of macrophage activating cytokine interferon-γ (IFN-γ) and IFN-γ in turn augments the synthesis of inflammatory cytokines tumour necrosis factor-α (TNF-α) and interlukin-1 (IL-1). TNF-α and IL-1 acts synergistically and instigate the production of many inflammatory and cytotoxic molecules in macrophages and vascular cells (Szabo et al., 2003). All these actions tend to promote atherosclerosis. Cytokines of the Th2 activation pathway are involved in anti-atherosclerotic immune reactions. However they are known to contribute to the formation of aneurysms by inducing the expression of elastolytic enzymes (Shimizu et al., 2004). T-cell cytokines further activates the downstream cytokine cascade which would eventually results in elevated levels of IL-6 and C-reactive proteins (CRP) in the peripheral circulation. In this way, the activation of a limited number of immune cells would result in the initiation of a potent inflammatory cascade resulting in the formation of an atherosclerotic lesion.

IL-10 and transforming growth factor-β (TGF-β) are the two anti-inflammatory cytokines. T-cell mediated immunity is under tonic inhibition by TGF-β and IL-10; and removal of these brakes was shown to accelerate the process of atherosclerosis (Robertson et al., 2003). Antibody producing B-cells and spleen B-cells were found to be effective inhibitors of atherosclerosis possibly because the antibodies produced by these cells are capable of recognising phosphorylcholine present in oxidised LDL and apoptotic cell membrane. They may thus contribute to the elimination of oxidised LDL and dead cells (Hansson, 2005a). The systemic indicators of inflammation include CRP and IL-6 which gets elevated in patients with unstable angina and myocardial infarction. Other inflammatory markers include fibrinogen, IL-7, IL-8, soluble CD-40 ligand and CRP related protein pentraxin (Galkina and Ley, 2009). Collectively, these findings suggest that inflammatory immune reactions in coronary arteries are capable of initiating acute coronary syndromes with circulating levels of inflammatory markers.

With increasing knowledge that atherosclerosis is an inflammatory disease several novel strategies have been offered for the prevention and treatment of cardiovascular diseases. Powerful immunosuppressants and anti-inflammatory drugs are increasingly being investigated as potential treatment strategies for acute coronary syndromes.

2.6. Infection as a risk factor for cardiovascular diseases:

Myocardial infarctions, strokes and peripheral vascular diseases constitute an enormous burden on the health care systems. Analysis of atherosclerotic plaques

reveals pools of cholesterol under a fibrous cap and heavy infiltration of monocytes and T-cells. This concentration of WBC within the plaque is influenced by several factors not yet fully known. One such influence is suggested to be infection (Dunne, 2000). The role of infection in atherosclerosis was first suggested over hundred years ago with the finding that acute infection with *Bacillus typhosus* resulted in fatty sclerotic changes in the arterial wall (Nieto, 1998). Research on the role of infection in atherosclerosis was renewed with the observation that patients with coronary artery disease were more likely to have elevated antibody titer to *Chlamydia pneumonia* (Saikku et al., 1988). Various proinflammatory mediators including cytokines released in response to bacterial lipopolysaccharides (LPS), heat shock proteins (HSP), immune complexes and possibly activated but uninfected mononuclear cells moves through the circulation and are capable of inciting an immune response in the arterial wall. The various immune responses include upregulation of endothelial cell surface receptors, enhancement of transendothelial migration of inflammatory cells and activation of WBC. These activated WBC's then oxidise LDL and release proteinases, which can destabilise the fibrous cap of atheroma.

Another route through which infection may result in the initiation or progression of an atherosclerotic lesion involves the dissemination of microorganisms from the site of infection directly to the endothelial cell surface. The organism may traffic to the site within an infected monocyte and can take advantage of the secondary host defense mechanism to infect the distal tissue. In the

endothelial surface, the organisms could drive a local inflammatory process (Kozarov et al., 2005).

A vast number of organisms has been associated with atherosclerosis (Danesh, 1999). Among viral pathogens are cytomegalovirus and *Herps simplex* virus and the bacterial pathogens involved are various dental organisms, *Helicobacter pylori*, *Chlamydia pneumoniae* and *Mycoplasma pneumonia*.

2.6.1. The role of oral microorganisms in CVD:

It has now become increasingly clear that oral cavity can act as site of dissemination of pathogenic organisms to distant body sites in immunocompromised hosts (Li et al., 2000). A number of epidemiological studies have suggested that oral infections may be a risk factor for systemic diseases. The anatomic closeness of these microfloras to the blood stream could facilitate bacteraemia and systemic spread of bacterial products and immune complexes. There are three theories linking oral infections to secondary non-oral diseases.

a) Metastatic infection: as a result of transient bacteraemia caused by oral infections and dental procedures microorganisms can gain entry into circulation. They are usually removed by the reticuloendothelial system and can lead to no other clinical problems other than a slight increase in body temperature. However if the disseminated organisms can find a favourable condition, it could settle at that particular site and can multiply (Thoden van Velzen et al., 1984).

b) Exotoxin injury: several Gram positive and Gram negative organisms are capable of producing diffusible proteins or exotoxins which include cytolytic

enzymes and dimeric toxins with A and B subunits. These exotoxins are among the most lethal toxins known (McGhee and Michalek, 1981).

c) Metastatic inflammation: soluble bacterial antigens entering the bloodstream react with circulating antibodies and forms macromolecular complex. These IC's are known to cause a variety of acute and chronic inflammatory reactions at the deposition site (Thoden van Velzen et al., 1984).

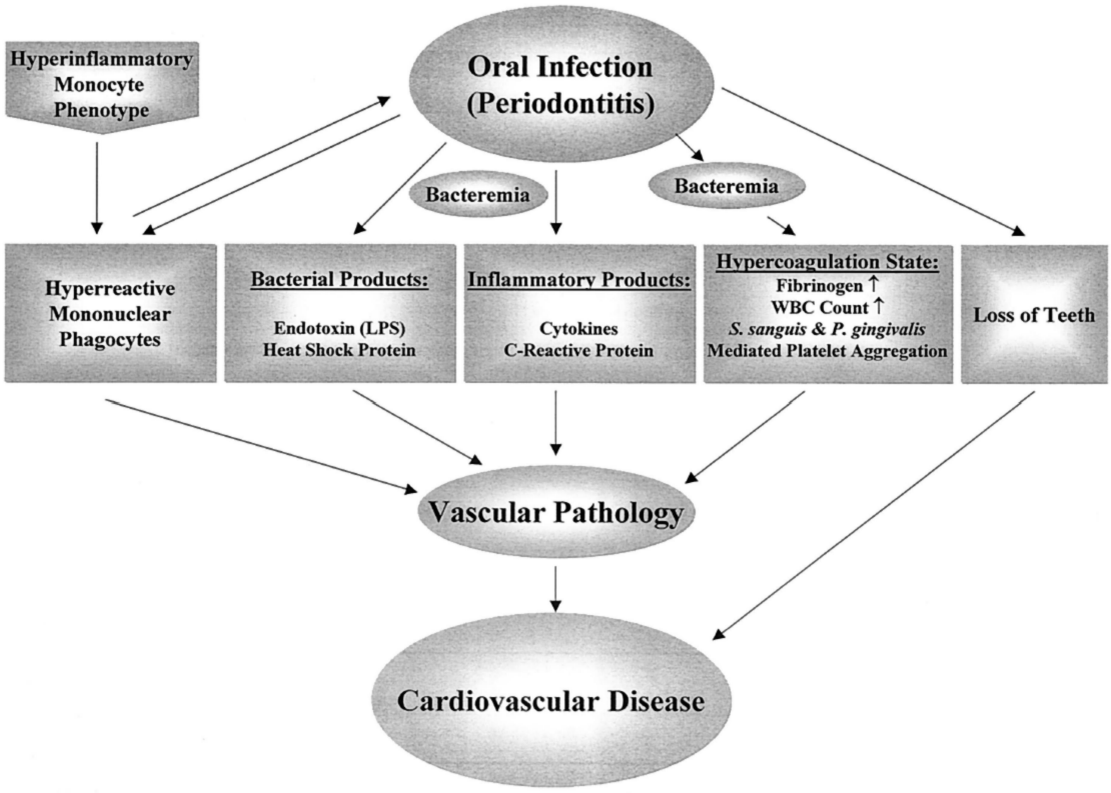

Fig:1. Proposed mechanism linking oral infections and CVD (Li et al., 2000).

The Gram negative oral microorganisms exert pro-atherogenic effects by contributing to low density lipoprotein oxidation, foam cell formation and thrombogenesis. The main receptor for LPS is CD-14 receptor which is a pattern recognition receptor and has been implicated in monocyte activation, leukocyte-

endothelial cell interaction and regulation of programmed cell death in both monocytes and endothelial cells (Arroyo-Espliguero et al., 2004). TLR-4, the most important CD-14 co-receptor is responsible for activating intracellular signalling pathways.

Theoretically, periodontal disease can effect systemic health by several possible mechanisms a) direct execution of infection from the periodontium into deeper, adjacent tissues, b) passage of inflammatory mediators from the periodontium into the circulation and thereby affecting distant sites (atherosclerosis), c) penetration of oral bacteria into the systemic circulation to cause infections to distant sites (endocarditis, thrombosis, atherosclerosis) and d) spread of oral bacteria, their products or host products to distal mucosal sites to promote or exacerbate disease (Scannapieco et al., 2003).

The evidences suggesting the direct effects of oral bacteria on atheroma formation include studies finding oral bacteria like *P gingivalis* and *S mutans* in carotid and coronary atheroma (Nakano et al., 2008 and Nakano et al., 2006a), the ability of *P gingivalis* to invade and proliferate in aortic and heart endothelial cells in vitro (Deshpande et al., 1998) and by induction of platelet aggregation associated with thrombus formation by Streptococcus and Porphromonas species (Herzberg and Meyer, 1996). Indirect effects occur by the induction of inflammatory response by oral infection that elevates the levels of acute phase proteins such as CRP and fibrinogen in circulation. Both CRP and fibrinogen are independent risk factors for coronary artery disease and their induction by oral infection may help explain the

link between oral infections and heart disease (Shklair et al., 1968 and Danesh et al., 1998).

Another theory suggests the role of autoantibodies recognising oral bacteria (Bachmaier et al., 1999). The antibodies to HSP of periodontal bacteria like *P gingivalis* can cross-react with mammalian HSP exposed in an injured endothelium or atheromatous plaque (Maeda et al., 1994). Elevated level of serum IgA class of antibody to two different oral pathogens *P gingivalis* and *A actinomycetemcomitans* were found to be associated with CVD incidence (Pussinen et al., 2005). Periodontal disease is characterised by significant increase in serum lipid profile (Ebersole et al., 1999) also. Studies now indicates that periodontitis or oral infections increase the pro-atherogenic properties of LDL and decreases the anti-atherogenic properties of HDL in vitro (Pussinen et al., 2004) which could be mediated by the oxidative stress caused by periodontal organisms (Dhotre et al., 2011).

2.6.2. *Streptococcus mutans* in cardiovascular diseases:

Two of the most common human diseases are the oral infections, dental caries and periodontitis. *Streptococcus mutans*, a Gram positive facultative anaerobic bacterium, is generally known to be pathogen of dental caries (Hamada and Slade, 1980). The theory of focal infection, which was promulgated during the 19th and early 20th centuries, stated that “foci” of sepsis were responsible for the initiation and progression of a variety of inflammatory diseases such as arthritis, peptic ulcers and various other systemic diseases (Hughes, 1994). In addition, more recent evidence has suggested a potential role of periodontal infections in more serious systemic diseases including cardiovascular disease, respiratory infections,

diabetes, and low birth weight complications (Li et al., 2000). Biochemical approaches identified three major unique properties of these organisms which appear to be important in disease initiation, aciduricity, acidogenesis and sucrose dependent colonization. Much attention has been focused on the mechanism by which *S mutans* colonizes tooth surfaces. Both biochemical and genetic studies suggest that this occurs via a two step process: a sugar independent attachment to the tooth pellicle modulated by *S mutans* adhesions, ionic or hydrophobic interactions, as well as impaction of the crevices on the tooth suface followed by a sucrose dependent enhancement of tooth colonization (Kuramitsu et al., 2001).

***S mutans* and bacteremia:**

Dissemination of oral bacteria into the blood stream is known to be induced by professional dental treatment and daily oral care practices, such as tooth brushing and flossing, and even food chewing (Seymour et al., 2000). Also the bacterial surface rhamnose glucose polymers (RGPs) which are composed of a backbone of rhamnose and side chains of α1,2, β1,2 and α1,3 glucosidic residues play an important role in resistance to phagocytosis by human polymorphonuclear phagocytes (Tsuda et al., 2000). Another lipoprotein prolyl cis/trans isomerase (PpiA), on the *S mutans* cell wall was also shown to contribute to anti-phagocytic activity (Mukouhara et al., 2011). The phagocytosis resistance is also induced by a cell surface protein antigen called PAc which is involved in initial attachment of cells to tooth surfaces and this protein was found to be immunodominant against human serum antibodies (Russell et al., 1992). It was reported that strains with PAc

protein expression could survive in the blood stream for a long causing the persistence of bacterimia and therby could result in IC induced diseases.

Role in cardiovascular diseases:

Oral streptococcal species, *Streptococcus mutans* are one of the major components of the oral microflora isolated from the blood of patients with infective endocarditis (IE) strongly suggesting a close relationship of the pathogen with infective endocarditis (Vose et al., 1987). Most of these studies are based on PCR methods using primers constructed with a species specific nucleotide alignment. IE occurs due to bacterial adherence to damaged valves. Recent studies of IE in Japan has shown that the most common causative organism for IE to be Streptococcus species (50%) followed with Staphylococcus species (32-37%) (Nakatani et al., 2013). *S mutans* were more frequently detected from vascular specimens than any other organism speculating that it could be a causative agent for cardiovascular diseases. In addition several other periodontitis-related organisms were also detected among which *T denticola* was the most frequently detected (Nakano et al., 2006b). *S mutans* were detected in the extirpated heart valve (69%) and atheromatous plaque samples (74%) while those related to periodontitis were detected in much lower frequencies (Nakano et al., 2006b). It has been suggested that the oxidative condition in the blood stream must also be considered as it gives survival advantages to oral streptococci when compared to that of the anaerobic counterparts. One of the most important steps for the development of atheromatous plaque lesion is the formation of foam cells, *Porphyromonas gingivalis* and *S mutans* strain GS5 were

shown to enhance foam cell formation (Kuramitsu et al., 2001). There are also reports of *S mutans* ability to bind to extracellular matrix molecules and fibrinogen (Beg et al., 2002) which is advantageous for plaque formation. *S mutans* were detected in 63.4-68.6% of heart valves extirpated from patients with a diagnosis other than IE (Nakano et al., 2007a). Although these organisms were detected from cardiovascular specimens, the source of these organisms in cardiovascular specimens were not yet clear.

Circulating streptococcus was capable of inducing platelet aggregation triggering the accumulation of platelets and fibrin into heart valve vegetations of endocarditis (Herzberg and Meyer, 1996). Oral streptococci induced platelet aggregation both in vitro and in vivo since these organisms express a platelet aggregation associated protein (PAAP) (Herzberg et al., 2005). In vitro induced platelet aggregation involved direct binding of bacteria to platelets through several surface components and two of the laboratory strains of *Streptococcus mutans*, GS5 and Xc induced platelet aggregation in an irreversible manner (Chia et al., 2004). The cell wall polysaccharides composed of rhamnose glucose polymers (RGPs) were able to induce platelet aggregation in presence of plasma (Chia et al., 2004). Endocarditis occurs by the ability of colonizing streptococci to induce the formation of vegetations, a fibrin platelet matrix inside which the bacteria are embedded and evade immune clearance by the host. Induction of platelet aggregation and bacterial thrombotic vegetation are considered important virulence traits in the pathogenesis of endocarditis (Herzberg and Meyer, 1996 and Sullam et al., 1996). Platelet aggregation by *S mutans* and other streptococcus species required the plasma

components and extracellular components including fibrinogen in addition to specific immunoglobulins IgG as cofactors to trigger aggregation (Sullam et al., 1988). *S mutans* were reported to bind to both rabbit and human platelets directly through serotype polysaccharides (Chia et al., 2004) without requirements of plasma components suggesting a receptor-ligand interaction. Bacteria binding to platelets at the site of cardiac valve lesions can lead to subsequent development of platelet fibrin matrices finally leading to enlargement of endocardial vegetations (Scheld et al., 1978). Very little information is available regarding the structural organization of the RGPs and how these polysaccharides are anchored to the bacterial cell wall. The PAAP protein of *Streptococcus sp.* which is known to induce platelet aggregation is known to be a RGP rich glycoprotein (Erickson and Herzberg, 1993) which are also known to cause platelet aggregation. Several Gram positive species including various species of Streptococcus and Staphylococcus are shown to induce platelet aggregation and specific antibody against these pathogens is required to activate the platelets by interacting with the Fcγ receptors on platelets. A high molecular weight protein antigen c (PAc), a surface antigen from *S mutans* is also having platelet aggregation property and most *S mutans* oral strains with PAc expression induce vegetation formation (Matsumoto-Nakano et al., 2009). Recently, analysis using coronary artery endothelial cells have shown that proteins like Cnm and Cbm from *S mutans* having collagen binding capability is playing an important role in invasion of endothelial cells indicating its possible contribution to cardiovascular infections and pathology (Nomura et al., 2012).

Antibodies recognizing cellular and extracellular antigens are present in patients with caries (Pucci et al., 1986). Several antigenic components of *S mutans* have been studied, as this organism is important in the development of dental caries. Two of the cellular antigens of molecular weight 59 kDa and 61 kDa and an extracellular antigen of 57 kDa were reactive towards serum components. One study has demonstrated significant secretory IgA response against *S mutans* (McGhee and Michalek, 1981). The predominant antigens reactive against IgA and IgG were proteins of molecular weight of around 63 and 60 kDa (Chia et al., 2000). Another extracellular protein antigen fraction of around 120 kDa was also detected with a similar intensity by antibodies.

2.7. Galectins:

Lectins are the class of proteins that can bind to specific carbohydrate structures and thereby can recognise vast array of glycoconjugates expressed in animal tissues. Most animal lectins are classified into four distinct families; C-type lectins (including the selectins); P-type lectins; pentraxins; and galectins formerly known S-type or S-Lac lectins (Barondes et al., 1994). Members of galectin family are defined by two properties: β-galactoside binding and sequence similarity in their overall carbohydrate binding domain. Galectins required the presence of β-mercaptoethanol in isolation buffers to maintain their activity suggesting the presence of one or more cysteine residues and hence this class of lectins was known as S-type lectins to denote their sulfydril dependency (Hirabayashi and Kasai, 1993). Presently four mammalian galectins (gal-1, -2, -3 and -4) have been well

characterised. Galectin-1 has been the most studied and well characterised galectin, since it was the first galectin discovered in 1975 (Teichberg et al., 1975). Currently around 14 mammalian galectins have been reported and many more are found in different organisms for example in vertebrates, invertebrates and protists

The first galectins found in vertebrates were isolated in 1976 from chick muscle as well as from extracts of calf heart and lung. Lactose was required for its isolation and is now termed galectin-1. The calf heart/lung galectin-1 is approximately 15 kDa in size and occurs as a non-covalent dimer. All of these galectins isolated exhibited hemagglutination activity against trypsinised rabbit erythrocytes which display more terminal galactose residues than human erythrocytes. Human erythrocytes require treatment with neuraminidase to enhance their agglutinability. The first galectin found was renamed galectin-1 (Barondes et al., 1994).

2.7.1. Different members of galectins:

The canonical carbohydrate binding domain (CRD) of galectins has approximately 130 aminoacids, although a small number of residues at the CRD originally come in contact with glycan ligands. A total of 15 galectins have been found in mammals, but only 12 galectin genes are found in humans including two for galectin-9. Galactins have been classified into three major groups (Hirabayashi and Kasai, 1993).

Prototypical galectins: which contain a single CRD that associate to form homodimers.

Chimeric galectins: galectin-3 is the only known member found in vertebrates. It is characterised by having a single CRD and a large amino terminal domain rich in proline, glycine and tyrosine residues. The amino terminal domain of this class of galectins is sensitive to metaloproteins such as MMP-2 and MMP-9. Chimeric galectins are more common in invertebrates.

Tandem repeat galectins: two CRD's occurs within a single polypeptide. The two CRD's are bridged by a small peptide domain. The link or bridge domain can range from 5 to more than 50 amino acids in length.

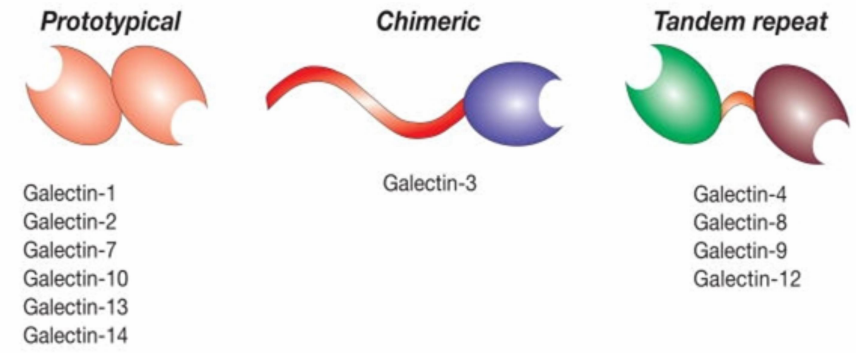

Fig: 2. Different types of galectins (Varkey,2009).

In addition to this, many of the galectin transcripts may be differentially spliced to generate many different isoforms. For example, atleast seven different mRNAs have been identified for human galectin-8. On the basis of sequence homologies two general subgroups of galectins have been distinguished; the galectin-1 subgroup which include galectin-1 and galectin-2 and the galectin-3 subgroup which includes all others.

Designation Structure	Species	Tissue/cell distribution	Architectural type	MM SDS/PAGE	AA residues
Gal-1 	human, rat, mouse, pig, hamster, monkey, bovine, ovine	muscle, heart, lung, placenta, brain, spleen, liver, lymph nodes, thymus, colon, prostate, macrophage	proto	14.5 kDa	134
Gal-2 	human, mouse	small intestine	proto	14.5 kDa	132
Gal-3 	human, rat, mouse, dog, hamster	macrophage, colon, leukemia cells, 3T3 fibroblast	chimera	29-35 kDa	>250
Gal-4 	human, rat, mouse, pig	alimentary tract, epithelial cells	tandem repeat	36 kDa	324
Gal-5 	rat	erythrocytes	proto	17-18 kDa	144
Gal-6 	mouse	gastro-intestine	tandem repeat	34 kDa	301
Gal-7 	human, rat	skin	proto	14.5 kDa	136
Gal-8 	human, rat	liver, lung, kidney	tandem repeat	34 kDa	316
Gal-9 	human, rat, mouse	kidney, thymus, Hodgkin's lymphoma	tandem repeat	35 kDa	322
Gal-10 	human	eosinophil, basophil	proto	17 kDa	142

Table: 2. Occurrence of different galectins (Rabinovich et al., 1999).

2.7.3. Functions of galectins:

Intra- and extracellular activities of galectin and its non-classical secretion:

Galectins are synthesised on cytosolic ribosomes and have features typical of cytosolic proteins. They have no signal peptide and have acetylated N-terminal. From the cytosol, galectins can be targeted to nucleus and other subcellular sites, as well as secreted by non-classical (non-endoplasmic reticulum-golgi) pathways. Galectins display a combination of intracellular and extracellular activities (Ochieng et al., 2004). For example, the same galectin-3 can play an important role in the regulation of RNA splicing in the nucleus (Patterson et al., 2004) and in cell adhesion and signalling outside the cell.

Non-classical secretion, a possible link between intra and extracellular activities is a characteristic feature of galectins and is not shared by most other lectins. The realization that certain proteins like nuclear factors can enter and exit cells by non-classical mechanisms and thereby mediate a novel way of intracellular communication is an exiting emerging area. Different members of galectins have been shown to exhibit contradictory effects on cell growth and proliferation (Rabinovich et al., 2002). Galectins are probably the most ancient class of glycan binding proteins and are found in organisms ranging from sponges and fungi to both invertebrates and vertebrates. Galectin signalling at cell surface can modulate various cellular functions. Intracellular galectins may interact with intracellular ligands and can contribute to some fundamental processes such as pre-mRNA splicing (Varkey, 2009).

Role of galectins in immune response and inflammation:

One of the major functions of galectins is to regulate immune and inflammatory diseases. Galectins are expressed by both T- and B-cells, regulatory T-cells, dendritic cells, macrophages, mast cells, eosinophils, monocytes/ macrophages and by neutrophils. Depending on the inflammatory stimulus, microenvironment and target cells, galectins can promote a pro- or anti-inflammatory response (Rabinovich et al., 2002). Response of galectins to immune cells also depends on the specific glycosylation of the surface glycoproteins in the immune cells to generate galectin ligands.

Galectin-1: Galectin-1 function is primarily associated with attenuating inflammatory responses by inducing the production of some anti-inflammatory cytokines such as IL-5, IL-10 and TGF-β and can also inhibit the production of pro-inflammatory cytokines such as IL-2, TNF-α and IFN-γ. Offner et al. (1990) provided clinical and histopathological evidence that galectin-1 is capable of preventing the development of experimental autoimmune encephalomyelitis. Galectin-1 knockout mice exhibited heightened sensitivity to the same and were found to be associated with elevated levels of Th1 and Th17 lymphocytes. Th1 and Th17 differentiated cells express non-sialylated glycan ligands that are essential for their binding to galectin-1 whereas Th2 cells may lack these ligands and express sialylated ligands that reduce galectin-1 binding and signalling (Toscano et al., 2007). There are also reports on the ability of galectin-1 in ameliorating the development and severity of graft versus host disease (Baum et al., 2003). All these

studies points towards the role of galectins in regulation of T-cell homeostasis in vivo. Galectin-1 is apparently immunosuppressive with regard to T-cell response, through induction of apoptosis, suppression of T-cell response or regulation of T-regulatory cell activities (Garín et al., 2007). With regard to other cell types, galectin-1 is involved in the inhibition of transendothelial migration and chemotaxis of neutrophils (La et al., 2003) as well as in the maturation and activation of dendritic cells (Fulcher et al., 2006).

Galectin-3: Galectin-3 recognises the T antigen Gal β1 → 3 GalNAc nearly 100 fold stronger than N-acetylactosamine (Ideo et al., 2002) and has been reported to be associated with T cell receptors (TCR) (Demetriou et al., 2001). Other immunoregulatory functions of galectin-3 included inhibition of IL-5 production by several immune cells, including eosinophils, activation of mast cells, neutrophils and monocytes, mediated by the production of reactive oxygen species. Galectin-1 and galectin-3 can also induce apoptosis in T-cells through cyt c release and caspase-3 activation (Fukumori et al., 2003). Galectin-3 functions as a pro-inflammatory mediator and are involved in regulating many aspects of the inflammatory response (Rabinovich et al., 2007).

Other galectins: Among other galectins, galectin-2 is involved in inducing T-cell apoptosis mediated by the activation of caspase-3 and caspase-9 and by the release of cyt c (Sturm et al., 2004). Galectin-4 was found to induce IL-6 production, an inflammatory cytokine and galectin-9 is identified as an eosinophil chemoattractant (Rabinovich et al., 2002).

Role of galectins in apoptosis and induction of cell surface phosphatidyl serine exposure:

Several galectins including galectin -1, -2, -3, -7, -8, -9 and -12 have been shown to induce apoptosis in some type of blood cells. Galectin-1 was reported to induce apoptosis in activated human T-cells as well as in certain human leukemia T-cell (Yang and Liu, 2003). Induction of apoptosis by galectin-1 involve cell surface glycoproteins including CD-7, CD-29 and CD-43 whereas induction of apoptosis in T-cells by galectin-3 involves CD-71 and CD-45 (Walzel et al., 1999). Overexpression of intracellular galectin-3 exhibits anti-apoptotic activity. This was attributed to the NWGR motif in galectin-3 which is also found in BCl-2 (a major apoptotic regulator protein). This motif was essential for this protein's apoptosis suppression activity (Yang and Liu, 2003). Other potential intracellular binding partners for galectin-3 include several apoptosis regulating proteins including BCl-2, Fas receptor (CD-95), synexin (Ca^{2+} and phospholipid binding protein) and Alg-2 (Yu et al., 2002). Apoptotic function of galectin-7 was mediated through c-Jun-N-terminal kinase (JNK), a known apoptotic regulator (Yang and Liu, 2003). In addition galectin such as galecin-1,-2 and -4 shows the unusual ability to induce the exposure of cell-surface phosphatidyl serine which acts as a therapeutic target to enhance phagocytosis of apoptotic cells (Schutters et al., 2013).

Role of galectins in cancer:

Expression of galectins have been documented in several different tumour types including astrocytoma, melanoma and prostrate, thyroid, colon, bladder and

overy carcinomas and their overexpression is often correlated with the aggressiveness of these tumours and the acquisition of metastatic phenotype (Rabinovich, 2005). Three galectins important in cancer progression and metastasis are galectin-1,-3 and -9. Galectin-1 could interact with oncogenic H-RAS and contribute to membrane anchorage of H-RAS (Paz et al., 2001). The effects of galectin-1 appear to be multifaceted. It can function in a carbohydrate dependent and independent manner and its effect can be positive or negative depending on the responder cell type or its subcellular localisation (Rabinovich, 2005). The immunosuppressive and apoptotic effects of galectin-1 can contribute to tumour survival where as decreased galectin-1 expression is associated with decreased tumour survival, due to increased survival of IFN-γ producing Th1 cells and heightened T-cell mediated tumour rejection. Studies using galectin-1 knockout cells have shown that expression of galectin-1 in tumour cell is essential for tumour angiogenesis (Thijssen et al., 2007). Galectins (galectin-1) can also play an important role in increasing the adhesion of cancer cells to the extracellular membrane (Rabinovich, 2005). Thus galectins play important roles in tumour progression and metastasis through indirect effects in regulating tumour immune responses and direct effects on tumour angiogenesis. Expression of galectin-3 may function as a histological tumour marker and its over-expression correlates well with neoplastic transformation and tumour progression towards metastasis (Liu and Rabinovich, 2005).

Role of galectins in animal development:

Galectins play important but rather subtle role in animal development. Galectin-3 knock out is associated with several phenotypic changes such as fatty liver disease, reduced mast cell function, reduced liver fibrosis upon induced liver damage and age dependent glomerular lesions. In contrast, lack of galectin-1 in mice is associated with decreased sensitivity to noxius thermal stimuli, altered primary afferent neural anatomy, aberrant topography of olfactory axons and reduced muscle regeneration ability after injury (Varkey, 2009).

Galectins in atherosclerosis:

It has now become known that galectins play an important role in the pathogenesis of cardiovascular diseases, in particular, atherosclerosis, stroke, myocardial infarction and heart failure. The new information holds great hope in the treatment of cardiovascular diseases by modulating galectin signalling (Al-Ansari et al., 2009). Moiseeva et al. (2003) have reported the involvement of galectin-1 in adhesion, migration and proliferation of vascular smooth muscle cell (SMC) which is considered integral to the development of atherosclerosis and restenosis. Enhancement of adhesion and migration of SMC by Gal-1 is mediated by its interaction with β1 integrins and ECM proteins laminin and cellular fibronectin (Moiseeva et al., 2000). In addition, Gal-1 is capable of binding to lipoprotein (a) [Lp(a)], a plausible mechanism by which Gal-1 might direct proliferation in the SMC compartment by recruiting Lp(a) via high affinity receptors towards SMCs causing accumulation of Lp(a) near the cell (Chellan et al., 2007). Another

mechanism by which Gal-1 can provoke SMC proliferation is by crosslinking receptor tyrosine phophatases which in turn cause activation of inhibitory phosphotyrosine residues in Src- like kinases. In addition to this IgA1 is also found to be a potent ligand for galectin-1 (Sangeetha and Appukuttan, 2005). Pathological significance of their recognition emerges from the observation that circulating immune complexes are increasingly being recognised as causative factors for vascular disorder including atherosclerosis (Galkina and Ley, 2009) and that antibodies against microbial surface antigens are heavily enriched in IgA (Paul et al., 2011). Galectin-3 (Gal-3) also plays an important role in atherosclerosis. Gal-3 is a mediator/modulator of cell ECM adhesive interactions and is well established as a regulator of angiogenesis, inflammation and autoimmune disorders (Le et al., 2005). Gal-3 is found to be overexpressed in human atherosclerotic lesions (Nachtigal et al., 1998). Conversely inactivating Gal-3 was effective in ameliorating the progression of pathology in apo(E) deficient mice. Gal-3 owes its involvement in atherosclerosis to its role in the transformation of macrophages into foam cells. There occurs an upregulation of Gal-3 when monocytes differentiate into macrophages (Liu et al., 1995). Expression and secretion of Gal-3 was tightly regulated and significantly differed among activated macrophages. Gal-3 was found to be a more distinctive descriptor of macrophage differentiation or activation than galectin-1 (Novak et al., 2012). In addition to activated macrophages, plaque foam cells may also secrete Gal-3 which act as a potent chemoattractant for monocytes and macrophages (Sano et al., 2000) and thus enhancing the recruitment of these cells to the artery wall. Chemotactic activity, binding and internalisation of

advanced glycation end (AGE) products, uptake of modified lipoproteins including Lp(a) and binding to lipopolysaccharides could possibly explain the role of galectins in atherogenesis (Al-Ansari et al., 2009).

Galectins in host microbe interaction:

Once galectins are exported to the extracellular space, they bind to their host ligands containing β-galactosides. Viruses utilises the protein synthesis machinery of host including glycosylation and acquire host type glycans on their surface. Galectin-1 is capable of binding to some enveloped viruses such as HIV-1 and nipah virus. In the case of nipah virus, galectin-1 inhibits its infection, while HIV-1 appears to exploit galectin-1 to stabilise virus attachment to CD4+ T-cells and macrophages and thus promote viral infections in these target sites (Ouellet et al., 2005). Galectins such as Gal-3 may function in stimulating macrophage uptake of pathogen materials for antigen presentation. The role of galectins in innate defense against microorganisms have been revealed by studying genetically engineered mice deficient in specific galectins (Rabinovich and Gruppi, 2005).

MATERIALS AND METHODS

3. Materials:

Polyethylene-glycol 6000 (PEG-6000), soybean trypsin inhibitor, sodium meta-periodate and 1-fluoro-2,4-dinitrobenzene (FDNB), benzamidine hydrochloride and potassium borohydride were purchased from Fluka, Buchs, Switzerland. Ortho-phenylenediamine (OPD), horse radish peroxidase (HRP), 1-O-methyl-α-D-glucopyranoside, 1-O-methyl-α-D-galactopyranoside, 1-O-methyl-α-D-mannoside, fluoroscein isothiocyanate (FITC), papain, iodoacetamide, Biogel P-4, dextran (15 kDa-400 kDa), melibiose, cellobiose, lactose, cellulose, celite, divinyl sulfone, acrylamide, N,N'-methylene bis-acrylamide, 2-mercaptoethanol, TEMED, fetuin, thyroglobulin, sulpho-NHS-LC-biotin, avidin-HRP, Tween-20, sodium cyanoborohydride, Comassie brilliant blue-G-250 and R-250, ditiothreitol, streptavidin, AMICON ultracentrifugal filter units 10 kDa MWCO , yeast-β-glucan (YBG) and anti-human IgG (Fc-specific) were purchased from Sigma-Aldrich , Bangalore, India. Polystyrene 96-well microplates (MAXISORP) were purchased from Nunc, Roskilde, Denmark. Polystyrene 96-well microplates were from Dynex Technologies, USA. Antibodies to human IgA, IgM, IgG and anti-apo(a) raised in rabbit were obtained from Dako, Denmark. Sephadex G-200 and Sepharose 4B and 6B were from Pharmacia Fine Chemicals, Uppsala, Sweden. Barbitone sodium and barbitone were purchased from BDH chemicals, Poole, England. Borate, ammonium persulphate, Triplex (sodium EDTA), hydrogen peroxide, potassium bromide, epichlorohydrin, solvents and buffer components were purchased from Merk, India. Outdated human plasma was obtained from the Department of Blood Transfusion Services of this Institute with Institutional Ethics Committee approval (IEC-511).

Streptococcus mutans (MTCC-890) was from Institute of Microbial Technology (IMTECH), Chandigarh, India. The seeds of *Arachis hypogaea* (peanut), *Artocarpus integrifolia* (jack fruit) and *Canavalia ensiformis* (jack beans) were obtained locally. Human placenta from mothers free from HIV, HPV and HCV infections was collected from the Obstetrics Department, Cosmopolitan Hospital, Thiruvananthapuram after prior approval from the Institutional Ethical Committee of that institution. Other chemicals used were of analytical grade and obtained from local purchases.

3.2. Methods:

3.2.1. Protein estimation by Bradford's method:

Coomassie brilliant blue G-250 dye solution was prepared as a 0.06% solution in 3% perchloric acid. The reagent was filtered through Whatman No.1 filter paper before use. The reagent and protein solutions were mixed in the ratio 1:1 and the absorbance at 620 nm measured immediately (Bradford, 1976).

3.2.2. Protein estimation by Lowry's method

Reagents

a. 2% sodium potassium tartarate
b. 1% copper sulphate
c. 2% sodium carbonate solution in 0.1 N sodium hydroxide.
d. Alkaline copper reagent: 1 ml of reagent 'a' and 'b' were mixed at the time of experiment and made up to 100 ml with reagent 'c'.
e. 1 N Folin Ciocalteau reagent.

0.5 ml of protein solution was mixed with 2.5 ml of alkaline copper reagent and incubated at 25^0C for 10 minutes. This was followed by the addition of 0.25 ml of 1 N Folin's reagent and incubation at 25^0C 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 of (DuBois et al., 1956) in a total volume of 5.5 ml with galactose as standard. The sample was made up to 0.5 ml with water and mixed with 1ml of 5% phenol, to this was added 4 ml of chilled sulphuric acid quickly and the mixture vortexed thoroughly. After 15 minutes incubation at room temperature, absorbance was measured at 485 nm.

3.2.4. SDS-PAGE:

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

3.3.5. Acid-PAGE

Acid PAGE was done as described by Reisfeld et al. (1962).

Reagents

Solution A:	1 N KOH	48 ml
(pH 4.3)	Glacial acetic acid	17.2 ml
	TEMED	4 ml
	Made up to 100 ml with water.	

Solution B :	1 N KOH	48 ml
(pH 6.6- 6.8)	Glacial acetic acid	2.87 ml
	TEMED	0.46 ml
	Made up to 100 ml with water.	
Solution C:	Acrylamide	30 g
	Bis acrylamide	0.8 g
	Made up to 100 ml with water.	
Solution D:	Acrylamide	20 g
	Bis acrylamide	0.5 g
	Made up to 100 ml with water.	

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

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

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

Gel preparation

A. Separating gel (5% acrylamide)

1 part A

1 part C

1 part water

3 parts G

B. Spacer Gel

1 part B

1 part D

1 part E

5 parts water

Polymerization was achieved under fluorescent light.

Reservoir buffer

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

3.2.6. Electroelution

The method described for electroelution of DNA from agarose gel (Ogden and Adams, 1987) was adapted for protein elution from acrylamide gel. For electro elution from acid PAGE tube gels, one of the tube gels was fixed, stained and destained to serve as a reference to cut out the required protein bands from other tube gels, which was kept at 4^0 C after the run. Gel slices containing the required protein band were minced with a scalpel blade and kept in tris-acetate buffer (5 mM Tris; 2.5 mM acetic acid, pH 8) in a dialysis bag and immersed in the same buffer in a horizontal electrophoresis chamber. A current of 100 V was applied across the bag for 2 hours. Electroelution was performed at 4^0C. Current was reversed for 5 min to detach the protein adhered to the sides of the dialysis bag facing the positive electrode. Contents of the bag were centrifuged at 1000 g and the supernatant containing protein was dialysed against PBS and concentrated by AMICON PM10 ultra filtration membrane.

3.2.7. Preparation of cross-linked guargum:

Soluble guar galactomannan was cross-linked to form an insoluble gel by a modification procedure described by Appukuttan *et al.* (1977). Guar gum powder (10 g) was mixed thoroughly with a finely dispersed emulsion of 2 ml

epicholorohydrin and 25 ml 3 N NaOH until the mixture became a solid cake. It was then left 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 until washings were neutral. The gel was then equilibrated with PBS and homogenized in a blender to obtain particles of about 300 μm size. Fine particles were discarded by repeated decantation.

3.2.8. Preparation of lactose-Sepharose 4B matrix:

Lactose was covalently attached to Sepharose using divinyl sulfone as the cross-linker. Lactose-divinyl sulfone Sepharose 4B was prepared as described by Dean et al. (1985). Sepharose 4B (20 g, moist weight) was washed in distilled water under suction and suspended in 20 ml of 1 M Na_2CO_3 pH 11.0. After adding 4 ml divinyl sulfone, the suspension was stirred with a magnetic bar for 1h at room temperature. Activated gel thus obtained was washed thoroughly in distilled water and its wet cake obtained by suction filtration over sintered glass funnel was added to 30 ml saturated solution of lactose in 1 M Na_2CO_3 and stirred overnight at room temperature as above. The reacted beads were washed successively with

1. 20 ml of 1 M Na_2CO_3 pH 11.0
2. 1000 ml of 0.2 M glycine-HCl, pH 3.0, containing 1 M NaCl to block unreacted activated groups in the gel.
3. 1000 ml of 1 M NaCl and
4. 1000 ml of distilled water.

The matrix was finally equilibrated in PBS 7.4 and packed into a chromatographic column of required dimension.

3.2.9. Preparation of jacalin:

Jacalin (jack fruit seed agglutinin) was isolated from the seeds of *Artocarpus integrifolia* (jack fruit seed) by the procedure described by Suresh Kumar et al. (1982). Thirty gram of jack fruit seeds were dehusked and soaked in PBS 6.5 for 12 h. The seeds were then cut into small pieces, homogenized in 300 ml PBS 6.5 and stirred 2 h at 4^0 C. The supernatant of homogenate obtained by centrifugation at 14,000 g for 20 min was subjected to 70 % ammonium sulphate saturation and stirred for 30 min at 4^0 C. The precipitated proteins recovered by a similar centrifugation were dissolved in PBS 6.5 and dialysed against the same buffer before loading on to cross-linked guar galactomannan column. The column was washed with PBS 6.5 and eluted with 0.15 M galactose in PBS 6.5. Fractions containing proteins were pooled and dialysed against PBS pH 7.4.

3.2.10. Isolation of galectin-1:

Galectin-1 from both bovine heart (BHL) and human placenta (HPL) was isolated as described by Sangeetha and Appukuttan (2005). Briefly, the tissue was homogenized in cold PBS (phosphate buffered saline, pH 7.4) containing 2-mercaptoethanol (5 mM), phenylmethylsulfonylflouride (PMSF; 0.2 mM), benzamidine hydrochloride (2 mM) and lactose (50 mM). The homogenate was centrifuged at 16000 g for 20 min. The supernatant proteins were then precipitated with ammonium sulfate at 70% saturation, dialyzed against PBS containing 2-

mercaptoethanol, and passed through a lactose-Sepharose column. The bound galectin-1 was eluted with PBS containing 150 mM lactose and 50 mM iodoacetamide and dialyzed against PBS. All operations were at 4^{o}C. The lectins were subsequently, either, conjugated with HRP or stored with 30% v/v glycerol at -20^{o}C until use. Lectin activity was assayed by hemagglutination with trypsinized human RBC.

3.2.11. Isolation of Concanavalin A (Con A):

Con A was isolated from the seeds of *Canavalia ensiformis* (Jack bean) as described by Surolia et al. (1973). Husk was removed from 850 g of jack beans after soaking in distilled water. These seeds were homogenised with 1700 ml of 1 M NaCl-0.01 M Tris HCl pH 7.4. The homogenate was stirred for 24 h at 4^{0}C and filtered through glass wool. The mixture was again stirred for 6 h with 1200 ml of 1 M NaCl-0.01 M Tris HCl pH 7.4 and filtered through glass wool. The filtrate was made to 1 M acetic acid concentration by adding concentrated acetic acid and stirred for 20 min at room temperature. The mixture was then centrifuged at 14500 g for 20 min. The supernatant was dialysed against 1 M NaCl-0.01 M Tris HCl pH 7.4, 1 mM Mg^{2+}, 1 mM Ca^{2+} and 1 mM Mn^{2+}. After dialysis the pH of the sample was made to 7-7.4 with Tris and NaOH. The sample was centrifuged and the soluble part was passed through Sephadex G-50 column. The column was washed with 1 M NaCl-0.01 M Tris HCl pH 7.4. The column was eluted with 0.1 M dextrose in 1 M NaCl-0.01 M Tris HCl pH 7.4, when the protein content of washing was below 75 µg/ml. Fractions having Con A activity were pooled and dialysed successively against 1 M NaCl in distilled water (3 changes), distilled water containing Mg^{2+},

Ca^{2+} and Mn^{2+} and finally in distilled water. After dialysis insoluble particles were removed by centrifugation and soluble part was lyophilised.

3.2.12. Isolation of human plasma anti-β-glucoside antibody (ABG):

ABG from human plasma was isolated as described by (Geetha et al., 2007). Affinity chromatography, dialysis and concentration were performed at 4 ^{0}C. Outdated frozen human plasma from healthy donors of age group 20-35 of the Department of Blood Transfusion Services of this institute was thawed, dialyzed thoroughly against PBS and centrifuged at 15,000 g. The supernatant (70 ml) was passed through a column (2 cm x 15 cm) consisting of a mixture of cellulose (microcrystalline) and celite (type 545, E. Merck, Germany) in 1:1 ratio (v/v). The column was washed with PBS till effluent was protein free. Bound protein was eluted using 0.2 M dialyzable dextrose (obtained by thorough dialysis of 20 ml 1 M dextrose in PBS against 80 ml PBS) into 3 ml fractions. Protein fractions were pooled concentrated by ultra filtration (10,000 MW cutoff membrane) and dialyzed against PBS to remove dextrose.

3.2.13. Preparation of serum dextran-binding immunoglobulin (DIg):

All steps were at 4^{0}C. Outdated human plasma (50 ml) from healthy male donors (25-40 years) of this institute was dialysed extensively against 20 mM potassium phosphate buffer containing 150 mM NaCl, pH 7.4 (PBS), centrifuged at 17415 g for 30 minutes and passed through a 2 cm x 30 cm column of Sephadex G-200 equilibrated in the same buffer. After washing out unbound proteins using PBS, bound protein (DIg) was eluted using 0.25 M dextrose in PBS. Fractions containing

protein were pooled, concentrated by ultra filtration (10,000 MW cut-off) and stored at 2-4^{0}C.

3.2.14. Isolation of anti-Gal:

Isolation of anti-Gal was done by method of Jaison et al. (1993). All steps were carried out at 4^{0}C. Outdated human plasma (50 ml) from healthy male donors (25-40 years) of this institute was dialysed extensively against 20 mM potassium phosphate buffer containing 5mM EDTA and 150 mM NaCl, pH 7.4 (PBS), centrifuged at 17415 g for 30 minutes and passed through a 2 cm x 30 cm column of soluble guar gum galactomannan which was cross-linked mediated by epichlorhydrin. Column was equilibrated in the same buffer. After washing out unbound proteins using PBS-EDTA, bound protein (anti-Gal) was eluted using 150 mM galactose in the same buffer in 3 ml fractions. Protein containing fractions were pooled, concentrated by ultra filtration (10,000 MW cut-off), dialysed against PBS to remove galactose and stored at 2-4^{0}C.

3.2.15. Isolation of immune complex (IC) from human serum/plasma and proteins from IC:

IC was isolated by precipitation with polyethylene glycol (PEG) 6000 as described in Hudson and Hay (1980).

Reagents:

a) Veronol buffered saline (VBS) stock solution contains 2 mM sodium barbitone, 3 mM barbituric acid and 140 mM NaCl pH 7.4 and 5 times dilute solution was used as working VBS buffer.

b) Polyethylene glycol 6000 (20% w/v) was prepared in working VBS buffer.

c) 0.2 M EDTA was adjusted to pH 7.6 with 0.1 N NaOH.

d) Working PEG solution (12%) was prepared by mixing PEG, EDTA and working VBS solution in the ratio 6:3:1.

e) Washing solution was prepared by mixing PEG, EDTA and VBS stock in the ratio 10:20:5 ratio.

To 1 ml serum/plasma appropriate volume of working solution of PEG solution was added in drops to attain final concentration of 2%, the contents were mixed well and incubated at 37^0C for 1 h and then overnight at 4^0C. Precipitated IC was collected by centrifugation at 4355 g for 20 min. The precipitate collected was washed thrice with washing solution and was redissolved in 500 µl PBS.

To isolate immunoglobulin from IC, the latter redissolved in PBS was treated with 100 M of the specific sugar (1-*O*-methyl α-D-Glc for DIg-IC) at 37^0C for 2 h and proteins precipitated using ammonium sulphate (45% saturation). After centrifugation the protein precipitate was redissolved in PBS of original serum volume and dialysed against PBS.

3.2.16. Preparation of apo(a):

Isolation of plasma lipoproteins or L1:

One ml plasma was taken and the density of the solution was made up to 1.24 g/cc with potassium bromide and subjected to ultracentrifugation at 535000 g for 4 h at 4^0C. Top 20% layer containing lipoproteins (L1) was collected and dialysed in PBS (7.4).

Preparation of apo(a) from L1:

L1 obtained was subjected to reduction using 4 mM DTT at 37^0C for 15 minutes. Density of the solution was made upto 1.24 g/cc with potassium bromide and subjected to ultracentrifugation at 535000 g for 4 h at 4^0C. Bottom 20 % containing apo(a) was collected and dialysed in PBS (7.4).

3.2.17. Conjugation of HRP to lectins/antibodies:

Periodate activation of HRP:

Horse radish peroxidise (2 mg) was dissolved in 0.2 ml freshly prepared 0.3 M $NaHCO_3$ and 10 µl flourodinitrobenzene (FDNB; 1% in absolute ethanol) was added, the contents were mixed and incubated for 1 h at room temperature (to prevent self coupling). To create reactive aldehyde groups on HRP the solution was treated with 0.2 ml sodium metaperiodate (0.06 M in water) for 30 min at room temperature, following which 0.2 ml of ethylene glycol (0.32 M in water; 32 µl made up to 1.78 ml with water) was added, mixed and incubated for 1 h at room temperature to remove excess periodic acid. Finally the solution was made up to 1 ml with 0.01 M carbonate buffer pH 9.5 (concentration is 2 mg/ml) and dialysed in 0.01 M carbonate buffer pH 9.5.

Conjugation of activated HRP to proteins:

Antibodies or lectins (1 mg) in 10 mM sodium bicarbonate buffer pH 9.5 (1 ml) was mixed with 0.67 mg periodate activated horse radish peroxidase in the same buffer and incubated at 25 °C for 2 h in the dark. Then potassium borohydride solution in distilled water (1%) was added to a final concentration of 0.1%. After 30

min. the mixture was dialysed against PBS with one change overnight. The labeled lectin/ antibody were stored in ice (Weir, 1986).

3.2.18. Biotin labelling of proteins:

Proteins (1 mg/ml) were dialysed in 0.1 M Na_2HPO_4 buffer pH 8. Sulfo NHS-biotin (0.002 M) was added and mixture incubated for 2 h at 4^0C. The biotinylated proteins were dialysed extensively in pH 7.4 to remove unreacted biotin. The labeled compound was stored at 4°C with 30 % glycerol v/v (Paul et al., 2011).

3.2.19. Preparation of neoglycoconjugates:

Lactose and melibiose were conjugated to protein soybean trypsin inhibitor by reductive amination using sodium cyanoborohydride. Soya bean trypsin inhibitor, melibiose/cellobiose, sodium cyanoborohydrate 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 contents were incubated at 25^0C for 14 days. The neoglycoconjugates thus formed were dialysed in PBS 7.4 and stored at -20^0C (Baues and Gray, 1977).

3.2.20. FITC conjugation of antibodies:

Fluorescein isothiocyanate (FITC) coupling to antibodies was done essentially as described by Hudson and Hay (1980). Antibody samples were concentrated to 1mg/ml, dialysed against 0.25 M carbonate-bicarbonate buffer (pH 9.0) and pre-incubated for 2 h with respective specific sugars (25 mM) to protect the antigen binding site from labeling. FITC (0.15 mg per mg protein) was then added and mixture kept overnight at 4^0C. Gel filtration using 25 ml Biogel-P4

column in PBS medium was used to separate the FITC labeled antibody from reagents.

3.2.21. Effect of specific ligands on antigen-induced increase in fluorescence of FITC– labeled antibody:

FITC-labeled antibody (6.25 μg protein in 25 μl PBS) preincubated with and without specific disaccharide (25 mM) was mixed with 15 μg of candidate antigen in 15 μl PBS and incubated overnight at 4^0 C. After diluting to 300 μl with PBS, fluorescence was measured in BIOTEK fluorescence reader using excitation at 485 nm and emission at 520 nm.

3.2.22. Determination of specific activity of antibody:

Specific activity of anti-Gal was defined as the ratio of thyroglobulin binding activity to immunoglobulin content of the same amount of antibody. These parameters were measured by ELISA. Polystyrene microwells (NUNC Immuno Break-apart, MAXISORB) were coated with thyroglobulin by 3h incubation at 37^0C with the protein (2 μg in 200 μl PBS). Wells were washed with PBS containing 0.05 % Tween-20 (PBS-T) and blocked with PBS containing 0.5 % Tween-20 at 37^0C for 30 minutes. After washing again with 0.05 % PBS-T, wells were incubated with 50 ng anti-Gal in 200 μl 0.05% PBS-T for 2 h at 4^0C, washed thrice with PBS-T (0.05%) and bound antibody assayed by probing with a mixture of HRP conjugates of anti-human IgG, IgM and IgA (all 1.5 μg antibodies per ml) in PBS-T (0.05%) and bound HRP activity was assayed by incubating for 15 minutes with 200 μl OPD (5 mg/ml) in citrate phosphate buffer, pH 5.0 containing 0.03 % H_2O_2, stopping the

reaction with 50 µl of 12.5% H_2SO_4 and reading the plates at 490 nm in BIOTEK ELISA reader, USA. To assay immunoglobulin content, anti-Gal (50 ng protein) directly coated on microwells as above were probed with the mixture of HRP conjugates of anti-human IgG, IgM and IgA used above and bound HRP activity assayed. The ratio of responses (OD at 490 nm) in the above two ELISA was taken as specific activity of the antibody.

3.2.23. Isolation of Fab from papain-digested FITC- labeled ABG:

Papain digestion was done essentially by the method by Hudson and Hay (1980). Fifty µl each of 1 M cysteine and 20 mM EDTA along with papain (25 µg in 25 µl) was added to 1 mg/ml antibody (ABG) in 1 ml 100 mM sodium acetate-acetic acid buffer (pH 5.5) and mixture incubated at 37 ^{0} C for 6 h. Iodoacetamide (13.8 mg) was added and incubation continued for 30 minutes to stop the reaction and mixture dialysed against PBS. The digested mixture containing Fab and Fc were then passed through a 20 ml of cellulose-celite column (1:1) and washed with PBS. The bound Fab was then eluted by using 250 mM dextrose and dialysed against PBS.

3.2.24. Total Immunoglobulins from plasma:

From 10 ml plasma, immune complexes and particulate matter were precipitated by 20% ammonium sulphate and removed by centrifugation at 17415 g for 30 minutes. Ammonium sulphate concentration in supernatant was raised to 45 % and the precipitate containing mostly immunoglobulins was collected by a similar centrifugation as above and dialysed against PBS.

3.2.25. Preparation of *Streptococcus mutans* binding proteins (SMBP) from plasma: All operations were at 4^0 C. Out-dated plasma (40 ml) was dialysed twice against 20 mM potassium phosphate buffer pH 7.4 containing 150 mM NaCl (PBS). After centrifugation at 17415 g for 20 minutes, the plasma was applied to a column of a mixture of Biogel P-4 (25 ml) and *Streptococcus mutans* cells (2 ml) cultured in Brain Heart Infusion Broth. After thorough washing to remove the unbound proteins the bound antibodies were eluted with a mixture of 250 mM dextrose and 150 mM galactose in PBS. The eluted proteins were then dialysed and concentrated using CENTRICON (Millipore) membrane concentrator (10,000 Da cut off).

3.2.26. Immunoglobulin distribution in SMBP: Polystyrene wells were coated with 50 ng of SMBP in 200 µl of PBS by overnight incubation at 4^0C. The wells were washed with 0.05% Tween-20 in PBS (PBS-T), blocked with 0.5% Tween- 20 in PBS for 30 min at 37^0C and again washed with PBS-T. Wells were then probed with 200 µl HRP- conjugated anti-human IgA, IgG or IgM (1.5 µg/ ml in PBS-T) for 2 h at 4 ^{0}C. After washing thrice with PBS-T, bound HRP was assayed by incubating 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 the absorbance read at 490 nm in BIOTEK (Winooski, VT,USA) microplate reader.

3.2.27. Specificity of SMBP: Dextran, yeast-β-glucan and TIM were coated on polystyrene wells by incubating 2 µg of each in 200 µl PBS on polystyrene wells overnight at 4^0 C. The wells were washed, blocked as described above and treated with SMBP (2 µg in 200 µl PBS) pre-incubated with or without specific sugars (25

mM each of 1-O-methyl-α-D-glucoside, cellobiose or 1-O-methyl-α-D-galactoside for dextran-, yeast-β-glucan- and TIM- coated wells respectively) and incubated for 2 hrs. After washing with PBS bound SMBP was probed with a mixture of HRP-conjugated anti-human IgA, IgG and IgM (1.5 µg/ml each in PBST). After washing thrice with PBST, bound HRP was assayed as described earlier.

3.2.28. Preparation of crude antigens from *Streptococcus mutans* cell culture: The cell harvest was sedimented and washed thrice with PBS by centrifugation at 17415 g. Cell suspension in PBS was subjected to three cycles of freezing and thawing and later to ultrasonication in probe sonicator (six 30 second treatments) at 25^0C. The antigens released in the supernatant were collected by centrifugation at 17415 g for 20 min and dialysed against PBS pH 7.4.

3.2.29. Preparation of positively charged protein antigen from *Streptococcus mutans* (PSMAg): Crude antigens from *S mutans* were subjected to electrophoresis in 7% polyacrylamide gel at pH 4.5 (Reisfeld et al., 1962). The lone protein that moved into the gel in 4h run was PSMAg and was electroeluted using the method described by Ogden and Adams (Ogden and Adams, 1987).

3.2.30. Preparation of negatively charged protein antigens from *Streptococcus mutans* (NSMAg): Ion exchange chromatography was used for the separation of negatively charged protein antigens from *Streptococcus mutans*. Initially the crude cell wall antigen (2 mg) prepared by freeze thawing and sonication was passed through CM-Sephadex A-50 (15 ml) in 100 mM sodium acetate-acetic acid buffer pH 5.0 for the removal of positively charged proteins. The unbound antigen

collected from above were dialysed against 10 mM Tris-HCl buffer pH 8.0 and passed through a 10 ml column of DEAE-Sephadex A-50 equilibrated in the same buffer. The bound protein (NSMAg) was then eluted using 200 mM NaCl, dialysed against water and concentrated by lyophilization.

3.2.31. Anti-carbohydrate antibody binding to different antigenic components from *S mutans*: Crude antigens from *S mutans* (PSMAg or NSMAg ; 2 µg in 200 µl PBS) were coated on polystyrene wells. After washing wells were probed with anti- carbohydrate antibodies (ABG, DIg and anti-α-galactoside antibody [anti-Gal] from human plasma; 2 ug in 200 ul PBS-T) pre-incubated for 1 h with or without specific sugars (25 mM each of cellobiose, 1-O-methyl-α-D-glucoside, and melibose respectively for ABG, DIg and anti-Gal). The bound antibody was detected using mixture of HRP-conjugated rabbit antibodies to human IgA, IgG and IgM (1.5 µg/ml each in 200 µl PBS-T). The bound HRP was then assayed as described earlier.

3.2.32. Incorporation of biotinylated antigen into IC fraction: Negatively charged *Streptococcus mutans* antigen (NSMAg) was biotinylated by method proposed by Paul et al. (2011) and used for the preparation of in vitro immune complex. High molecular weight components such as immune complexes from 20 ml healthy plasma were precipitated with 20% ammonium sulphate, supernatant dialysed in PBS (7.4), ultracentrifuged at 202,000 and bottom 30% volume collected as plasma immunoglobulin-rich (Ig-rich) fraction. Biotinylated antigen (50 µg) in 200 µl was added to 800 µl of the above fraction, mixture incubated for 2 h and

immune complexes formed were precipitated with 2% PEG. Plasma Ig-rich fraction incubated with a mixture of 25 mM each of 1-O-methyl-α-D-glucoside and 25 mM cellobiose (S1) or of 1-O-methyl-α-D-mannoside and melibiose (S2) was used as control. Streptavidin was coated on polystyrene wells by incubating the wells with 1 µg of the protein in 200 µl of PBS overnight at 4^0 C. The wells were washed and blocked as described earlier. Immune complexes dissolved in PBS (500 µl) were diluted 10X and 200 µl samples added to streptavidin-coated wells. After incubation at 4^0 C for 2 h followed by the washings with PBST, wells were probed with 200 µl HRP-conjugated mixture of anti-human IgA, IgG, IgM (1.5 µg/ ml each in PBS-T) for 2 h at 4 °C. After washing thrice with PBS-T bound HRP was assayed as described earlier. To prepare IC from plasma devoid of ABG and DIg, plasma passed through half its volume of a mixture consisting of cellulose, celite and Sephadex G-200 (1:1:2) in PBS was used. Plasma treated with cross-linked guar galactomannan (CLGG), a gel which does not offer ligands for ABG or DIg was used as control.

3.2.33. Immunoglobulin composition of NSMAg IC: Streptavidin was coated on polystyrene wells by incubating the wells with its solution (1 µg in 200 µl of PBS) overnight at 4^0 C. The wells were washed and blocked as described earlier. After incubation with 200 µl of 10X dilution of IC prepared using biotinylated NSMAg and washing with PBS-T wells were probed with 200 µl HRP-conjugated anti-human IgA, IgG or IgM (1.5 µg/ ml in PBS-T) for 2 h at 4 °C. After washing thrice with PBS-T, bound HRP was assayed as described earlier.

3.2.34. Presence of IgA in NSMAg IC: IC (50 ng) obtained by treating non-labeled NSMAg with plasma Ig-rich fraction as described earlier was coated on polystyrene wells (50 ng per well), wells washed with PBS-T and probed with 200 µl HRP conjugates of anti-human immunoglobulins IgG, IgA or IgM (1.5 µg/ml in PBS-T) for 2 h at 4^0C. After washing thrice with PBS-T, bound HRP was assayed as described earlier.

3.2.35. Recognition of IC by immobilized galectin-1: Galectin-1 from human placenta was immobilized by method described by Paul et al. (Paul et al., 2011). Briefly galectin-1 (1 mg in 1ml 20 mM potassium phosphate buffer, pH 8.0) preincubated with 25 mM lactose for 1 h to protect its binding sites was treated with 2 mM sulpho-NHS-LC-Biotin for 2 h at 4^0 C. After dialysis against PBS the biotinylated galectin-1 was immobilized by incubating its solution in PBS-T (5 µg in 200 µl) for 2 h on polystyrene microwells previously coated with streptavidin (1 µg per well) and washing out unbound protein. Affinity of immobilized galectin-1 to IC was checked by the ability of IC to inhibit the binding of fetuin-HRP to the immobilized galectin-1. IC was prepared from Ig-rich plasma fraction in presence or absence of NSMAg as described earlier. Fetuin-HRP (3 µg fetuin) incubated with or without 4X dilution of IC in 200 µl PBS-T was added to immobilized galectin-1 at 4^0C. After 2 h incubation and washing with PBS-T bound HRP was assayed as described earlier.

3.2.36. Increase in anti-Ig response of antibody (ABG and Anti-Gal) in presence of a ligand: TIC/ Tg (2 µg in 200 µl PBS) was coated on polystyrene wells and

ABG/ anti-Gal (250 ng ABG/ 100 ng anti-Gal in 200 µl PBS-T) pre-incubated with and without ligand [NSMAg for ABG 2 µg/ 200 µl PBS-T and apo(a) for anti-Gal 500 ng/ 200 µl PBS-T] was added. After 2 h incubation at 4^0 C and washing bound ABG/ anti-Gal was probed with a mixture of HRP conjugates of anti-human IgG, IgA and IgM (1.5 µg/ml). Bound HRP was assayed as described.

3.2.37. **The presence of free binding sites on ABG-NSMAg complex/ anti-Gal-apo(a) complex** : ABG/ anti-Gal (100 ng in 200 µl PBST) preincubated with 25 mM of specific (S2) and non-specific sugar (S1) was allowed to interact with biotin-labelled ligand (B-Ag) (500 ng B-NSMAg in 200 µl PBS-T for ABG and 4X dilution of B-apo(a) in 200 µl PBS-T for anti-Gal) and added to TIC/ Tg coated wells (2 µg in 200 µl PBS). The presence of Ab-Ag complex on coated wells were detected using avidin-HRP (2000X) and the bound HRP was determined as described.

3.2.38. Statistical analysis: Statistical analysis was done using Microsoft Excel 2000 version and Graphpad 5. Comparison between groups was done using Students t-test. P value of < 0.05 was considered significant.

Part 1:

Dietary and Microbial Antigens Offer Antigenic Epitopes for Naturally Occurring Antibodies of Healthy Human Plasma

Introduction

The immune system of normal unimmunised animals is characterised by the presence of B cells synthesising and secreting mainly polyreactive, but also monoreactive, natural antibodies that can react with a variety of self constituents (Avrameas, 1991). Several research groups have reported that the peripheral blood of healthy persons contain natural antibodies which recognise carbohydrate structures and they play a major role in providing protective host immunity (Casali and Schettino, 1996). These anti-carbohydrate antibodies are called natural antibodies since they are present in circulation without any deliberate immunisation (Shoenfeld and Isenberg, 1989). A majority of anti-carbohydrate antibodies are derived from the pool of natural antibodies produced already during foetal life, which do not need an antigenic stimulation (Avrameas, 1991). On the other hand a certain amount of anti-carbohydrate antibodies are produced during early stages of childhood when the gastrointestinal tract is colonised by bacteria. The presence of these antibodies thus shows the large variety of bacterial oligosaccharides to which the organism has been exposed to (Springer and Horton, 1969). Classic example for anti-carbohydrate antibodies include the anti- blood group antibodies and antibodies against Forssman (Fs) glycolipid antigen, anti-Gal, dextran-binding immunoglobulins (DIg), anti-β-glucan (ABG) antibodies, lactose binding immunoglobulins (LIg) and antibody against Thomson-Friedenreich antigen (T-antigen). Most of these antibodies act as biomarkers in inflammatory and autoimmune diseases. These antibodies are characterised by polyreactivity due to

which their antigen binding pockets are more flexible and can accommodate several antigens that too in different intensities (Notkins, 2004).

Glycan antigens are the most common and most abundant antigens expressed by commensal and pathogenic microbes. Dietary and microbial glycan antigens that enter circulation can get exposed to the naturally occurring anti-carbohydrate antibodies forming ICs that can persist for a prolonged period and are particularly enriched in polymeric IgA as reported in the case of ABG and DIg (Geetha et al., 2007 and Paul et al., 2011). Although erythrocytes bearing Fcγ receptors are involved in the clearance of IgG immune complexes via the reticuloendothelial pathway, they have very limited role in IgA-IC transport or processing (Matsuda et al., 1988) and is subjected to saturation (Haakenstad and Mannik, 1974). IgA-IC has been reported in several IC depository diseases (Timmermans et al., 2015). There is a high probability of these ICs remaining in circulation for a prolonged period of time.

High level of circulating IC has been correlated with vascular pathology and CVD. The evolving atherosclerotic plaque has all the characteristics of an inflammatory disease including the presence of immune cells as well as non-cellular components of immunity including immunoglobulins, complement and C-reactive proteins (Binder et al., 2005). Wide differences in the prevalence and incidence of CVD in different parts of the world led to the identification of several novel risk factors for CVD and with increasing knowledge of the role of inflammatory

reactions in CVD, infections including those caused by oral bacteria are implicated in CVD progression (Inaba and Amano, 2010).

Commonly encountered dietary and microbial antigens were used as part of present study. One of the common source of glycan antigen entry into the body is the dietary dextran synthesised from sucrose by *Leuconostoc mesentroides* and are known to contaminate sucrose during its manufacture (Sidebotham, 1974). Yet another source of polysaccharide and other glycoprotein infusion into the body is dental plaque-causing bacteria *Streptococcus mutans*. It is reported that oral infections with bacteria can increasingly predispose subjects to CVD including endocarditis (Abranches et al., 2009 and Nakano et al., 2007b). *S mutans* have been reported to produce and secrete α- and β-glucans of very high molecular weight (Sidebotham, 1974).

RESULTS:

A) Commercially available sugar is contaminated by dextran offering antigenic epitopes for naturally occurring anti-carbohydrate antibodies:

Dextran is produced by microorganisms like *Leuconostoc mesentroides*, a common inhabitant of wet muddy soil and is known to infect the cane and feed on sucrose thereby converting it to dextran (Kim et al., 2003). High molecular weight polysaccharides above 10,000 MW had been detected in edible sugar earlier (Paul et al., 2011). In order to retain polymers of size 10,000 Da or below, polysaccharides precipitated by 50 % ethanol was used in the present study. The presence of antigenic epitopes for naturally occurring ABG and DIg on polysaccharides thus

obtained was checked by coating 2 µg of the polysaccharides [methods] on polystyrene wells followed by addition of DIg or ABG and detection of bound antibody by HRP-labelled secondary antibody. Eventhough both ABG and DIg were recognising antigenic epitopes on these high molecular weight polysaccharides, DIg was slightly better than ABG in its recognition (Fig: 3). ConA binding was also checked as it is known to bind to terminal α-D-mannosyl and α-D-glucosyl group (Liener et al.,1986). Against ABG as well as DIg, the alcohol precipitated polysaccharides of edible sugar were more reactive than the undialysable polysaccharides reported earlier (Fig: 3 and 4). The specificity of these polysaccharides to DIg binding was further confirmed by the reversibility of DIg-HRP binding to dextran by alcohol-precipitated polysaccharides (Fig: 5).

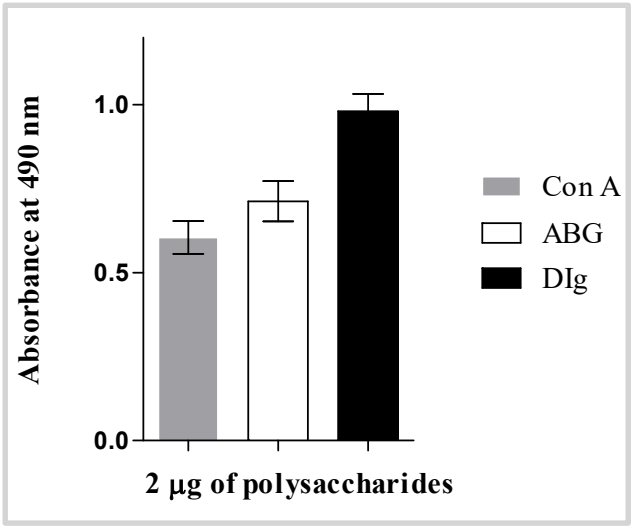

Fig:3. Con A, ABG and DIg binding to high molecular weight polysaccharides obtained by 50% alcohol precipitation from saturated solution of edible sugar: high molecular weight polysaccharides (2 µg in 200 µl PBS-T) was coated on

polystyrene wells and was followed by ConA (2 µg/well), DIg (1 µg/well) or ABG (1 µg/well). Bound Con A was detected using HRP (1 µg/well) and bound immunoglobulins using mixture of HRP-conjugated anti-human IgA, IgG and IgM (1.5 µg/ml each). The bound HRP was measured as mentioned earlier. Values are mean ± SD of six trials.

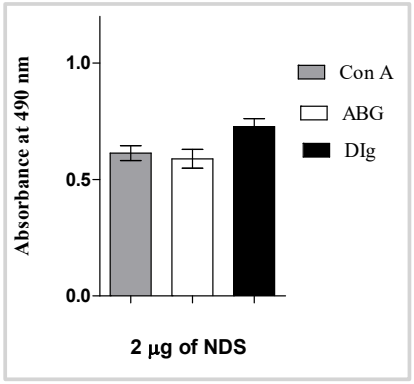

Fig:4. ConA, ABG and DIg binding to non dialyzable sugar (NDS) from edible sugar: NDS (2 µg in 200 µl) was coated on polystyrene wells and was followed by Con A (2 µg/well), DIg (1 µg/well) or ABG (1 µg/well). Bound Con A was detected using HRP (1 µg/well) and bound immunoglobulins using mixture of HRP-conjugated anti-human IgA, IgG and IgM (1.5 µg/ml each). The bound HRP was detected as mentioned. Values are mean ± SD of six trials.

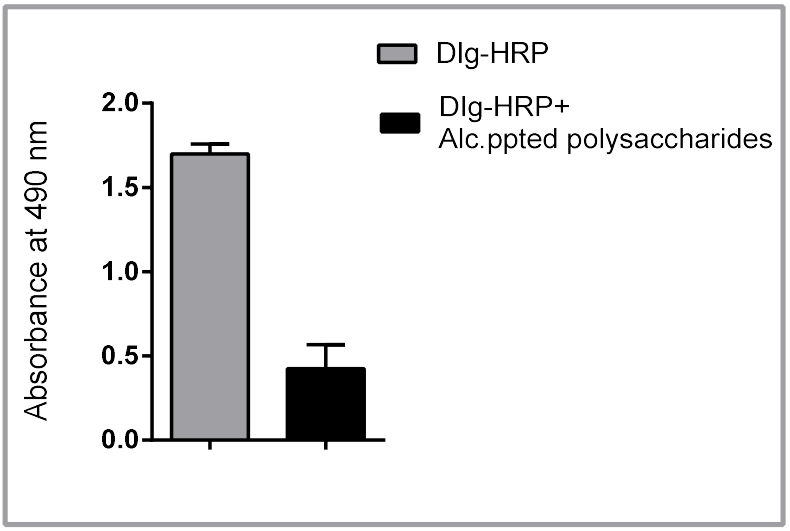

Fig: 5. Inhibition of DIg-HRP binding to coated dextran by alcohol precipitated polysaccharides. Dextran (2 µg in 200 µl PBS) was coated on polystyrene wells. After washing and blocking wells were treated with DIg-HRP (5 µg/ml in PBS-T) preincubated with or without alcohol precipitated polysaccharides (10 µg/ml). Bound HRP was assayed as mentioned. Values are mean±SD of 3 samples.

B) The α-Gal specificity of DIg varies from sample to sample:

DIg has primary specificity for α-linked glucosides (Kabat, 1960). Most natural antibodies are polyreactive as their antigen binding pockets can accommodate several different antigens, being more flexible and capable of assuming multiple three dimensional conformations before antigen contact (Notkins, 2004). Polyreactivity of natural antibodies could be attributed to lysine and arginine residues at the hypervariable regions forming salt bridges with complementary charged groups expressed on the surface of antigens (Avrameas, 1991). Some DIg samples had been reported from this laboratory to possess additional specificity to α-

galactosides (Paul et al., 2009). Simultaneous analysis of sixteen samples revealed that additional specificity toward α-galactosides varied widely among DIg samples (Fig: 6). This indicated that environmental antigens including those from diet that enter the body exert an affinity maturation effect on DIg.

In summary results shows that commercially available sugar contain dextran-like contaminants which offer antigenic epitopes more efficient than earlier reported for naturaly occurring anti-carbohydrate antibodies with DIg being slightly better than ABG in recognising them. Polyreactivity of these antibodies enables them to bind to α-Gal epitope bearing tissue and microbial surfaces.

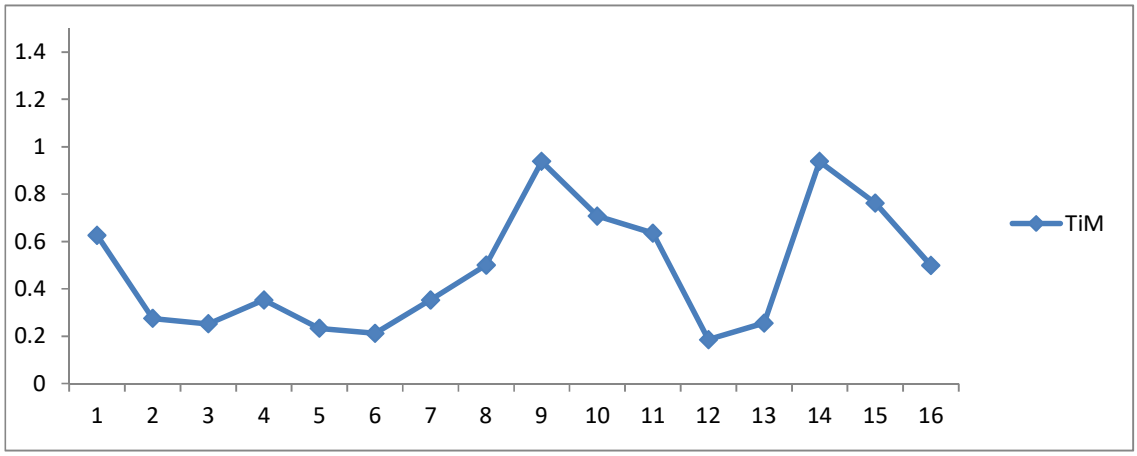

Fig: 6. Variation in the α-gal binding ability of different DIg samples. TIM (2 µg in 200 µl PBS) was coated on polystyrene wells. After washing and blocking DIg (1 µg in 200 µl PBS-T) was added. The bound DIg was probed using a mixture of HRP-conjugated anti-human IgA, IgG and IgM (1.5 µg/ml each). The bound HRP was assayed as mentioned. N=16

Naturally occurring anti-carbohydrate antibodies recognising *Streptococcus mutans* cell wall antigen:

Several reports have suggested that commonly occurring oral bacteria, *Streptococcus mutans* are associated with various diseases including infective endocarditis (McGhie et al., 1977), cardiovascular diseases (Nakano et al., 2008), diabetes (Southerland et al., 2005) and ulcerative colitis (Kojima et al., 2012). Apart from being the causative organism in dental caries *Streptococcus mutans* has been shown in recent investigations to contribute to inflammation, infection and disease throughout the cardiovascular system. Even as cardiovascular diseases are steadily on the rise worldwide epidemological studies suggest that only half of the disease incidence could be explained by classical risk factors so that novel risk factors for these diseases came to be identified (Mozaffarian et al., 2008), including microbial pathogens, corresponding antibodies and their immune complexes. Periodontitis is often associated with proinflammatory mediators which could be an indication of increased cardiovascular disease risk (De Nardin, 2001).

Streptococcus mutans was one of the most abundant bacterial species isolated from extrirpated heart valve (69%) and atheromatous plaque samples (74%), while other periodontal bacteria including *Porphyromonas gingivalis* was found at a much lower frequency (Nakano et al., 2006b), although the actual mechanism of tissue deposition of *S mutans* is not yet clear.

C) Anti-carbohydrate antibody in normal plasma recognizes *S mutans* cell wall antigens:

Heat-treated unbroken cells of *S mutans* mixed with celite for porosity were used as affinity matrix to isolate plasma carbohydrate-specific antibodies that recognize surface antigens of the bacteria. Use of solubilised and electrotransferred antigens for this purpose was avoided since this fraction might miss the polysaccharide as well as highly glycosylated protein antigens (Chia et al., 2000). Analysis of plasma proteins that were bound to the above matrix and were eluted with high concentrations of dextrose and galactose showed that carbohydrate-specific antibody populations of normal plasma that recognized *S mutans* (SMBP) were mostly IgG, followed by IgM and IgA (Fig: 7). SMBP consisted solely of immunoglobulin as shown by SDS electrophoresis (Fig: 8) in which the mobilities of three protein bands of SMBP were exactly identical with those of standard samples of IgA, IgG and IgM respectively. β-Glucans were best among inhibitors of SMBP recognition of *S mutans* antigens, followed by α-glucans indicating that ABG and DIg may be prominent antibodies in plasma recognizing *S mutans,* while anti-Gal may not be involved in recognition (Fig: 9). This conclusion was confirmed by the sugar specific recognition of ELISA plate-coated crude antigens solubilised from *S mutans* by both ABG and DIg nearly equally but poorly by anti-Gal (Fig:10)

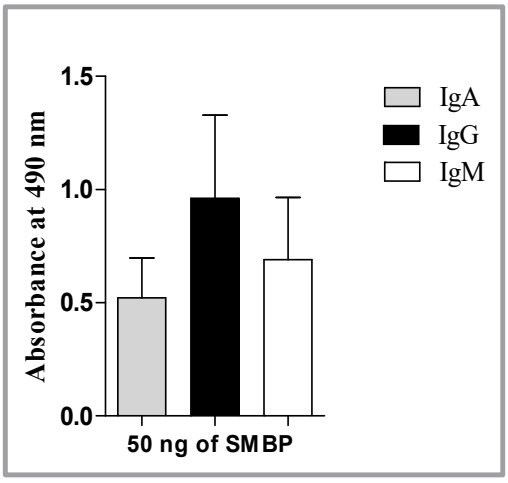

Fig: 7. Immunoglobulin distribution in SMBP. SMBP was coated on polystyrene wells (50 ng per well) and probed with HRP- conjugated anti-human IgG, IgM or IgA (1.5 µg of each antibody per ml). The bound HRP was assayed as described. Values are mean ± SD of six samples.

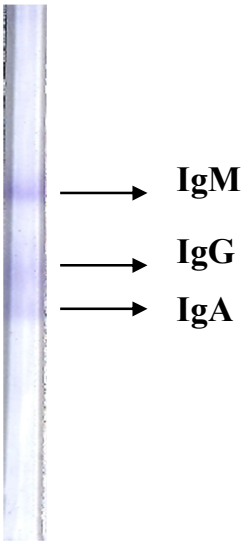

Fig:8. SDS-polyacrylamide gel electrophoresis of SMBP (25 µg). Horizontal arrows indicate positions of standard human immunoglobulin samples.

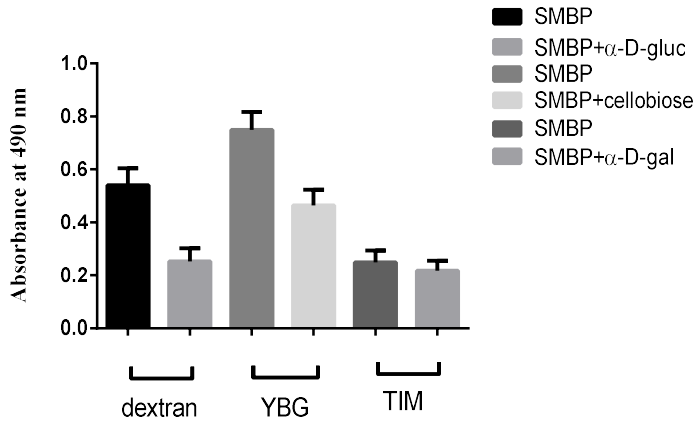

Fig:9. Sugar specificity of SMBP. Dextran, yeast-β-glucan (YBG) and TIM (2 µg each) were coated on polystyrene wells and SMBP (2 µg in 200 µl PBS-T) pre-incubated with or without respective specific sugar (25 mM of 1-O-methyl α-D-glucoside for dextran, cellobiose for YBG or 1-O-methyl α-D-galactoside for TIM) was added. After 2h incubation at 4^0C and washing bound SMBP was probed with a mixture of HRP conjugates of anti-human IgG, IgA and IgM (1.5 µg/ml). Bound HRP was assayed as described. Values are mean ± SD of six trials. P values for inhibition were: < 0.0001, < 0.0001 and 0.2034 respectively for the above three inhibitors.

D) Binding specificity of anti-carbohydrate antibodies to antigens isolated from *Streptococcus mutans.*

The three naturally occurring antibodies checked for *Streptococcus mutans* specificity included ABG, DIg and anti-Gal. There are several ways through which *S mutans* can gain access into circulation and can offer antigenic epitopes for naturally

occurring anti-carbohydrate antibodies. The crude antigen from *Streptococcus mutans* obtained by freeze thawing and sonication was found to be almost equally recognizable by both ABG and DIg although it was found to be poor ligand for anti-Gal (Fig:10).

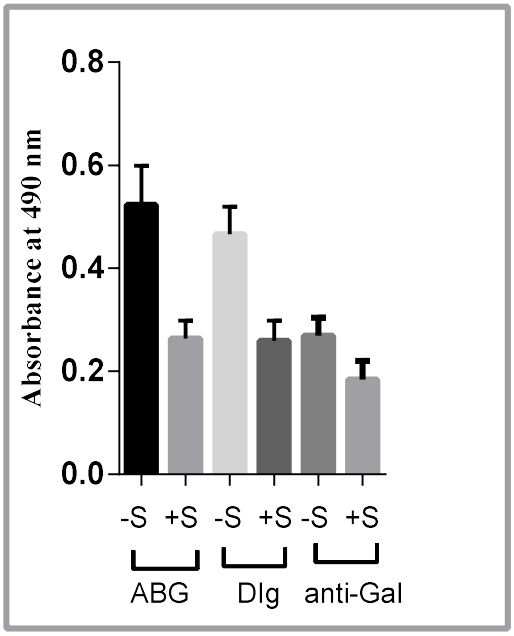

Fig:10. Anti-carbohydrate antibody binding to crude antigen from Streptococcus mutans. Streptococcus mutans crude antigen (2 µg) was coated on polystyrene wells and probed with anti-carbohydrate antibodies (2 µg of ABG, DIg or anti-Gal) pre-incubated with (+S) or without (-S) 25 mM specific sugar (cellobiose for ABG, 1-O-methyl-α-glucoside for DIg and 1-O-methyl-α-D galactoside for anti-Gal). Antibody bound to the antigen was detected using a mixture of HRP- conjugated IgG, IgA and IgM (1.5 µg per ml). Bound HRP was assayed as described. Values are mean ± SD

of six trials.. P values for the inhibition were: < 0.0001 (ABG), < 0.0001(DIg) and 0.002 (anti-Gal).

Streptococcus mutans cell wall antigen was further resolved into different components by acid-PAGE electrophoresis and ion exchange chromatography. Acid PAGE electrophoresis was done for the separation of positively charged proteins and ion-exchange chromatography for the separation of negatively charged protein antigens. Positively charged proteins (PSMAg) of *S mutans* extract moved as a single band in acid pH electrophoresis (Fig: 11). The electroeluted protein from the gel coated on microwells was recognized sugar-dependently by ABG and to a lesser extent by DIg (Fig: 12).

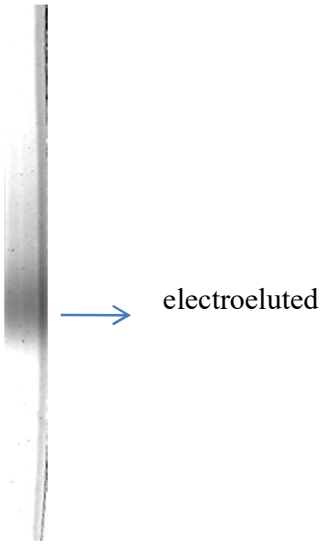

Fig:11. Acidic (pH 4.5) polyacrylamide gel electrophoresis of solubilised S mutans antigens (100 µg) in 7.5% gel.

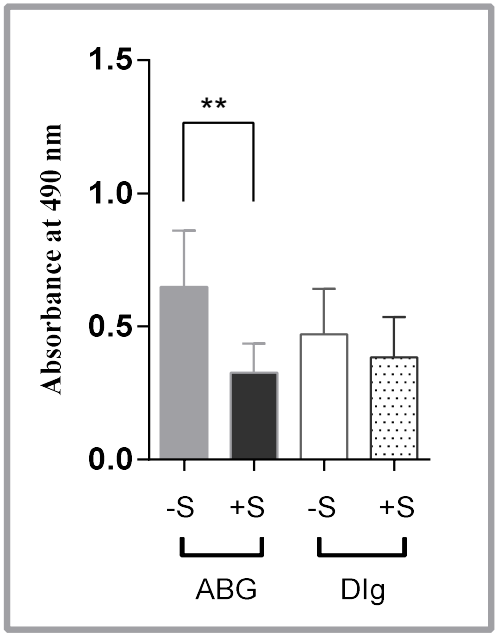

*Fig: 12. Anti-carbohydrate antibody binding to PSMAg. Polystyrene wells coated with PSMAg (2 µg per well) were blocked and probed with anti-carbohydrate antibodies (2 µg of ABG and DIg) pre-treated with (+S) or without (-S) 25 mM specific sugar (cellobiose for ABG and 1-O-methyl-α-D-glucoside for DIg). The antibody bound to coated antigen was detected using mixture of HRP-conjugated anti-human IgG, IgA or IgM (1.5 µg/ml). Bound HRP was assayed as described. Values are mean ± SD of four trials. P values for the inhibition of ABG and DIG binding to PSMAg were: 0.0050 (** ≤0.005) and 0.2767 respectively*

Negatively charged *S mutans* antigen (NSMAg) obtained by DEAE-Sephadex chromatography also moved as a single peak in SDS-polyacrylamide gel electrophoresis (Fig: 13). This protein in turn was also recognized both by ABG and DIg (Fig: 14). PSMAg or NSMAg recognition by ABG or DIg was poorly

inhibitable by specific mono- or disaccharide sugars. A reason may be that the low molecular weight mono or disaccharides used for inhibition were too different in structure from the antigenic epitopes that induced production of these antibodies. In addition, secondary interactions between protein components of the *S mutans* antigens and antibody binding site may render the antigen- antibody recognition harder for small sugar ligands to compete with.

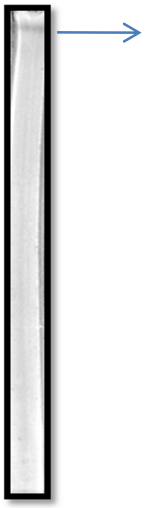

Fig: 13. Polyacrylamide (7.5 %)gel electrophoresis of NSMAg (50 µg) eluted from DEAE-Sephadex A-50.

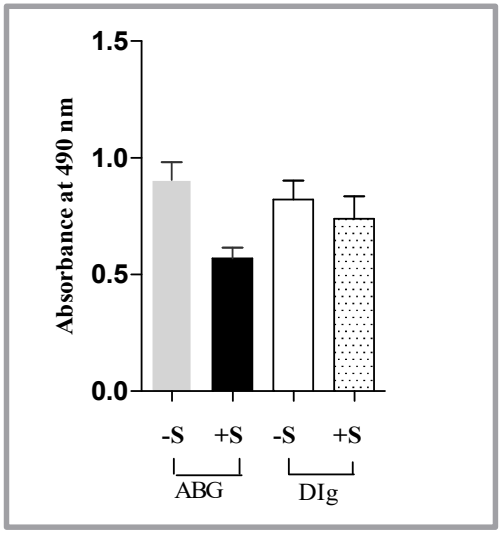

*Fig: 14. Anti-carbohydrate antibody binding to NSMAg: Polystyrene wells coated with PSMAg or NSMAg (2 µg per well) were blocked and probed with anti-carbohydrate antibodies (2 µg of ABG and DIg) pre-treated with (+S) or without (-S) 25 mM specific sugar (cellobiose for ABG and 1-O-methyl-α-D-glucoside for DIg). The antibody bound to coated antigen was detected using mixture of HRP-conjugated anti-human IgG, IgA or IgM (1.5 µg/ml). Bound HRP was assayed as described. Values are mean ± SD of six trials. P value for the inhibition of ABG binding to NSMAg with cellobiose = 0.0038 (**≤0.005).*

E) Lectin binding to *S mutans* antigens:

Concanavalin A (ConA) extracted from jackbean (*Canavalia ensiformis*) could recognise non-reducing terminal α-D-mannosyl and α-D glucosyl groups in glycoproteins (Liener et al., 1986) while jacalin, another plant lectin obtained from seeds of jack fruit (*Artocarpus integrifolia*) is specific for O-glycoproteins (Liener et al., 1986). ConA recognition of *S mutans* antigens (NSMAg and PSMAg) indicated

the presence of α-D glucosyl groups which could be attributed to the rhamnose glucan polymer (RGP) within their cell wall (Tsuda et al., 2000). A mild response to jacalin also suggested presence in both the above antigens of terminal α-galactosyl residues (Fig: 15 and 16).

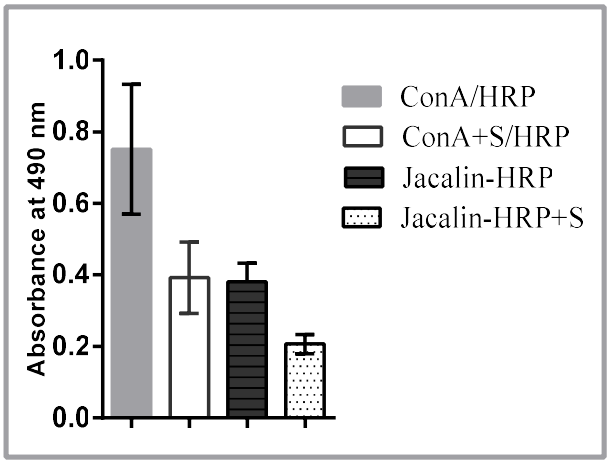

Fig: 15. Lectin binding to positively charged S mutans antigens (PSMAg): PSMAg (2 µg) was coated to polystyrene wells and probed with Con A (10 µg/ml) or jacalin-HRP (75 ng lectin per ml) pre-treated with or without specific sugar (25 mM 1-O-methyl-α-D mannoside for ConA and 25 mM 1-O-methyl-α-D-galactoside for jacalin-HRP). After washing, Con A wells were further treated with HRP (10 µg/ml) for 2h. All wells were washed and bound HRP was assayed as mentioned earlier. Values are mean ± SD of six trials.

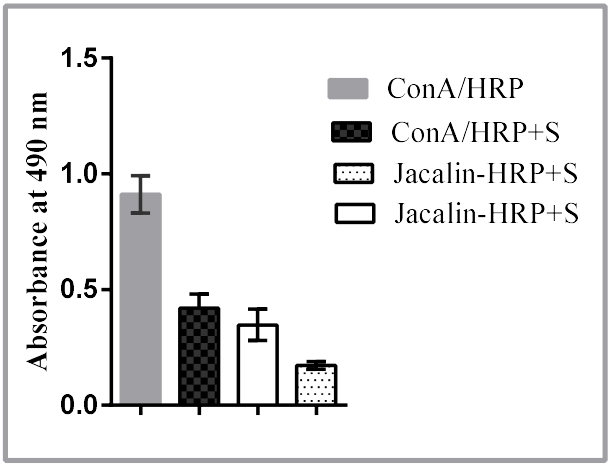

Fig: 16. Lectin binding to NSMAg: NSMAg (2 µg) was coated to polystyrene wells and probed with Con A (10 µg/ml) or jacalin-HRP (75 ng lectin per ml) pre-treated with or without specific sugar (25 mM 1-O-methyl-α-D mannoside for ConA and 25 mM 1-O-methyl-α-D-galactoside for jacalin-HRP). After washing, Con A wells were further treated with HRP (10 µg/ml) for 2h. All wells were washed and bound HRP was assayed as mentioned earlier. Values are mean ± SD of six trials.

Discussion:

Environmental microbial load offers abundant carbohydrate epitopes that can elicit the production of large quantities of anti-carbohydrate antibodies. Glycans constitute a major part of the antigens introduced into the host through dietary and microbial sources. Anti-carbohydrate antibodies including DIg, ABG, anti-Gal and LIg which are present in fairly high concentration in healthy human plasma could possibly encounter these antigens introduced into the body. These anti-carbohydrate antibodies are synthesised in response to microbial, environmental and dietary

antigens and are instrumental in innate immune response (Chacko and Appukuttan, 2003). Dextran which is present as a common contaminant in commercially available sucrose by *Leuconostoc mesentroides* contamination (Sidebotham, 1974) was used as an example of a dietary antigen to study its fate when exposed to plasma antibodies. Dextran provides a linear epitope for DIg and is an example of an antigen capable of eliciting a T-independent immune response. High molecular weight polysaccharides which are obtained by extensive dialysis or by alcohol precipitation of saturated sucrose was recognised by both ABG and DIg, the response of DIg being slightly better than that of ABG (Fig: 3 and Fig: 4) indicating the presence of dextran-like polysaccharides as contaminant in commercially available sucrose. Specificity of binding was confirmed by the reversibility of binding by inhibitory sugars. Earlier reports from our lab found that the anti-carbohydrate antibodies like ABG and DIg are particularly enriched in polymeric IgA (Geetha et al., 2007 and Paul et al., 2011) and that IgA was a potent ligand for Galectin-1 (Sangeetha and Appukuttan, 2005).

In healthy individuals dietary biomolecules have been shown to enter circulation and reported to exist in their native state or as immune complexes (Husby et al., 1986). Dextran-like high molecular weight polymers were found in all samples of commercially available sucrose samples tested and their indigestibility may cause them to enter circulation as an antigen. Thus they can act as a constant trigger for the formation of IC with naturally occurring anti-carbohydrate antibodies, DIg and ABG. The ICs formed by the addition of dextran was comprised mosly of IgM followed with IgG and IgA probably because antibodies involved in the

formation of these ICs belong to the natural antibody repertoire in which IgM is the most dominant immunoglobulin (Binder et al., 2005). The extremely high content of IgM in DIg-IC may point to the biological fate of these ICs. Complexes exceeding Ag_2Ab_2 in size were preferentially cleared by the hepatic reticuloendothelial system and the clearance of IgM ICs comes under this system. However saturation of the reticuloendothelial system can occur by the repeated administration of these complexes and this can result in the persistence of these ICs in circulation thereby increasing their likelihood of causing non-hepatic tissue deposition diseases (Haakenstad and Mannik, 1974). But IgG and IgA immune complexes are reported to be potentially proatherogenic (Burut et al., 2010). Thus the edible source of sucrose is a constant source of polysaccharide antigen to which the host immune system is constantly exposed to and could eventually lead to immune complex formation.

Although its primary ligand was α(1→6) linked glucans, DIg was found to be almost equally recognising terminal α-Gal bearing glycoproteins with wide variation in the α-Gal binding ability of different DIg samples (Fig: 6). The multiple specificity or dual specificity of DIg is significant since the pathogenic potential of terminal α-linked galactose (TAG) of endogenous or environmental origin is being increasingly recognised. The Gal α(1→3)Galβ(1→4)GlucNacR (i.e. α-Gal epitope) was found to be absent in humans, apes and old world monkeys because these species lack the glycosyltransferase enzyme that links the terminal galactose in an α(1→3) linkage to the N-acetyllactosaminyl of the carbohydrate chain (Galili et al., 1984). Reports from our lab has shown that these epitopes are present in

glycoproteins isolated from human brain grey matter (Jaison et al., 1993). Later atherosclerotic plaques in humans were also found to contain antibodies reactive against terminal α-galactosyl epitope (Mosedale et al., 2006). Serine- and threonine-rich peptide sequences (STPS) present in MUC-1 family of peptides or O-glycosylation sites of glycoproteins are reported to act as surrogate to TAG as a ligand for anti-Gal (Sandrin et al., 1997 and Geetha et al., 2014). Thus the presence of α-Gal binding DIg is of considerable pathological significance because DIg is often more numerous than anti-Gal in circulation. After binding to large antigens like dextran DIg may have additional free binding site as reported in the case of anti-Gal or LIg which after binding to large antigens like Lp(a) using one binding site of their IgG, use the other binding site to bind to other cell surface ligands (Mandagini et al., 2013 and Sabarinath and Appukuttan, 2015). Such ICs may initiate an inflammatory reaction at tissues with cells possessing appropriate antigens on their surface.

Another source of α-glucan-containing polymer to which immune system may be constantly exposed to are the plaque forming oral bacteria *S mutans*. Previous reports have suggested that infective pathogens play an important role in the pathogenesis of various diseases including atherosclerosis and several other disorders involving immune inflammatory reactions (Morré et al., 2000). Multiple pathogens are causally related to CAD while risk for CAD and other inflammatory diseases is related to the aggregate pathogen load (Epstein, 2002). Both viral and bacterial pathogens have been suspected in playing a part either directly or indirectly in the process leading to atherosclerosis (Cook and Lip, 1996). Chronic dental

bacterial infections are strongly correlated with progression of atherosclerosis and other inflammatory diseases (Morré et al., 2000). Researchers have suggested a link between the transfer of bacteria from the mouth to the blood stream on one hand and onset of various inflammatory reactions on the other (Burks, 2014). *Streptococcus mutans* is known to be one of the pathogens associated with inflammatory diseases although primarily it causes dental plaques and oral infections (Moreillon and Que, 2004). *S mutans* infecting the oral cavity can gain access into the circulation either through minor cuts and abrasions occurring during dental procedures or by routine dental procedures like brushing and flossing. Present results suggest that circulating antibodies that recognize *S mutans* surface antigens are mostly the α- and β- glucan-specific naturally occurring antibodies. The latter have been reported to be contributed by the anti-β-glucan (ABG) and dextan-binding (DIg) antibodies respectively (Geetha et al., 2007 and Paul et al., 2009).

Positively charged protein antigen from *S mutans* (PSMAg) isolated by electroelution after electrophoretic separation of S *mutans* antigens (Fig:11) and negatively charged *S mutans* antigen (NSMAg) bound to DEAE-Sephadex matrix and eluted by sodium chloride (Fig:13) were both recognized by circulating ABG and to a lesser extent by DIg. Both these recognitions were substantially inhibitable by small sugar antigens of respective antibodies suggesting glycan moieties of the protein antigens as ligands for antibody binding (Fig:12, 14). The ABG and DIg recognition of these cell wall glycoproteins from *Streptococcus mutans* could be attributed to the rhamnose glucan polymer (RGP) within their cell wall which contain a backbone of rhamnose sugar and side chains of β(1→2), α(1→2), and

α(1→3) glucose residues (Tsuda et al., 2000). Very little information is available regarding the structural organization of these RGPs within the *S mutans* cell wall. Erickson (1993) has reported that the PAAP protein in Streptococcus species which is known to induce platelet aggregation is an RGP rich glycoprotein.

Part II:

Antigen Induced Conformational Changes in Antibodies and the Occurrence of Free Binding Sites on ABG-NSMAg Complex:

Introduction:

Consequent to antigen binding at the hypervariable regions of their Fab chains antibodies are activated to mediate processes such as complement fixation, mast cell activation and triggering lymphocytes towards differentiation or tolerance. The latter function is attributed to conformational shift brought about in the Fc part in response to antigen binding (Schlessinger et al., 1975). Structural studies have shown that binding of antigen induces conformational changes in the antibody, antigen or both (Kuby et al., 2007; Brown and Koshland, 1975). Conformational changes in the binding site of the antibody has been proposed to result from an induced fit brought about by the antigen (Berger et al., 1999) leading to alteration in size, shape and charge distribution in the antigen binding pocket (Wilson and Stanfield, 1994). Antigen binding has been suggested to trigger a signal which is transmitted from the Fv region to the Fc in the form of conformational changes in the Fc portion (Davies and Cohen, 1996; Oda, 2003; Keskin, 2007). In support to this hypotheses smaller antigens capable of producing only local and limited conformational changes in the antigen binding site do not cause any change in the Fc part nor activate any effector function of the antibody (Oda, 2003). The mechanism of transmission of signal from Fab to Fc upon antigen binding is not yet clear though it has been assumed that inter-chain disulfide bond plays a major part.

Abe et al. (2011) reported a significant antigen-dependent fluorescence enhancement when fluorolabeled single chain variable region of antibody was used. In the present study we demonstrate using naturally occurring human plasma anti-

carbohydrate antibodies that FITC-labeled whole antibody shows significant increase in fluorescence upon antigen binding and that conformational changes in Fc part are largely responsible for this effect. We also show that increase in fluorescence of FITC-labeled antibody is proportional to a) affinity of antigen when various antigens bind to a given antibody and b) specific activity of the antibody when a given antigen binds to antibodies of varying specific activity from different individuals. Increase in fluorescence can be used as a measure of antigen binding affinity and to detect the presence of specific ligands. Naturally occurring anti-carbohydrate antibodies were used for the study since these are easily prepared in pure form and antigens and their analogues are readily available.

When using different *S mutans* antigenic components, NSMAg was most effective in inducing fluorescence shift in FITC-labeled ABG. ABG occupied by NSMAg can as well bind to a β-glucan ligand on macromolecule confirming the presence of free binding sites on these complexes and this could play an important role in facilitating their tissue deposition.

RESULTS:

A) **Fluorescence enhancement of FITC labeled antibodies upon antigen binding:**

Fluorescence of FITC-labeled antibodies, anti-Gal, DIg and ABG were enhanced upon binding of corresponding macromolecular antigen, viz bovine thyroglobulin, dextran 150 kDa and TIC respectively (Fig:17), percentage increase being 26, 34 and 32 % respectively. Further, enhancement of fluorescence following

binding of large antigens to each of the above antibodies was fully abolished by the presence of respective mono- or disaccharide antigens at 25 mM concentration. Trypsin inhibitor (TI) or human plasma albumin used as control proteins was ineffective in causing any change in fluorescence of FITC-labeled antibodies (data not shown), indicating that it takes a specific ligand as part of a macromolecule to cause fluorescence enhancement in antibodies. FITC labeling of antibodies did not cause any reduction in antigen binding activity of the antibodies or in their recognition by Fc-specific secondary antibodies (data not shown) owing primarily to protection of the binding site by small antigenic ligands during conjugation with FITC.

Total immunoglobulin fraction precipitated by 45% ammonium sulphate saturation of plasma was also coupled with FITC to check whether the increase in fluorescence observed was due to any colligative effect regardless of antigen specificity. No increase in fluorescence was produced by ligands like thyroglobulin, TIM or TIC on FITC-labeled total immunoglobulin (data not shown).

The change in fluorescence could be due to conformational changes produced in the antibody upon antigen binding (Schlessinger et al., 1975). The effector function of an antibody is activated by conformational changes in CH2 domain of the Fc part (Brown and Koshland, 1975; Sela-Culang et al., 2012) which in turn is located 80-100 A^0 away from the antigen binding site in the case of IgG (Connell and Porter, 1971) and much farther away in the case of IgM which assumes a staple conformation upon antigen binding (Kuby et al., 2007). Abe et al. (2011)

has concluded that the increase in fluorescence of fluorolabeled single chain variable region upon antigen binding is due to antigen dependent removal of quenching effect caused by the proximity of tryptophan residues in the antigen binding pocket. On the other hand Schlessinger et al. (1975) has shown that the change in CPL (circular polarization of luminescence) spectrum of antibody upon antigen binding was reversed by the removal of Fc by papain digestion. Most of the conformational changes in the antibody molecule take place away from the antigen binding site (Sela-Culang et al., 2012). Antigen binding has been shown to profoundly change the conformation of the Fc part of antibodies enabling it to fix complement as well as trigger effector functions in other cells (Schlessinger et al., 1975). These reports along with the fact that binding site at the variable region were protected from fluorescence labeling indicated that fluorescence increase caused by macromolecular ligands on FITC-labeled antibodies was mainly due to conformational changes in Fc part. Reversal of fluorescence shift by prior incubation of antibody with specific sugar confirmed that binding of macromolecular ligands was responsible for the observed fluorescence increase.

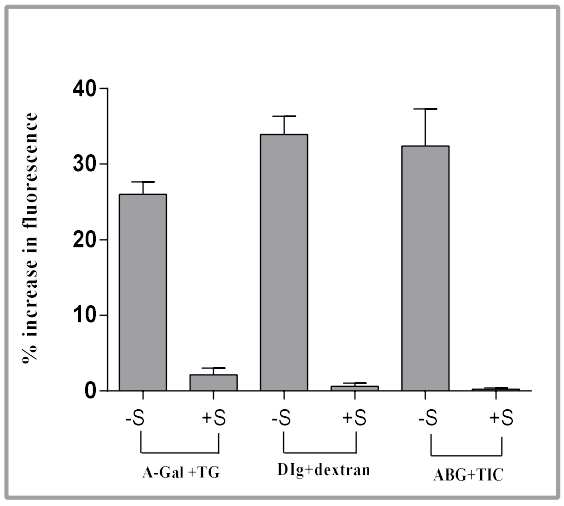

Fig:17. Increase in fluorescence of antibodies in presence of specific macromolecular antigen: Antibody (6.25 µg) was mixed with 15 µg of respective macromolecular antigen overnight before measuring fluorescence (details under methods). Values are mean ± SD of 7 anti-Gal samples, 5 DIg samples and 5 ABG samples. Percentage increase over that of free antibodies shown. Antibodies pre-incubated with specific sugars (S: 25 mM melibiose for anti-Gal, 25 mM 1-O-methyl-α-D-glucoside for DIg and 25 mM cellobiose for ABG) served as control. TG: bovine thyroglobulin; TIC: cellobiose coupled to soybean trypsin inhibitor.

B) **Fluorescence enhancement in FITC-labeled antibody increases with size of the macromolecular antigen:**

DIg was used to study the effect of molecular size of antigen on fluorescence enhancement in FITC-labeled antibody since dextrans of molecular mass ranging from 15 kDa to 400 kDa was available. DIg is a naturally occurring anti-carbohydrate antibody with specificity for α-linked glucosides (Kabat, 1960) which

are also the monomers of dextran. Same amount (15 µg neutral sugar) of different varieties of dextran with molecular masses of 15 kDa, 34-40 kDA, 150 kDa and 400 kDa produced fluorescence increase of 17.8 %, 25.8 %, 32.9 % and 34.9 % respectively in FITC-labeled DIg (Fig: 18). Results in Fig: 17 and Fig: 18 indicate that though monosaccharide moiety may be the basic sugar unit for anchoring of an antigen on a carbohydrate-binding antibody such as DIg, the larger antigens, unlike small sugars, engage in secondary interaction with regions in the antibody neighboring the binding site. Secondary interactions are likely to produce in the Fab region an induced fit akin to that produced by a substrate on an enzyme to an extent sufficient to produce a measurable conformational shift in the Fc region as well. Increase in fluorescence enhancement of FITC- antibody with increase in antigen size underlines this assumption. Our results agree with the findings of Oda (2003) that binding of small antigens are capable of producing only local changes in the binding site without activating effector functions. Further, Sela-Culang et al. (2012) has also shown that conformational changes in antibody takes place in the elbow angle between variable and constant domain as well as in a loop in the heavy chain constant domain and that these changes are significantly larger on binding of large antigens rather than that of small antigens.

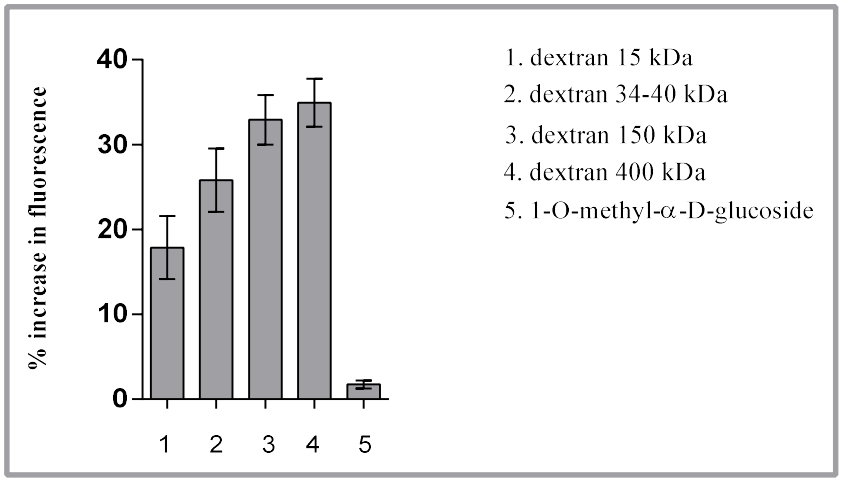

Fig: 18. Percentage increase in fluorescence of FITC-labeled DIg with dextran of different molecular weight. FITC-labeled DIg (6.25 µg) was mixed with 15 µg each of dextran of different molecular weight and the percentage increase in fluorescence from that of untreated FITC-labeled DIg was measured. Values are mean ± SD of four DIg samples for each dextran variety and 1-O-methyl-α-D-glucoside (25 mM) was used as control.

C) **Fluorescence enhancement in antigen-bound antibody is a measure of antigen binding affinity**

Anti-Gal that constitutes nearly 1% of circulating IgG is specific to α-linked terminal galactosides and is synthesized only by higher primates such as man, apes and old world monkeys (Galili et al., 1987). Using anti-Gal samples isolated from plasma of six individuals and varying in specific activity (methods) from 0.326 to 1.1 (Fig: 19) it was observed that increase in fluorescence enhancement was proportional to the specific activity of the antibody when the same amount of FITC-

anti-Gal and antigen (thyroglobulin) were used in all cases (Fig: 19). Apart from confirming antigen occupation at binding site as cause of fluorescence increase the results suggested that fluorescence enhancement upon antigen binding could be used as a tool to measure the antigen binding affinity of the antibody, a property that determines its specific activity

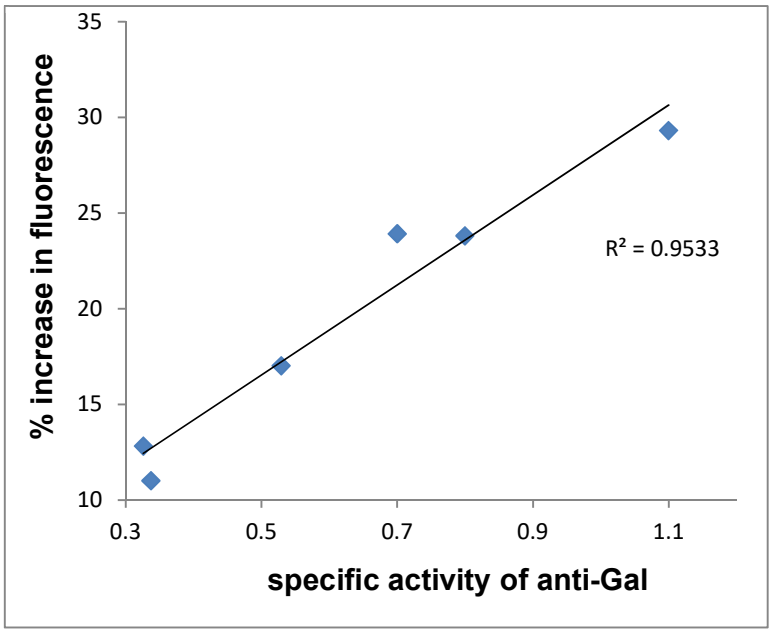

Fig: 19. Relation between fluorescence increase produced in FITC-labeled anti-Gal by thyroglobulin and specific activity of the antibody sample. FITC-anti-Gal (6.25 µg) samples differing in specific activity was mixed with thyroglobulin (15 ug) overnight and the percentage increase in fluorescence over untreated conjugate measured.

D) **Fc conformational changes upon antigen binding is responsible for fluorescence enhancement:**

To ascertain the contribution of conformational changes in Fc towards antigen induced fluorescence enhancement in FITC-labeled antibodies, effect of removal of Fc part of antibody by papain digestion was studied. Treatment with 2.5% papain under conditions described completely digested the antibody since the antibody sample purified by affinity chromatography after enzyme digestion had no intact IgG as shown by polyacrylamide gel electrophoresis in presence of SDS (Fig: 20). When ABG antibody subjected to papain digestion and later purified by affinity chromatography was captured on microplate coated antigen (TIC) and probed for presence of Fc region using HRP-labeled anti-Fc antibody, the response was considerably lower than that of intact antibody (Fig: 21). The marginal response towards anti-Fc antibody observed even after digestion with 5% and 10% papain concentrations could be attributed to less than absolute Fc specificity of the probing antibody used, rather than any remaining undigested antibody given the electrophoretic mobility in Fig: 20.

Antibody devoid of Fc part produced, an average, nearly 63 % less fluorescence than its native counterpart following binding of specific ligand (TIC) (Fig: 22).

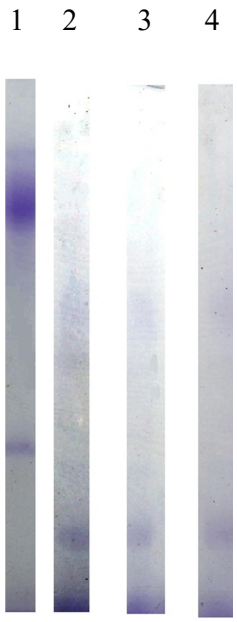

Fig: 20. 7.5 % SDS PAGE without β-ME treatment of intact anti-β-glucan antibody (1), Fab obtained by 10% papain digestion (2), Fab obtained by 5% papain digestion (3) and Fab obtained by 2.5% papain digestion (4).

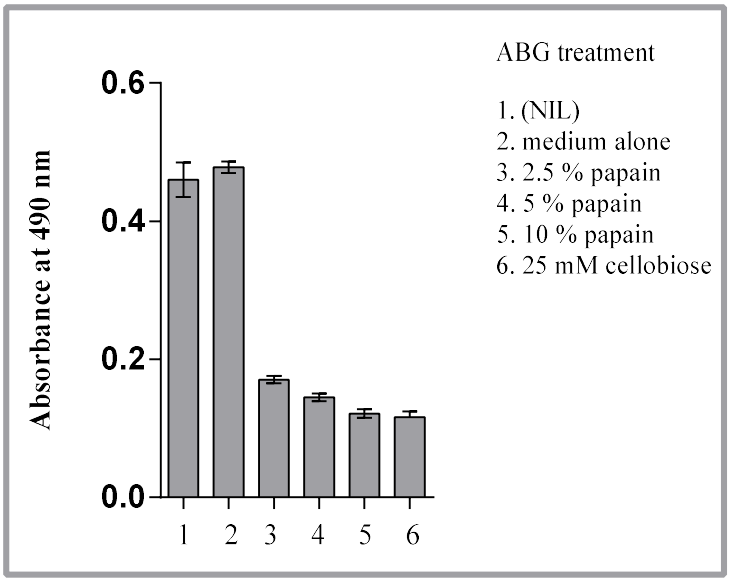

Fig: 21. Fc removal after papain digestion was checked by capturing the antibody (ABG) after enzyme digestion (2 ug in 200 µl PBS-T) on microplate-coated TIC (2 ug per well) and bound antibody probed using HRP conjugate of Fc-specific anti-human IgG. Untreated ABG with and without inhibitory sugar (25 mM cellobiose) were also used. (Values are mean ± SD of 3 samples).

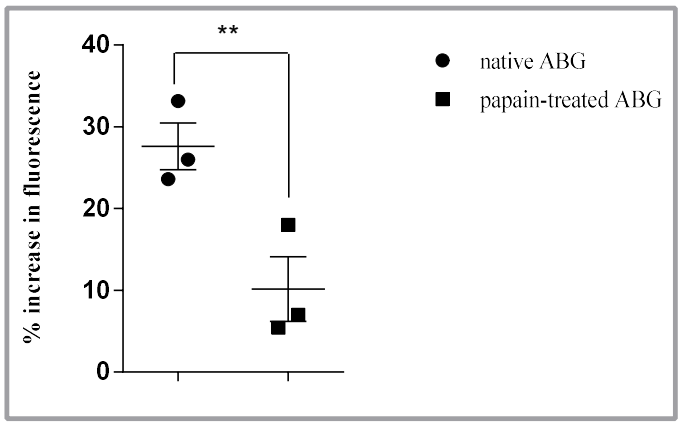

Fig: 22. Effect of papain digestion on fluorescence enhancement. ABG antibody equivalant to 3 µg Fab was mixed with 15 µg ligand and fluorescence enhancement was measured. Values are mean ± SD of 3 pairs. P value for the difference in fluorescence enhancement between intact antibody and Fab = 0.0045

E) **Conformational changes in FITC-labeled ABG upon binding to *Streptococcus mutans* antigen and the occurrence of free binding sites on *S mutans* antigen-ABG complex:**

The affinity of ABG towards *S mutans* antigen was measured by using FITC-labeled ABG. NSMAg was most effective in producing fluorescence enhancement upon binding to FITC-labeled ABG, producing 36.84% increase in fluorescence while same amount of TIC and *S mutans* crude antigen could produce only 28.6 and 27.34 % increase respectively (Fig: 23) and this increase was found to be sugar reversible (data not shown). The contribution of Fc towards ligand induced fluorescence increase was also checked by measuring fluorescence increase after removal of Fc by papain digestion. The fluorescence increase was marginal in

comparison with that produced in the native antibody following binding of specific ligand, NSMAg (data not shown).

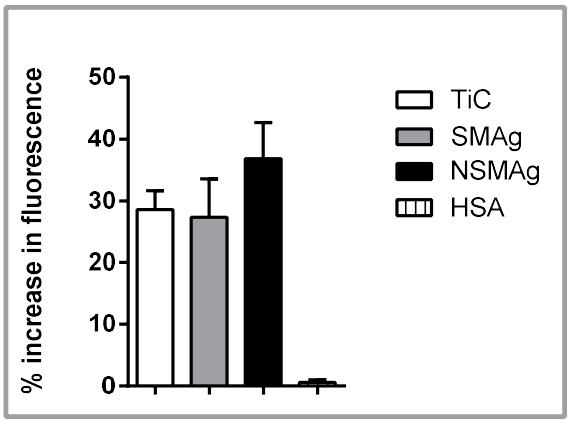

Fig: 23. Percentage increase in fluorescence of FITC-labeled ABG with different ligands: FITC-labeled ABG (6.25 µg) was mixed with 15 µg each of TIC, S mutans antigen, NSMAg and HSA and the percentage increase in fluorescence from that of untreated FITC-labeled ABG was measured. Values are mean ± SD of five ABG samples.

In addition to fluorescence increase, conformational changes in Fc upon NSMAg binding was confirmed by the increase in the anti-Ig response of ABG captured on plate-coated TIC if the antibody had been pretreated with NSMAg. While the preincubation of ABG with NSMAg was done with the intend of inhibiting ABG binding to plate-coated TIC, we observed instead of inhibition, an enhanced response with anti-human Ig-HRP (Fig: 24). A similar increase in anti-Ig response was observed with anti-Gal when apo(a) was used as a macromolecule soluble ligand for binding to the antibody before the latter was captured on plate coated TG (Fig: 25). Apo(a) subunit of Lp(a) had been reported to be efficient

ligand for anti-Gal (Geetha et al., 2014). In both the above cases the macromolecular antigen treated with the antibody in solution (NSMAg for ABG and apo(a) for anti-Gal) remains attached to the antibody which in turn binds to the plate-coated ligand. This suggested that the large antigen could occupy only one binding site of the antibody, since small saccharides capable of binding to all binding sites completely inhibited antibody binding to coated ligand (data reported earlier). The icrease in anti-Ig response can be attributed to two possibilities, a) macromolecular antigen binding potentiates the remaining binding site of antibody by allosteric activation and b) occupation of binding sites of antibodies by macromolecular ligands increases the Fc conformational changes.

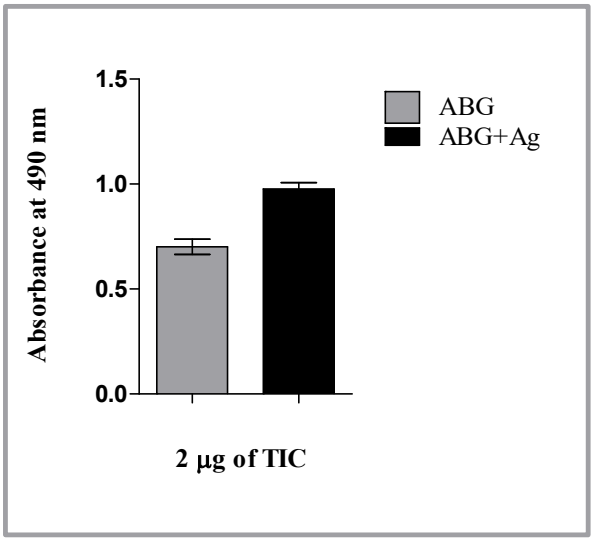

Fig: 24. Increase in anti-Ig response of ABG in presence of ligand. TIC (2 µg in 200 µl PBS) was coated on polystyrene wells and ABG (250 ng in 200 µl PBS-T) pre-incubated with or without NSMAg (2 µg) was added. After 2 h incubation at 4^0 C and washing bound ABG was probed with a mixture of HRP conjugates of anti-

human IgG, IgA and IgM (1.5 µg/ml). Bound HRP was assayed as described. Values are mean ± SD of 6 samples.

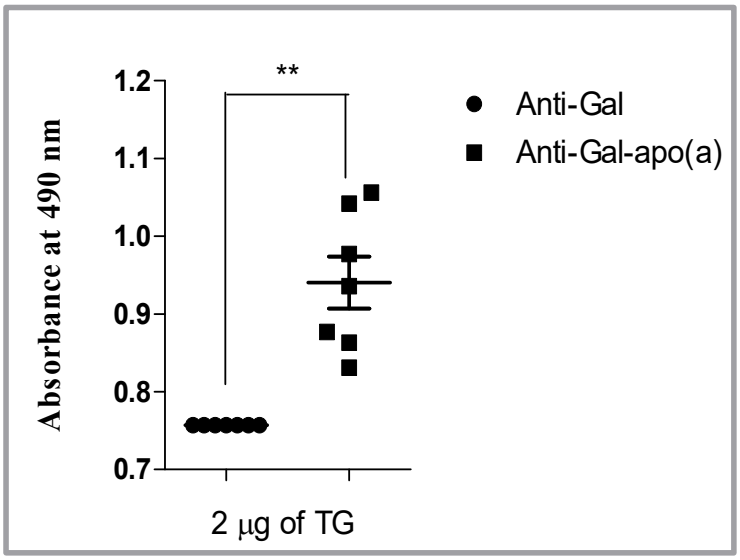

Fig: 25. Increase in anti-Ig response of anti-Gal in presence of apo(a): TG (2 µg in 200 µl PBS) was coated on polystyrene wells and anti-Gal (100 ng in 200 µl PBS-T) pre-incubated with or without apo(a) (500 ng) was added. After 2 h incubation at 4^0 C and washing bound anti-Gal was probed with a mixture of HRP conjugates of anti-human IgG, IgA and IgM (1.5 µg/ml). Bound HRP was assayed as described. Values are mean ± SD of 7 samples of apo(a). P value for the increase in anti-Ig response with apo(a)= 0.0015 (**).

F) **ABG bound to *Streptococcus mutans* cell wall antigen retains free binding sites**

The above results prove that in ELISA for either ABG or anti-Gal by capturing them to plate coated ligands response to anti-immunoglobulin-HRP

increased in case the antibody had been pre-incubated with macromolecular antigens, whereas low molecular weight sugar antigens are known to inhibit binding. Though increase in anti-Ig response was expected in antibodies upon macromolecular antigen binding, the above result also requires that even after macromolecular antigen occupation, the antibodies retain one unoccupied binding site with which they could in turn bind to the coated antigen. This possibility was examined experimentally. ABG pre-incubated with biotinylated NSMAg was found to bind in turn to TIC (a specific ligand for ABG), along with biotinylated antigen (Fig: 26) indicating the presence of free binding sites on ABG-NSMAg complex. Anti-Gal-apo(a) complexes also contained additional free binding sites (Fig:27). Recent studies from our lab have shown that, anti-Gal bound to Lp(a) posess free binding site with which they can bind to other epitopes (Mandagini et al., 2013). Further, LIg already occupied by desialylated Lp(a) could in turn bind to desialylated human RBC (Sabarinath and Appukuttan, 2015). The IC formed between multivalent antigens and antibodies forms lattice leaving some antigen binding sites on the antibody unoccupied due to steric hindrance and the unoccupied binding site on the IC will be available to accommodate a different antigen in different spatial arrangement (Mandagini et al., 2013).

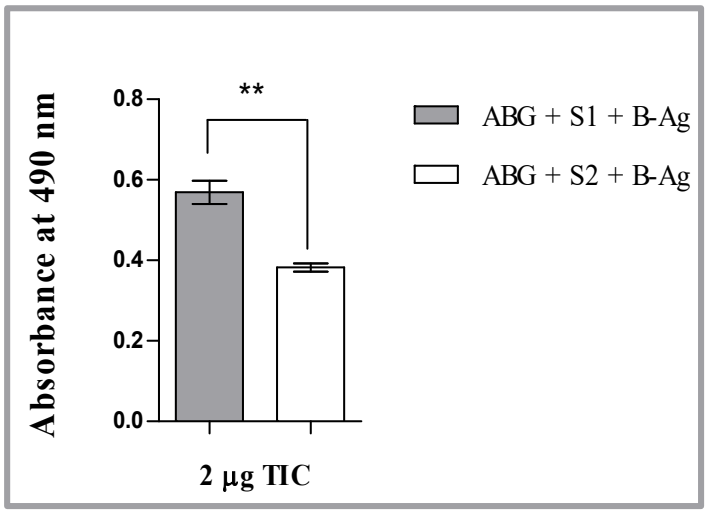

Fig: 26. The presence of free binding sites on ABG-NSMAg complex: ABG (100 ng in 200 µl PBST) preincubated with 25 mM of specific (S2) or non-specific sugar (S1) was allowed to interact with biotin-labelled NSMAg (B-Ag) (500 ng in 200 µl PBS-T) and added to TIC-coated wells (2 µg in 200 µl PBS). The presence of ABG-B-Ag complex on TIC-coated wells were detected using avidin-HRP (2000X) and the bound HRP was determined as described. Values are mean ± SD of four trials. P value=0.0040 (**)

S1=25 mM melibiose

S2=25 mM cellobiose

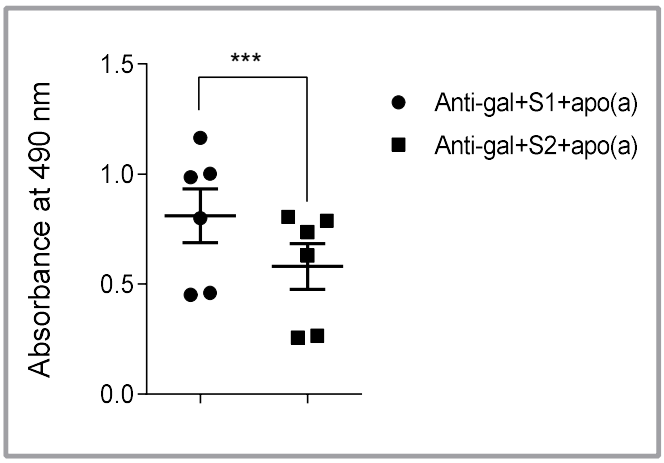

Fig: 27. The presence of free binding sites on anti-Gal-apo(a) complex: anti-Gal (100 ng in 200 µl PBS-T) preincubated with 25 mM of specific (S2) and non-specific sugar (S1) was allowed to interact with biotin-labelled apo(a) (500 ng in 200 µl PBS-T) and added to TG coated wells (2 µg in 200 µl PBS). The presence of anti-Gal-apo(a) complex on TG coated wells were detected using avidin-HRP (2000X) and the bound HRP was determined as described. Values are mean ± SD of six trials. P value=0.0005 (***)

S1=25 mM 1-O-methyl-α-D-glucoside

S2=25 mM 1-O-methyl-α-D-galactoside

Discussion:

Plasma anti-carbohydrate antibodies were used for this study for the following reasons: a) source of these antibodies (out-dated plasma from blood transfusion department) is readily available; b) plasma contains several anti-carbohydrate antibodies, each having distinct but defined specificity for respective carbohydrate antigens; c) mono- or divalent sugar antigens as well as macromolecular polysaccharide or glycoconjugate antigens are easily available for the three different plasma antibodies used; d) protection of antigen binding site by small mono- and disaccharide ligands enabled limiting of fluorescent labeling to Fc part; e) single step affinity chromatography protocol involving binding to natural or synthetic polysaccharide moieties and subsequent elution using dialyzable mono- or divalent sugars makes their isolation in pure form much easier than that of other antibodies, and finally f) being naturally occurring antibodies apparently involved in anti-microbial or anti-cancer defence of the body, characteristics of these antibodies elucidated here assumes clinical relevance. Fluorescence ligands attached covalently to amino acid residues at ligand binding sites have been used in the past to monitor ligand induced conformational changes in proteins. Aminophenoxazole maleimide coupled to cysteine group of ligand binding site has been used as fluorescent probe to detect conformational changes in β2 adrenergic receptor (Cohen et al., 2005). Recently fluorescence of antibody Fab fragment flurolabeled at its variable region has been shown to increase with antigen binding due to antigen- dependent release of fluorescence quenching (Abe et al., 2014).

Unlike the above examples present protocol measures fluorescence of FITC tags in one region of a protein consequent to ligand binding at a distantly located site in the same molecule. FITC coupling of each of the three antibodies used here was done in presence of specific sugar in order to protect the antigen binding site from accepting FITC moieties so that maximum amount of FITC coupling took place in the Fc part of antibody. Most of the conformational changes in the antibody upon antigen binding takes place away from the antigen binding site (Brown and Koshland, 1975). Oda, (2003) have reported that antigen binding causes allosteric conformational changes in the constant domains as well. The signals resulting from antigen binding have been shown to cause considerable conformational changes in CH1-CH2 domains (Oda, 2003 and Sela-Culang et al., 2012) which are distant from the antigen binding site. It is reasonable to conclude that these conformational changes are expressed as the fluorescence enhancement in FITC-labeled antibodies upon antigen binding. Fluorescent tags attached to proteins are known to change their emitted fluorescence in proportion to changes in their microenvironment following conformational shifts (Semisotnov et al., 1991).

Evidences for conformational change in Fc part consequent to occupation of binding site in Fab by large antigens include i) Fc of antigen-bound antibody getting receptive to complement and Fc receptors and ii) Fc:Fc association observed in immune complexes (Kusumi and Winkelhake, 1986). On any given antibody Fc activation resulting in complement fixation increased with the binding constant of the antigen used, indicating that energy of antigen binding was used to activate complement binding site of antibody (Brown and Koshland, 1975). Several lysine

residues in IgG1 heavy chain such as at positions 322 and 326 in the Fc region and at positions 234 and 235 at the hinge region are crucial in recognition of the antibody by complement (C1q) following antigen-induced activation (Gaboriaud et al., 2003). Since lysine residues are mainly used for covalent attachment of FITC it appears certain that conformational shift accompanying antigen binding would change the microenvironment around FITC moieties offering them more freedom to attain and dissipate energy resulting in greater fluorescence emission. Fluorescence enhancement upto about 10% in Fab following antigen binding observed above could arise from FITC labeling of amino acid residues on the Fab region beyond the binding site of the protecting saccharide present during labeling, since conformational change in Fab region is also reported to accompany antigen binding (Abe et al., 2011).

In the practical front the present results suggest that FITC-labeled antibodies may be used for detection as well as quantification of antigens in plasma or other biological fluids, therapeutic preparations or in culture supernatants of pathogens that secrete antigens. Further, data above also offer a way to compare the antigenic potential of candidate ligands present in food, pathogenic microbes or therapeutic preparations towards any given antibody. Naturally occurring antibodies induced apparently by gut bacteria are known to recognize complementary structures on macromolecules of dietary, bacterial or even host origin, forming immune complexes capable of causing inflammatory injury to host tissues (Springer and Horton, 1969 and Paul et al., 2011). Two variables that dictate this pathobiology viz. specific activity of antibody and structural heterogeneity of antigens can be

monitored by the present protocol. Our preliminary (unpublished) data reveal that the genetically determined size of human lipoprotein(a) determines its affinity for circulating anti-Gal antibody which is known to accommodate the serine- and threonine-rich peptide sequences of the lipoprotein as surrogate antigen (Mandagini et al., 2013).

The increase in anti-Ig response upon ligand binding (Fig: 24 and Fig: 25) could be due to either or both of a) Fc conformational changes induced by macromolecular ligands and b) potentiation of the remaining binding site of antibody by allosteric activation induced by a macromolecular ligand.

Streptococcus mutans as well as other Streptococcus species is known to cause coaggrgation of other anaerobic bacterial species including *Porphyromonas gingivalis* on the tooth surface as well as on cardiovascular tissues (Maeda et al., 2004). The presence of free binding sites on ABG-NSMAg complex (Fig: 26) suggest the possibility that such coaggregation could be mediated by the multivalent IgA and IgM antibodies that bind to *S mutans* and contain binding sites for other antigenic epitopes on other speicies or on host surface.

Part III:

Immune Complexes from Cell Wall Antigens of *S mutans* and Their Recognition by Autologous Galectin-1

Introduction:

Deposition of Ag-Ab immune complexes in tissues underlies the pathogenesis of a range of human diseases from glomerulonephritis, systemic lupus erythematosus (SLE), arthritis, transplantation rejection to rheumatic fever. It has been reported that neutrophils initiate IC induced inflammation (Mayadas et al., 2009). Presence of ABG, DIg and anti-Gal in significantly higher yield in normal healthy plasma points to the chances of these natural antibodies meeting their corresponding epitopes of microbial and dietary origin resulting in the formation of circulating ICs (CICs). Atherosclerosis which is now recognised as a chronic inflammatory disease is characterised by features of inflammation at all stages of its development and immune complexes may modulate the inflammatory reaction in atherosclerosis via Fc receptor signalling and complement activation (Burut et al., 2010). The rate of immune complex removal from circulation is a function of the mononuclear phagocyte system. The biological properties of ICs depends on the nature of antigens and antibodies and the lattice of ICs formed will influence their tissue deposition potential (Mannik, 1980). Kupffer cells which are effectively involved in the removal of large latticed ICs from circulation is saturable leading to prolonged circulation of complexes and enhanced deposition in tissues (Mannik, 1980).

Infections are being linked to atherosclerotic disease and may both augment the atherosclerotic process and contribute to later manifestations of overt clinical disease by facilitating plaque rupture and thrombosis (Schmidt et al., 2000).

Presence of antigenic epitopes for ABG and DIg in *S mutans* and the reports of presence of their antigens in atherosclerotic plaque samples and extirpated heart valve (Nakano et al., 2006a) suggest a possible role of these ICs in initiating an inflammatory reaction at the endothelial surface. ABG and DIg being particularly enriched in polymeric IgA (Geetha et al., 2007 and Paul et al., 2011) possibility of deposition of their IC at the tissue surface via Galectin-1 is a distinct possibilty since IgA in monomeric and polymeric form is a potent ligand for galectin-1 (Sangeetha and Appukuttan, 2005). In this section we have examined if *S mutans* antigen immune complex can bind to immobilized galectin-1 through recognition of glycans in IgA component of IC by the lectins. Galectin-1 obtained from bovine heart and human placenta was used for the study. Galectin-1 from bovine heart showed almost 87-92% identity with carbohydrate recognition domain of human galectin-1 (Ashraf et al., 2010).

RESULTS:

A) *Streptococcus mutans* cell wall antigen can form immune complexes when introduced in circulation:

In vitro studies were done to demonstrate the formation of immune complexes between NSMAg and natural antibodies from plasma. NSMAg was biotinylated and added to immunoglobulin rich fraction from which high molecular weight proteins like Lp(a) had been removed earlier by precipitation with 20% ammonium sulphate (refer methods) and the immune complex formed was precipitated by using 2% PEG which works on the principle of solvent exclusion.

ICs formed with biotinylated NSMAg were captured on streptavidin-coated wells and quantitated by probing with HRP-labeled anti-immunoglobulins. Results (Fig: 28) show IgM to be the leading antibody involved, closely followed by nearly equal amounts of IgA and IgG. This protocol was found to measure exclusively the antigen-specific IC since IC from antigen free plasma produced negligible response ($p<0.0001$, Fig: 29). Fig: 29 further suggests that plasma antibodies contributing to IC formation with NSMAg are almost totally contributed by α- or β-glucoside-specific antibodies such as ABG and DIg since sugars specific to these antibodies, unlike galactosides or mannosides were very efficient inhibitors of IC formation ($p<0.0001$, Fig: 29).

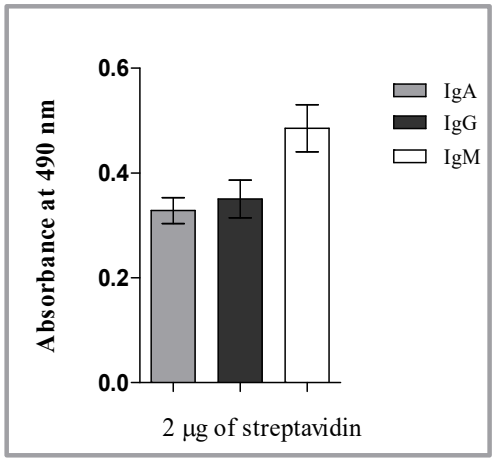

Fig: 28. Immunoglobulin distribution in IC precipitated with NSMAg from Streptococcus mutans. IC obtained after addition of biotin-labelled NSMAg to plasma Ig fraction as described under 'Methods' was diluted 10X and added to streptavidin-coated wells (2 µg) and bound IC detected using HRP- conjugates of

anti-human IgG, IgA and IgM separately (1.5 µg/ml). Bound HRP was assayed as described. Values are mean ± SD of six trials.

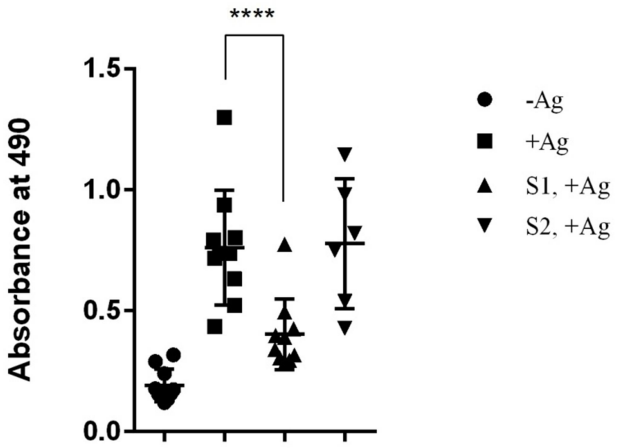

*Fig:29. Specificity of plasma antibodies that form IC with NSMAg. Fifty µg biotin-labeled NSMAg in 200 µl PBS was mixed with 800 µl human plasma Ig-rich fraction preincubated with specific sugars (S1+Ag), non-specific sugars (S2+Ag) or no sugar (+Ag) and IC formed was isolated as described (Methods). IC without antigen addition (-Ag) served as control. From IC dissolved in 500 µl 10X dilution (200 µl in PBS-T) was added to streptavidin-coated (2 µg per well) polystyrene wells. After washing bound IC was probed with a mixture of HRP-conjugated antibodies to human IgG, IgA and IgM. Bound HRP was determined as described. Values are mean ± SD of 10 samples; P values were: < 0.0001(****) for both +Ag vs -Ag and S1+Ag vs +Ag.*

S1: mixture of 25 mM cellobiose and 25 mM 1-O-methyl α-D-glucoside.

S2: mixture of 25 mM melibiose and 25 mM 1-O-methyl-α-D-mannoside.

B) ABG and DIg in circulation are the antibodies recognising the NSMAg when introduced into circulation:

The involvement of ABG and DIg in forming IC with *S mutans* antigen was further conformed by selective removal of ABG and DIg from plasma used for IC preparation. Removal of ABG and DIg by prior treatment with a column containing cellulose and Sephadex G-200 (dextran) resulted in significant reduction in IC formed by NSMAg in plasma Ig rich fraction (Fig: 30). Plasma passed through same amount of CLGG (cross linked guar galactomannan), which is an affinity matrix specific for anti-Gal was used as a positive control.

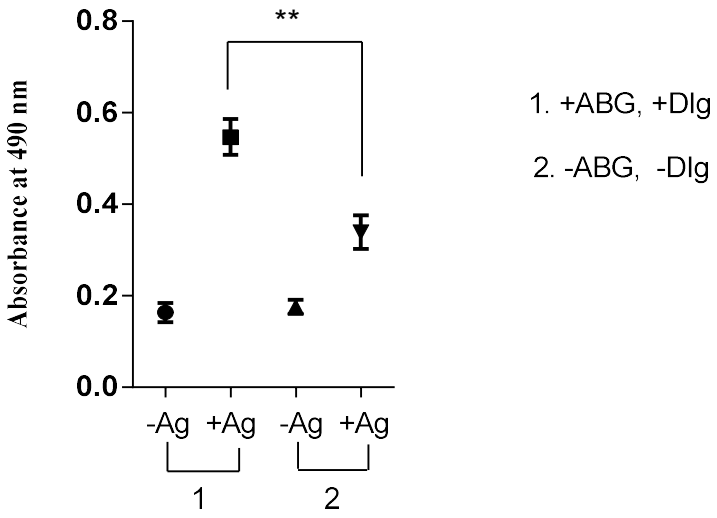

Fig: 30. Effect of withdrawal of ABG and DIg on IC formation by NSMAg. Plasma devoid of ABG and DIg (-ABG, -DIg) or containing these antibodies (+ABG, +DIg) was prepared as described (Methods) and used to prepare IC with (+Ag) or without (-Ag) biotinylated NSMAg. The IC was analysed by ELISA as described under

Fig:28. Values are mean ± SD of 6 trials. P value for IC formation by plasma samples (1 vs 2) with NSMAg = 0.0030 (**).

C) Elevated IgA content and galectin-1 anchoring of NSMAg IC

Fig: 31 Shows the relative contents of immunoglobulin types IgA, IgG and IgM in same protein amount (50 ng) of total immunoglobulin fraction (TIg) in normal plasma, immune complex from normal plasma [IC(-Ag)] and immune complex obtained after addition of NSMAg to normal plasma [IC(+Ag)]. There was significant increase in contents of all three immunoglobulins in NSMAg-IC (Table.3) in comparison to naturally occurring IC despite part of the proteins in IC being the NSMAg itself, indicating the substantial contribution of this antigen towards IC formation. Among the common immunoglobulin types IgA had been found to be far superior to IgG or IgM in offering ligands for galectin-1, due to the high O-glycan content of the former (Sangeetha and Appukuttan, 2005). Significant inhibition of fetuin-HRP binding to immobilized galectin-1 was effected by NSMAg-IC where as IC from plasma without antigen was hardly an inhibitor (Fig: 32) showing that the considerably elevated IgA content per unit protein in NSMAg-IC had facilitated its binding to the lectin. To check the possibility of NSMAg in the IC offering binding sites for galectin-1, free NSMAg dissolved in the same volume as for IC was also tried as an inhibitor of fetuin-HRP binding. Result (data not shown) indicated that NSMAg in itself was not an inhibitor.

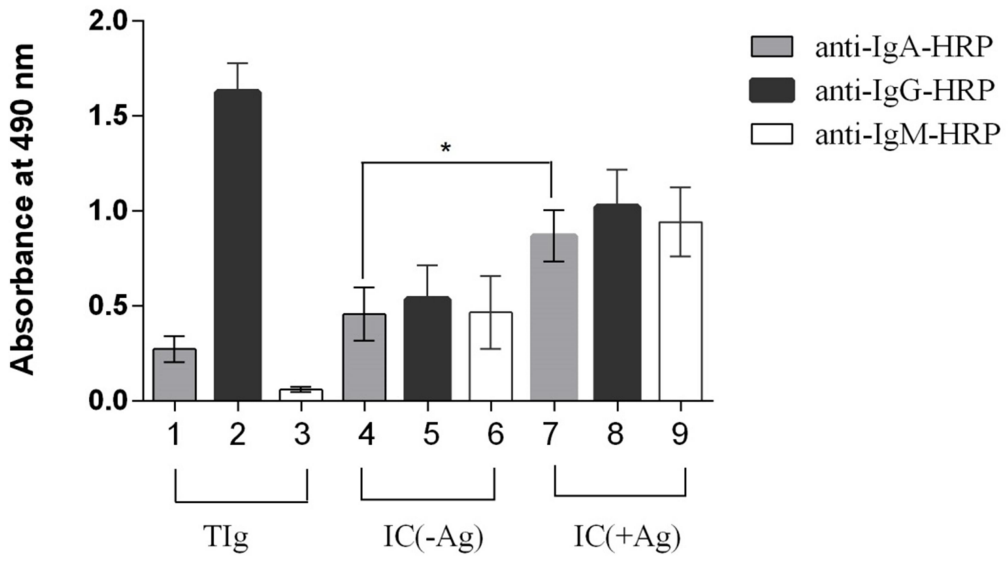

Fig: 31. Increase in IC immunoglobulins upon NSMAg addition to plasma immunoglobulins. From IC obtained by treating plasma Ig-rich fraction with (+Ag) or without (-Ag) NSMAg (Fig.28) 50 ng protein coated on polystyrene wells was probed with HRP conjugates of antibodies to human IgG, IgA or IgM as described earlier. Wells coated with total plasma immunoglobulins (TIg) served as control. Values are mean ± SD of 3 samples. P value for increase of IgA per unit IC protein = 0.0215 (<0.05).*

Ig Isotype	Percentage increase in IC with NSMAg
IgA	90.1
IgG	91.2
IgM	102.4

Table.3. Percentage increase in immunoglobulin content in IC precipitated from Ig rich plasma by adding NSMAg

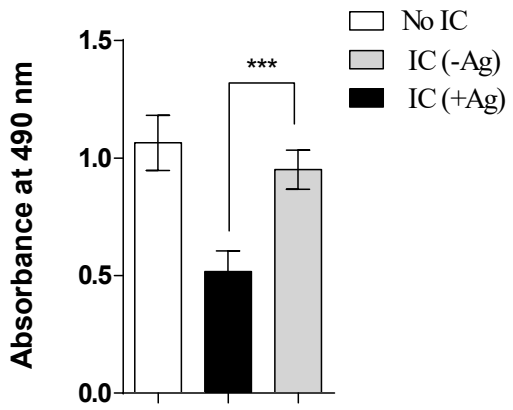

*Fig: 32. NSMAg-IC is a ligand for galectin-1. Biotinylated galectin-1 (5 µg in 200 µl PBS) was immobilised by incubation with streptavidin-coated (1 µg) polystyrene wells by incubation at 4^0 C for 2 h. Fetuin-HRP (3 µg) was treated with 4X diluted IC prepared as described under Fig: 28 with (+Ag) or without (-Ag) NSMAg in 200 µl PBS-T before addition to the immobilised galectin-1 and incubation at 4^0C for 2h. After washing, bound fetuin-HRP was assayed as described earlier. Untreated fetuin-HRP was control. P value for inhibition of fetuin-HRP binding (+Ag vs –Ag) was 0.0001 (***≤0.0005) ; n=6.*

Discussion:

IC formation is beneficial in facilitating the removal of antigens from circulation mainly through the reticuloendothelial system (RES) of the liver. Our current understanding of the functioning of ICs in the development of disease states began with the gradual realisation that the union of antigen and antibody in circulation does not always lead to beneficial effects. Factors that could influence IC formation and IC-mediated disease activity include IC characteristics and the state of the systems involved in IC clearance. Small ICs circulate for excessive periods of time and the half life for the clearance of such ICs approaches that of the immunoglobulin involved which is roughly three weeks for IgG (Schifferli and Taylor, 1989). Factors determining the localisation of circulating ICs (CIC) in vessel walls within a particular organ include the quantity, size and lattice structure of the CIC and it has been reported that ICs of intermediate size are more prone to cause tissue injuries such as glomerulonephritis. An excessive quantity of CIC can however lead to saturation of the RES also (Haakenstad and Mannik, 1974) resulting in enhanced deposition of large latticed soluble IC (Haakenstad et al., 1982). Saturation of the RES can lead to the persistence of ICs in circulation and thereby leading to enhanced likelihood of their non-hepatic tissue deposition. Erythrocyte-mediated handling of ICs may fail due to various reasons including excess IC formation and inherited CR1 deficiency, ICs may also form insoluble aggregates that do not bind to erythrocytes, but instead deposit in certain tissues and cause local damage (Schifferli and Taylor, 1989).

The present results suggest that *S mutans* which is a frequently reported organism in various inflammatory diseases can offer antigenic epitopes for naturally occurring anti-carbohydrate antibodies. Thus there is a chronic antigenic stimulation of the immune system mediated by pronounced levels of CIC. In most cases of bacterial endocarditis, the bacteria involved are of low virulence but when entrenched as vegetations on heart valves they are fully capable of inducing severe disease and abundant sequelae (Høiby et al., 1986).

De novo IC formed by NSMAg with plasma antibodies analysed using biotin-labeled NSMAg indicated IgM as leading antibody, closely followed by nearly equal amount of IgA and IgG (Fig: 28). Immunoglobulins of circulating ICs precipitated by 2% PEG contain comparable quantities of IgA, IgG and IgM in contrast to circulating free immunoglobulins which are predominantly IgG (Fig: 31). However IC precipitated by 2% PEG after addition of NSMAg to plasma contains twice as much IgA, IgG and IgM as does the same amount of circulating IC (Fig: 31). This indicated the significant contribution of *S mutans* antigens towards circulating IC in infected patients. Immunoglobulins that bind to killed *S mutans* cells as well as those precipitated by NSMAg from plasma contained IgG as leading antibody closely followed by nearly equal quantities of IgA and IgM. However anti-immunoglobulin probing of NSMAg-IC immobilized through its biotin tag indicated dominance of IgM. A possible reason for this difference is differential availability of Fc parts of immunoglobulins depending on the involvement of antibody per se in polystyrene binding. Antibodies were not polystyrene–bound in the latter protocol, unlike in earlier ones.

The increased IgA content of NSMAg IC compared to circulating immunoglobulins may account for the marked affinity of these ICs for galectin-1 as reflected in their capacity to inhibit fetuin-HRP binding to the lectin, where as circulating IC prepared without NSMAg addition was hardly inhibitory. Superiority of IgA over other immunoglobulins as ligand for galectin-1 had been demonstrated earlier (Sangeetha and Appukuttan, 2005). Moreover ICs formed by microbial polysaccharides had been reported to be recognized by immobilized galectin-1 mainly due to their high polymeric IgA content (Paul et al., 2011). ABG and DIg contain higher polymeric IgA content than total immunoglobulin fraction (Geetha et al., 2007 and Paul et al., 2011). Drickamer (1995) has reported that the increased sugar ligand density can cause enhanced lectin recognition of glycoproteins. Galectin-1 recognizes core-1 O-linked oligosaccharides, and IgA1 is the most abundant serum ligand for galectin-1 so that galectin-1 could be expected to play an important role in sequestering IgA immune complexes in immune complex mediated disorders (Sangeetha and Appukuttan, 2005). A major cell type in the subendothelial region involved in inflammation- and/or infection- mediated pathology are activated monocytes/macrophages in which expression of another lectin galectin-3 is several fold higher than that in resting monocytes (Novak et al., 2012). Incidentally galectin-3 affinity for O-glycans of the type present in IgA (core 1) is about two orders of magnitude higher than that of galectin-1 (Bian et al., 2011). This factor also underlines the pathological significance of IgA enrichment in bacterial antigen ICs.

Even though clearance of IgA-containing IC is generally mediated by Kupferr cells (Rifai and Mannik, 1984), involvement of such complexes has been reported in several immune complex-mediated diseases. Kauffmann et al. (1980) has reported the role of IgA-IC in initiating vasculitis of Henoch Schonlein purpura while Hall and Lawley (1985) have reported its role in Dermatitis Herpetiformis (DH). DH is characterized by deposits of IgA at the dermal epidermal junction and these patients had high levels of IgA-containing circulating immune complexes. Deposition of immune complexes in tissues has been associated with a wide range of other diseases like glomerulonephritis, arthritis, transplantation rejection and rheumatic fever (Mayadas et al., 2009). In addition infection-associated immune inflammatory reactions has been widely reported in various cardiovascular disorders including atherosclerosis (Hansson, 2005b). *Streptococcus mutans* had been detected in cardiovascular specimens including atheromatous plaque samples as well as in affected tissues of infective endocarditis patients (Nakano et al., 2006a and Nakano et al., 2007b). Although the reason for occurrence of these organisms in cardiovascular specimens were not clear, one possible mechanism could be galectin-mediated uptake of IgA-containing ICs formed by the organism in circulation. Incidentally IC formation followed by clearance by the reticuloendothelial cells is the major pathway for sequestration of pathogen-derived antigens from circulation (Haakenstad and Mannik, 1974).

Hyperglycemia accompanying diabetes has been reported to aggravate periodontal infections and related pathology (Janket et al., 2008) though the mechanism by which this is effected is hardly known. Glucoside-specific natural

antibodies had been shown to be inhibited by diabetic blood glucose concentrations, unlike normal levels (Chacko and Appukuttan, 2003). Since the above antibodies are also those primarily involved in IC formation with *S mutans* the present result suggest that inhibition of glucoside-specific scavenging antibodies caused by hyperglycemia may be a prime reason for increased susceptibility of diabetics to periodontal infections and ensuing pathology.

SUMMARY AND CONCLUSION

Environmental microbial load is known to offer abundant carbohydrate epitope that can elicit the production of large amount of anti-carbohydrate antibodies. The naturally occurring anti-carbohydrate antibodies present in fairly high concentration in normal healthy plasma could possibly encounter these antigens. The present study aims at understanding the ability of naturally occurring anti-carbohydrate antibodies to form immune complex with commonly encountered dietary and microbial antigens.

Dextran like high molecular weight polysaccharides had been reported in all samples of commercially available edible sugar samples tested (Paul et al, 2010) and their indigestibility may cause them to enter circulation as an antigen. Thus they may act as a constant source of IC's within circulation. In the present study we isolated a wider spectrum of polysaccharides from edible sugar samples using alcohol precipitation. Both ABG and DIg recognized the high molecular weight polysaccharides from commercially available sucrose samples, with DIg being slightly better than ABG in this respect. The de novo IC's formed by the addition of dextran was comprised mostly of IgM followed by IgG and IgA. A possible explanation for the finding is the fact that naturally occurring antibody repertoire is particularly enriched in IgM anibody. Thus the edible source of sucrose is a constant source of polysaccharide antigen to which the host immune system are constantly exposed to and could eventually lead to immune complex mediated diseases. Persistence of these IC's within circulation can occur by the saturation of the reticuloendothelial system which is involved in the clearance of IC's which thereby

can result in their non-hepatic tissue deposition probably contributing to the pathogenesis of tissue deposition diseases.

In addition to its primary specificity for α(1→3) linked glucan, DIg had been shown to possess additional specificity for terminal α-Gal-bearing epitopes. There was a wide variation in the α-Gal binding ability of different DIg samples analysed. The multiple specificity of DIg is significant because the pathogenic potential of terminal α-linked galactose (TAG) of endogenous or environmental origin is being increasingly recognised. Reports from our lab has shown that these epitopes (α-Gal) which were found to be absent in humans, apes and old world monkeys are present in human brain gray matter. Later it was reported that peptide sequences (STPS) present in MUC-1 family of peptides can act as surrogate to TAG as a ligand for anti-Gal. It will be interesting to study whether DIg with additional specificity for α-Gal can also use STPS sequence as a surrogate to TAG.

Another source of dextran like polymer to which our immune system is constantly exposed to are the plaque forming bacteria *S mutans*. Reports have suggested the role of infective pathogens in the pathogenesis of various diseases including atherosclerosis and several other disorders involving immune inflammatory reactions. Chronic dental bacterial infection are strongly correlated to atherosclerosis and various other CVD while infection associated risk for CVD and other inflammatory diseases is related to the aggregate pathogen load. Both α- and β- glucan specific antibodies (ABG and DIg) from normal healthy plasma were recognising cell wall antigen from *S mutans*. PSMAg and NSMAg obtained from *S*

mutans were recognised by circulating ABG and to a lesser extent by DIg. The recognition of *S mutans* cell wall antigen by ABG and DIg could be attributed to the rhamnose glucan polymer (RGP) within their cell wall which contain a backbone of rhamnose and side chains of β(1→2), α(1→2) and α(1→3) glucan chains.

A novel technique for measuring conformational shift produced by macromolecular antigen binding to antibodies, in terms of enhancement of fluorolabel at Fc is also demonstrated. This was done using naturally occurring human plasma anti-carbohydrate antibodies labelled with FITC while protecting the binding sites by small antigenic ligands. Increase in enhancement of fluorescence with affinity of antigen for a given antibody and with specific activity of antibody from different individuals for a given antigen validated this method.

The fluorescence studies suggest that FITC-labelled antibodies can be used for detection and quantification of antigens in plasma or other biological fluids, therapeutic preparations or in culture supernatents of pathogens that secrete antigens. Fluorescence enhancement of FITC-labelled antibody offer a way to compare the antigenic potential of candidate ligands present in food, pathogenic microbes or therapeutic preparations. Both specific activity of antibody and structural heterogeneity of antigens can be monitored by the present protocol.

Macromolecular ligands were used as prospective inhibitors of ABG or anti-Gal binding to microplate-coated glycoproteins bearing monosaccharide ligands for these antibodies, using anti-immunoglobulin-HRP to probe bound antibody. However macromolecular ligand produced an increased response in the ELISA.

Both ABG and anti-Gal were having additional free binding sites after binding to NSMAg or apo (a). The occurrence of free binding sites could be explained by steric constraints in accommodating two large ligands simultaneously on each of the Fab region of antibody.

The increase in anti-Ig response upon ligand binding could be attributed to the Fc conformational changes induced by macromolecular ligands or by the potentiation of the remaining binding site of antibody by allosteric activation induced by a macromolecular ligand. There are reports on the ability of *S mutans* in causing co-aggregation of other anaerobic bacterial species on the tooth surface as well as on cardiovascular tissues. The occurrence of free binding sites suggest the possibility that such coaggregation could be mediated by multivalent antibodies that after binding to *S mutans* contain additional binding sites for other antigenic epitopes on other species or on host surface.

Plasma antibodies were capable of forming de novo IC when precipitated with 2 % PEG after incubation with NSMAg. The precipitate obtained after NSMAg addition contained twice as much IgA, IgG and IgM as does the same amount of circulating IC which indicated the contribution of *S mutans* antigens towards circulating IC in infected people.

The increased IgA content of NSMAg-IC may account for the marked affinity of these IC's for galectin-1 as reflected in their capacity to inhibit binding of fetuin-HRP to immobilised galectin-1. A possible explanation for this could be abundance of polymeric IgA in both ABG and DIg. Polymeric IgA is reported to be

a better ligand for galectin-1. Involvement of IgA immune complexes had been reported in several IC mediated diseases. *S mutans* was detected in cardiovascular specimens including atherosclerotic plaque samples. One possible mechanism for the occurrence of these organisms in cardiovascular specimens could be galectin-mediated uptake of IgA containing IC's formed by the organism in circulation.

Periodontal infections and related pathology were reported to aggravate under hyperglycaemic condition accompanying diabetes though the actual mechanism is not clearly known. Glucoside specific natural antibodies were found to be inhibited by diabetic blood glucose concentrations and these antibodies being primarily involved in IC formation with *S mutans*, it could be a prime reason for increased susceptibility of diabetes to periodonttis and related pathology.

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List of Publications:

- Genu George, Mandagini Geetha & Padinjaradath S. Appukuttan (2015). "Antigen-Induced Activation of Antibody Measured by Fluorescence Enhancement of FITC Label at Fc". Journal of Fluorescence, 25(5): 1493-1499

- Genu George, Molly Antony, Jaisy Mathai and Padinjaradath S Appukuttan (2016). "Periodontitis and Inflammation: Plasma High Titer Naturally Occurring Anti-Glucan Antibodies Form Immune Complex with *Streptococcus mutans* Antigens". *Modern Research in Inflammation*, 5: 45-54.

- Binu Sheela, Genu George, Geetha Mandagini & Padinjaradath S. Appukuttan (2016). "Plasma anti-α-galactoside antibody mediates lipoprotein(a) binding to macrophages". *Glycoconjugate Journal.*