GLYCOBIOLOGICAL CHARACTERIZATION OF GLYCAN- ANTI-GLYCAN ANTIBODY IMMUNE COMPLEXES IN HUMAN SERUM INCLUDING ENZYMATIC MODULATION OF THEIR UPTAKE BY TISSUE LECTINS

A THESIS PRESENTED BY

ANU PAUL

TO

THE DEPARTMENT OF BIOCHEMISTRY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM – 695 011

The Thesis Entitled

GLYCOBIOLOGICAL CHARACTERIZATION OF GLYCAN- ANTI-GLYCAN ANTIBODY IMMUNE COMPLEXES IN HUMAN SERUM INCLUDING ENZYMATIC MODULATION OF THEIR UPTAKE BY TISSUE LECTINS

Submitted By

ANU PAUL

For the Degree of Doctor of Philosophy

of

Sree Chitra Tirunal Institute for

Medical Sciences and Technology

Thiruvananthapuram

Evaluated and Approved By

Dr. P.S. Appukuttan

Name of Guide

Name of Thesis Examiner

DECLARATION

I, **Anu Paul**, hereby declare that I had personally carried out the work depicted in the thesis entitled **"Glycobiological characterization of glycan- anti-glycan antibody immune complexes in human serum including enzymatic modulation of their uptake by tissue lectins"** 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, except where external help sought and acknowledged.

Anu Paul
27/03/2010

Dr. P.S Appukuttan,
Professor and Head,
Department of Biochemistry,
Sree Chitra Tirunal Institute for Medical Sciences & Technology,
Medical College P.O, Thiruvananthapuram,
Kerala, India- 695 011.

CERTIFICATE

This is to certify that **Ms. Anu Paul**, in the Department of Biochemistry of this institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The work relating to her thesis entitled **"Glycobiological characterization of glycan- anti-glycan antibody immune complexes in human serum including enzymatic modulation of their uptake by tissue lectins"** was carried out under my direct supervision.

Dr. P. S Appukuttan

27.03.2010

ACKNOWLEDGEMENT

It was my privilege to carry out my work under the guidance of Dr.P.S Appukuttan, Professor and Head, Department of Biochemistry, S.C.T.I.M.S.T, Thiruvananthapuram. I am greatly indebted to his constant encouragement, motivation, valuable inputs and enthusiastic guidance shown throughout the course of my study.

I specially thank Ms. Molly Antony, Scientist F, Dept. of Microbiology, S.C.T.I.M.S.T for providing me with microbial cultures throughout my study and also for her valuable suggestions as Doctoral Advisory Committee member that helped me in my work. Also I accord my gratitude to Dr. Prabha Balaram, Professor and Head, Research Division, Regional Cancer Centre, Thiruvananthapuram who was a member of my Doctoral Advisory Committee.

I am grateful to the Director, S.C.T.I.M.S.T, for providing me necessary facilities throughout the period of my study. Also I acknowledge the help offered by the Administrative Department, Medical illustration unit and Project Cell, S.C.T.I.M.S.T.

I would like to thank the Indian Council of Medical Research (ICMR) New Delhi, for financial assistance in the form of Junior and Senior Research Fellowships.

I take this opportunity to thank Dr. V. Mohanan Nair, Director, Indian Institute of Diabetes, Thiruvananthapuram for providing me blood samples. A special thanks to all staff of the Blood bank and the Central Clinical Laboratory, S.C.T.I.M.S.T for providing me blood and plasma samples all year round. Also I thank Obstetrics department, Cosmopolitan hospital, Thiruvananthapuram for providing human placenta.

I am thankful to Dr. Jayakumari, N, Professor, Dept. of Biochemistry and Dr. Srinivas G, Scientist D, Dept. of Biochemistry for all the support and encouragement they provided.

I record a special note of thanks for Ms. Annamma K.I (Junior Scientific Officer) for all the care and support. My sincere thanks to my department colleagues Ms. Geetha, M, Dr.Sangeetha S.R, Dr.Bijoy Chellan, Dr.Anuradha, Mr. Suboj Babykutty, Ms.Priya P.S, Mr.Vinod.V, Ms.Sumitha K.C, Mr.Bejoy Vijayan, Ms.Kalaivani.V, Ms.Soumyarani V.S, Mr.Sabarinath P.S, Ms.Padmakrishnan C.J and Ms.Genu George for every help and companionship they provided.

I specially thank Ms. Chandrika L, Ms. Shyamala TK and Mr.Sasidharan who assisted our lab during my period of work.

My sincere gratitude for everyone far and near, whose presence I experienced all way through which motivated and encouraged me to get going on to-date.

Anu Paul

CONTENTS

ABBREVIATIONS

ABG	Anti- β- Glucan
AGA	Anti-glycan antibody
AGAP	Anti-glycan antibody profile
AGA-PS-IC	Anti-glycan antibody –polysaccharide immune complex
AGRU	Anhydroglucose repeat units
Anti- Gal	Anti-α-galactoside antibody
APC	Antigen presenting cells
ASCA	Anti-Saccharomyces cerevisiae antibody
ASGPR	Asialoglycoprotein receptor
BBG	Barley β-glucan
BHL	Bovine heart lectin (galectin-1)
BSA	Bovine serum albumin
CCA	Candida cell wall antigen
CCA-IC	Candida cell wall antigen – immune complex
cIC	Circulating immune complex
CLGG	Cross-linked guar gum
CNBr	Cyanogen bromide
CRD	Carbohydrate Recognition Domain
DEAE	Diethylaminoethane
DIg	Dextran-binding immunoglobulin
ECM	Extracellular Matrix
EDTA	Ethylene diamine tetra acetic acid

ELISA	Enzyme linked immunosorbent assay
Gal	Galactose
Gal-1	Galectin-1
GalNAc	N-acetyl galactosamine
GC	Germinal centre
GG	Soluble guar gum
GIT	Gastrointestinal tract
Glc	Glucose
GlcNAc	N-acetyl glucosamine
HPL	Human Placental lectin (galectin-1)
HRP	Horse radish peroxidase
HSP	Henoch Schönlein purpura
IC	Immune complex
Ig	Immunoglobulin
IgA1	Immunoglobulin A1
IgA2	Immunoglobulin A2
LacNAc	N-acetyllactosamine
LPS	Lipopolysaccharide
1-O-methyl α-Glc	1-O methyl α-D glucopyranoside
1-O-methyl β-Glc	1-O methyl β-D glucopyranoside
mIgA	Monomeric IgA
MHC	Major Histocompatibility Complex
2ME	2-mercaptoethanol
MZ	Marginal Zone

NANA	N-acetyl neuraminic acid
NDS	Non- dialysable sugar
OPD	Ortho-phenylenediamine
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline (20 mM with 150 mM NaCl), pH 7.4
PBS-T	PBS containing 0.05% Tween 20
pIgA	Polymeric IgA
pIgA - IC	Polymeric Immunoglobulin A- Immune complex
PNA	Peanut agglutinin
PRR	Pathogen Recognition Receptor
T / TF antigen	Thomsen-Friedenreich antigen (Galβ 1, 3 GalNAc-)
TAG	Terminal-α-linked galactose
TEMED	N,N,N',N'- tetramethyl ethylene diamine
TD	T-cell dependent
TI	T-cell independent
URT	Upper Respiratory Tract
YBG	Yeast β-glucan
YGP	Yeast glycoprotein

SYNOPSIS

The formation of circulating antigen-antibody complexes is one manifestation of the natural course of immunologic response against soluble antigens. After an antigen-antibody reaction, the immune complexes (ICs) can be subjected to any of a number of responses including complement fixation, opsonization or phagocytosis.

Reticuloendothelial system (RES) plays major role in removal of IgG and IgM immune complexes that fix complement. Being not complement fixing, IgA-IC is not removed via RES. FcαR expressed on immune cells bind to Fc portion of IgA with very low affinity and hence the role of this receptor in IgA-IC catabolism remains controversial.

Among the IC-deposition-mediated diseases the least understood one is those attributed to IgA. There are several instances where circulating IgA gets deposited leading to pathogenicity as occurs in IgA nephropathy and Henoch Schönlein purpura (HSP). The mechanisms underlying such pathogenic situations are not fully delineated and the receptors responsible for such events are still unknown.

About 90% of serum IgA belongs to IgA1 subclass and the rest is IgA2. Eventhough IgA is synthesized at rate equal to that of IgG, maintenance of a circulating IgA titre at 20% of that of IgG suggests an efficient disposal mechanism. Hepatic ASGPR are involved more in the removal of IgA2 type and hence the catabolism of IgA1 that forms the bulk of serum IgA is not explained satisfactory.

The observation from this lab that IgA1 is the most prominent serum glycoprotein interacting with tissue galectin-1 is significant to both the normal biology of serum IgA1 and to IgA1-mediated immune pathology. A role for galectin-

1, well expressed and widely distributed in human tissues including the endothelial cells, in sequestering the most prominent T antigen bearing serum glycoprotein IgA1, seems very likely.

Glycan antigens entering human body through food, environment and microflora colonizing the skin, epithelia and gut, especially in humid tropical geographic regions is enormous. Anti-glycan antibodies (AGA) usually possess remarkably higher content of IgA1 especially in its polymeric form. This property is likely to dictate the fate of these antibodies and their immune complexes due to its binding to galectin-1.

The polymeric nature of the deposited IgA1 complexes especially in the desialylated form has been reported in diseases involving IgA-deposition. Preliminary studies on galectin-1 recognition of IgA1 have shed further light in this direction since polymeric IgA1 (pIgA1) in the desialylated form was found to bind the lectin more. IgA1 from IgA nephropathy patients was found to be relatively abundant in asialotype sugar chains.

High percentage of IgA (mostly IgA1) in Anti-β glucan (ABG) and Dextran binding immunoglobulin (DIg) and the observation that the ubiquitous lectin galectin-1, present also on vascular walls and underlying tissues recognizes exclusively IgA1 among immunoglobulins together underline the inflammatory potential of ABG/DIg-containing immune complexes.

AIMS AND OBJECTIVES OF THE STUDY

1) To characterize the immunoglobulin composition in; a).anti-α-Gal (specific for terminal α-linked galactose), b). DIg (specific for α 1→6 linked glucans like dextrans and c). ABG (specific for β 1→4/β 1→3 linked glucans).

2) To quantitatively assess the unit IgA content in polymeric IgA(pIgA) and monomeric IgA(mIgA) as well as demonstrating the recognition of IgA by tissue galectin-1.

3) To determine the effect of desialylation (by microbial neuraminidases) of IgA on its carbohydrate- dependant attachment to galectin-1 or other lectins.

4) To identify polysaccharides from common commensal and infectious microorganisms that offer antigenic epitopes for the human serum anti-glycan antibodies; ABG and DIg.

5) To demonstrate the presence in human serum of anti-glycan antibody polysaccharide (AGA-PS) immune complexes between either of the antibodies DIg, and ABG on the one hand and microbial polysaccharides on the other.

6) To verify if IgA in complexed or free form attach sugar-specifically to immobilized form of galectin-1.

RESULTS AND DISCUSSION

The presence of polymeric forms of IgA in the AGA which make up a large portion of the natural antibody repertoire is observed. It is possible that AGA is the major source of pIgA in serum.

Before ascertaining the difference between pIgA and mIgA in their capacity as ligands for lectins, anti-IgA recognition of the two was studied. Treatment with 2-mercaptoethanol (3mM) reduces the intersubunit disulphide bonds in pIgA to yield IgA monomers. This treatment helps to quantitatively assess its unit monomer content. ELISA experiments to assess HRP-labelled galectin-1 binding to immobilized IgA forms revealed that pIgA compared to mIgA and desialylated IgA compared to native IgA are far superior ligands for galectin-1 in terms of their unit IgA content. Notably, a plant lectin with sugar specificity similar to galectin-1, viz., Peanut agglutinin behaved quite similar to galectin-1 in relative affinities for IgA forms.

The presence of antigenic epitopes for ABG and DIg was checked in electrophoretically separated yeast glycoprotein (YGP) fractions. Both ABG and DIg recognize YGP, even though the response was higher in the case of ABG-HRP. Dextran is yet another polysaccharide that provides linear epitopes for natural antibody recognition. A high molecular weight polymeric fraction of commercial edible sugar was compared to standard dextran for presence of antigenic epitopes for ABG and DIg. Results suggest that high molecular weight portion of edible sugar contains epitopes for ABG and to a lesser extent for DIg. β-(1,3) Glucans from yeast

and barley were significantly less effective in capturing DIg than dextran which consists almost exclusively of α(1→6)- linked glucose moieties.

In vitro studies were done to demonstrate the formation of AGA-polysaccharide ICs between ABG on one hand and *Candida albicans* cellwall antigen (CCA) on the other. In order to isolate serum antibodies specifically reacting with CCA, increasing amounts of CCA were added to fixed volume of serum, ICs formed separated, and CCA-interacting antibodies thereof assayed by their capacity to bind to microplate coated CCA. Results show that, as the concentration of CCA added to serum increases, there is a corresponding increase in IC formation till the zone of equivalence is reached. There is a minimal level of CCA-binding antibodies in ICs prepared without addition of CCA showing that a minimum amount of circulating IC contributed by CCA-reactive antibodies occurs in normal serum.

Sugar inhibition studies suggests that ABG as well as anti-mannan antibodies which occur in the serum as natural antibodies are capable of forming ICs with the antigenic epitopes presented by *C.albicans*. Results prove that ABG-mediated ICs are present in normal individuals but are markedly elevated during fungal infections. IgA is the most prevalent Ig class in CCA- ICs unlike the natural ICs where IgG forms the major portion. Since β-glucans and related antigens reside on microbial cell surfaces and are released into host circulation, frequent exposure to these organisms as commensals or systemic infectants, experienced by even normal individuals could cause considerable increase in IgA-containing IC, contributed by ABG and related antibodies.

Serum to which glucose was added to a final concentration of 400 mg/dl which is often attained in diabetes showed marked reduction in the IC formation with CCA compared to controls. Results point towards the possibility that high sugar in serum prevents the ABG population from forming IC. It is reasonable to postulate that inhibition of natural and front-line defence mechanism mediated by antibodies like ABG by high serum blood glucose may be the primary reason for the high susceptibility of diabetics to infections.

In Type II diabetic serum samples, there is a notable surge of IC formation following the addition of CCA in comparison to control serum. Since the study showed that in normal sera artificially increased glucose concentrations keeps ABG from binding to added CCA, the pattern of CCA-IC formation in clinically confirmed diabetic patients was examined further.

Sugar inhibition pattern of CCA-precipitated antibodies varied widely among diabetic cases included for the study. In most cases there is only marginal inhibition by the sugars. Carbohydrate-independent antibodies seem to bring about the elevated response in diabetic serum.

Sugar inhibition studies shows that in most of these diabetic patients, who supposedly have combated the *Candida* infection, there was significant reduction in response to carbohydrate structures like β-glucan and mannoproteins but an elevated response is seen against an immunodominant antigen of 47 kDa (heat stable breakdown product of hsp90) which is sugar independently recognized.

The Ig composition of the antibody population binding to the 47kDA antigen in diabetics was characterized. The significantly high concentration of IgA in this antibody population indicates that IgA is the major Ig type that is involved in IC

formation in diabetics since the contribution of anti-carbohydrate antibodies in immune complex formation is limited.

IC formation in serum upon the addition of dextran was studied. Upon addition to serum, high molecular weight fractions of edible sugar could form immune complexes with antibodies such as DIg, as did pure dextrans.

Galectin-1 was immobilized by attachment of its biotinylated derivative on streptavidin coated plates. The binding of IgA1, but not IgG to immobilized galectin-1 was demonstrated. DIg consisting of polymeric IgA bound far more efficiently to immobilized galectin-1 than did anti-Gal that lacks pIgA. This leads to the conclusion that the high titre of pIgA and formation of pIgA-rich IC observed in diabetes pre-dispose these IC to more efficient binding to galectin-1 since the glycosylated part of IgA in IC is free. The results obtained from this thesis work suggest that high titre of anti-glycan antibodies in the serum of tropical individuals meet their epitopes resulting in a surge of immune complexes. The heavy microbial load of the tropical environment enhances the phenomenon.

IC from diabetic individuals were significantly better ligands than IC from normoglycemic individuals for immobilized galectin-1, thus offering an explanation for the IgA deposition in tissues in diabetes. The reported higher occurrence of pIgA as well as the asialo-nature of O-glycan in deposited IgA further supports this hypothesis.

To summarise, immune complexes, known to cause vascular damages and inflammation have been shown to be formed between several carbohydrate binding

serum antibodies on the one hand and carbohydrate antigens of dietary, environmental or microbial origin on the other. In particular, diabetic individuals have been shown to develop a quantitatively and qualitatively different antibody profile that exposes them to potential IC-induced damages. Polymeric IgA synthesized more in diabetes have been shown to be particularly susceptible to bind tissue galectin-1. Finally, in order to mimic the action of in vivo galectin-1 which is immobilized, a novel method to study the properties of immobilized tissue galectin-1 in active form has been developed.

An outcome of the study is the possibility of using galectin-1 inhibitors in mitigating inflammatory immune damage to vessel walls in diabetes. It was demonstrated in this laboratory that human RBC glycopeptides can efficiently reverse the binding of galectin-1 to its ligands. Further studies in this topic may result in development of therapeutics to block diseases involving IC- deposition.

CHAPTER I

INTRODUCTION

INTRODUCTION

The formation of circulating antigen-antibody complexes is one manifestation of the natural course of immunologic response against soluble antigens. After an antigen-antibody reaction, the immune complexes (IC) can be subjected to any of a number of responses including complement fixation, opsonization or phagocytosis.

Immune complexes consisting of IgM/IgG activates classical complement pathway. C3b bound to the Fc receptor of the immunoglobulins gets bound to complement receptor 1 (CR1) glycoprotein present on RBC. This keeps the IC in circulation until it is sequestered by the high affinity binding of Fc receptors present on kupffer cells as well as splenic macrophages. Their removal from plasma reduces the probability that those complement-coated IC will freely circulate in serum and be able to inadvertently damage vital organs and tissues.

Larger proportions of immune complexes formed in the absence of complement are deposited outside the reticuloendothelial system (Skogh and Stendahl 1983). Several physicochemical characteristics of immune complexes make them pathogenic. Circulating immune complexes has been discussed in literature as a causative agent of various conditions including cardiovascular diseases, Systemic Lupus Erythematosus etc.

Among the IC-deposition-mediated diseases the least understood one is those attributed to IgA. There are several instances where circulating IgA is getting deposited leading to pathogenicity as occurs in IgA nephropathy and Henoch Schönlein purpura (HSP). The mechanisms underlying such pathogenic situations are not fully delineated and the receptors responsible for such events are still unknown.

90% of serum IgA belongs to IgA1 subclass. Eventhough IgA is the second most prevalent Ig in circulation, high turn over rate maintains the homeostatic balance. Important function of serum IgA is to mediate the hepatobiliary excretion of corresponding circulating antigens (Russell, Brown et al. 1981). Removal of IgA-IC from circulation is mediated by a specific IgA receptor on Kupffer cells (Rifai and Mannik 1984). Since IgA is not a complement fixing antibody, RBC mediated removal of IgA-IC from circulation is minimal. Hepatic ASGPR are involved more in the removal of IgA2 type and hence the catabolism of IgA1 that forms the bulk of serum IgA is not explained satisfactorily.

There are several known receptors for IgA, although few were characterized in much detail. Only one Fc receptor belongs to the FcαR subgroup (Otten and van Egmond 2004), which is called FcαRI (or CD89) which is found on the surface of neutrophils, eosinophils, monocytes, some macrophages (including Kupffer cells), and some dendritic cells. But it has been reported that its binding affinity for ligand is very low ($Kd > 10^{-6}$ M). The IgA receptors on monocytes are constitutively expressed (Chevailler, Monteiro et al. 1989) and are involved in IgA-mediated phagocytosis (Maliszewski, Shen et al. 1985). Fc α/μ R can also bind IgA, although it has higher affinity for another antibody called IgM (Cho, Usui et al. 2006).

Other IgA receptors include the mesangial transferrin receptor (TfR) (Moura et al. 2001), a receptor for SIgA and pIgA on natural killer cells (Mota et al. 2003). Finally, there are a few lectin-like receptors that interact with specific glycan epitopes on IgA, including the asialoglycoprotein receptor (ASGPR) (Mestecky and McGhee

1987) and an IgA1/IgD receptor on T cells which is expressed during some pathogenic conditions (Rudd et al. 1994).

All of the above mentioned receptors are very limited in expression and even together cannot account for the high turnover rate of IgA1. It was suggested by Novak et al that ability of mesangial cell to bind IgA1 and IgA1-containing cIC *in vitro* was mediated by an IgA receptor that was different from CD89 (FcαR) or ASGP-R and had a higher affinity for IgA-cIC than for uncomplexed IgA (Novak et al. 2002).

Glycan antigens entering human body through food, environment and microflora colonizing the skin, epithelia and gut, especially in humid tropical geographic regions is enormous. Investigations on the varied immunological consequences of anti-glycan antibodies is pertinent since besides enabling host defence, these antibodies may give rise to immune complexes with soluble complementary glycans as well as cross react with host glycan structures due to polyspecificity which is an inherent attribute of many of these antibodies. Anti-glycan antibodies usually possess remarkably higher content of IgA1 especially in its polymeric form. This property is likely to dictate the fate of these antibodies and their immune complexes since the most ubiquitous and well –expressed carbohydrate-binding protein (lectin) in human tissue, galectin-1, has been found to recognize exclusively IgA1 among all immunoglobulin types (Sangeetha and Appukuttan 2005).

The levels of anti-carbohydrate antibodies represent a novel independent risk factor associated with coronary heart disease (Mosedale et al. 2006). In cross sectional studies IgA, but not IgG or IgM, has been shown to be elevated in patients with atherosclerosis (Muscari et al. 1988) or with a history of myocardial infarction

(Muscari et al. 1993). In prospective study examining dyslipidemic men who had a coronary event in the following five years, elevated levels of IgA, IgE and IgG, but not IgM, were shown to be higher in cases than controls (Kovanen et al. 1998). It was suggested that infection plays an important role in incident MI or death and that the risk posed by infection is independently related to the pathogen burden (Zhu et al. 2001).

The observation that IgA1 is the most prominent serum glycoprotein interacting with tissue galectin-1 is significant to both the normal biology of serum IgA1 and to IgA1-mediated immune pathology. Thus a role for galectin-1, well expressed and widely distributed in human tissues including the endothelial cells, in sequestering the most prominent T antigen bearing serum glycoprotein IgA1,seems very likely (Sangeetha and Appukuttan, 2005)

The polymeric nature of the deposited IgA1 complexes especially in the desialylated form has been reported in diseases involving IgA-deposition. Preliminary studies on galectin-1 recognition of IgA1 have shed further light in this direction since polymeric IgA1 in the desialylated form was found to bind the lectin more (Sangeetha and Appukuttan, 2005). IgA1 from IgA nephropathy patients was found to be relatively abundant in asialotype sugar chains (Iwase and Hiki., 1999).

Though anti-beta glucans (ABG) could not be detected even in yeast-infected patients in the West (Ballou, CE., 1982), presence of the antibody in plasma of all the samples studied in our lab (Geetha et al., 2007) indicates that environmental antigens either of microbial or of dietary origin may be instrumental in triggering ABG synthesis. In humid tropics where environmental load of fungi and yeast are

high as are reported levels of serum immune complexes (Greenwood and Whittle,1981), pathological role of ABG derived immune complexes is worth investigating. Infection-associated immune inflammation is increasingly implicated in vascular disorders including atherosclerosis (Hansson, 2005). ABG immune complexes are also relevant in the context of increased use of β-glucans as anti-cancer biological response modifiers (Thornton et al., 1996). The equally similar occurrence of Dextran-binding immunoglobulins in high concentrations in circulation in tropical individuals rather than Westerners (Chacko and Appukuttan.,2003) and its polymeric IgA content is noted before. Since these antibodies are present in high concentrations in sera their likely meeting with their corresponding epitopes can possibly result in pIgA containing IC.

High percentage of IgA (mostly IgA1) in ABG and DIg and the observation that the ubiquitous lectin galectin-1, present also on vascular walls and underlying tissues recognizes exclusively IgA1 among immunoglobulins (Sangeetha and Appukuttan, 2005) together underline the inflammatory potential of ABG/DIg-containing immune complexes.

Aims and objectives of the study

1) To characterize the immunoglobulin composition in; a) anti-Gal (specific for terminal α-linked galactose), b) Dextran binding immunoglobulin(D Ig) (specific for α 1→6 linked glucans like dextrans and c) anti-beta glucoside antibody (ABG) (specific for β 1→4 linked glucans like cellulose).

2) To quantitatively assess the unit IgA content in polymeric and monomeric IgA as well as demonstrating the recognition of IgA by tissue galectin-1.

3) To determine the effect of desialylation (by microbial neuraminidases) of IgA on its carbohydrate- dependant attachment to galectin-1 or other lectins.

4) To identify polysaccharides from common commensal and infectious microorganisms that offer antigenic epitopes for the human serum anti-glycan antibodies; ABG and DIg.

5) To demonstrate the presence in human serum of anti-glycan antibody polysaccharide (AGA-PS) immune complexes between either of the antibodies DIg, and ABG on the one hand and microbial polysaccharides on the other.

6) To verify if IgA in complexed or free form attach sugar-specifically to immobilized form of galectin-1.

CHAPTER II

REVIEW OF LITERATURE

Polysaccharide antigens

Glycans are predominant surface components of cells such as erythrocytes, immune cells and microorganisms and as such give rise to high levels of anti-glycan antibodies of all classes. Certainly both cellular and humoral immune responses rely heavily on interactions between glycans and glycan binding proteins, be it lectins involved in cell-cell interactions, lectins of the innate immune system, or antibodies recognising sugar-antigens on surfaces of pathogens. These antibodies have been shown to constitute a substantial fraction of total serum immunoglobulins in healthy individuals.

Recognition of polysaccharides as antigens began with the observation that when pneumococci are grown in fluid media, there is a substance in the culture fluid which precipitated specifically with anti-sera to the same pneumococcus (Dochez, A.R. and Avery, O.T. 1917). Later it was showed that this substance was polysaccharide and not protein as thought previously (Heidelberger, M. and Avery, O.T. 1923). Carbohydrates in the form of capsular polysaccharides and/or lipopolysaccharides are the major components on the surface of bacteria. These molecules are important virulence factors in many bacteria isolated from infected persons. Immunity against these components confers protection against the disease.

Several natural anti-carbohydrate antibodies are known to occur in normal sera (Huflejt, M.E., et al. 2009). They are called natural antibodies since they are present in sera of all individuals in the absence of deliberate immunization (Shoenfeld, Y. and Isenberg, D.A. 1989), and are thus a part of the innate immune system. Natural antibodies recognizing carbohydrates including blood group antigens are not found

during first weeks of life, and a hypothesis referred as "bacterial paradigm" has been proposed by G.F. Springer who postulated that anti-carbohydrate natural antibodies appear in response to stimulation of the immune system by bacterial O antigens and LPS of gastrointestinal bacteria (Springer, G.F. 1984, Springer, G.F. and Horton, R.E. 1969).

Anti-polysaccharide immune response is characterised by lack of T-lymphocye memory, isotype restriction and delayed ontogeny. In conventional thymus-dependent (TD) immune responses to protein antigens, B cell activation and antibody production occurs only after B cells have received two signals; one from antigen binding to the B cell receptor for antigen (BCR) and the other from T cells in the form of cell-cell contact and cytokine signalling i.e., rare primed T cells express T cell receptors that recognize antigen-derived peptides presented by the class II major histocompatibility complexes on the B cell. For several important classes of microorganisms, the second signal can come from specific components of pathogens themselves, enabling antibody production in the absence of T cells. Given the feature of host immune responses induced by polysaccharides, they were classified as the "T-independent"(TI) antigen. The concept of T-independency arose from the observations that neonatally thymectomized (Humphrey, J.H., et al. 1964) and nude mice (Weissman, I.L., et al. 1976) gave unimpaired antibody responses to large polymeric molecules, although they were not able to mount a humoral response to T-dependent (TD) antigens, such as proteins. The antigens that could not induce immune responses in T cell- deficient animals were designated as T-dependent antigens. Structurally, T-dependent antigens are usually complex proteins with large

numbers of different, non-repetitive, antigenic determinants. In many species there may be a continuous gradation from T dependence to T independence.

T-independent antigens are further divided into type I (TI-I) and type II (TI-II), based on the ability to elicit antibodies in the CBA/N mouse strain, with an X-chromosome linked immunodeficiency (xid) (Scher, I. 1982) based on their interactions with B lymphocytes (Mosier, D.E., et al. 1977). TI type 1 are defined as antigens capable of inducing proliferation and differentiation of both naïve and mature B lymphocytes (e.g., lipopolysaccharides (LPS) of gram-negative bacteria). TI type 2 antigens are of high molecular mass repetitive polysaccharide structures that exhibit no intrinsic B lymphocyte stimulating activity. eg., capsular polysaccharides of Gram positive bacteria and exopolysaccharides, which induces no response in *xid* strains. These antigens are also characterised by their poor in vivo degradability and inability to stimulate MHC class II restricted T lymphocyte help. TI type 2 antigens will activate only mature B lymphocytes and most likely act by cross-linking the cell surface immunoglobulin of specific, mature B lymphocytes. This results in the production of antigen- specific antibodies.

One of the most critical properties of this group of antigens is their ability to deliver prolonged and persistent signalling to the B cell. This by itself is not however sufficient to stimulate immunoglobulin synthesis and they must therefore stimulate non-T cells to interact with the B cells either directly or indirectly via cytokine production. There is evidence implicating the natural killer (NK cell) and T-cell is playing important role in response to TI antigens. While T cells may regulate the response to TI antigens it is not via the CD40 ligand (Foy, T.M., et al. 1993). Cytokines such as IL-3, GMCSF, and IFN-gamma significantly enhance antibody

production by these antigens. The nature of the B cell activating signal is critical in determining the quantitative and qualitative profile of Ig isotype production. Different types of antigens tend to elicit distinct profiles of immunoglobulin subclasses. The antibody produced in response to stimulation with T-independent antigens is reported to be predominantly IgM (Bos, N.A., et al. 1989). Later observations led to the suggestion that the immunoregulatory pathways that regulate isotype restriction patterns stimulated by polysaccharide antigens are complex, and no single pattern can be unequivocally defined (Mond, J.J., et al. 1995).

It has been suggested that B cells activated by TI antigens behave differently than B cells activated by TD antigens. Following TD antigen challenge many of the proliferating B cells within the PALS would enter the follicles and initiate a germinal centre (GC) reaction, while most B cells proliferating in response to a TI antigen would differentiate into plasma cells outside the follicle. MZ-B cells play an important role in TI antibody responses, particularly to TI- II antigens (Fagarasan, S. and Honjo, T. 2000). In GC, B cells may undergo somatic hypermutation, Ig class-switching and memory cell induction (Berek, C. and Ziegner, M. 1993, Kroese, F.G., et al. 1990). Eventhough TI- antigens are reported to induce only minimal or no GC development it was observed that immunization of mice with α(1→6) dextran, a native polysaccharide, induced the formation of germinal centres (Wang, D., et al. 1994). But germinal centre reaction is found to be less productive for a TI antigen than for a TD antigen (Sverremark, E. and Fernandez, C. 1998). This is probably due to inefficient T cell help and lack of different co-stimulatory signals during the GC reaction.

Complement plays an important role in B cell activation and in immune responses driven by TI antigens. This regulatory role of complement is mediated by CR2, the receptor for C3d which is expressed on the majority of mature resting B cells but not on immature B cells (Iida, K., et al. 1983). In addition, C3b and C3d coupled to Ag targets the complexes to CR3- and CR-4 expressing marginal zone (MZ) macrophages. The high antigen concentration in the splenic MZ is key to inducing marginal zone B cells to proliferate, form B cell foci and produce IgM antibodies independent of T-cell help (Ochsenbein, A.F. and Zinkernagel, R.M. 2000). The time at which the human immune system acquires responsiveness to polysaccharide antigens coincides with maturation of the marginal zone B cell, which occurs at approximately two years.

The nature of the B cell activator not only influences the pattern of cytokines secreted by the B cell, but also determines the induction of Ig secretion and class switching in response to a given set of cytokines (Mond, J.J., et al. 1995). IFN-γ secreted by NK cells regulates both B cell maturation to Ig secretion and Ig class switching in immune responses to TI antigens.

Eventhough early experiments abolished the possibility of immunologic memory in TI antigens, later experiments demonstrated that memory responses could be elicited in response to these antigens (Zhang, J., et al. 1988). These secondary antibody responses are not only greater in magnitude than the primary responses, but display a different pattern of Ig classes with a preponderance of IgG and IgA antibodies produced. The capacity to generate memory to these TI-type I antigens was associated with the appearance of antigen –specific B cells in the MZ of the spleen.

The single undisputed fact that remains about TI-antigens is that they cannot bind to MHC class II restriction elements.

B-1 cells play a major role in responses to TI-2 antigens (Pecquet, S.S., et al. 1992). These cells, originally defined by the surface expression of CD5 and high levels of IgM, arise early during ontogeny, home predominantly to the peritoneal and pleural cavities, have a capacity for self-renewal, and display different receptor specificities. They differ from conventional B cells (B2 cells) in their phenotype, specificity repertoire, and mode of replenishement. They have been shown to be responsible for the production of many multireactive and autoreactive IgM antibodies that are encoded by germ line sequences. Many of these IgM antibodies are directed to such epitopes on microorganisms as phosphatidylcholine, dextran, phosphorylcholine, and certain determinants on *Escherichia coli*.

IgA switching differs among purified B cell subsets, suggesting that individual B cell populations could contribute differentially to IgA expression in vivo, depending on available stimuli. MZ-B1 cells undergo class switching to IgA in minimal culture conditions at a higher frequency than follicular B2 cells (Kaminski, D.A. and Stavnezer, J. 2006). It was reported that monoclonal Immunoglobulin A derived from peritoneal B cells is encoded by both germ line and somatically mutated V_H genes and is reactive with commensal bacteria (Bos, N.A., et al. 1996). B1 cell-derived IgA antibodies play an important role in host defenses at the mucosal surface, preventing systemic penetration of commensal bacteria.

Many of the anti-carbohydrate antibodies that make up the front-line defense system of our body show multiple specificity or polyreactivity. Polyreactive antibodies are a major component of the natural antibody repertoire (Zhou, Z.H., et al.

2007) produced in the apparent absence of antigenic stimulation (Tauber, A.I. and Podolsky, S.H. 1994). The most likely explanation for their occurrence is that they are the result of the millions of normally occurring VDJ rearrangements and exist in the germline or near-germline configuration. These antibodies are highly represented in humans (Chen, Z.J., et al. 1998). These polyreactive antibodies bind to antigens with low affinity as compared to monoreactive antibodies and each polyreactive antibody has a distinct binding pattern that can vary for different antigens by as much as 1000 fold. Contrary to the classic "lock and key" rigid-structure hypothesis of antigen-antibody interaction, the antigen-binding pocket of polyreactive antibodies, perhaps because of their germline configuration, are believed to be more flexible and therefore can accommodate different antigenic configurations (Notkins, A.L. 2004).

In contrast to the nonpolyreactive antibodies, the great number and diversity of polyreactive antibodies, with the ability of many of them to recognize the same bacteria and multiple antigens on those bacteria, make them a major component of the natural antibody repertoire and potentially an important first line of defense. These antibodies represent the humoral component of the innate immune system. Infact, the exposure of the pathogens to the polyreactive antibodies in the presence of the complement, could result in the lysis of bacteria, which in turn could lead to the release of ligands (e.g., CpG DNA) that bind to Toll-like receptors. In this way the innate immune system could be activated (Krieg, A.M. 2002). The initial encounter of polyreactive antibodies with pathogens could serve as a first line of defense and give the adaptive immune system to be activated.

Naturally occurring serum anti-glycan antibodies are good candidates for biomarkers in inflammatory and auto immune diseases (Dotan, N., et al. 2006). The

anti-glycan antibody profile (AGAP) magnitude of subclasses IgG and IgA were higher than that of IgM (Dotan, N., et al. 2006). Anti-glycan antibodies are used to diagnose Inflammatory Bowel Disease (IBD), and discriminate between Crohn's disease (CD) and Ulcerative colitis (UC). Anti-Saccharomyces cerevisiae antibody (ASCA) are directed against oligomannosidic residues on the polysaccharide mannan in the cell walls of the yeast *Saccharomyces cerevisiae* and have a prevalence of 48-69% among CD patients and 15% among UC patients (Sendid, B., et al. 1996).

Natural antibodies are characterized by the presence of many arginine and lysine residues in their hypervariable regions (Avrameas, S. 1991) and many of them in fact tend to bind negatively charged antigens (Lekakh, I.V., et al. 2001).

Classical examples of anti-carbohydrate antibodies include anti-blood group antibodies (Milland, J. and Sandrin, M.S. 2006) and antibodies against Forssman (Fs) glycolipid antigen GalNAc α 1-3GalNAc β 1-3Gal α 1-4Gal β 1-4Glc (Kano, K. and Milgrom, F. 1977, Young, W.W., Jr., et al. 1979). The theoretical and methodological advances in the field of immunology and affinity chromatography have led to the identification and characterisation of several new naturally occurring carbohydrate binding immunoglobulins in the invertebrate plasma. Among the latter are anti-α-galactoside antibody (Galili, U., et al. 1984), anti-mannan antibody (Summerfield, J.A. and Taylor, M.E. 1986) anti-β-glucan (Schwarz, M., et al. 2003) and dextran binding immunoglobulin (Chacko and Appukuttan, 2003).

Anti-carbohydrate natural antibodies appear in number of pathologies, the group of particular attention includes antibodies to tumor-associated carbohydrate antigens such as Gal β 1-3GalNAc α (TF, Thomsen–Friedenreich antigen), GalNAc α 1- O Ser/Thr (Tn) (Springer, G.F. 1984), and several related ones (Lloyd, K.O. 1991).

β-glucan

The cell walls of saprophytic and pathogenic fungi are composed of proteins, lipids, and carbohydrates, which are structurally distinct from cell wall components produced by higher species, including mammals (Kapteyn, J.C., et al. 1995). The major cell wall components of fungi are mannoprotein (20-30%), β-(1→3) glucans (25-35%), β-(1→6) glucans (35-45%), chitin (0.6-2.7%), protein (5-15%) and lipid (2-5%) (Cassone, A. 1989, Shepherd, M.G. 1987). Glucans, major carbohydrate constituents of fungal cell walls, are polymers generally composed of a (1→3)-β-D-linked linear backbone containing anhydroglucose repeat units (AGRUs) with a glycosidic linkage between the 1 and 3 positions (Kapteyn, J.C., et al. 2000, Klis, F.M., et al. 2001, Shepherd, M.G. 1987). Some but not all, glucan polysaccharides exhibit side chain AGRUs which branch exclusively from the 6 position of the backbone AGRU (Kim, Y.T., et al. 2000).

In fungi, β-glucan is a key component of the cell walls of most families, including medically important fungi such as *Candida* and *Aspergillus*, but not Zygomycetes. It is also one of the major components of the cyst wall of *Pneumocystis carinii*. It plays an important role in the mechanical strength and rigidity of fungal cellwalls (Cabib, E., et al. 1988). This polymer has been shown to be produced by a variety of organisms, including algae (laminarin), bacteria (curdlan), and plants (callose). In plants, β-glucan is observed to be sythesized in apical meristem, in special vascular connections called plasmodesmata, in wound response tissue synthesis (Jacobs, A.K., et al. 2003), and in specialized structures and in seeds (Yim, K.O. and Bradford, K.J. 1998). β-glucan from barley is a mixture of approximately 70% β- 1, 4- glucan and 30% β- 1, 3-glucan.

As traces of plant and fungal matter are ubiquitous in virtually all environments, β- glucan is present, at some level, almost everywhere. Human exposure occurs through breathing, ingestion, fungal colonization and infection, systemic administration of β-glucan containing pharmaceuticals, and through invasive use of cellulosic medical devices such as surgical sponges, gauze packings, and dialysis membrane. The product types observed to have β-glucan contamination include drugs, blood fractionation products, biologics, and cellulose-containing medical devices (Usami, M., et al. 2002).

Animals including humans do not synthesize β-glucan in the body or absorb it through intact intestinal walls. Plasma β-glucan concentrations in normal subjects are less than 10pg/ml (Obayashi, T., et al. 1995). Blood β-glucan concentration may increase to as high as approximately 1ng/mL in systemic fungal infections and have been shown to be high in autopsy–proven deep mycoses, fungemias and anti-mycotic responsive febrile episodes. Thus far, fungi reported to be involved in the elevation of β-glucan in the blood include *Aspergillus, Candida, Trichosporon, Cryptococcus, Saccharomyces, Fusarium, Acremonium* and *Ochroconis gallopavum*. However pulmonary cryptococcosis rarely seems to be associated with hyper-β-glucanemia unless it is invasive or extensive. This may be due to a possible low content of β-glucan in the cell walls of *Cryptococcus*. Animal studies show that most of the β-glucan induced into the blood stream is incorporated into the liver and spleen, and that the β- glucan level lingers above normal for a prolonged period of time after the acute fall from the peak formed after injection. Thus β-glucan appears to be constantly released into the circulation from the reticuloendothelial system, once it has been saturated with the polysaccharide.

Vertebrates do not possess specific β glucan hydrolases and (1→3)-β-D glucan is broken down by slow oxidative degradation (Nono, I., et al. 1991) by active oxygen or nitrite ions that are produced by macrophages or polymorphonuclear (PMN) leukocyte although the mechanism of this response remain unclear. It may thus be retained intracellularly for prolonged periods of time (Ohno, N., et al. 1999). However, considering their long retention time in the body, both metabolism and degradation are thought to occur extremely slowly.

β-glucan may occur with variable molecular weight, branching, substitution, and quaternary structure. β- glucan has become the object of increasing interest due to a growing understanding of its role as a biological response modifier. β-glucans elicit potent biological responses across several kingdoms, including plants, insects, and animals (Brown, G.D., et al. 2003).

Nuclear magnetic resonance spectroscopy have proven to be particularly useful for the characterization of water soluble and insoluble β-(1→3)-glucans (Kim, Y.T., et al. 2000, Williams, D.L., et al. 1991). In general, glucans are water insoluble microparticulates upon initial isolation from the cell wall or culture medium. The glucan isolated from *S. cerevisiae* is a water insoluble microparticulate with molecular weight 105 kDa that can be converted to a water soluble form with minimal reduction in molecular weight (Ensley, H.E., et al. 1994). Glucan exposure in environmental settings is almost certainly not as pure glucans, but as macromolecular cell wall complexes that contain varying amounts of glucan.

Although the particulate form of (1→3)-β-D-glucan is found in nature, many labs have reported the effects of solubilized (1→3)-β-D-glucans. Particulate and

soluble β-glucans have the same primary structures. Activity of particulate β-glucans is thought to differ from soluble β-glucans because of their physical properties rather than their primary structures. However, the toxicological characteristics of soluble (1→3)-β-D-glucans may not be comparable with the original particulate form of (1→3)-β-D-glucans. Four major types of methodologies have been used to solubilize particulate (1→3)-β-D-glucans. These methods include: heating, solubilization in acid, solubilization in base, or use of organic solvents.

Glucan can assume a number of solution conformations depending upon the solvent system (Young, S.H., et al. 2003). For water soluble glucans, the two predominant conformations are single helix and/or triple helix. The single helical conformation is characterised as a semi-flexible coil, while the triple helix exists as a complex of three intertwined single helices that are stabilized by extensive hydrogen bonding involving the C2 hydroxyl group, located at the centre of the helix. The predominant form of β-glucan is a triple helical structure (Kopecka, M. and Kreger, D.R. 1986). The triple helical form may be converted to the singe helical form by a variety of techniques, including exposure to high pH, solvents, and heat (Saito, H., et al. 1990).

The biological activity of glucans is dependent on several factors: types of bond linkages, molecular weight, degree of branching (DB) and conformation. (1→3)-β types of bond linkages stimulate macrophages most effectively than other linkage types. The immune response is in part determined by size rather than by chemical structure. The anti-tumor activity could be completely lost when degree of polymerization (DP) of a linear (1→3)-β-D-glucan is less than 38, as in the case of laminarin (Saito, H., et al. 1991). An increased degree of (1→6) side chain

substitution not only increases the solubility in water but also affects the biological activity of glucans. The most active polymers for anti-tumor activity have a DB between 0.20 and 0.33.

β-glucan; A Pathogen Associated Molecular Pattern

The innate immune system has evolved a complex network of receptors, which rapidly identify microorganisms based on invariant molecular structures (lipids, proteins, and/or carbohydrates) that are shared by a variety of microbes (Akira, S. 2001). These invariant molecular structures are called pathogen associated molecular patterns (PAMPs). They include glucan, lipoteichoic acid, peptidoglycan, lipoproteins, lipoarabinomannans, and other products (Medzhitov, R. and Janeway, C.A., Jr. 1997). The first step in the modulation of cellular activity by glucans is the recognition and binding of the glucan polymer by pattern recognition receptors (PRRs) located on cell membranes.

For a number of years, it was thought that the Type 3 complement receptor (CR3) was the β-glucan binding site on macrophages, neutrophils and NK cells in mammals ((Ross, G.D., et al. 1987, Thornton, B.P., et al. 1996, Vetvicka, V., et al. 1996). β- glucan also binds a non-CR3 site on neutrophil cell line (Michalek, M., et al. 1998). Human promonocytic cells specifically bind glucans through scavenger receptors which are widely distributed (Rice, P.J., et al. 2002). Glucan specific receptors have been identified on primary cultures of normal human dermal fibroblasts (Kougias, P., et al. 2001) normal human vascular endothelial cells (Lowe, E.P., et al. 2002), human epithelial cells (Ahren, I.L., et al. 2001) and human anterior pituitary cells (Breuel, K.F., et al. 2004).

Dectin-1 which is highly expressed on alveolar and inflammatory macrophages recognizes (1→3)-β and (1→6)-β- linked glucans, and it also recognizes intact *S.cerevisiae* and *Candida albicans* in a glucan-dependent fashion. (Brown, G.D. and Gordon, S. 2001). Dectin-1 is broadly expressed with the highest expression levels found on monocytes and neutrophils in the blood, bonemarrow, and spleen and in low levels in dendritic cells and a subpopulation of T cells (Taylor, P.R., et al. 2002). Human homologue of Dectin-1 is reported to be a type II transmembrane receptor with a single CRD and an immunotyrosine –based activation motif (ITAM motif) (Willment, J.A., et al. 2001).

Glucans are recognized by Dectin-1 and the glucan stimulatory signal is transduced into the cell through TLR2 dependent mechanisms. Dectin-1 and TLRs trigger independent and cooperative inflammatory responses (Brown, G.D., et al. 2003).

Immunomodulatory roles of β-glucan

β- glucan showed various biological activities triggering the activation of complement, and production of various biological mediators like leukotriene, TNF-α and so on. It is possible that β-(1→3) glucan has some influence on, and can be a parameter which reflects the immune response and inflammatory reactions of the host. β-glucan may enhance the activation state of both monocyte (Czop, J.K. and Austen, K.F. 1985) and PMN or NK cells (Vetvicka, V., et al. 1996). β-glucan in some way stimulates the antigen presenting cell involved to produce IL-4 which is essential for the development of Th2 cells. Th2 cells play a key role in allergic immune response (Del Prete, G., et al. 1988). (1→3)-β-D- glucan may increase the induction of allergy to substances in the indoor environment, by shifting the Th1/Th2 balance of the

immune response towards a Th2 polarization and the production of specific IgE. This reaction represents a suppression of the host defences, and it is possible that this renders the host more susceptible not only to inflammation but also to infection and allergens, resulting in atopy. In the case of decreased Th1 cell function, namely, decreased IL-2 production, the resistance of the host to *C.albicans* decreases and recovery from candidiasis is delayed (Farah, C.S., et al. 2002, Farah, C.S., et al. 2001).

Numerous medical applications have been proposed and developed for β-glucans. Among these, anti-tumor applications have been a particularly strong focus, especially in Japan (Ohno, N., et al. 1986). Other applications of β-glucans include the stimulation of general immunity and immuno-protective effects (Hong, F., et al. 2003) and wound healing (Wei, D., et al. 2002). Commercial exploitation of β-glucan of alginic origin (Laminarin) for industrial applications, and β-glucan of bacterial origin (curdlan) as an additive in the processed food industry, are also well established (Spicer, E.J., et al. 1999).

Anti β - glucan

The first reported evidence of antibody specific to (1→6)-branched (1→3)-beta-D-glucan was prepared in rodents using grifolan conjugated with bovine serum albumin as an immunogen. The hapten site of the antibody was the monoglucosyl branched moiety of (1→3)-beta-D-glucan (Adachi, Y., et al. 1994). Serum from mice immunized with *Candida albicans* showed higher reactivity than non-immune, normal mice to solubilized candida cell wall β-glucan (CSBG). These facts suggested that CSBG included epitopes of the specific antibody in *Candida* immune mice (Uchiyama, M., et al. 2000). Anti- β-glucan has protective role against *Candida*

albicans infection in mice (Bromuro, C., et al. 2002). The presence of a novel cellulose-binding immunoglobulin present in human serum was detected using carbohydrate array (Schwarz, M., et al. 2003). The anti-cellulose antibody is specific to β4 linked saccharides with a preference for glucopyranose over galactopyranose residues. Masuzawa et al., (2003) reported the presence of antibodies in normal human serum against soluble cell wall β-glucan. When a search was conducted by expanding the range to include clinical patients, markedly decreased levels of this antibody were demonstrated in patients with rheumatic disease and vasculitis, and the degree of variation increased among patients with malignant tumors (Masuzawa et al., 2003). When anti-β-glucan antibody titre was examined in other animals, high titres were detected in inbred strains DBA1 and DBA2 mice (Harada, T., et al. 2003). The presence of this antibody in such a wide range of animals alludes to a strong connection between β-glucans and immune function.

Anti-β -glucan antibody in normal human and normal mouse sera showed reactivity to pathogenic fungal *Aspergillus* and *Candida* cell wall glucan (Ishibashi, K., et al. 2005). The anti-β -glucan antibody titer of DBA/2 mice intravenously administered with either *Candida* or *Aspergillus* solubilized cell wall β -glucan decreased remarkably dependent on dose. Moreover the decrease of the anti-β -glucan antibody titer in deep mycosis patients led to the suggestion that the anti-β -glucan antibody formed an antigen–antibody complex and participated in the immune response as a molecule recognizing pathogenic fungi (Ishibashi, K., et al. 2005).

Anti-β-glucan antibodies elicited by a laminarin-conjugate vaccine confer cross-protection to mice challenged with major fungal pathogens such as *Candida*

albicans, Aspergillus fumigatus and *Cryptococcus neoformans* (Torosantucci, A., et al. 2005).

The anti-β-glucan which is a normal human plasma antibody that had three times higher IgA to IgG ratio and substantially higher polymeric IgA content than total serum immunoglobulins was prepared using affinity chromatography on cellulose column in this laboratory (Geetha, M., et al. 2007). It also recognized polystyrene well-coated beta1→3 linked glycans of *Saccharomyces cerevisiae*, *Candida albicans* and of barley in decreasing order of affinity. Its sugar-binding site could thus accommodate beta-glucoside with or without substitution at C4 and C3. The high titres of these antibodies in the nonimmune state can be attributed to spontaneous sensitization by substances containing β-glucans in the environment.

Recently it was reported that anti-β-glucan antibody repertoire in normal, healthy Italian adults consists of various isotypes and subclasses, mostly being IgG2. They are prevalently directed against glucans with β (1→6) rather than β (1→3) configuration (Chiani, P., et al. 2009).

The isotype of anti-β-glucan antibodies may affect details of the β-glucan epitopes recognized, and this may be associated with a differing ability to inhibit virulence attributes of the fungus and confer protection *in vivo* (Torosantucci, A., et al. 2009). The protective IgG2b selectively bound to β1, 3-linked (laminarin-like) glucose sequences whereas the non-protective IgM bound to β1,6- and β1,4-linked glucose sequences in addition to β1,3-linked ones.

It is possible that anti-β glucan antibody plays a definite role in the initial immune response and defence against opportunistic infections. Anti- β-glucan may be able to interact with fungal cell wall or extra cellular β-glucan and modify the host

defence system. It was suggested that anti-β-glucan antibody could play a role as β-glucan recognition molecule and induce clearance of pathogenic fungi and biological activity by collaboration with other recognition molecules such as β-glucan receptor or complement in man.

Dextran

Dextran is a collective name for high-molecular-weight polymers ≥1,000 Dalton composed of D-glucose units connected with α-1,6 linkages and various amounts of side branches linked with α-1,2, α-1,3, or α-1,4 to the main chains. Technically the molecular weight (M_w) can range between 1500 and several million; therefore, a dextran of 1 million M_w has potentially thousands of possible structures due to its branched nature. Historically dextrans had been long recognized as contaminants in sugar processing and other food production. The formation of dextran in wine was shown by Pasteur to be due to the activity of microbes. The name dextran was created by Scheibler in 1874, who demonstrated dextran was a carbohydrate with the formula $(C_6H_{10}O_6)_n$ and a positive optical rotation. Three classes of dextrans can be differentiated by their structural features. Class 1 dextrans contain the α (1→6)-linked D-glucopyranosyl backbone modified with small side chains of D-glucose branches with α(1→2), α (1→3), and α (1→4)-linkage. The class 1 dextrans vary in their molecular weight, spatial arrangement, type and degree of branching, and length of branch chains depending on the microbial producing strains and cultivation conditions (Kim, D., et al. 2003). Isomaltose and isomaltotriose are oligosaccharides with the class 1 dextran backbone structure. Class 2 dextrans (alterans) contain a backbone structure of alternating α(1→3) and α(1→6)-linked D-glucopyranosyl units with α(1→3)-linked branches. Class 3 dextrans (mutans) have a backbone structure of consecutive α (1→3)-

linked D-glucopyranosyl units with α(1→6)-linked branches. (Maina, N.H., et al. 2008).

Dextrans are found as bacterial extracellular polysaccharides. They are synthesized from sucrose by beneficial lactic acid bacteria, such as *Leuconostoc mesenteroides* and *Lactobacillus brevis,* but also by the dental plaque-forming species *Streptococcus mutans, S. sanguis*, *S. sobrinus*, *S. cricetus*, and *S. rattus* and by the mold *Rhizopus* spp. The enzymes that synthesize dextrans from sucrose are known by the generic term dextran-sucrase (Sidebotham, R.L. 1974). Other dextran-producing bacteria, *Acetobacter capsulatus* (renamed *Gluconobacter oxydans*) and *Acetobacter viscous*, produce dextrin-dextranase that converts dextrin to dextran. Bacteria employ dextran in biofilm formation (Banas, J.A. and Vickerman, M.M. 2003) or as protective coatings, e.g., to evade host phagocytes in the case of pathogenic bacteria (Meddens, M.J., et al. 1984). Dextrans are believed to be responsible for the formation of dental plaque and the induction of caries on the surface of teeth and have, therefore, been a subject of numerous studies. The secretion of dextrans provides an opportunity for bacteria to modulate adhesion, e.g. in tooth decay, by having a softer or more rigid bacterial cell surface, depending on the polysaccharide itself and the pH and ionic strength.

The *Leuconostoc mesenteroides* strains are inducible and require sucrose in the medium for the biosynthesis of dextrans. *Streptococcus* species are generally constitutive and do not require sucrose in the growth media for enzyme expression (Eifuku, H., et al. 1989). Dextran-sucrase catalyzes the synthesis of glucan, which contains 50% or more α-1, 6 glucosidic bonds within the main chain. The structures and properties of bacterial dextrans vary between microbial strains and according to

growth rate and reaction conditions. Dextran from *Weisella confusa* was found to be more linear than that of L. mesenteroides, which contained about 4.1% alpha-(1→3)-linked branches (Maina, N.H., et al. 2008).

The position of the branch linkages, the degree of branching, the length of branch chains, and molecular weight distribution affect the physicochemical properties of dextrans (Abbott, D., et al. 1966). For example, the degree of solubility in water decreases when the degree of branching is increased. In fact, dextrans with >43% branching through 1, 3-α linkages have been considered water insoluble (Mehvar, R. 2000).

Dextran in raw sugar

Dextran is produced by microorganisms which infect the cane and feed on the sucrose; therefore, the presence of dextran immediately indicates lost sugar. The bacteria are mainly *Leuconostoc* species and are ubiquitous in the soil. They enter the cane at places of exposed tissue caused by machine harvesting, cutting, burning, growth, freezing, disease and pests. Any delay in the kill-to-mill time allows the bacteria to proliferate and the dextran levels to soar, especially in wet muddy cane.

Applications of dextrans

Dextran is used commonly by microsurgeons to decrease vascular thrombosis. The antithrombotic effect of dextran is mediated through its binding of erythrocytes, platelets, and vascular endothelium, increasing their electronegativity and thus reducing erythrocyte aggregation and platelet adhesiveness.

Larger dextrans, which do not pass out of the vessels, are potent osmotic agents, and thus have been used urgently to treat hypovolemia. Dextran in intravenous solution provides an osmotically neutral fluid that once in the body is

digested by cells into glucose and free water. The hemodilution caused by volume expansion with dextran use improves blood flow. In medicine, clinical grades of dextrans with a molecular weight range of 75-100 kDa have been used as blood-plasma volume expanders in transfusions (Terg, R., et al. 1996). Dextran infusion is a safe, effective, and low-cost replacement therapy in patients with cirrhotic ascites treated by Large volume Paracentesis (Acharya, S.K., et al. 1992).

The larger dextrans are excreted poorly from the kidney and therefore remain in the blood for as long as weeks until they are metabolized. Dextran is used in some eye drops as a lubricant, and in certain intravenous fluids to solubilise other factors, e.g. iron (iron dextran). It also increases blood sugar levels.

The long history of the safety of dextrans has allowed them to be used as additives to food and chemicals, and in pharmaceutical and cosmetics manufacturing (Kato, I., et al. 2005). Dextrans have been investigated for the targeted and sustained delivery of drugs, proteins, enzymes, and imaging agents (Mehvar, R. 2000).

Immune response to dextran

Native and clinical dextrans derived from strains of microorganisms synthesizing products with different ratios of 1→6 to non 1→6 linkages have been found to be antigenic in man. The antigenicity of dextran in man, its occurrence in commercial sugar and its elaboration by microorganisms in the gastrointestinal tract appear to provide an explanation for the occurrence of systemic allergic reactions in man on infusion of dextran and for the occurrence of small quantities of anti-dextran

and of cutaneous sensitivity in individuals not previously injected with dextran (Kabat, E.A. and Berg, D. 1953).

Difference in antigenicity with differing molecular weights of dextran was noted by Kabat and Bezer. A significant drop in capacity to elicit antibody formation in man was found with injected dextran fractions of average molecular weight of 51,300 or below (Kabat, E.A. and Bezer, A.E. 1958).

The reactions of all specific anti-dextrans have been thought to occur at the nonreducing ends of glucose chains with the terminal α (1-6)-linked D glucopyranosyl residue being immunodominant. Quantitative precipitin reactions with branched and linear dextrans suggested that alpha (1- 6)-specific human anti-dextrans are mixtures of molecules having terminal and non-terminal specificities and that the fraction of each type can vary among individuals (Cisar, J., et al. 1975).

The response to polysaccharide antigens, such as dextran (α1-6 glucose polysaccharide), is predominated by antibodies of the IgG2 subclass (Yount, W.J., et al. 1968). IgG and to a lesser extent IgM class anti-dextran antibody was detected in chronic liver diseases such as liver cirrhosis and chronic hepatitis. Morito et al., suggested that the damaged hepatocytes in the process of the liver disease may release the dextran antigen into the patient's circulation which is responsible for the formation of anti-dextran antibodies by the patients with liver diseases (Morito, T., et al. 1985).

Dextran or dextran-like material, which inhibited the binding of anti-dextran serum to dextran, was reported in sera of several patients with various gastrointestinal diseases, especially gastrointestinal ulcers, and also often in sera of aged people.

Absorption of dextrans from food or their production by intestinal bacteria may be facilitated in various diseases like gastric and duodenal ulcers, ulcerative colitis, Crohn's disease, colorectal carcinomas and rheumatoid arthritis (Palosuo, T. and Milgrom, F. 1981).

Abnormally high humoral responses have been described in IgA nephropathy (IgAN) towards antigens commonly involved in infectious events or food intolerance. In a comparative analysis of humoral responses to a common environmental antigen, dextran B 512, present both in non-pathogenic microorganisms and normal diet, anti-dextran IgA and IgG were at significantly higher levels in IgAN patients than in controls (Kennel, A., et al. 1995).

Dextran-binding antibody isolated in high yield from plasma of blood donors was purified by a single step affinity chromatography on Sephadex G100 using 1-*O*-methyl α-D -glucoside as eluant in this laboratory. Analysis of protein peaks obtained in size exclusion high pressure liquid chromatography (HPLC) suggested the presence of polymeric IgA in this antibody. The dextran-binding antibody bound sugar-specifically to glycoconjugates from different bacterial populations. Binding of the antibody from dialysed plasma to immobilized dextran was lowered only marginally in presence of glucose at 4.5 mM (which nears normal serum glucose concentrations), but substantially in presence of the sugar at 20 mM and above which are reached in diabetic sera (Chacko, BK and Appukuttan ,PS, 2003).

IgA responses known to strictly depend on the participation of helper T cells (Luzzati, A.L. and Jacobson, E.B. 1972) were seen to be produced against dextran antigens. B cells activated by dextran switch directly from IgM to IgA secretion

(Coutinho, A. and Forni, L. 1982). The response to the TI-2 antigen dextran, although largely T cell independent, may be modulated by T cells. Dextran -specific antibody of IgM, IgG3, and IgA isotypes can be detected in sera of euthymic dextran-immune mice, whereas in athymic mice IgG3 and IgA antibodies are undetectable. Furthermore, plasma cells producing dextran-specific antibody of IgM, IgG3, and IgA isotypes might be localized in lymphoid organs other than spleen, which would explain why these isotypes are present in sufficiently high amounts to be detected.

Anti-α-galactoside antibody

The α-gal epitope is a unique carbohydrate structure that is absent in humans but is naturally produced on glycolipids and glycoproteins in non-primate mammals, prosimians and New World monkeys. Antibodies specific for α-Gal are produced in the host without intentional immunization and are termed natural antibodies (Galili, U., et al. 1984).

The α-galactosyl or α-gal epitope is formed by the action of the enzyme α-1-3 galactosyl transferase, which adds a terminal galactose residue on to Galβ (1-4) GlcNAc- moieties of glycoproteins in the golgi of most mammalian cells (Basu, M. and Basu, S. 1973). However, in man, Old world monkeys and apes this enzyme is inactive, due to a gene modification leading to a premature transcriptional stop codon (Lanteri, M., et al. 2002). Higher primates therefore have no terminal Gal α (1-3) Gal residues, but instead have substantial quantities of an antibody that interacts specifically with the α-Gal epitope via anti-Gal (Galili, U., et al. 1984, Galili,U et al., 1987). This natural antibody, termed anti-gal, comprises 1% of total serum IgG, but is also present in IgA and IgM forms (Hamadeh, R.M., et al. 1995).

The continuous production of anti-gal throughout life suggests that this antibody is produced in response to chronic antigenic stimulation. As a result of the understanding of the carbohydrate structure of blood group antigens, it became apparent that natural anti-blood group antibodies interact with a variety of carbohydrate structures shared between bacteria and some blood group antigen. Furthermore, ingestion or inhalation of E.coli strain 086 was reported to increase anti-blood group B antibody activity in the individuals studied (Springer, G.F. and Horton, R.E. 1969). Anti-gal readily bound to a variety of *E.coli, Klebsiella, Serratia* and *Salmonella* isolates from normal stool (Galili, U., et al. 1988). All these bacterial epitopes were reported to contain Gal α 1-terminal structures linked to various penultimate sugar units. Although the exact bacterial structures that stimulate the human immune system to produce anti-Gal have not been identified as yet, it is likely that anti-Gal is produced as part of the immune response to a variety of bacterial antigens containing α-galactosyl structures.

Some of the organisms that make α-gal epitopes have been associated with infectious diseases, for example leishmaniasis (Towbin, H., et al. 1987) and Yersinia (Hammer, M., et al. 1990). Moreover, levels of the natural anti-gal antibody have been shown to be raised in patients with autoimmune diseases such as Graves's disease and other autoimmune diseases of the thyroid. Studies on anti-Gal IgA have shown that in Henoch Sch□nlein purpura there is a major increase in titer of anti-Gal IgA antibody within the serum (Davin, J.C., et al. 1987).

The specificity of anti-Gal slightly differs in individuals of various blood groups. In blood group A and O individuals, anti-Gal can also interact with blood group B antigen, whereas in individuals of blood groups B or AB, anti-Gal interacts

with α-Gal epitopes. The affinity of anti-Gal is low and ranges between $2x10^5$ and $5x10^6$ M^{-1}. This low affinity is because of the lack of ionic bonds between anti-Gal and α-gal epitope.

Anti-gal undergoes isotype switch according to the general pattern of antibody response. This was demonstrated in studies on anti-gal production in new born baboons that were heterotopically transplanted with baby pig hearts (Galili, U., et al. 1997). The immune system of the neonatal baboon responds to α-gal epitopes, first producing anti-gal IgM and subsequently anti-gal IgG antibody molecules. Somatic mutations were observed in the VH genes utilized by these hybridomas as well as human VH genes encoding α-Gal specific antibodies. α-Gal-specific antibodies were thought to be largely T cell independent due to the structural nature of the epitope on glycoproteins and glycolipids, which should allow efficient cross-linking of the B cell antigen receptor. Later it was confirmed that antibodies specific for α - Gal have T cell-dependent component (Cretin, N., et al. 2002).

The concentration and affinity of anti-Gal greatly increase after transplantation of xenografts into humans and monkeys. It is possible that within a short period post-transplantation, B lymphocyte clones containing somatic mutations that confer high affinity to this antibody proliferate within the lymph nodes and spleen and produce increased amounts of high affinity anti-Gal, as observed in recipients of xenografts (Galili, U. and Anaraki, F. 1995, Galili, U. and LaTemple, D.C. 1997, Galili, U., et al. 1997).

Pathophysiology of Immune Complex (IC) - mediated diseases

After an immunogenic stimulus, antibodies are produced that combine with the evoking antigenic determinants wherever they are encountered, thereby forming immune complexes (ICs). This process is usually of benefit to the host, since it results in the neutralization or elimination of the antigens. Most ICs are of little pathologic significance because they are rapidly cleared by hepatic and splenic phagocytes (Schifferli, J.A., et al. 1982). However, ICs may, under certain conditions, localize in vascular structures, thereby inciting inflammatory responses. The localization may result either from formation of the complex at the site as in the Arthus reaction or from deposition of circulating ICs. It is this latter circumstance, in which complexes from the circulation produce lesions in multiple sites is generally referred to as "immune complex disease" (Theofilopoulos, A.N. and Dixon, F.J. 1980).

The formation, fate, and biologic activities of circulating ICs depend on the nature of the antibodies and antigens involved as well as on the molar ratio of the two reactants. Of the features that are important to the formation and function of ICs, first is the immunoglobulin (Ig) class, which determines the antibody's valence for a specific antigen as well as its ability to bind to cellular Fc receptors and to activate the complement(C) system. Second is the association constant for the union of specific antibody and antigen. For antigens, valence, size, and chemical composition are important factors in IC formation. Monovalent antigens do not form lattices with their corresponding antibodies; therefore, the complexes they develop remain in the circulation for long periods without depositing in tissues. In contrast, multivalent antigens such as polysaccharides and proteins do combine with their specific

antibodies so as to form lattices and later ICs of varying composition, depending on the molar ratio of the reactants.

IgG-ICs are eliminated from the circulation mainly by the non-parenchymal liver cells (Mannik, M. and Arend, W.P. 1971, Skogh, T. 1982). Interaction with Fc-receptors is considered to be the most important mechanism of IgG-IC entrapment in the liver (Finbloom, D.S., et al. 1980, Leslie, R.G. 1980). Asialoglycoprotein receptor mediates internalization of glycoproteins, including Ig, resulting in intracellular degradation of the ligand and recycling of the receptor. If the reticuloendothelial system (RES) is strained by IC handling, the complexes may be deposited outside the RES (Haakenstad, A.O. and Mannik, M. 1974). This may occur in tissues such as skin, muscle, joints, and kidneys, and is probably mediated by mechanisms other than Fc-receptor interaction (Schrieber, L. and Penny, R. 1982). Deposition of ICs outside the RES is common in many disease states (Theofilopoulos, A.N. and Dixon, F.J. 1980), and it is probable that this contributes to the onset of active disease.

ICs activate the C system through both the classical and alternative pathways. It appears that IgG and IgM activate primarily the classical C pathway, whereas IgA and IgE activate the alternative C pathway. Mannan Binding lectin (MBL) recognizes and binds to glycoproteins, including Igs, with aberrant glycosylation and/or denaturation, and possibly plays a role in the elimination of altered self-components such as aberrantly glycosylated IgG and immune complexes (Saevarsdottir, S., et al. 2004). Once the complement system is activated, several biologic activities are generated that play a role in IC-induced diseases. These include a) the immune adherence phenomenon through C3b receptors, b) chemotaxis of leukocytes through C5a and possibly the C567 complex, c) anaphylaxis through the binding of C3a and

C5a on mast cells and basophils, d) macrophage activation through the Bb fragment of factor B of the alternative C pathway, and e) solubilization of ICs via activation of the alternative C pathway and intercalation of C3b into the complexes. Moreover, ICs can activate a variety of cell types by interacting with their Fc and C receptors. Complement may increase the solubility of ICs by inhibiting non-specific interaction with other serum proteins and by preventing Fc-Fc interaction of immune complexes.

C3b coated (opsonized) IC attach to cells bearing C3b receptors (CR1) in the circulation, in particular to the erythrocytes, since in humans 80-90% of CR1 in the blood is located on these cells. This immune adherence reaction appears to be a physiological system that allows IC to be transported through the circulation to the fixed macrophages of the Mononuclear Phagocytic System (MPS) where they are safely eliminated. On these cells, IgG Fc receptors mediate both binding and ingestion of particulate complexes. Phagocytosis of ICs by macrophages results in the release of various hydrolytic enzymes. Eosinophils mediate antibody-dependent cellular cytotoxicity (ADCC) against parasites. Basophils and mast cells degranulate after binding to IC or complement components followed by a release of various biologically active substances such as heparin, histamine, slow reacting substance of anaphylaxis and platelet activating factor.

Factors affecting the immobilisation and deposition of circulating immune complexes

The initial stages of circulating immune complex deposition require a sequence of events, which enables circulating IC and inflammatory cells to cross the endothelial barrier. The first step in tissue-deposition of IC is likely to be the interaction with vascular receptors. C1q receptors, expressed by endothelial cells, and

the Fc receptors, expressed on the renal interstitium and by damaged endothelium could play a role in IC immobilization.

The immobilized IC is then able to interact with circulating cells expressing Fcγ receptors. Human Fc γ RIIA and Fc γRIIIB expressed on neutrophils play important context dependent roles in IC-induced neutrophil recruitment and subsequent tissue injury (Mayadas et al., 2009). When the FcγRIIIB-mediated removal of IC that takes place under homeostatic conditions is overwhelmed or defective, ICs may translocate and accumulate in extravascular tissue. There, they may persist and activate resident cells to release inflammatory mediators like LTB4 and TNF-α that lead to FcγRIIIB shedding enhance FcγRIIA-dependent recruitment and activity, and thus instigate a cycle of vessel damage that predisposes to IC-mediated disease. Also PMN activation is followed by release of mediators, such as platelet-activating factor (PAF) and interleukin (IL)-1. PAF induces vasodilation and activates platelets, which form aggregates and release vasoactive amines. The resulting increased vascular permeability allows circulating IC to cross the endothelial barrier. The frequent involvement of the kidneys in IC-associated disease may be a consequence of the existence of C3b receptors in the renal glomerular epithelial cells, Fc receptors in the renal interstitium, and a collagen-rich structure (the basement membrane), which can also be involved in non-specific interactions with antigens or antibodies.

Interaction of extravascular IC with associated C3b delivers additional activation signals to already primed granulocytes, resulting in the release of metalloproteinases, oxygen active radicals, and nitric oxide. These compounds can

cause tissue damage and can further increase vascular permeability, and in doing so contribute to the perpetuation of an inflammatory reaction.

Factors affecting immune complex deposition

1) Physico-chemical characteristics of pathogenic immune complexes

Size, affinity of the Ag.Ab reaction, and class and subclass of antibodies involved in IC formation are among the most important determinants of the pathogenic significance of IC. In the case of circulating IC (cIC) formed at very great antibody excess, very large Ag.Ab aggregates containing IgG1 or IgG3 antibodies will activate complement very effectively and are rapidly phagocytosed and thus are of limited pathogenicity. This is due to the combination of facts: very avid ingestion and degradation by phagocytic cells, and difficulty in diffusing across the endothelial barrier. ICs formed in great antigen excess are very small complexes (Ag1.Ab1-3), even while involving IgG1 and IgG3 antibodies, are able to diffuse easily into the extravascular compartment, but are usually non-pathogenic because of their inability to activate complement. The greatest pathologic potential seems to lie between these two extremes of complex size, ie, in ICs formed when antigen excess is modest. These ICs are of intermediate size (Ag2-3.Ab 2-6) and soluble; so they circulate and disseminate widely. Since they are large enough to fix C, they can exert inflammatory effects at many sites. Under the appropriate circumstances, these IC may be deposited in the subendothelial space and trigger inflammatory reactions. On the other hand, IC formed in situ between tissue-fixed antigens and freely diffusible antibodies of the IgG1 and IgG3 class are always likely to be proinflammatory. Due to their large size, IgM antibodies are rarely involved in formation of IC in tissues. ICs composed of

cation antigen have a higher potential to activate and bind complement C1q (Michelin MA et al., 2002).

2) Hydrostatic Pressure

IC deposition seems to depend greatly on hemodynamic factors and the anatomy of particular sites. The glomerulus, choroid plexus, synovium, skin, and uveal tract all sustain a high degree of blood flow per unit mass of tissue and thereby are exposed to and can trap large quantities of ICs in their vascular walls. Consequently, these sites are particularly subjected to IC-associated disorders. Acute glomerulonephritis is found mainly in glomeruli situated close to the medulla and this may be due to a higher intraglomerular hydrostatic pressure in these glomeruli than in those in the outer parts of the cortex.

3) Vascular permeability

Lines of evidence in humans and animal models suggest that circulating ICs first localize within the vasculature and then translocate into extravascular tissue (Cream, JJ et al., 1971). Changes in vascular permeability induced by cytokines and/or lipid mediators secreted by hematopoietic cells and mast cells (Jancar S and Sanchez Crespo M., 2005) are likely the initial trigger for IC deposition. In addition, ICs themselves promote vascular leakage in "permeability"-susceptible tissues such as the joint tissue, which may explain why circulating ICs can promote tissue specific disease such as rheumatoid arthritis (Kyburz D and Corr M., 2003). However, permeability is likely an amplifier rather than a prerequisite for localization of arthritogenic antibody.

The mechanisms responsible for IC deposition involve the release of vasoactive amines followed by increased vascular permeability. Human platelets bear receptors for IgG Fc and Clq, and they aggregate and release nucleotides and vasoactive amines in response to ICs. The events that follow the activation of C, in particular, the cleavage of C3 and C5, underlie much of the inflammatory response evoked by ICs deposited along vascular basement membranes (Cochrane CG and Dixon FJ, 1978)

4) Time

If a host's exposure to antigen is of limited duration, even though some complexes form, tissue injury and other clinical manifestations are transient, as in classical acute serum sickness. In contrast, if the antigen remains long in the circulation or if there is a continuous supply of antigenic material for protracted periods, as with chronic infections or autoantigens, the potential exists for continuing IC formation. The closest experimental approximation to such chronic IC disease in man is "chronic" serum sickness (Theophilopoulose and Dixon, 1980).

5) Complement deficiency

The deposition of immune complexes outside the RES may be explained by poor ability to keep the ICs soluble, circulating, and harmless, awaiting physiological clearance by the RES (Skogh and Stendahl., 1983). C3b covalently linked to ICs solubilizes ICs by interfering with Fc-Fc interactions. Moreover, C3b-based convertases activate C5, which can in turn promote IC deposition. Complement can also enhance degradation of soluble immune complexes. It has also been shown that complement may prevent soluble immune complexes from precipitating (Schifferli and Peters, 1982). This would consequently keep the immune complexes circulating,

preventing non-specific deposition outside the RES, but allowing specific elimination by the non-parenchymal liver cells after Fc-receptor and/or C3 receptor interaction. In autoallergic disease states such as systematic lupus erythematosus (SLE), serum complement concentrations are generally reduced during active disease. This is not merely the result of complement consumption; it may to a greater extent be explained by reduced synthesis of C3. As a consequence of the low complement levels, the immune complex solubilizing properties are reduced in these sera as compared to normal controls.

6) Role of Fc γ receptors and Complement Receptors (CR): Affinity and number of available receptors

The affinity and number of available Fcγ receptors on professional phagocytic cells (PMN leucocytes, monocytes, and macrophages) are important in the expression of IC disease. If IC is predominantly taken up by those cells in tissues where they abound, such as the liver and spleen, the likelihood of developing tissue inflammation is limited. Patients with SLE and rheumatoid arthritis have decreased ability to clear antibody-sensitized RBC, indicating a general inability to clear circulating IC. In these IC mediated diseases, the number of CR1 on the surface of red cells is found to be less which contributes to decreased IC clearance.

8) Host factors that influence the development of IC- mediated diseases

The development of IC disease in experimental animals is clearly dependent on host factors like age, weight, and sex of the immunized animal. The magnitude of the response primarily depends on genetic factors.

Glycoproteins

Glycoproteins are compounds containing carbohydrate (glycan) covalently linked to protein (Spiro, R.G. 1973). The carbohydrate may be in the form of a monosaccharide, disaccharide(s), oligosaccharide(s), polysaccharide(s), or their derivatives (e.g. sulfo- or phospho-substituted). Apparently most of the proteins in nature are glycoproteins. Glycoproteins are found in soluble form in the extracellular fluids and in insoluble form in membrane and intercellular matrix components. Of all the biologically occurring macromolecules, the glycoproteins represent the most diverse group, ranging from substances in which the carbohydrate component represents less than 1% of the total weight to those in which it represents over 80% of the total (Kornfeld, R. and Kornfeld, S. 1976). Glycosylation is the most common co-translational and post-translational modifications of proteins (Sharon, N. 1986). More than half of known protein sequences can potentially be glycosylated. Glycoproteins are widely distributed in all three domains of life viz, archaea, bacteria and eukaryotes.

This class of compounds includes enzymes, hormones, immunoglobulins, lectins, toxins, carrier and structural proteins. Although nearly 200 different monosaccharides are found in nature, only 13 are known to occur in glycoproteins (Sharon, N. and Lis, H. 1982). The sugars that commonly occur in glycoproteins include galactose, mannose, glucose, N-acetylglucosamine, N-acetylgalactosamine, sialic acids, fucose, and xylose. Proteoglycans are a diverse group of proteins containing a large number of glycosaminoglycan side chains and is distinct from glycoproteins in that they contain repeating disaccharide units characteristic of the

glycosaminoglycan chains. The proteoglycans contain various uronic and sulfated amino sugars.

Glycosylation types in glycoproteins

Glycoproteins have their carbohydrate side chains either N- or O- linked as defined by the type of linkage between the carbohydrate chain and the peptide backbone (Iwase, H. 1988, Jentoft, N. 1990, Kornfeld, R. and Kornfeld, S. 1985).

N-glycosylation

The N-glycosidic linkage is between the anomeric carbon atom of N-acetyl-D-glucosamine and the amide nitrogen of asparagines in the polypeptide chain (Kornfeld, R. and Kornfeld, S. 1976). The oligosaccharide chain is attached by oligosaccharyl-transferases (via N-acetyl-D-glucosamine residue) to the amide group of an asparagine (Asn) in the consensus sequence Asn-X-Ser/Thr, where X is any aminoacid residue but a proline or aspartic acid (Marshall, R.D. 1972, Petrescu, A.J., et al. 2004). This sequence is known as a glycosylation *sequon*. Since glycosylation occurs co-translationally, once the protein has folded potential glycosylation sites are no longer accessible to the glycosyltransferases (Marshall, R.D. 1972). The presence of N-linked protein glycosylation systems in all three domains of life that build on same principles suggests that this post translational modification process is a very ancient one.

Only about one third of the potential Asn-X-Ser/Thr sites in proteins are actually glycosylated. It has been suggested that rapid folding of the nascent polypeptide is responsible for the lack of glycan chains at potential glycosylation sites (Marshall, R.D. 1972). The carbohydrate units linked to the asparagines contain a

common pentasaccharide core: Man α-1→6 (Man α-1→3) Man β 1→ 4 GlcNAc β 1→ 4 GlcNAc (Montreuil, J. 1980). Three types of *N*-linked oligosaccharide chains are observed: (a) those containing mannose only, referred to as high-mannose, attached to the common pentasaccharide inner core, (b) those containing galactose, *N*-acetylglucosamine, fucose, and neuraminic acid in addition to the inner core, known as complex or *N*-acetyllactosaminic type, (c) those containing both high mannose and complex type, the hybrid type. The total number of mannose residues in high mannose type ranges from 6 to 12 and the chains are often branched. The complex type contains the disaccharide N-acetyl lactosamine (Gal β1→4GlcNAc) attached to the core (Tai, T., et al. 1977). Sialic acid residues may or may not be linked to Gal. Most hybrid molecules contain a "bisecting" N-acetyl glucosamine linked β1→4 to the β-linked mannose residue, although some exceptions exist (Hunt, L.A. 1983).

O-glycosylation

The second major type of saccharide-peptide linkage occurring in glycoproteins is the O-glycosidic linkage, which is found in mucins, blood group active glycoproteins, certain plasma glycoproteins and membrane glycoproteins. The O-linked oligosaccharides of glycoproteins are usually clustered within heavily glycosylated regions of the peptide chain. Steric interactions between carbohydrate and peptide within these clusters induce the peptide core to adopt a stiff and extended conformation and this conformational effect appears to represent a major function of O-glycosylation (Jentoft, N. 1990).

In O-glycans, carbohydrate is attached to hydroxyl groups of amino acids, serine or threonine. These include (i) mucins (Klein, A., et al. 1992) containing D-GalNAc α-1,3-linked to the hydroxyl oxygen of Ser or Thr residues by the enzyme

UDP-N-acetyl-D-galactosamine (ii) proteoglycans, acidic mucopolysaccharides joined though D-Xyl in β-1,3-linkages to L-Ser (Kjellen, L. and Lindahl, U. 1991), (iii) collagens, including D-Gal β-1,5-linked to 5-hydroxy-D-Lys, and (iv) the plant glycoproteins which include extensin and arabinogalactan proteins, (AGPs) (L-Arab to 4-hydroxy-L-Pro).

Examples of (i) are serum immunoglobulin IgA1 (Iwase, H., et al. 1999), submaxillary and bronchial mucin (Mawhinney, T.P., et al. 1992), fetuin and many others, present in plasma cell membranes and biological fluids (Maemura and Fukuda, 1994), Proteoglycans include acid mucopolysaccharides (or glycosaminoglycans, GAGs) chondroitin, keratan, and dermatan and heparin sulphates found in connective tissue, skin and blood (Fransson, L.A., et al. 1985). The proteoglycans are named so because the glycan part is by mass the dominating part (Hook, M., et al. 1984). Also, O-linked N-acetylglucosamines (GlcNAc) have been found in several cytoplasmic and nucleoplasmic glycoproteins and its importance as a regulatory signalling mechanism comparable to, and sometimes mutually exclusive with phosphorylation has been realized (Hayes and Hart, 1994). Several other types of O-linkages exist, including Man-α-O-Ser/Thr in yeast and mammalian glycoproteins and Glc β-O-Ser/Thr and Fuc α-O-Ser/Thr in blood clotting factors.

Main type O-glycans are the major constituents of mucin glycoproteins. Epithelial cells and specialized mucin-producing cells synthesize cell surface-bound and secreted mucin glycoproteins that carry a heterogeneous variety of GalNAc-Ser/Thr O-glycans comprising more than 50% of the molecular weight. These mucins have lubricating and protective functions. The expression of mucin genes as well as the mucin-type O-glycan structures are often altered in cancer and other diseases, and

the new and unusual carbohydrate and peptide epitopes can be useful for the diagnosis, prognosis, the monitoring of the disease progress, or for immunization against cancer.

Many cell adhesion molecules are mucin-like membrane bound glycoproteins that also have Ser/Thr/Pro-rich O-glycosylated domains. Secreted mucins form a viscous gel that functions as a protective layer over the epithelium and can trap particles and microbes. Many of the O-glycans represent specific receptors for bacteria. Depending on their O-glycosylation pattern, mucins control the functions, adhesiveness, and antigenicity of the cell surface and participate in the control of the immune system. Cell adhesion mediated by integrin and E-cadherin can be blocked by mucins. Mucins can block natural killer cell-mediated cell lysis and the action of cytotoxic lymphocytes (Varki, A, Kannagi, R and Toole, BP., 2009).

Depending on which saccharide groups are subsequently attached to the protein-linked GalNAc residue, mucin O-glycans are divided into four major subtypes (Schachter and Brockhausen, 1992). (i) Core 1, Gal β1-3GalNAc α -, in its unsubstituted form is the Thomsen-Friedenreich (T or TF) antigen that occurs in many cancer cells. Core 1 may be substituted by sialic acid in α 2-3 linkage to Gal and in α 2-6 linkage to GalNAc to form sialyl-T antigens (Springer, G.F. and Desai, P.R. 1982). GalNAc can also be directly substituted by sialic acid to form the sialyl Tn antigen (STn), which is not used for further elongation reactions. Both the Tn and STn occur only in a restricted number of normal glycoproteins but are often found in cancer and are associated with poor prognosis. (ii) Core 1 may be branched by a GlcNAc in β 1-6 linkage to GalNAc to form core 2. (iii) The core 3 structure is formed by the addition of GlcNAc to form GlcNAc β1-3 GalNAc- Ser/Thr and can be

substituted with sialic acid α 2-6GalNAc. (iv) The core 4 structure requires the core 3 structure as substrate and is formed by the addition of GlcNAc to form GlcNAc β 1-3 (GlcNAc β1-6) GalNAc- Ser/Thr. Other modifications to the core GalNAc structure have also been found but appear to be uncommon. Commonly the core 2 and the core 4 branches are elongated with one or multiple lactosamine structures (Gal β1→4GlcNAc) (Lowe, 2001). Of the four main core O-glycan structures, the core 1 and 2 structures are widely distributed while the core 3 and core 4 structures are less common and expression has been mostly associated with mucin producing tissue of the digestive tract.

Core 1, O-glycan or T-antigen was discovered about 70 years ago by Thomsen and Friedenreich as a laboratory curiosity. The phenomenon of panagglutinability acquired by bacterially contaminated human red blood cells led to the discovery of T-antigen (Friedenreich, V., 1930).

Biological significance of glycosylation

A given glycan can have different roles in different tissues or at different times in development (intrinsic functions) or in different environmental contexts (extrinsic functions). As a broad generalization, it can be stated that terminal sequences, unusual structures, and modifications of glycans probably mediate the more specific biological roles within the organism. However, such glycans or modifications are also more likely to be targets for pathogens and toxins. Perhaps as a consequence, intraspecies and interspecies variations in glycosylation are relatively common, and at least some of the diversity of glycans in nature may represent the signatures of past or current host-pathogen interactions.

Almost all cells carry carbohydrates on their surfaces in the form of glycoproteins, glycolipids and polysaccharides (Cook, G.M. 1986). The greatest variation in glycosylation pattern tends to be found among the outermost (non-reducing terminal) regions of glycans on cell surfaces and extracellular molecules (Lis, H. and Sharon, N. 1993). Glycans are best positioned to mediate recognition by carbohydrate-binding proteins (Baenziger, J.U. 1985) and hence they mediate many important biological roles. The biological roles of oligosaccharides appear to span the spectrum from those that are trivial, to those that are crucial for the development, growth, function or survival of an organism. These include a purely structural role, an aid in the conformation and stability of proteins, the provision of target structures for microorganisms, toxins and antibodies, the masking of such target structures, control of the half-life of proteins and cells, the modulation of protein functions, and the provision of ligands for specific binding events mediating protein targeting, cell-matrix interactions or cell-cell interactions (Varki, A. 1993). Glycoproteins rich in sugar are relatively resistant to proteolysis (Gottschalk, A. and Thomas, M.A. 1961). The protection against proteolytic degradation is attributed to steric hindrance by the carbohydrate as well as the more stable conformation adorned by the glycosylated protein.

Another well-accepted function of oligosaccharide units of glycoproteins is that they are involved in the initiation of the correct polypeptide folding in the rough endoplasmic reticulum (ER), and subsequent maintenance of protein solubility and conformation. Thus, many proteins that are incorrectly glycosylated fail to fold properly and/or fail to exit the ER, and are being consigned instead to degradation in proteasomes (Varki, A. 1993).

Many enzymes are glycosylated proteins; though deglycosylation in most cases does not lead to the loss of enzyme activity. When the hormone human β-chorionic gonadotropin (β-HCG) is deglycosylated, it still binds to its receptor with similar affinity, but fails to transmit a signal through stimulation of adenylate cyclase (Bahl, O.P., et al. 1974).

Since all cells are covered with a dense coating of sugars, it has long been predicted that oligosaccharides must be critical determinants of 'cell-cell interactions'. O-glycans are critically involved in the regulation of cell adhesion and are receptors for mammalian lectins. Perhaps the best-documented example is that of the selectin family of receptor proteins that mediate the adhesion of leukocytes to endothelial cells (L-selectin), the recognition of leukocytes by stimulated or wounded endothelium (E-selectin), and the interactions of activated platelets or endothelium with leukocytes (P-selectin. The E-selectin binding to O-glycans play an important role in the binding of cancer cells to the endothelium.

The mammalian egg coat (the zona pellucida) contains a large number of O-glycans, as well as some N-glycans. Removal of egg N- glycans by glycosidase treatment does not destroy sperm binding, but loss of O-glycans following mild alkali treatment ablates sperm binding (Bleil, J.D. and Wassarman, P.M. 1988, Florman, H.M. and Wassarman, P.M. 1985).

The glycosylation of a protein is not directly specified in the genome but is determined by levels of glycosyltransferase expression, accessibility of glycan attachment sites, concentrations of nucleotide sugar donors and other environmental factors. Though variable, the glycosylation of a protein is often characteristic of the cell type where it was synthesised. The carbohydrates of glycoproteins modify the

physicochemical properties of proteins by changing their hydrophobicity, electrical charge, mass and size. Proteoglycans and the collagens are important in the physical maintenance of tissue structure, integrity and porosity.

The attachment of sugars to proteins is known to increase the solubility of the latter. Sialic acids in salivary glycoproteins are responsible for the high viscosity of the mucous solutions (Gottschalk, A. and Thomas, M.A. 1961). Negatively charged acylneuraminic acid residues impart physical strength to cell membranes because of their mutual repulsion and influence the mutual adhesion of cells in organ structure. The antifreeze glycoprotein of Antarctic fish depends on the integrity of the disaccharide Gal β (l-3) GalNAc units for their activity. The ability of these glycoproteins to depress the freezing point of water is lost on removal or modification of the saccharide side chains (Vandenheede, J.R., et al. 1972).

The classical work of Ashwell and his co-workers have demonstrated that removal of sialic acid from circulating glycoproteins leads to a dramatic enhancement in the rate of glycoprotein clearance from the circulatory system (Ashwell, G. and Morell, A.G. 1974). Carbohydrates also serve as important recognition markers. Cell surface glycoproteins are the immunodeterminant structures of blood group A, B, H and M/N specificities (Watkins, W.M. and Morgan, W.T. 1952).

They are also receptors for many plant and bacterial toxins, and serve as antigens for autoimmune and alloimmune reactions. In most of these instances, there is exquisite specificity for the sequence of the oligosaccharide involved. Thus, for example, the influenza viral haemagglutinins specifically recognize the type of sialic acid, its modifications and its linkage to the underlying sugar chain. Certain

oligosaccharides can act as highly specific receptors for a variety of viruses, bacteria and parasites.

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

The extracellular matrix consists of a variety of glycoconjugates, each of which has been shown to have binding sites for various types of sugar chains, e.g. the heparin-binding domains of fibronectin and collagen. Such oligosaccharide-binding domains has role in the organization of the matrix.

Oligosaccharide sequences on soluble glycoconjugates such as the mucins can also act as 'decoys' for microorganisms and parasites. Thus, pathogenic organisms attempting to gain access to mucosal membranes might first encounter their cognate oligosaccharide ligands attached to soluble mucins. Upon binding to these sequences, they could then be swept away by ciliary action, leaving the mucosal cells untouched. In these cases, the host may successfully turn the specificity of the pathogen receptor to its own advantage.

Glycosylation pattern of Immunoglobulin A1

The oligosaccharides attached to the immunoglobulins are large (≈2kDa each) and, when not constrained by their location, are flexible, particularly around the N-glycosidic linkage. The glycans are multifunctional, playing crucial structural roles and participating in binding events, for example to serum lectins such as Mannan Binding Lectin (MBL) (Malhotra, R., et al. 1995). Other important roles for immunoglobulin glycans include maintaining solubility and conformation (Mimura, Y., et al. 2000); facilitating subcellular transport, secretion and clearance ; (Gala, F.A. and Morrison, S.L. 2002) and maintaining effector functions by ensuring optimal binding of the Fc to Fc receptors (Mimura, Y., et al. 2001). Although the five classes of human immunoglobulins differ greatly in their content of carbohydrate and in its distribution along the polypeptide chain, the carbohydrate often is in homologous positions and usually seems to lie in between the compact domains of the heavy chain or on the surface of a domain. The location and nature of the five oligosaccharides of the μ chain of human IgM has been reported (Putnam, F.W., et al. 1973, Shimizu, A., et al. 1971). In contrast, the γ chain of human IgG has a single oligosaccharide (Edelman, G.M., et al. 1969), and the ε chain of human IgE is reported to have six (von Bahr-Lindstrom, H. and Bennich, H. 1974). In all these cases the oligosaccharide contains glucosamine (GlcN), which is attached to asparagine by an N-glycosidic linkage. The IgA1 subclass is unusual among glycoproteins in having two types of linkage to the polypeptide chain, the N-glycosidic linkage of GlcN to asparagine and an O-glycosidic linkage of galactosamine (GalN) to serine (Baenziger, J. and Kornfeld, S. 1974, Baenziger, J. and Kornfeld, S. 1974). IgA1 is relatively heavily glycosylated (8% carbohydrate).

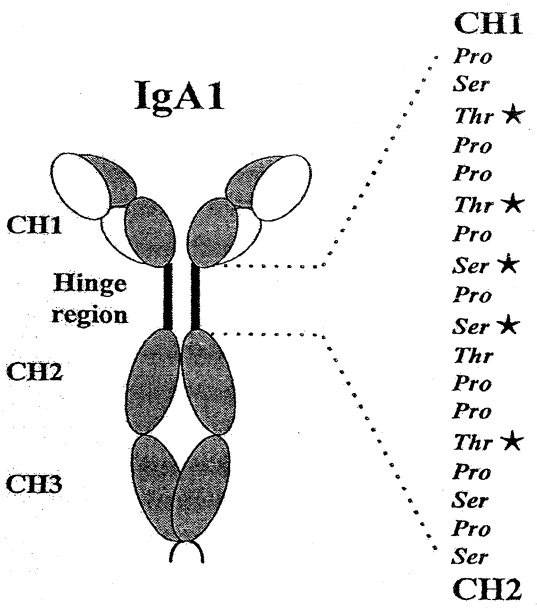

Figure 1. Position of IgA1 hinge region O-glycosylation sites. On the left is the human IgA1 molecule, showing the location of the hinge region between the CH1 and CH2 domains. On the right is the amino acid sequence of the hinge region. All of the serine and threonine residues are potential O-glycosylation sites, but in a recent study, only those marked were found to be occupied.

There are a variable number of N-linked glycans on IgA depending on both the isotype and allotype. All IgAs contain an N-linked carbohydrate at position 263 in C_H2 and at position 459 in the tail-piece extension of C_H3. All IgA2s contain additional glycans at position 166 in C_H1 and 337 in C_H2. IgA2m(2) and IgA2(n) contain a fifth glycan at position 211 in C_H1 (Yoo, E.M. and Morrison, S.L. 2005). Analysis of pooled monomeric serum IgA1 showed that over 80% of the N-glycan biantennary complex oligosaccharides to which sialic acids were attached was exclusively in the α (2–6) linkage. In addition, approximately 13.6% of tri- and tetra antennary structures were reported (Baenziger, J. and Kornfeld, S. 1974).

Serum IgA consists mainly of IgA1 and circulates at around 1mg/ml, and although it is predominantly monomeric, it also forms dimers and higher polymers. The glycosylation of IgA has been widely reported. The galactose-terminating

glycans are ligands for the asialoglycoprotein receptor (ASGP-R), which mediates the clearance of IgA from the serum (Basset, C., et al. 1999, Stockert, R.J. 1995).

IgA1 contains a proline-rich hinge sequence (23 aminoacid sequence) between the Fab and Fc regions of the glycoprotein (Figure 1). Within this sequence, there are nine potential *O*-glycosylation sites/α-chain (18 sites/molecule) of which five residues (Thr-228, Ser-230, Ser-232, Thr-225, and Thr-236) (Baenziger, J. and Kornfeld, S. 1974) are fully or partially occupied. A sixth sugar at Ser-224, Thr-233, Ser-238 or Ser-240 is present in 5-10% of the glycoforms (Tarelli, E., et al. 2004). There is no well-defined motif for the acceptor site other than the proximity of Proline residues and a single enzyme, UDP-N-acetyl-α-D-galactosamine: polypeptide N-acetyl galactosaminyl transferase 2, appears to be responsible for transferring the initial N-acetylgalactosamine (GalNAc) to all of the different sites within the hinge peptide (Iwasaki, H., et al. 2003). The sugars protect the hinge from protease digestion, allowing it to adopt several conformations depending on the structures of sugars. The O-glycans within the hinge of IgA1 assume many different structures (Fig.2), with the most abundant being monosialylated T antigen (NeuAcα2-3Galβ1-3GalNAc). However, larger neutral and sialylated tetrasaccharide structures (Galβ1-4GlcNAcβ1-6Galβ1-3GalNAc) were also present (Mattu, T.S., et al. 1998).

The four major *O*-glycan forms of human IgA1.

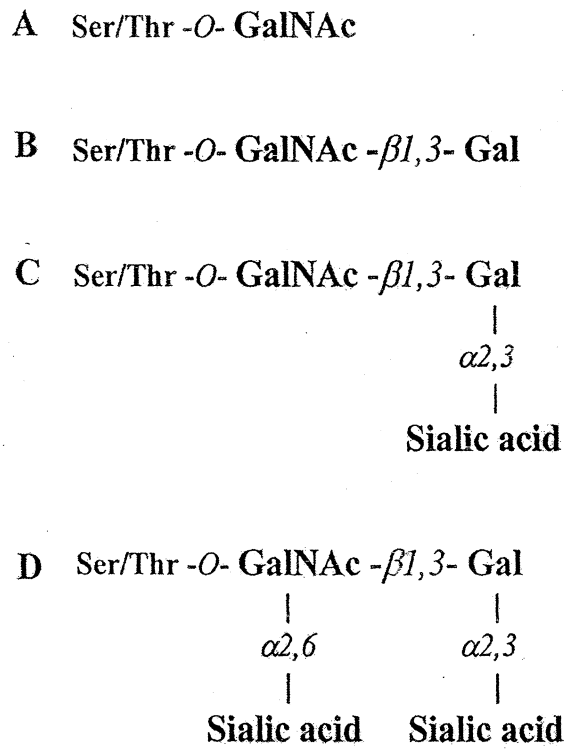

Fig.2 The IgA1 *O*-glycans are all based on a core *N*-acetyl galactosamine (GalNAc) unit in *O*-linkage with serine or threonine. This may occur alone (form A) or may be extended with β1,3-linked Gal (form B). Further extension with sialic acid in α 2,3 linkage with Gal can then occur (form C), and finally, addition of sialic acid in α 2,6-linkage with GalNAc makes form D.

O-glycosidically linked oligosaccharide units are characteristic of the IgA1 subtype and are not found in the IgA2 subtype due to a deletion of the regions in which they occur (Frangione, B. and Wolfenstein-Todel, C. 1972). The O-linked sugars isolated from colostrum secretory IgA (sIgA) appear to be more complex and heterogeneous, containing sialic acid, fucose and N-acetylglucosamine (Pierce-Cretel, A., et al. 1984).

Aberrant glycosylation has been linked to disease in which there is reduced clearance of IgA. The asialoglycoprotein receptor removes IgA from serum by binding to terminal galactose residues on the glycans (Stockert, R.J. 1995). In IgA

nephropathy circulating IgA1 gets deposited in the glomeruli of the kidney mesangium leading to renal failure. Aberrant O-glycosylation of serum IgA1 in patients is ascribed to a decrease in terminal galactosylation and sialylation. Reduced levels of terminal galactose lead to diminished clearance by the liver ASGP-R of both IgA and serum immune complexes containing IgA1, thus promoting their deposition in the kidney. In addition several possible roles have been proposed for the carbohydrate in immunoglobulins, but none has been established. It has been suggested (Marshall, R.D. 1972) that carbohydrates increase solubility, facilitate secretion, and act as spacers between the domains.

Whereas the role of secretory IgA is established in mucosal immunology, the function of serum IgA antibodies is mostly unknown. Studies on the ability of IgA antibodies to regulate humoral response are scarce. IgA was shown in only one report to enhance the induction of immunological memory to soluble antigen (Klaus, G.G. 1979). The specificity of serum IgA in the human antibody repertoire and IgA antigen selection remain poorly defined. Serum IgA is considered a "discrete housekeeper" because IgA immune complexes can be removed by the phagocytic system with little or no resulting inflammation. Moreover, monomeric serum IgA displays anti-inflammatory activity and is capable of inhibiting functions such as IgG-induced phagocytosis, bactericidal activity, oxidative burst, and cytokine release. IgA deficiency is associated with allergy and autoimmunity. Polymeric IgA and IgA-containing immune complexes, in contrast, can efficiently trigger immune effector functions on blood leukocytes through IgA Fc receptors.

Most intracellular IgA occurs in monomeric form even in cells that secrete predominantly polymeric IgA (Bargellesi, A., et al. 1972, Kutteh, W.H., et al. 1982, Kutteh, W.H., et al. 1982, Parkhouse, R.M., et al. 1971). In view of these findings, it was proposed that polymerization occurs shortly before or at the time of secretion of the assembled IgA from the cells. These studies have indicated that IgA-producing cells in bone marrow secrete predominantly monomers, while in the gut both forms are produced, with a marked predominance of pIgA. In other tissues, such as lymph nodes or spleen, IgA is present in both forms in variable proportions (Kutteh, W.H., et al. 1982).

Galectins

Galectins, formerly known as S-type lectins or galaptins are a phylogenetically conserved family of lectins defined in 1994 as a shared consensus of amino-acid sequences of about 130 amino acids and the carbohydrate recognition domain (CRD) responsible for β-galactoside binding (Barondes, S.H., et al. 1994, Caron, M., et al. 1990, Gabius, H.J. 1994, Hirabayashi, J. and Kasai, K. 1993, Hirabayashi, J., et al. 1996). Although initially described in vertebrate taxa, their presence has since been documented in prochordates, invertebrates, and fungi (Cooper, D.N., et al. 1997, Hirabayashi, J., et al. 1996, Pfeifer, K., et al. 1993).

Historical background

First galectin was originally described in 1975 during studies on the possible presence of lectins in the electric organs of the electric eel (Teichberg, V.I., et al. 1975). The protein, termed electrolectin, had hemagglutinating activity on trypsinized rabbit erythrocytes that was inhibitable by β-galactosides and could be isolated by affinity chromatography on β-galactoside supports. Notably, this protein required the inclusion of 2-mercaptoethanol (sulfhydryl-containing compounds) in isolation buffers to maintain its activity, suggesting the presence of one or more free cysteine residues.

The first mammalian galectin was purified from calf heart and lung (de Waard, A., et al. 1976). This was followed by purification of this lectin from chick muscle (Den, H. and Malinzak, D.A. 1977, Nowak, T.P. and Barondes, S.H. 1977).

Like electrolectin, those from other sources were dimers with subunit molecular weights of about 15,000; and in each case, activity was only retained in buffers with sulfhydryl-containing compounds like 2ME or dithiothreitol for maintenance of their hemagglutinating activity (Barondes, S.H. 1984). Evidence on other members of the family came from studies on chick intestine (Beyer, E.C., et al. 1980), mouse 3T3 fibroblasts (Roff, C.F. and Wang, J.L. 1983), human fibroblasts (Roff, C.F., et al. 1983) etc. Their biochemical properties and histological distributions were studied in relation to development and differentiation.

These lectins were isolated by affinity chromatography on asialofetuin-Sepharose column using lactose as eluant. Kurt Drickamer named them "S-type lectins" to denote their sulfhydryl dependency (Drickamer, K. 1988). All of these proteins demonstrated hemagglutinin activity. Trypsinized rabbit erythrocytes, which display more terminal galactose residues than human erythrocytes, are readily agglutinated by most of these lectins, whereas human erythrocytes require treatment with neuraminidase to enhance their agglutinability.

Later it became clear that all members of this family are not "sulfhydryl-dependent" in activity. Electrolectin, unlike most other β-galactoside lectins, does not contain cysteine residues, but its key tryptophan residue in the binding site of its CRD can be oxidized to form an oxindole derivative, causing loss of activity (Levi, G. and Teichberg, V.I. 1981).

Alkylation of galaptin (present galectin-1) with iodoacetamide yields carboxamidomethyl-galaptin, which is fully active and stable to atmospheric oxygen in the absence of disulphide-reducing reagents (Whitney, P.L., et al. 1986). IgE Binding protein (present galectin-3) does not require a thiol-reducing reagent for

maintenance of activity (Frigeri, L.G., et al. 1990). Finally, Hirabayashi and Kasai, proved no cysteine residue is required for the sugar binding function by means of site directed mutagenesis, substituting one of the six Cys with Ser with no change in sugar binding activity (Hirabayashi, J. and Kasai, K. 1991).

The nomenclature for galectins was systematized in 1994 (Barondes, S.H., et al. 1994) in the joint statement published in *Cell* and the term S-type lectins was abandoned in favour of the general term 'galectin'. The membership in galectin family required fulfilment of two criteria: "affinity for β-galactosides and significant sequence similarity in the carbohydrate-binding site, the relevant amino acid residues of which have been determined by X-ray crystallography".

The first galectin found, a 14kDa homodimer (variously termed electrolectin, β-galactoside-binding lectin, galaptin, L-14, etc., depending on its source) was renamed galectin-1. Its nearest homolog was termed galectin-2. CBP35, CBH30, Epsilon Binding Protein, Mac-2, L-29, and L-34 were termed galectin-3, and other members of this family were numbered consecutively by order of discovery.

Classification and occurrence of galectins

The carbohydrate-recognition domain (CRD) of most galectins takes approximately 130 amino acids, and this is indicated by the oval domain. A total of 15 galectins have now been found in mammals, but only 12 galectin genes are found in humans, including two for galectin-9. Galectins have been classified into three groups according to their structure: prototypical, chimeric, and tandem repeat (Hirabayashi, J. and Kasai, K. 1993).

On the basis of sequence homologies, two general subgroups of galectins can be distinguished: the galectin-1 subgroup, which includes galectin-1 and galectin-2, and the galectin-3 subgroup, which includes all others.

Many of the galectin transcripts may be differentially spliced to generate many different isoforms. For example, at least seven different mRNAs have been identified for human galectin-8, some encoding a tandem-repeat form and others a prototypical form. Galectin-5 (prototypical) and galectin-6 (tandem-repeat) are found in rodents, but not humans, and galectin-11 (ovagal11; prototypical) has been reported in sheep. Confusingly, the GRIFIN (Galectin related inter-fibre protein), which in mammals is expressed in the eye lens and may not bind to carbohydrates, has also been termed galectin-11 due to its high sequence similarity to galectins (Ogden, A.T., et al. 1998).

There are also a number of galectin-related proteins that have homology with galectins, but they may not bind carbohydrates, or at least not bind to typical β-galactosides. For example, galectin-10 expressed in eosinophil granules in a crystalline form, known as the Charcot–Leyden crystal protein appears to bind better to β-mannosides than to β-galactosides. Another galectin-related protein (GRP) is HSPC159, which is expressed in human haematopoietic stem cell precursors, but lacks a critical tryptophan residue that is highly conserved in all other galectins; thus, it may be unable to bind β-galactosides. Subunits of legume lectins (Sharon, N. 1993) and serum amyloid P component (a pentraxin with lectin activity) (Emsley, J., et al. 1994) have the same topology and very similar tertiary structure as the galectin carbohydrate-binding domains have but show no sequence homology. This suggests

that galectins are a subset of a larger group of proteins sharing conserved folding motifs.

Galectins are found in virtually all organisms. Galectin is reported in sponges, birds, amphibians, fish etc. Galectin-like proteins are even expressed in some viruses that infect pigs and fish, including porcine adenovirus and lymphocystis disease virus. Galectins are also expressed in *Drosophila melanogaster* and *Caenorhabditis elegans* (26 candidate genes). Galectin-like sequences are predicted from the plant genome (*Arabidopsis thaliana).*

Galectin-1 is expressed in many tissues, predominantly of mesodermal origin, such as skeletal and smooth muscle, liver, lung, heart, skin, spleen, placenta, intestine, and kidneys. In addition, expression is also found on central and peripheral nervous tissue. During the development, galectin-1 is mainly restricted to the brain tissue and olfactory system, whereas with maturation it is extended to the peripheral nervous tissues. Galectin-2 is expressed in hepatomas. Galectin-3 is abundant in various epithelial cells and in macrophages; galectin-4 is confined to the epithelial cells of the alimentary tract and galectin-7 is found in epidermis (Colnot, C., et al. 1996, Cooper, D.N. and Barondes, S.H. 1999, Gitt, M.A., et al. 1998, Timmons, P.M., et al. 1999). Galectin-3 surface expression has been shown in normal human monocytes and its level increases as monocytes differentiate into macrophages (Liu, F.T., et al. 1995). Galectin expression can also be induced by various stimuli. From several studies it is apparent that each cell might express most of galectins; yet, during development or in various differentiation stages or under different physiological or pathological conditions, one or more galectins are preferentially expressed in each cell type. This

implies a fine control of gene expression and suggests that such control should be coordinated (Chiariotti, L., et al. 1999). For example, inflammatory mediators modulate galectin-3 (Cherayil, B.J., et al. 1989, Liu, F.T. 1993, Liu, F.T., et al. 1995) and galectin-9 that acts as a potent chemoattractant selectively for eosinophils is induced by allergic stimulation in monocytes (Matsumoto, R., et al. 1998). Galectin expression can also be induced by cell developmental dynamics and amounts of various binding partners such as glycans.The evolutionary conservation of galectins likely reflects the essential roles of galectins in development and function of multicellular organisms including cell adhesion, migration, differentiation and death (Perillo, N.L., et al. 1998).

Structure of galectin

The general designation of the genes encoding galectins is LGALS (lectin, galactoside binding, soluble) and gene numbering is being kept consistent with the numbering of the proteins so that LGALS1 encodes galectin-1 and so on. There is likely to be only one gene for the soluble 14kDa galactoside binding lectin in each species (Abbott, W.M. and Feizi, T. 1989). In humans LGALS1 and LGALS2 have been mapped to the q12-q13 region of the chromosome 22 (Mehrabian, M., et al. 1993). In the mammalian galectin genes, the CRDs are encoded by three consecutive exons (Barondes, S.H., et al. 1994, Gitt, M.A. and Barondes, S.H. 1991). The portion of the core sequence which represents the carbohydrate recognition domain (CRD) is contained between about residues 30 and 90, a segment generally encoded by a single exon in the middle (Barondes, S.H., et al. 1994, Lobsanov, Y.D., et al. 1993).

Immunochemical studies with conventional antisera and amino acid analysis have suggested that the β -galactoside binding lectins among phylogenetically related species are antigenically and structurally related (Childs, R.A. and Feizi, T. 1979, Levi, G. and Teichberg, V.I. 1982). The molecular properties of vertebrate galactose-binding lectins are strikingly similar from chicken to cow to man. The amino acid identity in the carbohydrate-binding domains among differrent known galectins from mammalian species ranges from about 20 to 40% (Oda, Y., et al. 1993). The amino acid sequence homology of the same galectin from different mammalian species is 80-90%.

Comparison of their amino acid sequences and mutagenesis studies have suggested the functional importance of some conservative hydrophilic residues (His44, Asn46, Arg48, Glu71 and Arg73 of human 14 kDa lectin). Several non-charged residues (Gly14, Phe45, Pro47, Phe49, Val59, Trp68, Pro78 and Phe79) are also well conserved, and are probably important to maintain the structural framework of these proteins. A consideration of molecular evolution suggests that lectins belonging to this family probably existed in the Precambrian era. Ubiquitous occurrence of these homologous lectins with shared sugar specificity suggests that they are involved in 'essential minimum' functions of multicellular animals, possibly in cooperation with their partner glycoconjugates (Hirabayashi, J. and Kasai, K. 1993).

X-ray crystallography studies show that the CRD of galectin subunits is composed of five (F1-F5) and six (S1-S6)-stranded antiparallel β-sheets arranged in a β-sandwich or jelly-roll configuration that completely lacks α-helix. The conserved carbohydrate-binding aminoacids are in strands S4-S6 (Loris, R. 2002, Rini, J.M. and

Lobsanov, Y.D. 1999). The monomer folds of all CRDs are remarkably similar between/among all galectins. In the dimeric proteins, such as galectin-1, -2, and -7, the subunits are related by a twofold rotational axis perpendicular to the plane of the β-sheets. The glycan-binding sites in the CRD are located at opposite ends of the dimer. The compactly arranged structure of the CRD partly explains the protease resistance of the galectin CRD.

Unlike other galectins, galectin-1 contains six cysteine residues and displays carbohydrate binding activity only when the three intramolecular disulfide bonds (Cys2- Cys130, Cys16-Cys88, and Cys42-Cys60) are reduced (Hirabayashi, J. and Kasai, K. 1991, Tracey, B.M., et al. 1992). This suggests that disulphide bond formation somehow distorts aspects of the galectin-1 monomer fold that are necessary for carbohydrate binding. Human Gal-1 exists as a dimer in solution. The integrity of this dimer is maintained principally by interactions between the monomers at the interface and through the well-conserved hydrophobic core, a factor which explains the observed stability of the dimer in molecular terms (Lopez-Lucendo, M.F., et al. 2004). Nevertheless, one of the main characteristics of the homodimeric galectin-1 protein is that it spontaneously dissociates at low concentrations (Kd ~ 7 μm) into a monomeric form that is still able to bind to carbohydrates (Cho, M. and Cummings, R.D. 1995), but with a lower level of affinity (Leppanen, A., et al. 2005). Galectin-1 may, hence be dimeric and cross-link ligands if present at a high enough concentration, which is well within the range found in nature. But it may also act as a monomer at lower concentrations as suggested (Blaser, C., et al. 1998). Galectin-1 can also exist in an oxidized form, that is, a form that lacks lectin activity (Outenreath, R.L. and Jones, A.L. 1992).

Biosynthesis and export of galectins

The mRNA for the protein is translated on free polysomes in the cytoplasm. They also hold many features of cytoplasmic proteins i.e., have no disulphide bridges, no sugar chains or typical transmembrane segments and possess acetylated N-termini. Newly synthesized galectins isolated directly from the cytoplasm of cells are functional in binding β-galactosides, indicating that they are potentially functional in that compartment. There is compelling evidence for galectins having non-carbohydrate-binding partners in the cytoplasm. Galectins are probably unique among all types of animal lectins in that they can be found in the nucleus, cytoplasm, outer plasma membrane, and extracellular matrix (Barondes, S.H. 1984, Zanetta, J.P., et al. 1984).

The nascent peptide chains of galectins do not contain a signal peptide sequence or a hydrophobic peptide cluster required for entry into the classical secretory pathway (Cleves, A.E., et al. 1996). While many types of cells involved in immunity store galectins in their cytosol without exporting them, some of those cells start secreting galectins (without compromising membrane integrity) when differentiated or activated by receptor engagement or cytokines (Rubartelli, A., et al. 1992). This secretion occurs through a "leaderless" secretory pathway (non classical export), which is also used by fibroblast growth factors 1 and 2, and IL-1β. Galectin-1 is secreted by activated B cells, activated but not quiescent T cells,CD4+ CD25+ regulatory T cells, activated macrophages, and certain epithelial cells (Baum, L.G., et al. 1995, Blaser, C., et al. 1998, Garin, M.I., et al. 2007, Rabinovich, G.A., et al. 1998, Zuniga, E., et al. 2001).

Whether different transport membrane proteins occur in animal cells for galectin export is unknown. An alternative pathway for secretion being considered is membrane blebbing involving membranous structures (Mehul, B. and Hughes, R.C. 1997). When galectin-1 secretion starts during muscle cell development, it is first accumulated underneath the plasma membrane, then in small membrane evaginations, the blebs, which are about 2μm in diameter. Galectin-1 appears to exit from myoblasts via blebs, which pinch off to form lectin-enriched vesicles (Cooper, D.N. and Barondes, S.H. 1990). Similarly, galectin-3 assembles into patches that eventually appear to underlie the plasma membrane as a prelude to deposition in the extracellular space, possibly through vesicular extravasation. A unique N-terminal domain of 11 amino acids in galectin-3 acts as a determinant for its secretion (Menon, R.P. and Hughes, R.C. 1999).

Functional relationships of galectins

Extracellular functions

Extracellular functions of galectins depends on their binding to and cross-linking of various glycan groups of glycoproteins and/or glycolipids on the surface of various cell types (Brewer, C.F., et al. 2002). The minimal saccharide unit recognised by galectins is the galactose residue. Though initially called β-galactoside specific due to its high affinity for lactose, rigorous examination of anomer specificity has revealed that galectin-1 recognizes α-linked galactose moieties better than β-anomers (Appukuttan, P.S. 2002). Thomsen-Friedenreich antigen with or without sialic acid cover (T antigen) has also been found to be a potent ligand for galectin-1 (Sangeetha, S.R. and Appukuttan, P.S. 2005). In this respect galectin-1 resembles jacalin

(Sureshkumar, 1992) and peanut agglutinin (Chacko, B.K. and Appukuttan, P.S. 2001), both of which prefer α- to β- anomer of galactose and accommodate T antigen with high affinity. Thus among serum IgA (Sangeetha, S.R. and Appukuttan, P.S. 2005) and lipoproteins (Chellan, B., et al. 2007) , T-antigen containing O-linked oligosaccharides rather than N-acetyl lactosamine-containing N-linked oligosaccharides were recognized by galectin-1.

Binding studies using purified or synthetic oligosaccharides indicate that galectin-1 has relatively high affinity for core 2 *O*-glycans compared with core 1 *O*-glycans; solution binding assays demonstrated that galectin-1 affinity for core 1 *O*-glycans was 125-fold lower than for Gal β 1,4 GlcNAc sequences in core 2 branches (Leffler, H. and Barondes, S.H. 1986, Leppanen, A., et al. 2005). Galectin-1 can bind to a glycoprotein receptor, CD43, bearing only core 1 *O*-glycans and lacking lactosamine sequences; in this case, low affinity/high avidity binding to a highly abundant but less preferred glycan ligand T-antigen structure is sufficient to induce T cell death (Hernandez, J.D., et al. 2006).

In short, the binding sites of galectins may be viewed as containing several subsites: one for galactose, another for *N*-acetylglucosamine and still other sub-sites that may be filled by other sugars and the aglycone moiety, such as a peptide or lipid.

Co-crystallization of galectins with simple β-galactose-containing disaccharides has revealed that the galactose residue is most tightly bound and interacts with the protein along one side through hydrogen bonds involving C-4 and C-6 hydroxyls, the ring O, and van der Waals interaction between the hydrophobic patch formed by CH 3-5 and a Trp or Tyr in the protein (Camby, I., et al. 2006). The glucose residue also interacts, mainly via OH3 and is significant, since to most

galectins lactose binds with about 100 fold higher affinities (K_d 0.5 mm) than galactose alone (Leffler, H. and Barondes, S.H. 1986). However there is some room for variation of the Glc residue. If the C-2 hydroxyl of the latter is replaced by acetamido group to form GlcNAc, the affinity goes up by a factor of about ten for some galectins but not others, due to interactions involving the NAc group. Gal bound β 1→3 to GlcNAc also binds well because here OH4 of GlcNAc takes the steric place of OH3 of GlcNAc in LacNAc (Gal β 1, 4 GlcNAc) (Loris, R. 2002). The affinity of each galectin member to its potential ligands varies depending on substitutions in this core β-galactoside, which results in subtle yet significant differences in the CRD. For example, α-N-acetylgalactosamine modification of Gal residues significantly decreases the affinity of galectin-1 for this residue, but increases the affinity of galectin-3 (Hirabayashi, J., et al. 2002). Alternatively, modification with α-2,6 linked sialic acid, but not α -2,3 linked sialic acid, reduces the affinity mainly of galectin-1, as well as of galectin-3 (Amano, M., et al. 2003, Barondes, S.H., et al. 1994, Hirabayashi, J., et al. 2002, Sato, M., et al. 1992, Sparrow, C.P., et al. 1987).

The binding of galectins to individual lactosamine units is characterized by relatively low levels of affinity ($K_d \sim 50$ μm) (Ahmad, N., et al. 2004). In contrast, galectin binding to natural glycoconjugate ligands expressed on cell surfaces or in the extracellular matrix is usually of much higher affinity (i.e., in the μM or sub mM range). It is the arrangement of lactosamine disaccharides in multiantennary repeating chains (up to three branches) that increases the binding avidity ($K_d \sim 4$ μm) and in contrast, there is no increase in avidity when the recognition unit is repeated in a string (poly-N-lactosamine) (Ahmad, N., et al. 2004). In polysaccharides, galectin-1 does not bind glycans that lack a terminal non-reducing unmodified N-

acetyllactosamine (Stowell, S.R., et al. 2004). At the same time, human galectin-1 shows a preference to polyacetyllactosamine with terminal Gal β-GlcNAc.

While Th1 and Th17 cells express the repertoire of cell-surface glycans (poly-N-acetyllactosamine units), which are critical for galectin-1 binding and cell death Th2 cells are protected from galectin-1 binding through α 2, 6 sialylation of cell surface glycoproteins (Toscano, M.A., et al. 2007). This results in galectin-1 dependent skewing of T cells toward a Th2 cytokine profile.

The crystal structure of bovine galectin-1 was derived for the protein in complex with a bi-antennary N-glycan containing two terminal β-galactose residues. In this extended crystal structure, the N-glycan is bridged between two galectin dimers, thus effectively creating a crystal latticework. This type of crystal latticework may be unique among galectins in regard to vertebrate galectins and may be critical for their signalling and adhesive functions.

The affinity of galectins' CRD toward their glycan ligands is often lower ($\approx 10^{-6}$ M) than those observed in typical protein-protein interactions ($\approx 10^{-8}$ M) (Hirabayashi, J., et al. 2002). Despite a weak affinity of their CRD, galectins achieve a stable interaction with their ligands through their multivalency, as binding to multiple ligand leads to increases in their avidity (Ahmad, N., et al. 2004, Brewer, C.F., et al. 2002). Soluble monomeric galectin-1 also binds weakly to lactosamine residues compared to dimeric galectin-1 (Leppanen, A., et al. 2005). Importantly, the affinity for galectin ligands is similar and independent of the number of CRDs in the molecule when the galectins are immobilized to a solid surface (Hirabayashi, J., et al. 2002).

Considerable diversity was observed for individual galectins in binding specificity in terms of (1) branching of N-glycans, (2) repeating of N-acetyllactosamine units, or (3) substitutions at 2-OH or 3-OH groups of nonreducing terminal Gal. Although most galectins showed moderately enhanced affinity for branched N-glycans or repeated N-acetyllactosamine, some of them had extremely enhanced affinity for either of these multivalent glycans. Some galectins also showed particular preference for alpha1-2Fuc-, alpha1-3Gal-, alpha1-3GalNAc-, or alpha2-3NeuAc-modified glycans. To summarize, galectins have evolved their sugar-binding specificity by enhancing affinity to either "branched", "repeated", or "substituted" glycans, while conserving their ability to recognize basic disaccharide units, Gal beta 1-3/4GlcNAc (Hirabayashi, J., et al. 2002).

Complex glycans found on the surface of cells may infact interact with galectin aminoacid residues and their formed surface patches are not within, or are more distant from, the "lactose disaccharide defined" carbohydrate- binding domain. Moreover, differences in self-association profiles, organisation of different galectins on the same glycoconjugates, and the possibility of galectin heterooligomerisation (Miyanishi, N., et al. 2007) could all contribute to functional differentiation.

The functional importance of the galectin-1 oligomeric state has been more clearly demonstrated by Nishioka T et al., who reported a naturally occurring form of galectin-1 (galectin -1β) that lacks the first 6 N-terminal residues (Nishioka, T., et al. 2002). Galectins form "cross-linked" complexes with specific glycoproteins. For example, galectin-1 forms 3D complexes with asialofetuin, a 48kDa monomeric glycoprotein possessing three triantennary N-linked complex carbohydrates with

terminal galactose residues (Gupta, D., et al. 1996, Mandal, D.K. and Brewer, C.F. 1992).

Galectins appear to bind selectively to some cell-surface and extracellular matrix ligands. However, the precise physiological roles of these interactions with each galectin are not well understood. Potential ligands for galectin-1 and galectin-3 include basement membrane proteins (such as laminin and fibronectin), membrane receptors (such as integrins α7β1 and α1β1, CD43, CD7, and CD45), lysosome-associated membrane proteins (LAMP-1 and LAMP-2), vitronectin, and fibronectin. Galectin-1 binding to T-cell surface glycoproteins result in a striking redistribution of these glycoproteins into segregated membrane microdomains on the cell surface, which apparently triggers apoptosis (Pace, K.E., et al. 1999). CD3 and CD45 co-clustered as did CD7 and CD43, but these assemblies were segregated from each other. CD7 is essential for galectin-1 induced cell death, while CD45 regulates the process in a fashion that is dependent on its glycosylation state (Hernandez, J.D. and Baum, L.G. 2002).

Intracellular functions

The intra-cellular functions of galectins likely involve interactions with intracellular proteins, and they could be lectin-carbohydrate or lectin-peptide interactions (Liu, F.T. 2002). The intracellular functions of galectins have been suggested by the finding that both galectin-1 and -3 are a required factor in the splicing of nuclear pre-mRNA in a cell-free system (Vyakarnam, A., et al. 1998).

Protein ligands for galectins include Gemin4, oncogenic H-Ras, OCA-B, pre-B cell receptor (human, not murine system).

Biological roles of galectins

Regulation of the immune system

Galectins in general and galectin-1 in particular, are known to be deeply involved in the initiation, amplification, and resolution of inflammatory responses. Galectins are expressed by activated T and B cells, regulatory T cells, dendritic cells, mast cells, eosinophils, monocytes/macrophages, and neutrophils. Immune cell responses to galectins also depend on the specific glycosylation of surface glycoproteins in those cells to generate galectin ligands. Since the majority of the members of the galectin family are multivalent in glycan binding, they can cross-link specific ligands to induce various activities such as cell-cell or cell-pathogen interactions, signal transduction through receptor clustering , or the formation of a multivalent galectin– glycoprotein lattice on the cell surface (Nieminen, J., et al. 2007, Sato, M., et al. 2002, Sato, S. and Nieminen, J. 2004).

T-cell homeostasis and survival

Galectin-1 has been documented to negatively regulate cell growth and apoptosis of activated but not resting T-cells, suggesting the potential role of this protein in restoring T-cell homeostasis by apoptosis (Blaser, C., et al. 1998, Goldstone, S.D. and Lavin, M.F. 1991, He, J. and Baum, L.G. 2004, Matarrese, P., et al. 2005, Perillo, N.L., et al. 1995, Rabinovich, G.A., et al. 1998). Several galectins (including galectin-1, -2, -3, -7, -8, -9, and -12) have been shown to induce apoptosis in some types of blood cells. For galectin-1, this activity has been studied most in

human T cells, where apoptotic pathways may involve cell-surface glycoproteins including CD7, CD29, and CD43.

T-cell immune disorders and chronic inflammation

Galectins can regulate inflammatory responses; depending on the inflammatory stimulus, microenvironment, and target cells. Galectin-1 has powerful immunoregulatory effects through its ability to inhibit T-cell effector functions (van der Leij, J., et al. 2004).

Galectin-1 function is generally associated with attenuating inflammatory responses. In contrast, galectin-3 has a proinflammatory role. Galectin-1 suppresses the secretion of the pro-inflammatory cytokine interleukin-2 (IL-2) tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) (Rabinovich, G.A., et al. 1999) and favours the secretion of the anti-inflammatory cytokines IL-5, IL-10, and transforming growth factor-β (TGF-β) in activated T cells (van der Leij, J., et al. 2004). Also, galectin-1 positively influences migration of human monocyte-derived dendritic cells (Fulcher, J.A., et al. 2006, Perone, M.J., et al. 2006).

Galectin-1 can contribute to the balance between Th1 and Th2 immune responses, which are characterized by the types of cytokines produced. Although Th1- and Th-17-differentiated cells expressed the repertoire of cell surface glycans critical for galectin-1-induced cell death, Th2 cells were protected from galectin-1 through differential sialylation of cell surface glycoproteins (Toscano et al., 2007). Gal-1 treatment increases T-cell susceptibility to activation-induced apoptosis and promotes a shift from a Th1 to a Th2-polarised immune response (Rabinovich et al., 1999a).

Galectin-1 has been shown to be a key mediator of suppressive activity of T regulatory cells (Garin, M.I., et al. 2007). Galectin-1 plays a key role in the induction and maintenance of foetal survival in vivo through modulation of multiple tolerogenic mechanisms (Blois, S.M., et al. 2007).

Galectin-1 has been shown to inhibit T-cell adhesion to extracellular matrix glycoproteins, such as fibronectin and laminin (Rabinovich, G.A., et al. 1999b). As T cells migrate through endothelial monolayers, galectin-1 expressed on the surface of the latter cells can retard the process (He, J. and Baum, L.G. 2006). In total, galectin-1 is apparently immunosuppressive with regard to the T-cell response, through induction of apoptosis, suppression of the T-cell response, or the mediation of the T-regulatory cell activities.

Both galectin-1 and -3 activate neutrophils, leading to the secretion of superoxide and IL-8 (a chemokine for neutrophils) (Nieminen, J., et al. 2005, Yamaoka, A., et al. 1995). In contrast to galectin-3, galectin-1 suppresses both *in vivo* acute neutrophil migration and *in vivo* transmigration of neutrophils across endothelial cells (La, M., et al. 2003, Rabinovich, G.A., et al. 2000).

Role of Galectins in Apoptosis and Induction of Cell-Surface Phosphatidylserine Exposure

Cummings and colleagues reported that galectin-1,-2 and -4 induce surface exposure of phosphatidylserine on activated neutrophils without inducing cell death by apoptosis (Dias-Baruffi, M., et al. 2003, Karmakar, S., et al. 2005, Stowell, S.R., et al. 2007). Notably, probably due to the high contents of phosphatidylserine on the

surface, galectin-1 treated neutrophils are phagocytosed by activated macrophages to a level similar to aged neutrophils resulting in the secretion of anti-inflammatory cytokines, such as TGF-β and IL-10 (Erwig, L.P. and Henson, P.M. 2007). Galectin-1 increases arginase activity and decreases LPS- induced reactive nitrogen species (RNS) production by macrophages (Correa, S.G., et al. 2003). In addition, MHC-II expression and antigen-presenting activity by macrophages was decreased when galectin-1 is present *in vivo* and *in vitro* (Barrionuevo, P., et al. 2007). Thus galectin-1 appears to skew macrophages to states of "alternative activation" or "deactivation" (Gordon, S. and Taylor, P.R. 2005).

Galectins in host-microbe interactions

The expression of galectin-1 is markedly upregulated after parasite or virus infection (Giordanengo, L., et al. 2001, Lim, J.W., et al. 2003, Zuniga, E., et al. 2001). Enveloped viruses acquire host-type glycans on their surface as viruses utilize the host protein synthesis machinery, including glycosylation. Indeed, galectin-1 binds to some enveloped viruses, such as HIV-1 and Nipah virus. For Nipah virus, galectin-1 inhibits its infection (Levroney, E.L., et al. 2005) while HIV-1 appears to exploit galectin-1 to stabilize viral attachment to CD4+ T lymphocytes and macrophages, thus promoting viral infection in these target cells (Ouellet, M., et al. 2005) and altered T cell surface glycosylations in HIV-1 infection results in an increased susceptibility to Gal-1-induced cell death (Lanteri, M., et al. 2003).

Action on the nervous system

Galectin-1 is abundantly expressed in a variety of vertebrate organs including rat and human brain. In rat brain, this lectin is predominantly expressed inside the neuron cytosol and in neuritic processes. It is also transiently secreted and found on the surface of neuronal cells. It participates in intracellular traffic of glycosylated molecules in nerves. It is possible that cytosolic lectin also plays a role in the construction of the cytoskeleton since the lectin was shown to bind sugar-independently to actin molecules (Joubert, R., et al. 1992). Galectin-1 in its oxidized form, a form that lacks lectin activity, promotes neurite outgrowth (Outenreath, R.L. and Jones, A.L. 1992) and enhances axonal regeneration in peripheral (Horie, H., et al. 1999), and central nerves even at relatively low concentrations (pico M range) (Horie, H., et al. 2000). Various reports suggest a relation between galectin-1 expression (or altered expression) and neurological diseases. Galectin-1 expression by neuronal and glial cells is closely correlated with regenerative success after injury (Wada, M., et al. 2003) and the level of autoantibodies to galectin-1 is significantly higher in patients with neurological disorders than in healthy controls (Lutomski, D., et al. 1997). Galectin-1 induces astrocyte differentiation, with the subsequently differentiated astrocytes greatly enhancing their production of the Brain-Derived Neurotrophic factor (BDNF) that, in turn, plays an important role in the survival, differentiation, and synaptic plasticity of neurons (Sasaki, T., et al. 2004). In this context galectin-1 may thus be considered as a means for the prevention of neuronal loss in cases of injury to the central nervous system (Egnaczyk, G.F., et al. 2003). Oxidised galectin-1 stimulates the migration of Schwann cells from both the proximal

and the distal stumps of transected nerves and promotes axonal regeneration after peripheral nerve injury (Fukaya, K., et al. 2003).

Roles of Galectins in Animal Development

Galectins play important, but rather subtle, roles in animal development. Lack of galectin-1 in mice is associated with a different set of interesting phenotypic changes, including decreased sensitivity to noxious thermal stimuli, altered primary afferent neural anatomy, aberrant topography of olfactory axons, and reduced muscle regeneration ability after injury.

It is possible that the redundancy of galectin family members contributes to survival of these null mutants or that these particular galectins are involved only in post developmental processes, such as immune regulation. Galectin-1 expression has been reported in male and female gonads (Wollina, U., et al. 1999). In the uterus galectin-1 expression is restricted to the endometrium and varies during the menstrual cycle and the early phases of gestation (von Wolff, M., et al. 2005). Galectin-1 is externalized during adipocyte differentiation (Wang, J.L., et al. 2004) and is able to modulate osteoblastic differentiation (Andersen, H., et al. 2003) as well as the proliferation and death of haematopoietic stem and progenitor cells (Vas, V., et al. 2005).

The effect of galectin-1 on cell signalling pathways

Galectin-1 is mitogenic for various types of normal or pathological murine and human cells (Moiseeva, E.P., et al. 2000, Symons, A., et al. 2000). Galectin-1 inhibits the growth of other cell types such as neuroblastoma (Kopitz, J., et al. 2001) and stromal bone marrow cells (Andersen, H., et al. 2003). While high concentrations ($\approx$1μM) of recombinant galectin-1 inhibit cell proliferation independently of galectin-

1 sugar binding activity, low levels (≈1 nM) are mitogenic and are susceptible to inhibition by lactose (Adams, L., et al. 1996, Vas, V., et al. 2005). Furthermore, galectin-1 can also regulate cell cycle progression in human mammary tumor cells (Wells, V., et al. 1999). The seemingly paradoxical positive and negative effects of galectin-1 on cell growth are highly dependent on cell type and cell activation status, and might also be influenced by the relative distribution of monomeric versus dimeric, or intracellular versus extracellular, forms.

Roles of Galectins in Cancer

The galectin-1 expression or over expression in tumors or the tissue surrounding them must be considered a sign of their malignant progression resulting in poor prognosis for large number of cancer patients. Three galectins that have shown importance in cancer progression and metastasis are galectin-1, -3, and -9. Expression of galectin-1 has been well documented in many different tumor types including astrocytoma, melanoma, head and neck, prostrate, thyroid, colon, bladder and ovarian cancer.

Galectin-1 modifies each of the three cell migration-related processes; adhesion, motility, and invasion. Galectin-1 can potentiate or inhibit adhesion of various normal and cancer cells to extra cellular matrix (ECM) via cross-linking of glycoproteins (integrins) exposed on cell surfaces with carbohydrate moieties of ECM components such as laminin and fibronectin (Andre, S., et al. 1999, Ellerhorst, J., et al. 1999, Moiseeva, E.P., et al. 1999, Moiseeva, E.P., et al. 2003, van den Brule, F., et al. 2003). In addition galectin-1 can also mediate homotypical cell interactions, so favouring the aggregation of human melanoma cells (Tinari, N., et al. 2001) and heterotypical cell interactions as between cancer and endothelial cells, which in turn

favour the dispersion of tumour cells (Clausse, N., et al. 1999, Glinskii, O.V., et al. 2005, Lotan, R., et al. 1994).

Cell motility includes dynamic remodelling of the actin cytoskeleton as well as the microtubule network. The stable knockdown of galectin-1 certainly alters the expression of a number of genes that are either directly or indirectly involved in actin polymerisation in glioblastoma cells (Camby, I., et al. 2005).

Galectin-1, galectin-3 and galectin-8 seem to be involved in tumour astrocyte invasion of the brain parenchyma as their levels of expression are higher in the invasive parts of the xenografted glioblastomas than in their less invasive parts (Camby, I., et al. 2001).

The abundance of proapoptotic galectin-1 in privileged immune sites such as the placenta (Hirabayashi, J., et al. 1989), the brain (Joubert, R., et al. 1992) and the reproductive organs (Wollina, U., et al. 1999) along with the expression of galectin-1 in the stromal tissue around tumors (Berberat, P.O., et al. 2001, Gillenwater, A., et al. 1996, Sanjuan, X., et al. 1997, Shimonishi, T., et al. 2001) or in the endothelial cells from capillaries infiltrating them, rather than in adjacent non tumoral stroma (Clausse, N., et al. 1999) suggest that galectin-1 might trigger the death of infiltrating T cells and protect these sites from the tissue damage induced by T-cell derived pro-inflammatory cytokines.

The immunomodulatory effects of galectin-1 and the correlation between galectin-1 expression in cancer cells and their aggressiveness suggest the hypothesis that tumor cells may impair T-cell effector functions through the secretion of galectin-1 and that this mechanism may contribute toward tilting the balance in favour of an immunosuppressive environment at tumor site. The blockade of the biological activity

of galectin-1 in melanoma tissue resulted in a reduced tumor mass and stimulated the *in vivo* generation of a tumor-specific T-cell response (Rubinstein, N., et al. 2004).

Galectin-1 could be involved in tumor angiogenesis because both vascular smooth muscle and endothelial cells express it (Moiseeva, E.P., et al. 2000, Moiseeva, E.P., et al. 2003, Clausse, N., et al. 1999)). Furthermore , although the vessel walls of normal lymphoid tissues do not express galectin-1, the blood vessel walls of lymphomas do so in relation to their vascular density (D'Haene, N., et al. 2005). Treatment with galectin-1 specific anti-sense oligodeoxynucleotides or polyclonal antigalectin-1 antibodies resulted in the inhibition of endothelial cell proliferation and migration, which suggests an essential role for galectin-1 in angiogenesis (Thijssen, V.L., et al. 2006).

Galectin-1 and atherosclerosis

Galectin-1 is least heard-of in atherosclerosis research so far. Nevertheless, two reports had highlighted the role of galectin-1 in smooth muscle cell (SMC) proliferation, an important step in atherogenesis (Moiseeva, E.P., et al. 2000, Moiseeva, E.P., et al. 1999). Galectin-1 binds lipoprotein (a) (Chellan et al., 2007), a modified low density lipoprotein molecule implicated in atherogenesis. Recent reports have shown that galectin-1 may be involved in chemoattractant at sites of inflammation in vivo and may contribute to disease processes such as atherosclerosis (Malik, R. K et al., 2009).

Functional antagonists of galectins

Anti-galectin compounds could have therapeutic value as anti-inflammatory (Liu, F.T., et al. 2002) and as anti-cancer agents (Ingrassia, L., et al. 2006). Most

types of antagonists are based on derivatives of lactose, in one way or another. The multivalent design approach was exploited by Rabinovich et al., who synthesized lactulose amine compounds (essentially polymethylene-spaced dilactosamine derivatives) that demonstrated apparently selective effects in different events linked to tumor cell apoptosis, cell aggregation, and endothelial cell morphogenesis, suggesting that subtle differences in carbohydrate structures may be potentially useful to block tumor growth and metastasis (Rabinovich, G.A., et al. 2006). Galectin-binding affinities for most of these reported compounds are relatively weak. Secondly, there still remains good cross-reactivity, bringing the question of specificity to the forefront. The probability of eliciting various unwanted side effects is also there. Peptides have been identified as potential galectin antagonists. Peptides bind somewhere on the surface of the galectin and directly on the carbohydrate-binding site, especially because both peptides carry a net positive charge like the actual carbohydrate-binding site. A modified natural polysaccharide, modified citrus pectin (MCP), is of interest as an anti-cancer agent (Nangia-Makker, P., et al. 2002). The smaller complex oligosaccharide units of MCP can combine with the carbohydrate-binding domain of galectin-3 (Inohara, H. and Raz, A. 1994) and interfere with its binding to specific cell surface receptors. Besides MCP and GCS100, a similar pectin derivative (Chauhan, D., et al. 2005), other therapeutic strategies include dietary polysaccharides (Sathisha, U.V., et al. 2007) and peptide mimetics (Andre, S., et al. 2007, Zou, J., et al. 2005).

CHAPTER III

MATERIALS AND METHODS

Materials

Neuraminidase from *Clostridium perfringens,* Standard human immunoglobulin types (human IgA, IgG, IgM), orthophenylene diamine (OPD), 1-O-methyl-α-D-glucoside, 1-O-methyl β-D glucoside, cellobiose, mannose, glucose, galactose, lactose, soluble guar gum, bovine serum albumin, ovalbumin, amido black, divinyl sulphone, cyanogen bromide, Tween-20, acrylamide, N, N`-methylene bisacrylamide, TEMED, agarose, 2-mercaptoethanol, TEMED, Iodoacetamide, fetuin, Sephadex G-200, dextran (molecular weight 400,000-500,000 kDa), horse radish peroxidase (HRP) type II, Streptavidin, Sulpho-NHS-LC Biotin, Coomassie brilliant blue G, polyethylene glycol 6000 (PEG) , yeast (*Saccharomyces cerevisiae*) β-glucan (YBG) and barley β-glucan (BBG) were purchased from Sigma-Aldrich (India), Bangalore. *C. albicans* strain (ATCC No.10231) was obtained from Dept. of Microbiology, S.C.T.I.M.S.T.

Polystyrene 96 well microplates (MAXISORP) were purchased from Nunc, Denmark. Antibodies to human IgA, IgG and IgM raised in goat were purchased from Dako, Denmark. Sepharose 4B and 6B were the products of Pharmacia Fine Chemicals, Uppsala, Sweden. Coomassie brilliant blue R-250 was purchased from Pierce Chemical Co. USA. Phenyl methyl sulfonyl fluoride, benzamidine hydrochloride and potassium borohydride were obtained from Fluka, Buchs, Switzerland. Other chemicals used were of analytical grade and obtained from local sources. The seeds of *Arachis hypogeae* was obtained locally and peanut agglutinin (PNA) were prepared.

Human IgG (Sigma Chemicals, U.S.A.) was freed from traces of IgA by passing its solution in PBS, pH 6.5 (10 mg in 5 ml) through a 5 ml jacalin-Sepharose 4B column in the same buffer at 4°C. Unbound fraction (free from IgA as shown by ELISA was used as IgG.

Human placenta and umbilical chord, immediately after delivery, was collected from the Obstetrics Department, Cosmopolitan Hospital, Thiruvananthapuram. The tissue was washed extensively in running water to remove blood clots and debris and then washed in ice cold PBS 7.4. Placenta was then cut into pieces (50 gm) and kept frozen at -20° C before use. Fasting serum and blood samples were collected from the Central Clinical Laboratory and Blood Bank of this institute. The collection of all biological samples had the prior approval of the institute. Diabetic serum samples were collected from Indian Institute of Diabetes, Pulayanarkotta.

Methods

Protein estimation by Bradford's method

Reagent: 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.

Procedure: The reagent and protein solutions were mixed in the ratio 1:1 and the absorbance at 620 nm measured immediately (Bradford, M.M. 1976)].

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).

Protein was also estimated by a modified Lowry's method, by including 0.5% SDS in the alkali reagent, to estimate protein in presence of the non-ionic detergent, SDS (Dulley, J.R. and Grieve, P.A. 1975).

Carbohydrate estimation by phenol-sulphuric acid method

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

Reagents

a. Sulphuric acid

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

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.

Electrophoresis

Alkaline-PAGE

Alkaline-PAGE at pH 8.3 on 7% tube gel was done as described by Davis (Davis, B.J. 1964)].

Reagents

a. One hundred ml Tris (1.5 M) containing 24 ml 1 N HCl and 0.12 ml TEMED, pH 8.8.
b. One hundred ml Tris (0.5 M) containing 48 ml 1 N HCl and 0.46 ml TEMED, pH 6.8.
c. 28 g acrylamide and 0.735 g bis acrylamide dissolved in 100 ml distilled water.
d. 20 g acrylamide and 5 g bis acrylamide dissolved in 100 ml distilled H_2O.
e. 4 mg riboflavin dissolved in 100 ml distilled water.
f. 14 mg ammonium persulphate dissolved in 10 ml distilled water.

Separating gel (Acrylamide 7%)

One part 'a' was mixed with one part 'c'. To this mixture, an equal volume of 'f' was added and mixed.

Spacer gel

One part 'b', one part'd', one part 'e' and 5 parts distilled water were mixed.

Reservoir buffer

0.05 M Tris/0.38 M glycine, pH adjusted to 8.3.

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

Acid-PAGE

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

Reagents

Solution A :	1 N KOH	48ml
(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	0.46ml
	Made up to 100 ml with water.	
Solution C:	Acrylamide	30g
	Bis acrylamide	0.8g
	Made up to 100 ml with water.	
Solution D:	Acrylamide	20g
	Bis acrylamide	0.5g

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.

Electroelution

The method described for electroelution of DNA from agarose gel (Ogden, R.C. and Adams, D.A. 1987) was adapted for protein elution from acrylamide gel. For electro elution from alkaline PAGE tube gels, one of the tube gels was fixed, stained

and destained to serve as a reference to cut out the required protein bands from other tube gels, which was kept at 4^0 C after the run. Gel slices containing the required protein band were minced with a scalpel blade and kept in tris-acetate buffer (5 mM Tris; 2.5mM 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 1000g and the supernatant containing protein was dialysed against PBS and concentrated by AMICON PM10 ultra filtration membrane.

Preparation of matrices

1. Cross-linked guar gum (CLGG)

Soluble guar galactomannan was cross-linked to form an insoluble gel by a modification of the procedure described by Appukuttan *et al.* (Appukuttan, P.S., et al. 1977)]. Guar gum powder (10 g) was mixed thoroughly with a finely dispersed emulsion of 2ml epicholorohydrin and 25 ml 3 N NaOH until the mixture became a solid cake. It was 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.

2. Lactosyl-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. (Dean, P.D.G., 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. 500ml of 0.2 M glycine-HCl, pH 3.0, containing 1 M NaCl to block unreacted activated groups in the gel.
3. 500ml of 1 M NaCl and
4. 500 ml of distilled water.

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

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

Immobilization of proteins on Sepharose was achieved through cyanogen bromide method according to Lowe (Lowe, J.B. 2001). Sepharose 4B (40 ml) (Pharmacia Biotech, Sweden) was activated using cyanogen bromide by the method

of (Cuatrecasas, P. and Anfinsen, C.B. 1971)] in Na_2CO_3 (2 N) at 8 °C for 5 min. and washed with 0.1 M $NaHCO_3$ buffer pH 8.5. The protein sample in 0.1 M $NaHCO_3$ was added to the activated gel (2 mg/ml gel) and stirred gently overnight at 4 °C followed by incubating the gel in 0.1 M ethanolamine hydrochloride to block the unconjugated activated groups on the gel. The coupled gel was washed successively with 20 times the gel volume of

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

The protein-Sepharose 4B affinity matrix can be stored at 4 °C with 0.02% sodium azide or packed into a column of required size for chromatography. Control used for anti-IgA-Sepharose was cyanogen bromide-activated Sepharose coupled to ethanolamine (as 100 mM ethanolamine hydrochloride) instead of protein.

Preparation of PNA

All preparations were done at 4°C. Fifty gram dehusked peanut soaked for 24 h in 20 mM phoshate buffer, pH 6.5 containing 150 mM NaCl was homogenized in the same buffer using a POLYTRON homogeniser and stirred for 1 h. After 15,000 g centrifugation the lipid slab on top was removed and proteins from supernatant, precipitated by 70% ammonium sulfate saturation, were redissolved in and dialysed against PBS 6.5. From 15,000 g supernatant of the dialysate one half was passed through a 40 ml CLGG column equilibrated in the same buffer. After washing

out unbound proteins using PBS 6.5, bound proteins were eluted using 0.15 M lactose in the same buffer and concentrated using AMICON PM 10 membrane.

Preparation of jacalin

Jacalin (Jack fruit seed agglutinin) was isolated from the seeds of *Artocarpus integrifolia* (jack fruit seed) by the procedure described by Sureshkumar et al (1982). Thirty g of Jack fruit seeds were dehusked and soaked in PBS 6.5 for 12 h. The seeds were then cut into small pieces, homogenized in 300 ml PBS 6.5 and stirred 2 h at 4^0 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.

Tissue Collection

Human placenta, immediately after delivery, was collected from the Obstetrics Department, Cosmopolitan Hospital, Thiruvananthapuram. The tissue was washed extensively in running water to remove blood clots and debris and then washed in ice cold PBS 7.4. Placenta was then cut into pieces (50 gm) and kept frozen at -20^0 C before use. Bovine heart tissues were collected from slaughter houses within two hours of slaughter. After removing fat deposits, bovine heart muscle was washed in PBS 7.4 and kept frozen at -20°C until use.

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 (5mM), phenylmethylsulfonylflouride (PMSF; 0.2mM), 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 sufate 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°C. The lectins were subsequently, either, conjugated with HRP or stored with 30% v/v glycerol at -20°C until use. Lectin activity was assayed by hemagglutination with trypsinized human RBC.

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 (2cm x 15cm) 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 0.2M dialyzable

dextrose (obtained by thorough dialysis of 20ml 1M dextrose in PBS against 80ml PBS) into 3ml fractions. Protein fractions were pooled concentrated by ultra filtration (10,000MW cutoff membrane) and dialyzed against PBS to remove dextrose.

Preparation of serum dextran-binding immunoglobulin (DIg)

All steps were at 4^0C. 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 12,000 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 (10,000MW cut-off) and stored at 2-4^0C.

Isolation of anti-Gal

Isolation of anti-Gal was done by method of Jaison et al., (222).All steps were carried out at 4^0C. 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 12,000 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 (10,000MW cut-off), dialysed against PBS to remove galactose and stored at 2-4^{0}C.

Conjugation of Horse Radish Peroxidase (HRP) to antibodies / lectins.

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 (Heyderman et al., 1989).

Enzyme treatment of glycoproteins on microwells.

For coating to polystyrene wells 200 µl PBS containing the specified amount of glycoprotein was added to a 96 well ELISA plate (Nunc Maxisorp) and incubated at 37°C for 3h. Wells were then washed with PBST, blocked with PBS containing 0.5% Tween 20 for 30 min. at 37°C and again washed with PBST. Enzyme treatment of polystyrene well coated glycoproteins was uniformly in 0.5% Tween 20 at 37°C with 5 mU neuraminidase for 1h.

Enzyme-labeled antibody binding to polystyrene-well coated proteins, protein conjugates or polysaccharides

Polystyrene microwell coating (96 well NUNC MAXISORP) of fungal /yeast polysaccharides, followed by washing and Tween-20 blocking of wells was done as described earlier. Wells were then incubated at 4^{0}C with the specified dilution

of HRP conjugates of antibody in 200µl PBS –T for 2h and washed thrice with the same buffer. To assay the bound HRP, wells were then incubated at 25^0C for 30 min with 200 µl ortho phenylene diamine (0.5mg/ml) in 0.1M citrate-phosphate buffer, pH 5.0 containing 0.03% H_2O_2, followed by addition of 50µl 12.5% H_2SO_4 and absorbance measurement at 490nm in a BIOTECH (USA) ELISA reader. Alternately, after blocking of wells, specified quantity of ABG or its components in 200µl PBS-T was added, wells incubated at 4^0C for 2h and washed thrice with PBS-T. To quantitate bound antibody, wells were incubated at 4^0C with 200µl PBS-T containing HRP conjugates of goat antiserum (IgG fraction) against human IgG, IgM or IgA or a mixture of three conjugates at 1.5 µg/ml of each antibody.

Sugar inhibition of anti-glycan antibody binding to plate -coated *C.albicans* polysaccharide

Polystyrene microwells were coated with *C.albicans* polysaccharides (2 µg/well) and blocked as described above. Wells were then treated at 4^0 C for 2 h with 200 µl PBS-T containing 2 µg of AGA previously incubated for 1 h at 4^0C with 50 mM cellobiose/ mannose. Following washing with PBS-T, bound AGA was assayed using anti-human Ig HRP as described previously.

Isolation of polymeric IgA and monomeric IgA from ABG/DIg

Anti β glucan/DIg was subjected to acid PAGE at pH 4.5 for 8 hours and gel cut into 16 equal fractions. Proteins electroeluted from each fraction was coated on NUNC Maxisorp wells (1 µg/well) and probed with HRP conjugates of anti-human immunoglobulins. Protein electroeluted from the gel sections corresponding to the

first peak of IgA was pooled together and taken as polymeric IgA fraction while the second peak is taken as monomeric IgA.

Quantitation of IgA monomer content in polymeric and monomeric IgA

Equal amounts of polymeric and monomeric IgA (1 μg/well) treated with 3 mM 2-ME at 37°C for 1h and coated on NUNC microtitre plates at 4^0C for 1 hour. Probing was done with anti-IgA –HRP conjugate.

Preparation of *Candida albicans* antigen (CAA)

C. albicans strain (ATCC No.10231) was cultured in Sabouraud's dextrose agar in Roux bottle and cells grown were enriched in Sabouraud's dextrose broth.

Media composition of Sabouraud Agar, Modified

Enzymatic Digest of Casein	10.0 g
Dextrose	20.0 g
Agar	20.0 g

Suspend the powder in 1 L of purified water:

The cell harvest was sedimented and washed thrice with PBS by centrifugation at 15,000g. Suspension of the pellet in PBS (10ml) was homogenized using POLYTRON homogenizer and later subjected to ultrasonication (six 30 second treatments) at 25^0C. The polysaccharides released to the supernatant were collected by centrifugation at 15,000g for 30min and dialysed against PBS pH 7.4.

Electrophoretic separation of *Candida albicans* antigen.

CAA was subjected to polyacrylamide gel electrophoresis at alkaline pH (8.3) for 2 h.The gels was cut into 8 segments of equal length. Electro-eluted sample from each

segment was coated on to microtitre wells after ten times dilution and probed using HRP conjugates of horse antibodies to human immunoglobulins IgA, IgG and IgM. The supernatant was dialysed against PBS pH 7.4.

Isolation of yeast glycoproteins.

Baker's yeast (5 g) suspended in 25 ml PBS was subjected to three successive freezing and thawing, homogenized in a POLTRON homogenizer. The suspension was then sonicated for six 30 s bouts. The sample was stirred for 30 min at 4^0c, centrifuged at 12,000xg and supernatant collected. The supernatant was dialysed against PBS pH 7.4.

Electrophoretic separation of yeast glycoproteins

Undialysible portion that consists of yeast glycoproteins were subjected to polyacrylamide tube gel electrophoresis at pH 8.3 in 7% gel. Ten bands, which were seen in electrophoresis, were cut, electroeluted and protein assayed using Bradford's method.

Isolation of non-dialysable fraction of table sugar (NDS).

Table sugar samples from local grocery shops dissolved in PBS (35% w/v) were dialysed extensively against the same buffer in 10,000 MW cut-off dialysis membrane and non-dialysable components in the bag saved.

Electrophoretic separation of NDS.

NDS was subjected to polyacrylamide tube gel (5%) electrophoresis at alkaline pH (8.3) for 2 h. The gels were cut into 8 segments of equal length. Polysaccharides from each crushed segment were eluted into 4 times its volume of

PBS by overnight incubation at 4^0C. Each eluate was diluted ten times and 200 µl coated on 96 well ELISA plates.

Preparation of immune complex (IC) from serum and proteins from IC.

IC was precipitated with 2% PEG 6000 in veronal buffer (2 mM sodium barbitone, 3 mM barbituric acid and 140 mM sodium chloride) pH 7.4 by a modification of the procedure by Hudson and Hay (1980). Briefly, 1 ml serum was treated with 40 µg non-dialysable sugar (NDS) / 100 µg dextran /20 µg CCA for 3 hours at 37^0C. PEG 6000 (100 µl of 12% solution in veronal buffer) was then added and solutions kept at 4^0C overnight. Precipitated IC was collected by centrifugation at 2000 g for 20 minutes, washed once with 2% PEG 6000 in veronal buffer by centrifugation as above and redissolved in 500µl PBS.

To isolate immunoglobulin from IC, the latter redissolved in PBS was treated with 100M of the specific sugar (1-*O*-methyl α-D-Glc for DIg-IC and cellobiose for ABG) 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.

Immobilization of galectin-1 by biotinylation.

Galectin-1 was biotinylated by incubating 3 mg/ml of galectin with 2mM sulpho NHS-Biotin at pH 8.0 for 2 hours at 4°C in the presence of 25mM lactose. Unconjugated Sulpho-NHS Biotin and free lactose were separated from galectin by dialysis in PBS pH 7.4. To Nunc Maxisorb wells coated with 1 µg streptavidin and blocked with 0.05% T-20 PBS, 10X dilution of biotinylated galectin was added and

kept at 4°C for two hours. Immobilization of galectin was confirmed by addition of fetuin-HRP (100X) in the presence and absence of lactose 50mM.

Demonstration of binding of IgA to immobilized galectin-1

IgA (from Colostrum); 30 µg/100 µl and IgG (36 µg/100 µl) was added to NUNC plate coated immobilized galectin-1 (as mentioned above) simultaneously along with 100 µl of fetuin-HRP (100X). Reduction in fetuin-HRP binding to immobilised galectin-1 in these wells in comparison to the control wells is checked.

Statistics

For statistical analysis, Microsoft Excel program was used. For comparison between two groups, the Student's *t*-test was used. Statistical significance was considered as $P < 0{\cdot}05$.

CHAPTER 4

RESULTS AND DISCUSSION

Part I

Anti- polysaccharide antibodies are rich in polymeric IgA which make it better ligands for tissue galectin-1

A well-equipped repertoire of carbohydrate binding antibodies forms the first line of defense against pathogens. These immunoglobulins belong to IgG, IgM, IgA, and IgD subtypes and are called natural antibodies, since they are present in sera of all individuals in the absence of deliberate immunization (Shoenfeld, Y. and Isenberg, D.A. 1989) and are thus a part of the innate immune system. Natural antibodies that recognize carbohydrates including blood group antigens are not found during first weeks of life, and a hypothesis referred as "bacterial paradigm" has been proposed by G.F. Springer who postulated that anti-carbohydrate natural antibodies appear in response to stimulation of the immune system by bacterial O antigens and LPS of gastrointestinal bacteria (Springer, G.F. and Horton, R.E. 1969). The best known and studied natural carbohydrate antibodies are directed to blood group antigens A and B (Milland, J. and Sandrin, M.S. 2006) , terminal α-Galactoside structure(Gal α (1-3)Gal β (1-4)GlcNAc) (Cooper, D.K. 1998, Galili, U., et al. 1984) and Forssman (Fs) glycolipid antigen GalNAc α (1- 3)GalNAc β (1-3)Gal α (1-4)Gal β (1-4)Glc (Kano, K. and Milgrom, F. 1977, Young, W.W., Jr., et al. 1979). Anti-carbohydrate antibodies appear in number of pathologies, the group of particular attention includes antibodies to tumor-associated carbohydrate antigens such as Gal (α 1-3)GalNAc-α (TF, Thomsen–Friedenreich antigen), GalNAc-α-1-OSer/Thr (Tn) (Springer, G.F. 1984), and several related ones (Lloyd, K.O. 1991).

Anti-polysaccharide immune response is characterized by lack of T-lymphocye memory and isotype restriction. Still there is evidence implicating the natural killer (NK cell) and T-cell playing important role in response to T-Independent (TI) antigens. While T cells may regulate the response to TI antigens it is not via the CD40 ligand (Foy et al., 1993). The nature of the B cell activating signal is

critical in determining the quantitative and qualitative profile of Ig isotype production. The immunoregulatory pathways that regulate isotype restriction patterns stimulated by polysaccharide antigens are complex, and no single pattern can be unequivocally defined (Mond et al., 1995). Also many carbohydrate structures presented as part of a glycoprotein elicits T-dependent response, e.g., TAG epitope, mannoprotein etc. Their antibody profile is characteristic of antibody response to protein involving IgG as the major component.

B-1 cells play a major role in responses to TI-2 antigens (Pecquet et al., 1992).These cells, originally defined by the surface expression of CD5 and high levels of IgM, home predominantly to the peritoneal and pleural cavities, have a capacity for self-renewal, and display different receptor specificities. The marginal zone (MZ) macrophages of the spleen were regarded as critical components for TI-2 responses. Monoclonal IgA derived from peritoneal B Cells is encoded by both germ line and somatically mutated V_H genes and is reactive with commensal bacteria (Bos et al., 1996). Significant numbers of IgA plasma cells can be derived from B1 cells, which reside in the peritoneal cavity. IgA switching differs among purified B cell subsets, suggesting that individual B cell populations could contribute differentially to IgA expression in vivo, depending on available stimuli. Non follicular MZ and B1 cells undergo class switching to IgA in minimal culture conditions at a higher frequency than follicular B2 cells. (Kaminski et al., 2006). IgA is synthesized in a variety of different sizes, including monomers, dimers, tetramers, and even higher polymeric forms. A single clone of cells can synthesize different forms of IgA (Moldoveanu et al., 1984; Peppard and Jackson, 1987), and it has been suggested that IgA secreting

cells lodged in different organs can produce IgA with different size profiles (Kutteh et al., 1982).

Glycan antigens entering the human body through food, environment and microflora colonizing the skin, epithelia and gut, especially in humid tropics is enormous making it pertinent to characterize the anti-glycan antibodies and their immune complexes. Anti-glycan antibodies are rich in polymeric IgA content making it potential source of high quantities of pIgA-IC in serum. 90% of serum IgA is IgA1 in nature with the characteristic Core I O-glycan in its hinge region. Systemic mechanisms for clearance of IgA-IC and IgA in human beings include mainly phagocytosis by kupffer cells. Hepatic asialoglycoprotein receptors (ASGPR) mediate the endocytosis of desialylated IgA. Because the systemic mechanisms for clearance of IgA-IC are saturable((Rifai, A. and Mannik, M. 1984, Russell, M.W., et al. 1981), high concentrations of IgA-IC in circulation may lead to their deposition in extrahepatic tissues, as has been implicated in the pathogenesis of IgA nephropathy.

Different anti-glucan antibodies are used in this study to characterise their amount of polymeric IgA content. Relative IgA content is likely to dictate the fate of these antibodies and their immune complexes since the most ubiquitous and well expressed tissue lectin (galectin-1) has been found to recognize exclusively IgA1 among all immunoglobulin types (Sangeetha and Appukuttan,2005).

The anti-β-glucan, specific to β (1→3)/β (1→4) linked glucose is a normal human plasma antibody that had three times higher IgA to IgG ratio and substantially higher polymeric IgA content than total serum immunoglobulins. Anti β-glucan is prepared from normal healthy human plasma using cellulose: celite affinity chromatography column and elution with dextrose (Geetha, M., et al. 2007). Recently

it was demonstrated using microchip format glycan array that β 1,4 linked glucan binding antibodies are commonly seen in healthy donors (Huflejt, M.E., et al. 2009). Anti-β-glucan is subjected to electrophoresis at acid pH and the separated fractions were analyzed for its immunoglobulin composition by ELISA.

Anti-dextran human antibodies had been produced earlier by immunization with derivatized dextran ((Kabat, E.A. and Berg, D. 1953). Sporadic cases of naturally occurring serum anti-dextran antibodies had been reported in some Western population (Kraft, D., et al. 1982, Anastase,S., et al., 1996). It was reported from our lab that dextran-binding immunoglobulin (DIg) is naturally occurring in all blood donors tested in the province of Kerala (Chacko and Appukuttan, 2003). Dextran-binding immunoglobulin (DIg) having specificity to α (1,6) linked glucose is isolated from plasma of healthy human donors by affinity chromatography on Sephadex G-200.

Sephadex is selected as affinity matrix for purification of DIg since this gel is obtained by cross-linking dextran which is a polymer formed exclusively of glucose in α (1→6) linkage (Sidebotham, R.L. 1974). Marked inhibition of the antibody by (a) dextran (b) sucrose followed by maltose and Melibiose (c) para-nitro phenyl and methyl α –glucosides suggest that DIg is produced primarily against α-glucosides containing unmodified C4 hydroxyl group (Chacko and Appukuttan, 2003).

Anti-α –galactoside antibody (anti-Gal) is a polyclonal antibody present exclusively in the sera of man, apes and old world monkeys, constituting nearly 1% of their serum IgG content (Galili, U., et al. 1984). Majority of α-Gal-specific Abs are T cell dependent in nature (Cretin, N., et al. 2002). It recognizes the terminal α-galactoside epitopes with the notable exception of blood group B antigen (Galili, U.,

et al. 1987). Anti-Gal is believed to be produced in the above animals in response to immune stimulation by enteric bacteria that bear α- galactosyl antigenic epitopes (Galili, U., et al. 1988). Human serum anti-Gal was prepared by affinity chromatography using guar-galactomannan column (Jaison and Appukuttan, 1992).

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

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

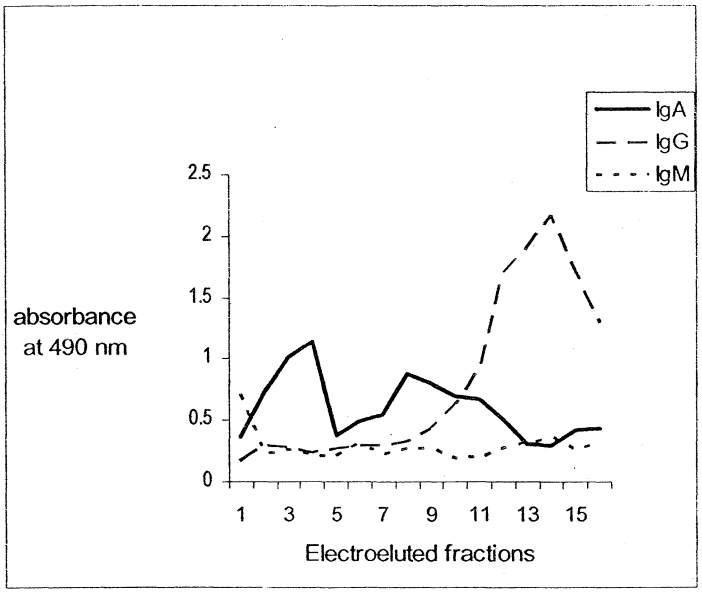

Fig.3. Immunoglobulin composition in anti-β-glucan. Anti β glucan was subjected to acid PAGE at pH 4.5 for 8 hours and gel cut into 16 equal fractions. Proteins electroeluted from each fraction was coated on NUNC Maxisorp wells (1 μg/well) and probed with HRP conjugates of anti-human immunoglobulins.

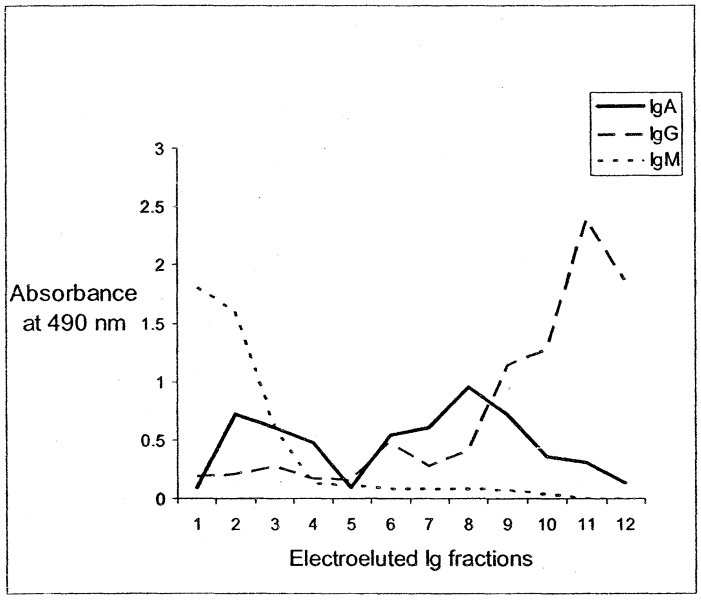

Fig.4. **Immunoglobulin composition of Dextran binding immunoglobulin.** Dextran binding immunoglobulin was subjected to acid PAGE at pH 4.5 for 8 hours and gel cut into 12 equal fractions. Proteins electroeluted from each fraction coated on NUNC Maxisorp wells (1 μg/well) and probed with HRP conjugates of anti-human immunoglobulins.

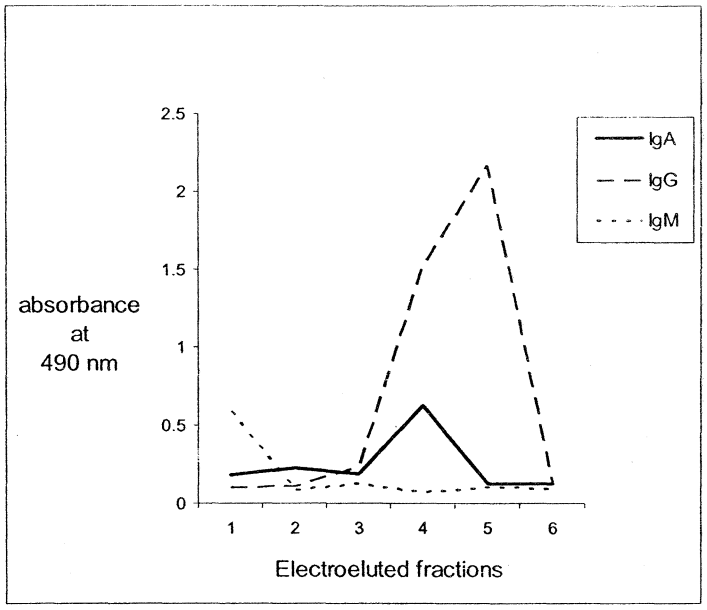

Fig.5. **Immunoglobulin composition of Anti α-galactoside antibody.** Anti α-galactoside was subjected to acid PAGE at pH 4.5 for 8 hours and gel cut into 16 equal fractions. Proteins electroeluted from each fraction was coated on NUNC Maxisorp wells (1 µg/well) and probed with HRP conjugates of anti-human immunoglobulins.

ABG was isolated at a yield of i.e., 4-7 mg/100 ml plasma. ABG has significantly higher IgA content in comparison with total serum Ig leading to a three times higher IgA to IgG ratio (Geetha et al., 2007). Electrophoretic pattern shows that anti-β glucan consists of IgA in polymeric as well as monomeric form (Fig.3).

High circulating concentrations of dextran-binding immunoglobulin (3-6 mg/100 ml plasma) is seen in donors who had no history of exposure to dextran infusions. DIg consists of IgA in the polymeric and monomeric form eventhough IgM forms the major immunoglobulin component (Fig.4).

Anti α- galactoside was isolated at a yield of i.e., 0.8-1 mg/100 ml plasma. Anti α- galactoside present in IgG, IgA and IgM forms (Hamadeh et al., 1995) are devoid of any polymeric IgA content (Fig.5). The above results indicate that presence of polymeric forms of IgA in the anti-glycan antibodies which make up a large

portion of the natural antibody repertoire is observed. It is possible that anti-glycan antibodies that constitute such a high concentration are the major source of polymeric IgA in serum.

The presence and/or the intensity of the stimulating signal would play a more important role in p-IgA production than the route of antigenic exposure (Mascart-Lemone, 1987). Also T-dependency is another factor that determines isotype switching. Even though terminal α-galactoside epitope is a carbohydrate antigen, being presented as part of glycoproteins or glycolipids its immune response is T-dependent, and hence IgG class forms the main component in the antibody population (Cretin, N., et al. 2002). Eventhough direct T-cell help is reportedly absent in eliciting DIg and ABG, non-cognate T cell help directs the B cell to get class switched to IgA and IgG. The linear and repetitive nature of antigenic determinants in dextran and β-glucan which offer epitopes to these antibodies can be another reason for production of antibodies with multiple valencies as that occurs in polymeric IgA. Anti-Gal, on the other hand is elicited by a single terminal α-linked galactose moiety.

Since these natural antibodies occur in such a high concentration, immune complex formation with their corresponding antigenic epitopes can possibly result high pIgA-IC in circulation.

Quantitation of IgA monomer content in polymeric and monomeric IgA

Direct IgA inter monomeric contact can occur to some extent since IgA polymers lacking J chain have been observed (Tomasi and Czerwinski, 1976). J chain is disulfide-bonded to only two of the subunits of polymeric IgA and remaining subunits in the higher polymers are disulfide-bonded one to the other. Limited

reduction of serum polymeric IgA using 2ME does not bring out dissociations into H, L, or J chains, suggesting that the interchain disulfide bridges between H--H, L--H, and H--J were intact and that 2ME produced selective cleavage of intersubunit bonds (Hauptman and Tomasi,1975).

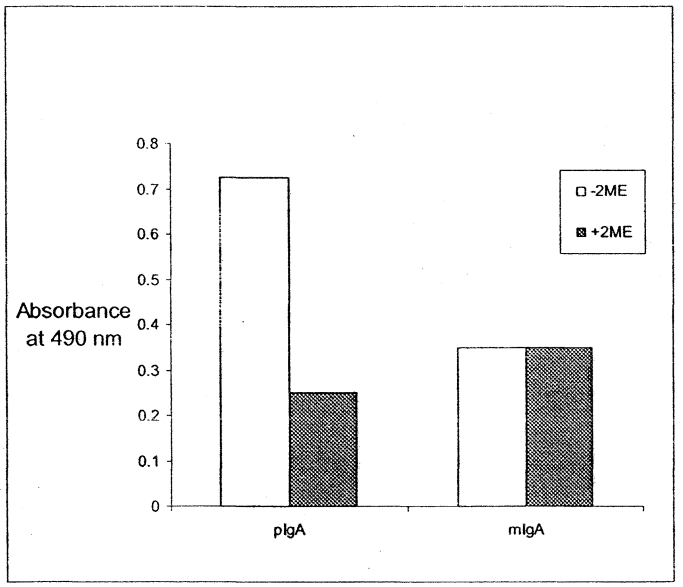

Fig.6.Quantitation of unit monomer content in polymeric IgA. One μg of the electroeluted protein solution corresponding to Polymeric and monomeric IgA (Obtained from ABG by acid PAGE and ELISA) was treated with 3mM of 2-ME at 37°C for 1h and coated on NUNC-Maxisorp wells and was probed with anti-IgA – HRP (500X).

Before ascertaining the difference between polymeric IgA and monomeric IgA in their capacity as ligands for lectins, anti-IgA recognition of the two was studied. Polystyrene well coated polymeric IgA was far superior to its monomeric form (coated after reduction of the polymer with 3 mM 2ME). Antibody response of originally monomeric IgA coated without prior 2ME reduction was comparable to that of reduced polymeric IgA (Fig.6). Higher antibody binding to polymeric Ig may be a result of subunit clustering. In this study, treatment with 2ME (3mM) reduces the intersubunit disulphide bonds and cleaves apart polymeric IgA subunits which helps

to quantitatively assess its unit monomer content. Results suggest that the response of polymeric IgA to anti-IgA HRP is 2.4 times more than that of the monomeric form (Fig.6).

IgA recognition by lectins in terms of unit monomer content.

It was reported from this laboratory that the presence of core-I O-glycan structure in the hinge region of IgA1 makes it a potential ligand for tissue expressed galectin-1 (Sangeetha and Appukuttan, 2005). Neuraminidase enzyme produced by various pathogens like influenza virus cleaves the α-linked N-acetyl neuraminic acid exposing the T antigen (Galβ-1,3GalNAc-α) which is an efficient ligand for galectin-1. Neuraminidase produced by *C. perfringens* that cleaves α -2-3 linked sialic acid residues is used in this study. Sialidase activity together with free sialic acid has been detected in the serum of patients with acute post streptococcal glomerulonephritis (Rodrigues-Iturbe et al., 1981).

Before polymeric IgA and monomeric IgA were compared in terms of their capacity to act as ligands for galectin-1, activity of another similar lectin i.e., peanut agglutinin (PNA) was checked. PNA is remarkable in its specificity for T antigen provided this group is not substituted by sialic acid moieties (Chacko and Appukuttan, 2001).

PNA-HRP response for unit monomeric IgA is 1.6 times more in the case of polymeric IgA compared to monomeric IgA fraction (Fig.7). Sialic acid substitution fully abolishes binding of this lectin to T antigen. PNA resembles galectin-1 in being more reactive to α-anomer than to β-anomer among methyl and para-nitrophenyl derivatives of galactose and the recognition of O-linked rather than N-linked

oligosaccharides among glycoconjugates (Chacko, B.K. and Appukuttan, P.S. 2001). Enhancement of the recognition by PNA-HRP upon neuraminidase treatment is 2.77 times more in the case of polymeric IgA than in the case of monomeric IgA (Fig.8). Desialylation increases PNA binding by 1520% for polymeric IgA and 750% for monomeric IgA.

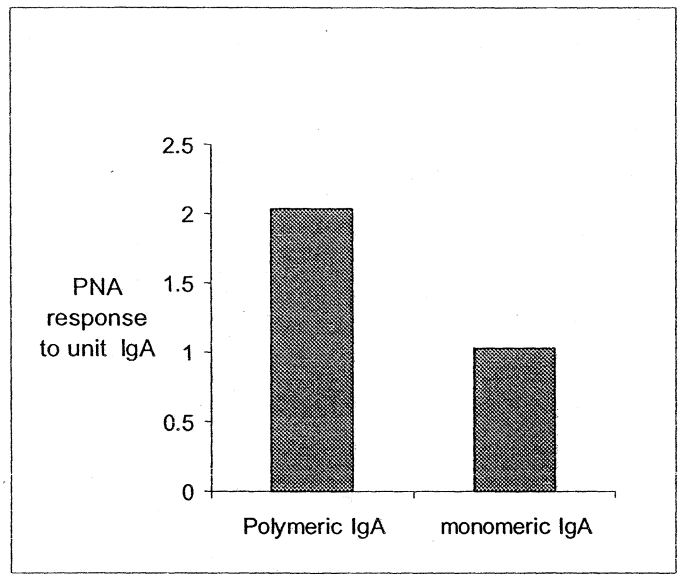

Fig.7. Peanut agglutination (PNA) response to unit monomeric IgA. One µg/ml monomeric and polymeric IgA (Obtained from ABG by acid PAGE and ELISA) coated on microtitre wells, probed with PNA-HRP. Response for unit monomeric IgA calculated using the formula,

PNA response for unit IgA

$$= \frac{\text{Absorbance value after neuraminidase treatment}}{\text{Absorbance value for mIgA/pIgA after 2ME treatment}}$$

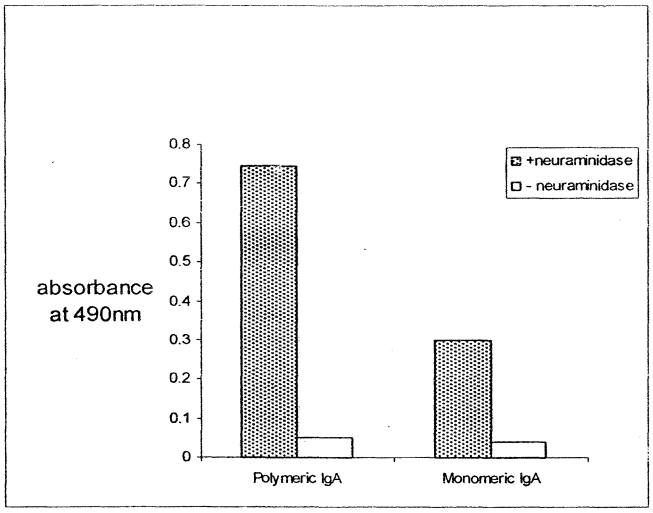

Fig.8. Effect of desialylation of immobilized IgA on its recognition by Peanut agglutinin. One µg of the electroeluted protein solution corresponding to polymeric and monomeric IgA (Obtained from ABG by acid PAGE and ELISA) treated with and without 5 mU neuraminidase coated on microtitre wells, probed with PNA-HRP (200X).

Since many infectious pathogens secrete the enzyme neuraminidase, such infections are likely to cause desialylation of IgA or its immune complexes. This event can enhance the recognition of IgA by galectin-1 (Sangeetha and Appukuttan, 2005). Since anti-polysaccharide antibodies are rich in polymeric forms of IgA, binding affinities of galectin-1 to monomeric and polymeric IgA in native and desialylated state were compared. Galectin-1 isolated from human placenta using lactose Sepharose column was used for the study. Binding of HRP-conjugated galectin-1 to polystyrene plate-coated IgA was studied. Results (Fig.9) shows that in native (non-desialylated) form, polymeric IgA was 1.71 times more efficient than monomeric as a ligand for galectin-1, when same protein contents of both were coated. Enhancement of the recognition by human placental lectin-HRP upon neuraminidase treatment is 2.66 times more in the case of polymeric IgA than for monomeric IgA (Fig.7).

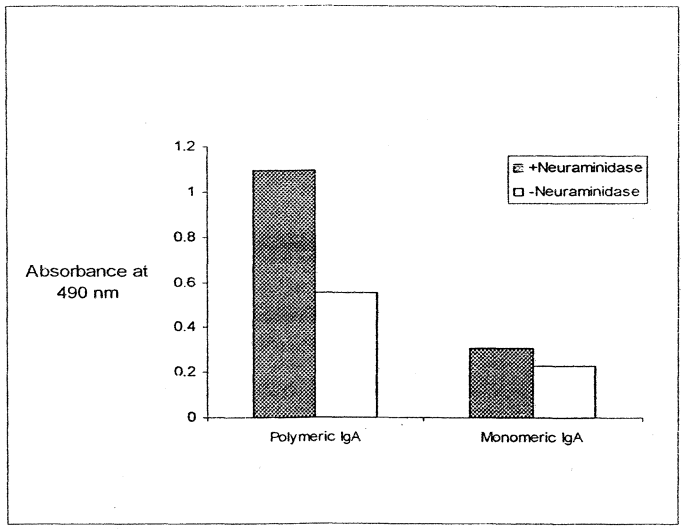

Fig.9. Effect of desialylation of immobilized IgA on galectin-1 attachment. 1μg/well of the electroeluted protein solution corresponding to polymeric and monomeric IgA coated on wells, treated with and without 5mU neuraminidase, probed with human placental lectin- HRP.

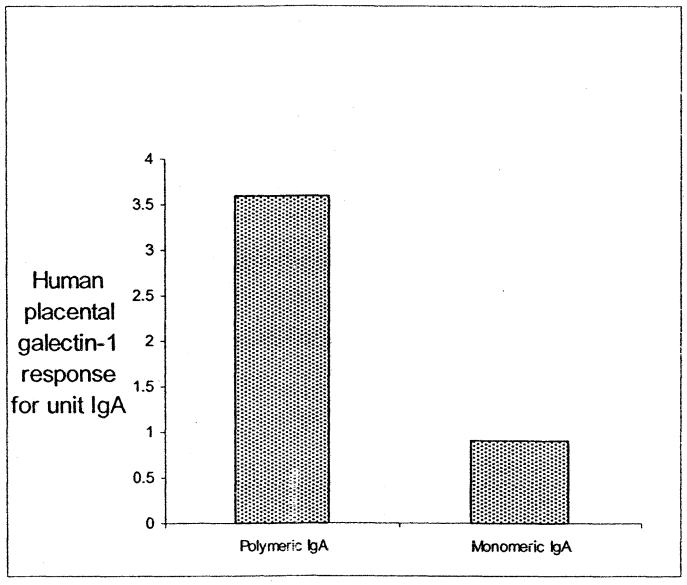

Fig.10. Human placental lectin (galectin-1) response for unit monomeric IgA. 1 μg/well of the electro eluted protein solution corresponding to polymeric and monomeric IgA treated with and without 5mU neuraminidase was coated on wells, probed with human placental lectin- HRP. Response for unit monomeric IgA calculated using the formula,

$$\text{HPL response for unit IgA} = \frac{\text{Absorbance value after neuraminidase treatment}}{\text{Absorbance value after 2ME treatment}}$$

HPL-HRP response for unit IgA after desialylation is 2.7 times more in the case of polymeric IgA compared to monomeric IgA fraction (Fig.10).An increase in polymeric content may follow many infections and diseases like diabetes. Desialylation is also known to accompany infections and diabetes. Since both an increase in polymeric IgA content and desialylation enhance IgA recognition by galectin-1, the result points towards the chance of IgA1-rich immune complexes to get attached to tissue galectin-1 more efficiently in the above disease states.

Discussion

Circulating immune complexes with a molecular composition exceeding Ag_2Ab_2 are preferentially cleared by the hepatic reticuloendothelial system. Opsonized immune complexes bind to red cells through CR1 and this process facilitates the transport of complexes within the circulation. As the amount of serum complexes increases further, saturation of the reticuloendothelial system occurs and thereby the likelihood of their non-hepatic tissue deposition is enhanced (Haakenstad and Mannik., 1974). Since IgA does not activate complement through the classical complement pathway, erythrocytes have a very limited role in the binding of IgA-IC (Matsuda, S., et al. 1988). The existence of specific receptors on Kupffer cells mediates the binding and clearance of IgA-IC. The galactose-terminating glycans are ligands for the asialoglycoprotein receptor (ASGP-R), which mediates the clearance of IgA from the serum (Basset.C et al., 1999, Stockert.R.J., 1995). N-glycans alone mediate the removal of glycoproteins through ASGPR. IgA exposed to free neuraminidase would be denuded of sialic acid and cleared by hepatic asialoglycoprotein receptor (ASGPR) (Bhatia, A and Kast, 2006). In man, liver

disease is frequently associated with marked elevations in serum IgA including pIgA. But the removal through ASGPR accounts for turnover of a very minor fraction of IgA1 unlike in the case of IgA2 though serum half lives of both are similar (Kerr, M.A, 1990).

Fc α RI receptors present on the monocytes, neutrophils as well as macrophages in tissues have a role in the catabolism of IgA-IC (Otten, M.A. and van Egmond, M. 2004). Mesangial cell receptors reportedly have a role in IgA1 catabolism. Novak et al., suggested that mesangial cells possess receptors that are different from both FcαRI (CD89) and ASGP-R and had higher affinity for circulating immune complexes than for uncomplexed IgA (Novak et al., 2002).

Other known receptors for IgA are polymeric Ig receptor on epithelial cells in the mucosa, Fcα/μR on the majority of B lymphocytes and macrophages and transferrin receptor. Saturation or dysfunction of those receptors might contribute to the increased serum levels and glomerular deposition of circulating, soluble IgA-IC reported in patients with IgA nephropathy, Henoch -Sc□hnlein purpura, and glomerulonephritis associated with alcoholic liver cirrhosis. Reports say that in IgA Nephropathy, IgA molecules found in the glomerular mesangium are polymeric IgA1 (Tomino et al., 1982). Results show that sialylation increases the binding nature and more so in the polymeric IgA. IgA1 from patients was found to be relatively abundant in asialotype sugar chains (Iwase and Hiki., 2002). The IgA deposited in glomerular and other lesion sites was found to be exclusively of IgA1 class (Mestecky et al., 1986). When injected into experimental rats desialylated IgA1 in contrast to native IgA1 accumulated in glomeruli (Sano et al., 2002).

In the glomerulus of kidney blood is filtered through slit membrane between foot protrusions of endothelial cells, basement membrane and epicyte. Galectin-1 (Gal-1) is a newly identified component of the slit diaphragm, binding to the ectodomain of nephrin. It is speculated that podocytes are a major site of biosynthesis of Gal-1 in the glomerulus, either as a free molecule or in complex with its interacting components. (Shimizu,M et al., 2009). The occurrence of galectin-1 in glomerulus at which IgA1 gets deposited especially in the desialylated form led us to investigate the possibility of sugar-dependent anchoring of IgA1 on galectin-1.

Since the results described in this chapter show that both polymeric and desialylated state of IgA1 are factors leading to its far higher binding to galectin-1, the evidences cited above suggest a role for galectin-1 in IgA deposition in tissues. Significantly, a higher binding by IgA1 to form immune complexes does not engage its carbohydrate side chains.

Circulating IC have been noted as a major predisposing factor for cardiovascular diseases (Mustafa, A., et al. 2000, Szondy,E et al., 1981). IgA levels have been found to be significantly higher in subjects with severe atherosclerosis than in controls (Muscari et al., 1989). The increase in total serum IgA in the presence of severe atherosclerosis is secondary to the occurrence of major ischemic events, possibly reflecting a protracted production of antibodies against antigens generated by the ischemic process (Muscari et al., 1993). The atherosclerotic intima contains a small amount of tissue-bound IgG and IgA (Hollander et al., 1979). Indeed, circulating immune complexes have been noted as a major factor predisposing patients to cardiovascular disorders (Mustafa A., 2000). Prevalence of seropositivity of *Chlamydia pneumonia* was higher in patients with atherosclerotic plaque and one

of its markers *Chlamydia pneumonia* IgA may be a predictor of atherosclerosis in these vessels (Jha et al., 2008). Chronic active infection has a role in atherosclerosis and Cp.IgA is a predictor of plaque formation. Association between anti-milk IgA antibodies and atherosclerosis was shown by Muscari et al (1989).

Vascular inflammation and endothelial activation are noted as important mediators of elevated CHD risk (Rowley, K et al., 2003). Galectin-1 is expressed on endothelial cell surfaces with enhanced expression upon activation of the cells (Baum, LG., 1998). Even though the significance of galectin-1 expressed in endothelium on T-cell biology is studied well, its capability to capture IgA as a receptor and mediate further inflammation is not done yet.

Tissue fibronectin, as well as laminin, serve as endogenous ligands for galectin-1, suggesting that galectin-1 may play a role in assembly of the extracellular matrix, or in the control of cell adhesion based on lectin-extracellular matrix interaction (Ozeiki,Y et al., 1995).

Altogether it can be inferred that galectin-1 could play important roles in the catabolism as well as pathogenesis of IgA in free or complexed form.

The observation that IgA1 in the polymeric form acts as better ligand for galectin-1 is significant to both the normal biology as well as the IgA-mediated immune pathology. Incidently, though IgA is the most synthesized and most turned over immunoglobulin type in serum, biological fate of IgA1 subtype that makes up more than 85% of total IgA is much less clear than that of IgG or IgA2 (Monteiro and van de Winkel, 2003). A role for galectin-1, well expressed and widely distributed in human tissues including the endothelial cells and mesangium, in sequestering the most prominent T antigen-bearing serum glycoprotein, IgA1, seems very likely.

Part II

Identification of polysaccharides from commensal and infectious microorganisms that offer antigenic epitopes for anti-glycan antibodies (AGA) and form immune complex(IC) *in vitro*

Environmental microbial load offers abundant carbohydrate epitopes that can elicit the production of large quantities of anti-carbohydrate antibodies. Warm and humid tropical climate is favourable for the survival of microorganisms including fungi in large numbers which possess carbohydrate epitopes like β- glucans on their cell wall structures. Glycan epitopes in the form of polysaccharides, glycoproteins or glycolipids are not only abundant in microbial, dietary or environmental antigens, but are also easily available for recognition by cognate biomolecules due to their hydrophilicity and exposure to the extra cellular environment. As a result anti-carbohydrate antibodies of varying specificities form a considerable part of circulating human immunoglobulins (Chacko, B.K. and Appukuttan, P.S. 2003, Geetha, M., et al. 2007, Springer, G.F. 1984).

The high titre of ABG, DIg and Anti-gal isolated from normal human plasma indicates chances of their meeting with corresponding epitopes to form circulating immune complexes (cIC) *in vivo*. Immune complex (IC) formation mediated by natural antibodies represents front-line immune defense mechanism against pathogens. Due to the high representation of pIgA in anti-glycan antibodies like ABG and DIg, the AGA-IC are enriched in pIgA, eventhough their physiological relevance in combating infection is less clear than that of ICs formed of the complement fixing IgG/IgM. Under normal physiological conditions, ICs are removed effectively from circulation by the RBC mediated transport of IC to macrophages in spleen and liver. Erythrocytes have a very limited role in binding IgA immune complexes (Matsuda, S., et al. 1988). Reports indicate that kupffer cells contribute to the clearance of IgA-IC by phagocytosis (Rifai, A. and Mannik, M. 1984). Since systemic mechanisms for clearance of IgA-IC are saturable, high concentrations of IgA-IC in the circulation

may lead to their deposition in extrahepatic tissues, as implicated in the pathogenesis of IgA nephropathy (Emancipator, S.N. and Lamm, M.E. 1989). In this context it is relevant to investigate the pathogenic potential of the overburden of IgA-IC in the context of emerging reports on IgA deposition-mediated diseases.

Commonly occurring environmental sources that can offer antigenic epitopes to these antibodies were sought as part of the study. Fungal β- glucans are variably but commonly present in food, environment and in human-commensal microorganisms, mostly complexed with other constituents, in particular with proteins (Ishibashi et al., 2005). β-glucan enters the bloodstream from food containing baker's yeast. *Saccharomyces cerevisiae*, the common 'bakers's or 'brewer's yeast consists of β-glucan as part of its cell wall. *S. cerevisiae* is a common colonizer of mucosal surfaces and part of the normal flora of the gastrointestinal tract, the respiratory tract, and the vagina. Its presence in normally sterile fluids has been classically described in patients with rupture of the local barriers or with very high fungal loads. Portals of entry include translocation of ingested microorganisms from the enteric or oral mucosa and contamination of intravenous catheter insertion sites. The most consistent risk factor for *S. cerevisiae* fungemia is the use of probiotics (Munoz, P., et al. 2005). Crohn's disease, an inflammatory bowel disease is characterised by the presence of large quantities of anti-*S. cerevisiae* antibody in serum (Walker, L.J., et al. 2004). It has been noted that limiting the uptake of *S.cerevisiae* containing food can improve the prognosis of this disease. Cell wall of *S. cerevisiae* consists of mannoproetins of varying molecular weights to which β-glucan is attached via phosphodiester linkages.

Fungal pathogens like *Aspergillus* Spp. which consists of β –glucan in the cell wall is abundantly present in tropical atmosphere. *Candida albicans* is another prospective source of this glycan epitope and hence used as a main tool for this study.

C. albicans is an opportunistic pathogen in humans that takes a lead role in infection once the immune system gets compromised. The major components (80-90%) of the cell wall of *C. albicans* are carbohydrates ;(1) mannan or polymers of mannose covalently associated with proteins to form glycoproteins, also referred to as mannoproteins; (ii) β-glucans and (iii) chitin. Protein (6 to 25%) and lipids (1 to 7%) are present as minor cell wall constituents. Yeast cells and germ tubes are similar in their cell wall composition, although the relative amounts of β-glucans, chitin and mannan may vary with the morphology. Quantitatively β-glucans constitute 47 to 60% by weight of the cell wall. Evidences indicate that mannoproteins may establish covalent associations with β (1→3) - and β (1→6) –glucans through phosphodiester linkages. A complex array of protein-containing components has been solubilized from isolated cell wall preparations and from intact cells of both candidal growth forms. Several studies have identified 20-40 polypeptide species in the medium-to-low-molecular-mass (from 80 to 15 kDa) range. Several mannoprotein species within a molecular mass range of 58 to 180 kDa are also detected. Increasing or persistently high glucan levels (inspite of antifungal treatment) appear to be indicators of more severe outcome (Obayashi, T., et al. 1995). Candida albicans glucan has a higher percentage of β-1,6 crosslinks than that seen in *S. Cerevisiae*.

While β-glucan is in itself a very poor immunogen, its presence as a moiety in complex, microbial cellwall molecules, particularly proteins (Klis, F.M., et al. 2001), makes it a natural glycoconjugate, eventually enhancing its immunogenicity. While

there is plenty of data on the presence in human serum of high levels of antibodies against fungal mannans or mannoprotein (Greenfield, R.A., et al. 1983) very few data have been so far generated about the presence of anti- β -glucan antibodies (Ishibashi, K., et al. 2005, Kondori, N., et al. 2003).

A high quantity of β-glucan in serum is considered as a marker of fungemia. It has been reported that even the sera of normal healthy adults contains 10-20 pg/ml of β–glucan. Earlier studies failed to demonstrate presence of anti-β-glucan antibodies even in yeast-infected persons and led to the conclusion that β-glucan is not exposed on *Candida* cell surface (Ballou, CE.1982). Recent studies on the physiological roles of ABG do not focus on IgA, mostly owing to the fact that IgA does not mediate effector functions like IgG/IgM.

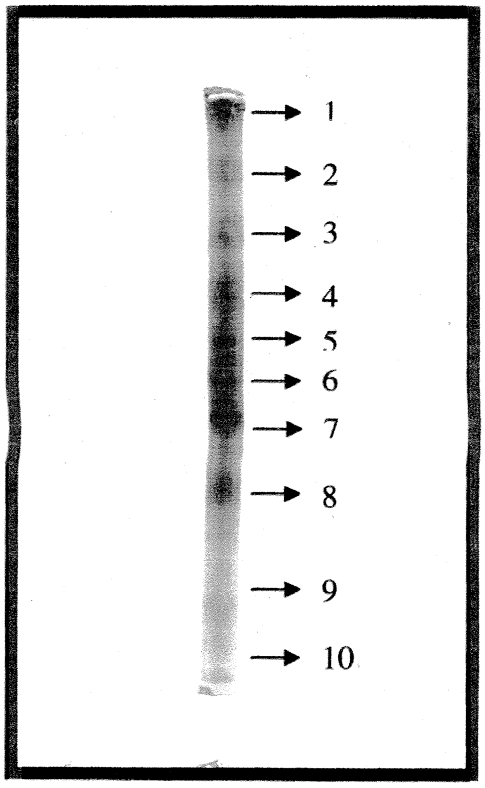

Fig.11. Separation of yeast glycoproteins (YGP) by alkaline PAGE. Cell surface glycoproteins from *S. cerevisiae* were isolated as described in Methods and alkaline PAGE was done at pH 8.3 in 7% gel.

Ten bands were seen in electrophoresis (Fig.11) and their corresponding protein portion was cut from unstained gels, and protein obtained by electroelution.

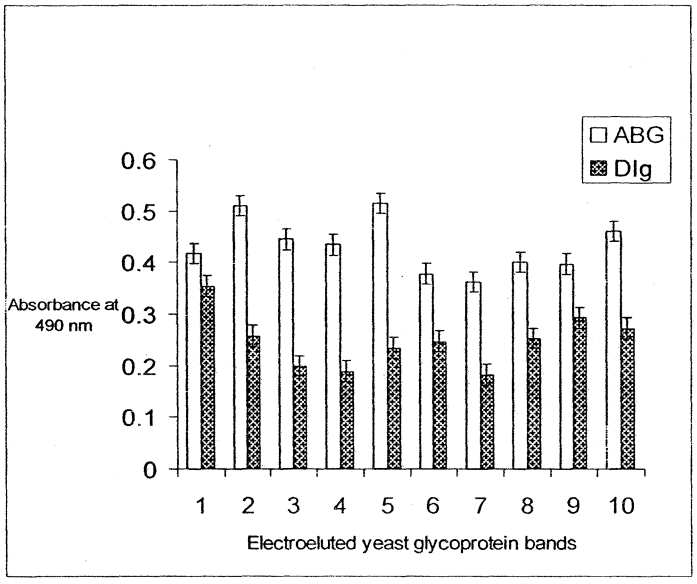

Fig.12. Carbohydrate –dependent recognition of yeast glycoproteins by ABG-HRP and DIg -HRP. One microgram each of the ten electroeluted yeast glycoproteins was coated on NUNC ELISA plate wells and probed using ABG-HRP (3μg antibody/ml) and DIg-HRP (3μg antibody/ml). Values are mean ± S.D of six trials.

The presence of antigenic epitopes for ABG and DIg was checked in the electrophoresed YGP. Both of these anti-carbohydrate antibodies recognize yeast glycoproteins (Fig.12), even though the response was higher in the case of ABG-HRP. This is expected since β-linked glucans form majority of the cell wall of *S.cerevisisae.* The recognition of YGP by DIg can be attributed to the multiple specificity of this α (1,6) linked glucan specific antibody to β (1,3) linked glucans also.

The carbohydrate dependency of the YBG recognition by ABG was confirmed by inhibiting the binding of ABG to coated Candida Cellwall Antigens (CCA) using different fractions of yeast glycoproteins (Table 1.)

No. of the YGP protein band	Concentration required for 50%inhibition
Band No.1	4.6μg/ml
Band No.2	1.8μg/ml
1-*O*-Methyl β-D glucoside	**1164 μg/ml**
Cellobiose	**194 μg/ml**

Table 1. Inhibition of ABG binding to *Candida* cellwall antigen (CCA) by yeast glycoproteins. Two micrograms CCA coated on ELISA plates were treated with ABG (2 μg/well) which had been pre-incubated for 1h at 4^0 C with and without serial dilutions of yeast glycoproteins and bound ABG estimated using a mixture of HRP conjugates of goat anti-human immunoglobulin.

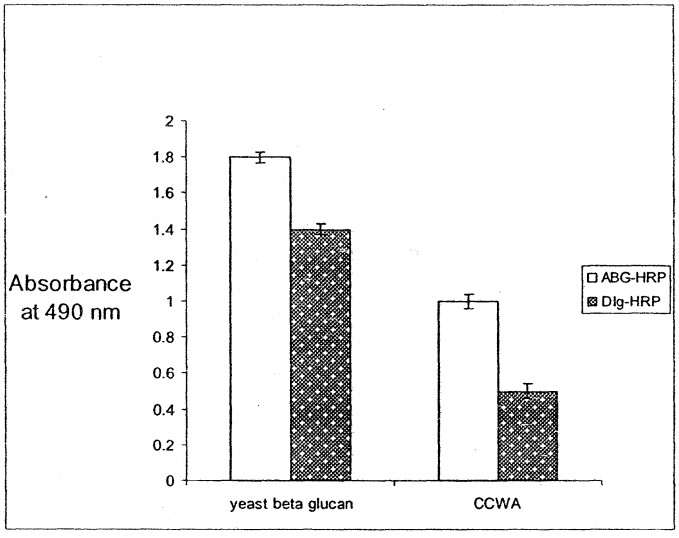

Fig.13. Comparison of binding of ABG-HRP and DIg-HRP to coated yeast β-glucan and CCA. CCA (1μg/well) (2) and yeast β-glucan (0.5μg/well) coated on ELISA plate wells were probed with DIg-HRP and ABG-HRP (3 μg/ml antibody). Values are mean ± S.D of six trials.

Glycoconjugates present in wild yeast offers efficient epitopes for the two anti-carbohydrate antibodies studied (Table 1). ABG was found to be more efficient than DIg in recognizing the glycoconjugates of *Candida albicans*. Comparative study shows that yeast β-glucan is far better than CCA in ABG/DIg binding (Fig.13). Though CCA used is crude isolate while yeast β-glucan used for the study is a

purified form purchased from Sigma. The former has been found to be >95% carbohydrate by mass. Results in table 1 suggest that as ligands for ABG, yeast polysaccharides are superior to the best mono- and disaccharide of the antibody by orders of 10^3 and 10^2 respectively. The yeast polysaccharide from fractions 1 and 2 (Table 1) were used for comparison since they were leading constituents of YBG.

Dextran-like glucans form part of bacterial, yeast and fungal surface antigens (Sutherland, I.W. 1985). Dextran is yet another polysaccharide that provides linear epitopes for natural antibody recognition. It is a typical example for T-independent type 2 antigen. Dextran is produced from sucrose by *L.mesenteroides* which contaminates the sugar cane during processing. To detect dextran-like molecules in edible sugar, non-dialysable portion that was left after removal of sucrose by extensive was isolated as described in Methods. Commercially available high molecular weight dextran was used as standard. Presence of antigenic epitopes for ABG and DIg in non-dialysable sugar (NDS) was checked.

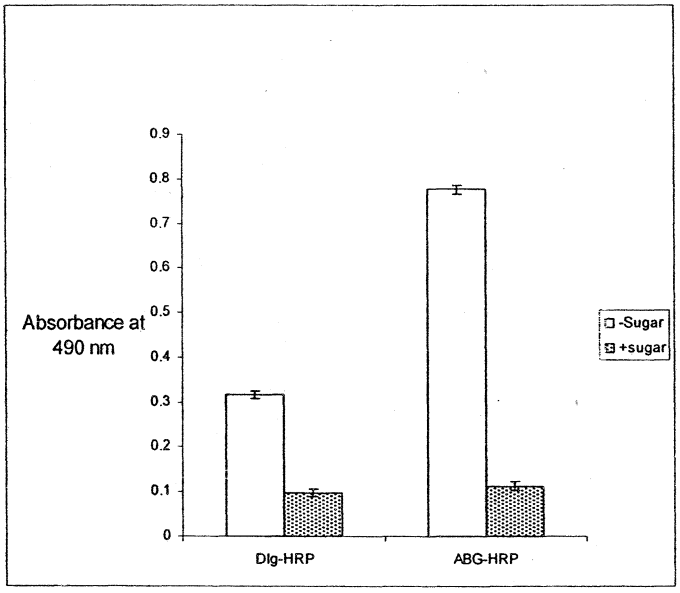

Fig.14. Antigenic epitopes for ABG and DIg from edible sugar. Two µg/well NDS coated on microtitre plates, probed with DIg-HRP and ABG –HRP with and without sugar inhibition (50 mM 1-0-Methyl alpha D- glucoside for DIg and 50 mM Cellobiose for ABG) . Values are mean ± S.D of six trials.

Results suggest that non-dialysable portion of the edible sugar contains epitopes for ABG and to a lesser extent for DIg (Fig.14). Reversibility of the binding by inhibitory sugars confirms the specificity of binding. Even though these antibodies show anomer preference, their multiple specificity, which is characteristic of natural antibodies, enables them to bind to various epitopes present on microbial cell surfaces.

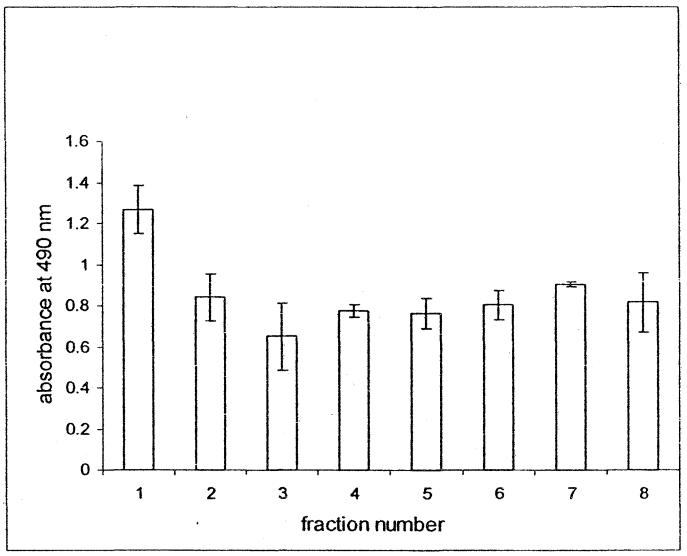

Fig.15. Binding of DIg to non-dialysable fraction from table sugar. Non-dialysable sugar (NDS) fractions isolated by polyacrylamide tube gel electrophoresis at alkaline pH and passive elution (Methods) were coated on to polystyrene microtitre wells after ten times dilution and probed using DIg (1 µg per well) followed by HRP conjugates of goat antibodies to human immunoglobulins IgA, IgG and IgM. Values are mean ±S.D of six trials.

Leuconostoc mesenteroides that contaminates unhygienically processed sugar cane extract has been reported to synthesize dextrans using sucrose (Sidebotham 1974). NDS was present in all of 10 consecutive table sugar samples collected from grocery shops of this region and varied between 0.03 to 0.1 % (w/w) of total sugar

(Data not shown). NDS was separated into 8 components by electrophoresis. All the components were recognised by DIg indicating the potential of impure dietary sugar to form DIg-mediated immune complexes (Figure 15).

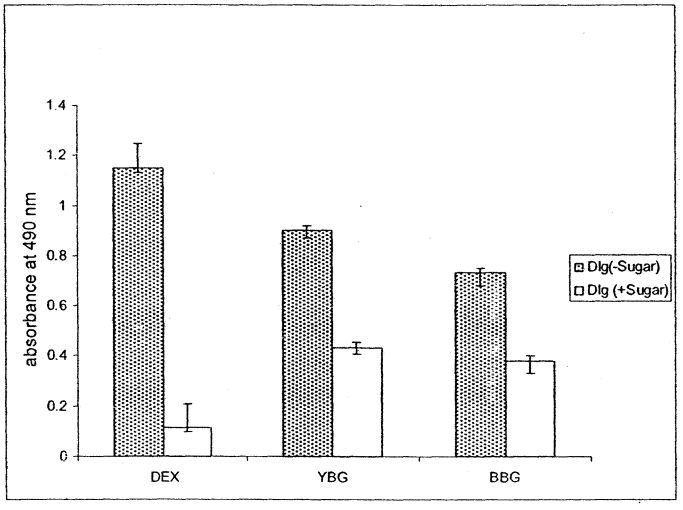

Fig.16. Relative affinity of DIg binding to dextran and β-glucans from yeast (YBG) and barley (BBG). Each sample was coated on NUNC ELISA plate wells (2 μg/well). DIg (1 μg) previously incubated with or without 50 mM αMG at 4^0C for 1 h was added to each well. After incubation for two hours at 4^0C, bound DIg was assayed using HRP conjugates of anti-human IgA, IgG and IgM. Values are mean ±S.D of six trials. P = 0.04

The affinity of DIg for α-linked glucose moiety was evident from the efficiency of various polysaccharides coated on polystyrene wells to capture the antibody (Figure 16). β-1,3 Glucans from yeast and barley were significantly less effective in capturing DIg than dextran which consists almost exclusively of α(1→6)- linked glucose moieties. Notably the reversibility of DIg binding with α MG also decreased from dextran to yeast and barley β –glucan (Figure 16). DIg is a natural antibody and hence multiple specificity is expected. Multiple specificity of an antibody is thought to be caused by the flexibility of the antigen binding pocket (Notkins, 2004). A well known example of a protein with specificity for two different carbohydrate epitopes is serum mannan binding protein (MBP) which accommodates both mannose and glucose

moieties (Summerfield and Taylor, 1986). In humans of A or O (H) blood group, synthesis of anti-blood group B antibody is not precluded so that their serum anti-Gal antibody has been reported to accommodate either α(1→3)- linked or α(1→4)- linked galactose (Galili et al.,1987a). DIg purified using an exclusively α (1→6)- linked glucose polymer is unique in accommodating the structurally distant β (1→3)- linked glucose in glycans.

Polyreactive antibodies are said to be more of IgM class. DIg preparation usin Sephadex G-100 consisted mostly of IgG and relatively little IgM. Since the occurrence c IgM was suspected more in DIg, modified affinity chromatography using Sephadex G-20 gel that offers pores that can accommodate IgM molecules as well was employed to prepa DIg.

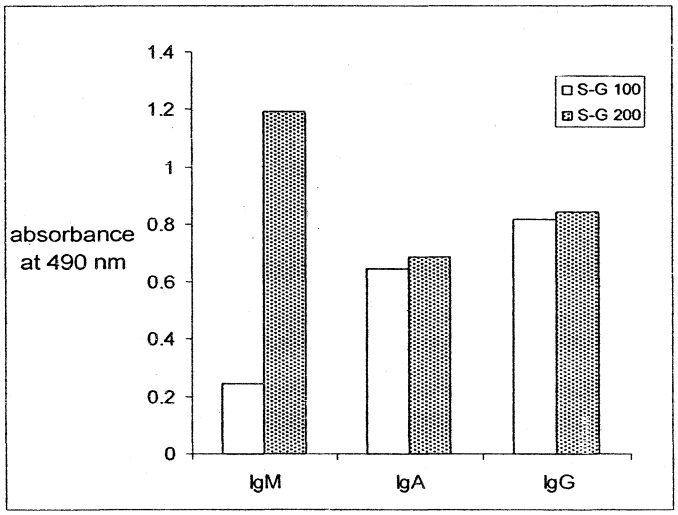

Fig.17. Comparison of immunoglobulin class in DIg prepared on Sephadex G-100 an Sephadex G-200. DIg was isolated from plasma of same donor using Sephadex G-100 ar Sephadex G-200 column. Anti-human IgA/IgG/IgM was coated on Nunc Polystyrene wel (0.5 μg/well). DIg isolated from both affinity chromatography columns was added to th wells (1 μg/ml) and probed using anti-human immunoglobulins separately (3 μg each/wel as per the coating.

The comparison study shows that there is tremendous increase in the yield of IgM in the DIg population prepared using Sephadex G-200 (Fig.17). Though usually used as a gel filtration column Sephadex here is used as an affinity matrix and is made up of dextran crosslinked by epichlorhydrin. Sephadex G-100 acts as a molecular sieve and molecules of larger dimension i.e. above 150,000 Da do not penetrate the matrices of swollen gel. Lower yield using Sephadex G-100 must have resulted from restricted access into the relatively smaller pores of this gel for the pentameric IgM that forms bulk of DIg. But Sephadex G-200 permits even IgM to pass through.

Results show that IgM yield is six times higher in Sephadex G-200. Yield of DIg employing Sephadex G-200 as affinity matrix was in the range of 4-6 mg from 70 ml plasma each from 12 donors, as against 0.9 to 1.4 mg obtained using Sephadex G-100 as matrix earlier (Chacko, B.K. and Appukuttan, P.S. 2003).

Demonstration of the formation of Glycan-Anti-glycan immune complexes using *Candida albicans* cellwall antigen (CCA).

In vitro studies were done to demonstrate the formation of anti-glycan antibody polysaccharide immune complexes between anti-β- glucan antibody on one hand and fungal polysaccharides on the other. The dimorphic fungus *Candida albicans* is both a commensal and opportunistic pathogen of humans. Candida species are found as the normal flora of skin, mouth, intestine and mucocutaneous junctions. Predisposing factors for Candidiasis include immunosuppressive, antibiotic and cytotoxic therapies, the presence of intravenous catheters and indwelling devices, very low birth weight, AIDS, diabetes, and drug abuse. Depending on the underlying host effect, this microorganism is able to cause a variety of infections that range from

mucosal candidiasis to life- threatening disseminated candidiasis (Martinez, J.P., et al. 1998).

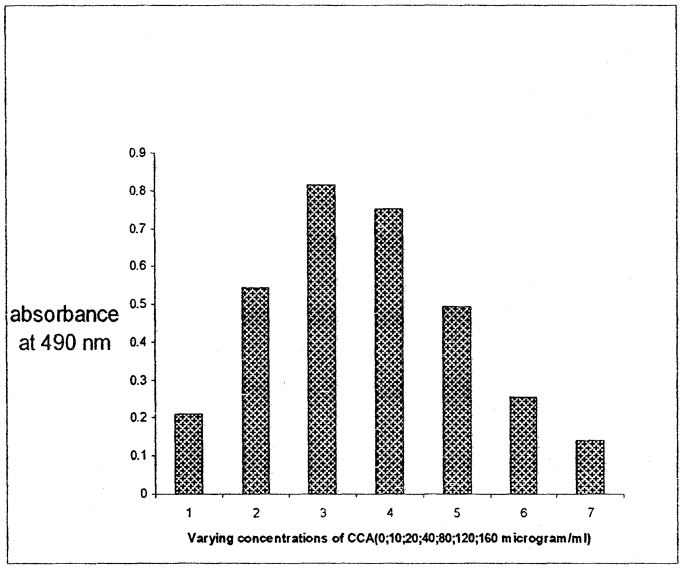

Fig.18. Immune complex formation in serum upon the addition of varying concentrations of CCA. Seven different concentrations of CCA (0, 10, 20, 40, 80 and 160 μg/ml respectively) were added to normoglycemic serum and processed as described in Methods. Two microgram per well coated CCA was treated with 20 x dilution of the IC-pellet and the bound antibodies were probed with a 1:1:1 mixture of HRP conjugates of anti-human Ig.

In order to isolate serum antibodies specifically reacting with CCA, increasing amounts of CCA were added to fixed volume of serum and immune complexes formed separated, and antibodies thereof analysed in terms of their capacity to bind to microplate coated CCA. Results show that, as the concentration of CCA increases, there is a corresponding increase in immune complex formation till the zone of equivalence is reached (Fig.18).

The cell wall of *Candida albicans* is not only the structure in which many biological functions essential for the fungal cells reside but also is a significant source of candidal antigens. Although cell-mediated immunity is often considered to be the

most important line of defense against candidiasis, cell wall protein and glycoprotein components also elicit a potent humoral response from the host that may include some protective antibodies. Thus, coating of fungal cells with host antibodies has the potential to influence profoundly the host-parasite interaction by affecting antibody-mediated functions such as opsonin-enhanced phagocytosis and blocking the binding activity of fungal adhesins for host ligands. The addition of CCA to serum from normal healthy individuals results in formation of ICs which can be precipitated by 2% Polyethylene glycol (PEG). Even though PEG precipitation is a non-specific method of IC isolation which works on the principle of solvent exclusion, the low concentration of PEG used in this study reduces the sensitivity and hence increases the specificity. CCA coated NUNC polystyrene wells used for the ELISA further enhances the specificity of the test. There is a minimal absorbance value seen even in the control wells were IC not incubated with CCA is added. This shows that a minimum amount of circulating IC contributed by CCA-reactive antibodies occurs in serum even without the intentional addition of the antigen. This result is not unexpected in the tropical climatic conditions where moist and warm atmosphere favours the growth of various saprophytic as well as pathogenic fungi. It should be noted that normal population has a minimum level of 10-20 pg/ml β-glucan in blood.

The specificity of the newly formed ICs was confirmed by adding glycogen as a control antigen to an aliquot of the serum tested (Fig.19). Result suggests that glycogen precipitates no more CCA-reactive antibodies than where no antigen was added and the hike in the response in cases of CCA addition was structure-specific.

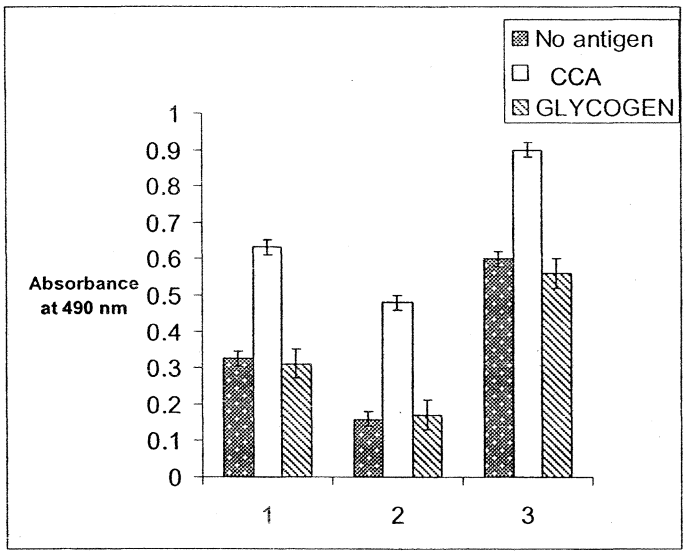

Fig.19. Comparison of immune complexes formed upon the addition of CCA and glycogen to serum. CCA and glycogen 20 µg/ml each was added to normoglycemic serum aliquots and processed as described in Methods. To Two microgram per well coated CCA 20 x dilution of the IC-pellet was added and the bound antibodies were probed with a 1:1:1 mixture of HRP conjugates of anti-human Ig. (3 µg of each). Mean +/-S.D of triplicate trials shown. $P < 0.05$.

The *C. albicans* cell wall may be envisaged as a dynamic and plastic, multilayered structure located external to the plasma membrane. The cell wall is the structure responsible for maintaining the shape that characterizes each growth form (yeast and hyphae) of the fungus. In addition it mediates the initial interaction between the microorganism and the environment.

Antibody production may be stimulated as a result of gastrointestinal colonization or other types of infection, and this may explain the presence of anti-Candida antibodies in the sera of healthy individuals (Martinez, J.P., et al. 1998). The occupation of the gastrointestinal tract by *C. albicans* result in release of candida cell wall carbohydrates like β glucan into circulation, which elicits the production of ABG. Most cases of systemic candidiasis are endogenous in origin with a continuum from colonisation to infection.

Effect of cellobiose and mannose on the binding of immune complexes to coated CCA was checked. Immune complexes to CCA were precipitated and inhibition studies were done as described in Methods.

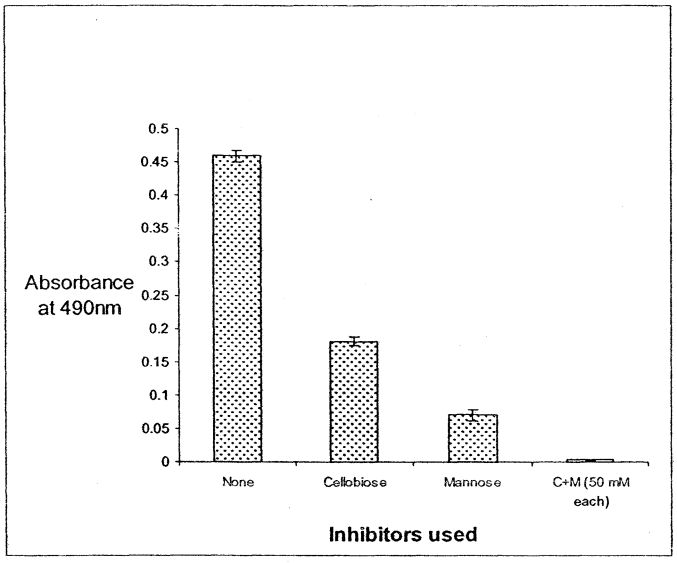

Fig.20. **Sugar-dependent binding of antibodies to CCA.** One ml serum to which 20 µg/ml CCA was added was kept at 37^0 C for 3 hours. After PEG precipitation and separation of proteins as described in Methods, 20X dilutions of the samples were added to Two µg CCA-coated wells with or without previous inhibition with the sugars and probed with HRP conjugates of antibodies to human IgA, IgG and IgM separately (3 µg each). (1) no inhibition, (2) inhibition with cellobiose 50 mM (3) inhibition with mannose 50 mM (4) inhibitions with both sugars together, each 50 mM. Mean +/-S.D of triplicate trials shown. $P < 0.05$.

Sugar inhibition using 50 mM cellobiose as well as 50 mM mannose brings about remarkable reduction in the binding of IC to the CCA-coated wells (Fig.20). This suggests that anti-β-glucan as well as anti-mannan antibodies which occur in the serum as natural antibodies are capable of forming immune complexes with the antigenic epitopes presented by *C.albicans*. Also both sugars together will bring about a synergistic effect depicting the presence of two types of antibodies. Anti-mannan antibodies were shown to be ubiquitous in human sera, presumably because the immune system can be stimulated as a result of colonization by *C.albicans* in the

absence of disease (Domer, J.E. 1989). Along with the anti-mannan antibodies, anti-β –glucan antibodies also have a major role in precipitating the immune complexes as evident from the above result. The role of anti-β-glucan in defence against fungal infections like *C.albicans* and *Aspergillus* Spp. was suggested by Ishibashi et al (Ishibashi, K., et al. 2005).

It was reported that interplay between protective and inhibitory antibodies dictates the outcome of experimentally disseminated Candidiasis in recipients of a *Candida albicans* vaccine (Bromuro, C., et al. 2002).They demonstrated that some anti-Candida antibodies i.e., anti-mannoprotein antibodies can block the protective potential of the immune serum, a potential to which anti-β-glucan antibodies appear to contribute.

Both laminarin (linear β-1,3 linked glucose polymer with occasional β-1,6 branches) and pustulan (linear β-1,6 linked glucose polymer)-like molecular structures are commonly present in β -glucans of most pathogenic fungi (Torosantucci, A., et al. 2005). The balance between antibodies against these two specificities is strongly influenced by the nature of the adjuvant and route of administration. The protective β-glucan epitope has a linear, β -(1,3), laminarin-like configuration (Chiani, P., et al. 2009). Anti-beta-glucan antibodies elicited by a laminarin-conjugate vaccine confer cross-protection to mice challenged with major fungal pathogens such as *Candida albicans, Aspergillus fumigatus* and *Cryptococcus neoformans*. The protective IgG2b selectively bound to β 1,3-linked (laminarin-like) glucose sequences whereas the non-protective IgM bound to β 1,6- and β 1,4-linked glucose sequences in addition to β 1,3-linked ones (Chiani, P., et al. 2009). The isotype of anti- β-glucan antibodies may affect the β-glucan epitopes recognized, and this may be associated with differing abilities to inhibit virulence attributes of the fungus and confer protection in vivo.

Altogether, anti-β -glucan antibodies are indeed commonly present in human serum, although with rather large individual subject variations. Different exposure to the different sources of this antigen, coupled with the possible different immunogenicity of the β-glucans present in the different sources, could account for the wide differences in individual antibody response. In human candidiasis, the value of anti-mannan and anti-β -glucan antibodies, and their interaction in terms of protection from fungal disease, are debated (Cassone, A., et al. 2005).

Results shown above prove that ABG-mediated immune complexes are present in normal individuals and are markedly elevated during fungal infections. Immunoglobulin class prevalence in CCA immune complexes in comparison with natural immune complexes was done for further characterisation of the immune complexes.

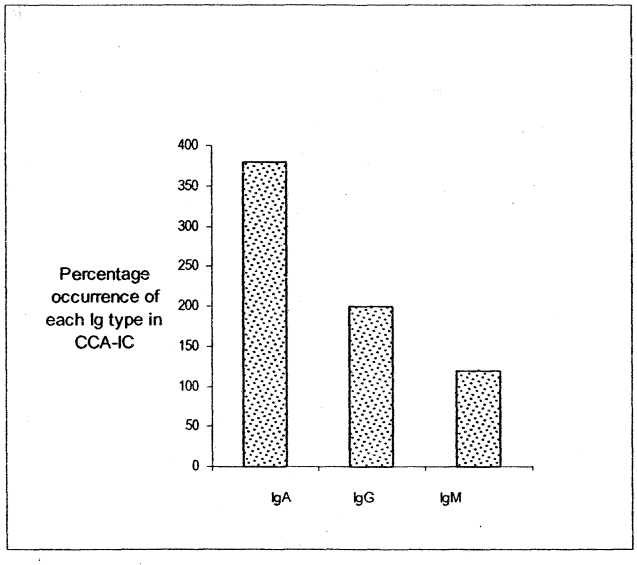

Fig.21. Immunoglobulin class composition of CCA-IC. To one ml serum 20 µg/ml CCA or PBS was added and incubated at 37^0 C for 3 hours. After PEG precipitation and separation of proteins as described in Methods, 20X dilutions of the samples were added to CCA -coated wells and probed with HRP conjugates of antibodies to human IgA, IgG and IgM separately.

Percentage increase in O.D was obtained as;

$$\frac{\text{(Absorbance for CCA-IC-absorbance for control IC)X100}}{\text{Absorbance for control IC}}$$

Percentage increase in each Ig type in IC formed upon addition of CCA compared to normal IC has been determined (Fig.21). Result suggests that IgA is the most prevalent immunoglobulin class in CCA- ICs unlike the natural immune complexes where IgG forms the major portion. Anti-mannan antibodies are formed T-dependently and are devoid of IgA in remarkable amounts (Durandy, A., et al. 1983). Hence it can be concluded that the high IgA in CCA-IC corresponds mostly to ABG.

Since β-glucans and related antigens reside on cell surfaces and are released into host circulation, frequent exposure to these organisms as commensals or systemic infectants, experienced by even normal individuals leads to a considerable increase in IgA-containing IC, contributed by ABG and related antibodies.

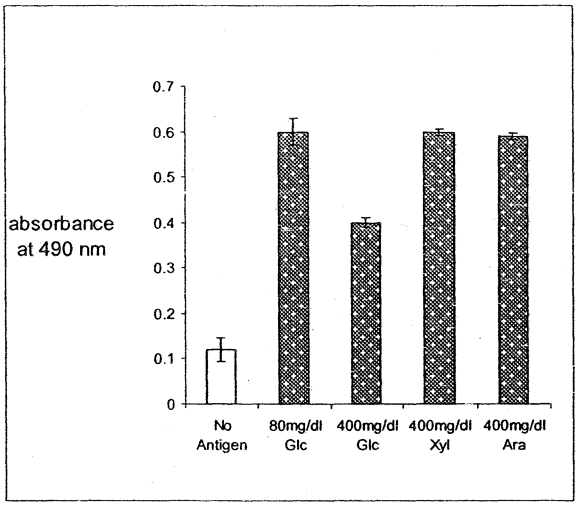

Fig.22. Effect of high serum glucose on CCA-IC formation. (Shaded blocks represent CCA added samples whereas unshaded one is the control.) Twenty μg/ml CCA was added to sera, which was pre-treated with (3^{rd},4^{th} and 5th blocks) and without(2^{nd} block) sugar. [1) No antigen added, 2) serum glucose level; 80mg/dl), 3) serum glucose level; 400mg/dl), 4) serum glucose 80mg/dl + xylose 320mg/dl, 5) serum glucose 80mg/dl+ 320mg/dl arabinose]. Two micrograms of coated CCA was treated with 20 x dilutions of the immune complex pellet and the bound antibodies were probed with a 1:1:1 mixture of HRP conjugates of anti- human Ig. Values are mean ± S.D of six trials. $P<0.05$

Inhibition of ABG binding to microplate-coated fungal polysaccharides by common sugars had been reported earlier from this laboratory (Geetha, M., et al. 2007). In the present study, inhibition of CCA-mediated IC-formation in serum by glucose and control sugars at different concentrations was attempted.

To rule out any colligative effect by high amounts of inhibitory sugar, equally high concentrations of arabinose and xylose to which ABG does not show specificity is used as controls (Fig.22). The minimal inhibition mediated by this non-specific sugar clearly indicates that very high glucose concentration is inhibitory to ABG. Serum to which glucose was added to a final concentration of 400 mg/dl which is usually attained in diabetes showed marked reduction in the IC formation compared to controls. Results point towards the possibility that high sugar in serum prevents the ABG population from forming IC. The marked reduction in IC formation in high serum sugar conditions indicates that glucose concentrations achieved in diabetic conditions can inhibit the ABG from taking part in IC formation. It is reasonable to postulate that inhibition of natural and front-line defence mechanism mediated by antibodies like ABG by high serum blood glucose may be the primary reason for the high susceptibility of diabetics to infections.

Diabetic serum samples were collected from Indian Institute of Diabetes Thiruvananthapuram, for further studies after obtaining informed consent from patients in prescribed format.

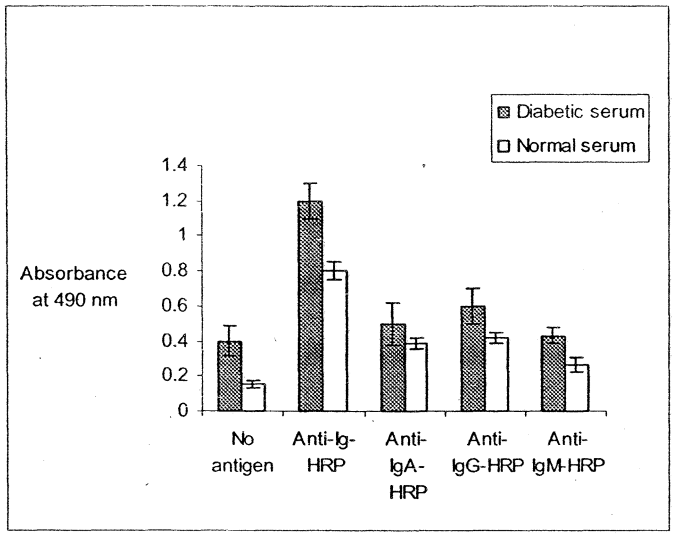

Fig.23. Comparison of CCA-IC from diabetic and normal serum To one ml serum 20 µg/ml CCA was added and incubated at 37^0 C for 3 hours. After PEG precipitation and separation of proteins as described in Method, 20X dilutions of the samples were added to CCA -coated wells and probed with HRP conjugates of antibodies to human IgA, IgG and IgM separately. Values are mean ± S.D of thirty trials. $P<0.05$

In diabetes there is a notable surge of IC formation following the addition of CCA in comparison to that in normal serum (Fig.23). Since previous data (Fig.22) showed that in normal sera artificially increased glucose concentrations keeps ABG from binding to added CCA, the pattern of CCA-IC formation in clinically confirmed diabetic patients was examined further.

Sugar inhibition pattern of CCA-precipitated antibodies varied widely among diabetic cases included for the study. In most cases there is only marginal inhibition by the sugars (Fig.24). Carbohydrate independent antibodies seem to bring about the elevated response in diabetic serum. Further characterisation of the CCA-IC was done by studying the IC-interaction with electrophoretically separated CCA fractions.

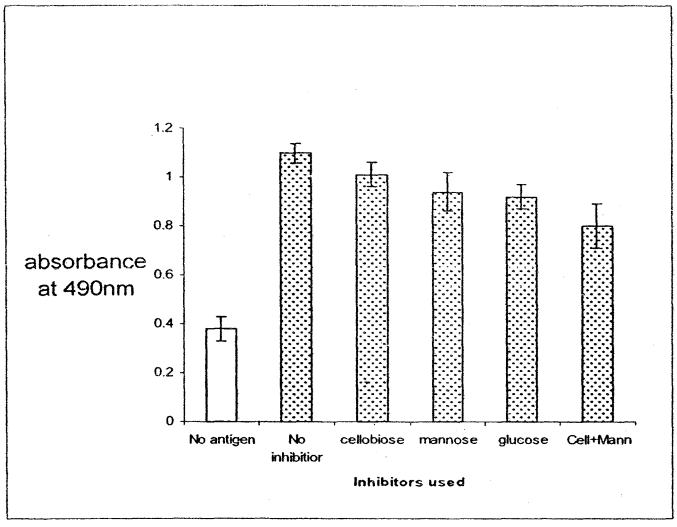

Fig.24. Immune complex formation mediated by CCA addition to diabetic serum and its characteristic sugar independency. To one ml serum 20 µg/ml CCA was added and the mixture incubated at 37^0 C for 3 hours. After PEG precipitation and separation of proteins as described in Method, 20X dilutions of IC pre-incubated with or without sugars (50 mM) were added to CCA -coated wells and probed with HRP conjugates of antibodies to human IgA, IgG and IgM separately. [Shaded blocks represent CCA-IC whereas unshaded one is the control where no antigen is added].

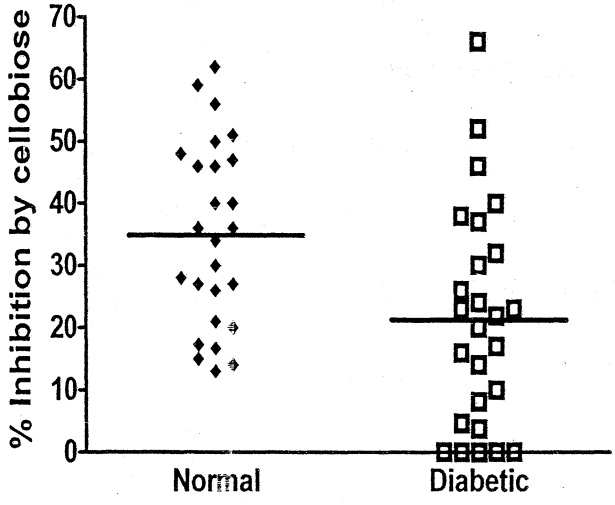

Fig.25. Inhibition pattern of CCA-IC to plate coated CCA by cellobiose in normal and diabetic serum. To one ml serum 20 µg/ml CCA was added and incubated at 37^0 C for 3 hours. After PEG precipitation and separation of proteins as described in Method, 20X dilutions of IC pre-incubated with or without sugars were added to CCA -coated wells and probed with HRP conjugates of antibodies to human IgA, IgG and IgM. {No. of samples in each group =26}. Graphical representation was done using GraphPad Prism software.

The inhibition pattern of CCA-IC to plate coated CCA by cellobiose in normal and diabetic serum was studied. The mean percentage inhibition of CCA-IC from normal and diabetic serum samples using 50 mM cellobiose was 35% and 21% respectively (Fig.25).

Eventhough high glucose concentration was found to be inhibitory for ABG (Fig.20) the results as a whole suggest that clinical status of the subject is relevant while studying the pattern of the antibody response. Even as present results indicate glucose mediated inhibition of ABG type antibodies as reason, the predisposition of diabetic patients to infection by pathogenic *Candida* Spp. has also been explained in terms of enhancement of yeast growth by elevated tissue fluid glucose levels (Knight, L. and Fletcher, J. 1971). Studies have shown that prevalence of yeasts were greater among diabetics than among normal subjects (Barlow, A.J. and Chattaway, F.W. 1969). Because germ-tube formation is generally considered to be an essential step in the pathogenesis of *C.albicans* infection and a fundamental component of the invasive process, enhanced yeast-mycelial conversion that occurs in diabetic serum could potentially augment the ability of C.albicans to establish infection in patients with type II diabetes (Plotkin, B.J., et al. 1996). Both PMNLs and macrophages play an essential role in the early response to infection with *C.albicans* (Diamond, R.D. 1989). Hyperglycemia and hyperlipidemia have been shown to affect the phagocytic capability of granulocytes (Stanley, V.C. and Hurley, R. 1969). Oral yeast colony levels in diabetic patients rapidly fell within few days as their hyperglycemia was controlled (Odds, F.C., et al. 1978).

It has been reported that if blood is collected in the early stages of infection, before the development of humoral response, then the tests to detect antibodies may

yield negative results (Mitsutake, K., et al. 1996). Study on the behaviour of ABG antibody titer in blood on administration of glucan to DBA/2 mice showed that on the administration of pathogenic fungal cell wall glucan, a remarkable decrease in the antibody titer was specifically observed, which suggested the formation of an Ag-Ab complex and prompt clearance from the blood. ABG antibody also showed great reactivity to the pathogenic fungal cell wall glucan *in vivo*. Furthermore, in deep mycosis patients, whose sera was positive for β -(1,3)-glucan, the ABG antibody titer decreased and this decrease correlated with the clinical symptoms. It was suggested that ABG antibody could play a role for β -glucan recognition molecule, and induce clearance of pathogenic fungi and biological activity by collaboration with other recognition molecule such as β -glucan receptor or complement in humans (Ishibashi, K., et al. 2005).

Since the diabetics are at higher risk of fungal infection, the decrease in ABG in diabetics, as observed from the sugar inhibition pattern can also be attributed the possible entry of ABG in circulation to form IC with the Candidal antigens. CCA was electrophoresed and redissolved IC was added to the separated fractions for further characterization of diabetic and normal CCA-IC.

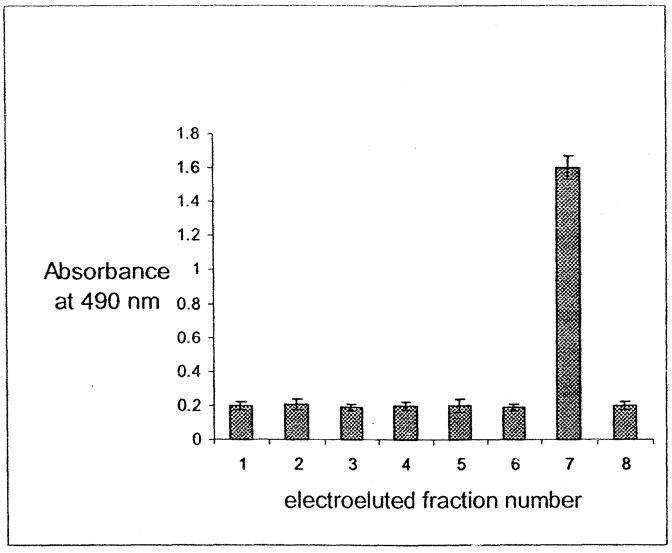

Fig.26. Nature of CCA-IC from diabetic serum binding to electrophoretically separated CCA. CCA was electrophoresed in 3.5% alkaline tube gel for 2 hours. The tube gels were cut into 8 sections and CCA fractions were passively eluted to PBS pH 7.4 and was coated on to microtitre wells and 20x dilution of the CCA –IC prepared from diabetic serum was added to the wells. This was probed with HRP conjugates of anti- immunoglobulin mixtures of IgA, IgM and IgG.

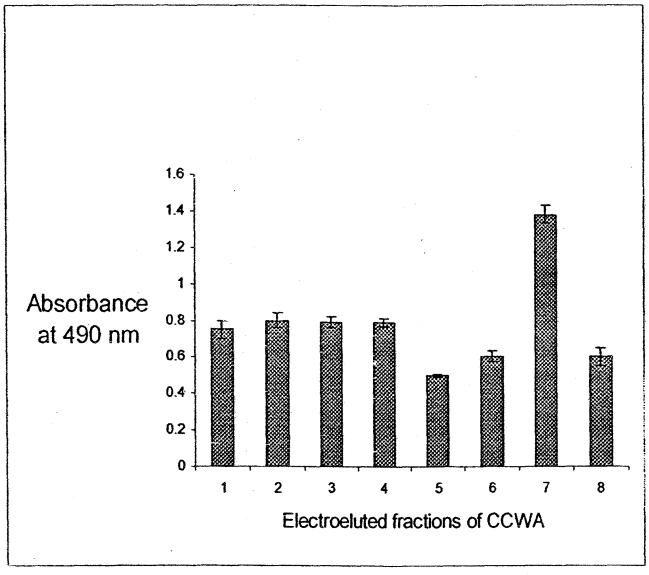

Fig.27. Nature of CCA-IC from normal serum binding to electrophoretically separated CCA. CCA was electrophoresed in 3.5% alkaline tube gel for 2 hours. The tube gels were cut into 8 sections and sections from the same regions were chopped and CCA fractions were passively eluted to PBS pH 7.4.The CCA eluate from each section was coated on to microtitre wells and 20x dilution of the normoglycemic CCA –IC was added to the wells. This was probed with HRP conjugates of anti-immunoglobulin mixtures of IgA, IgM and IgG.

Out of the six diabetic cases studied, having fasting blood glucose level above 300 mg/dl, binding of immunoglobulins from redissolved CCA-IC is restricted to a particular portion alone in four of the cases, i.e. the seventh fraction of the electrophoresed CCA eluate (Fig.26).

Results show that in all six normoglycemic cases studied, having fasting blood glucose level 80 mg/dl, binding of immunoglobulins from CCA-IC occurs to all regions, maximum being seen to the 7th fraction (Fig.27).

Carbohydrate portion of many candidal cell wall surface adhesions and receptors is important in fungal cell adherence to host epithelial cells and proteins (Casanova, M., et al. 1992, Chaffin, W.L. and Stocco, D.M. 1983). Circulating antibodies specific for this fraction correlated with an increased resistance to disseminated candidiasis. Such antibodies may alter the adherence of Candida cells *in vivo* or may enhance phagocytosis (Matthews, R. and Burnie, J. 1996).

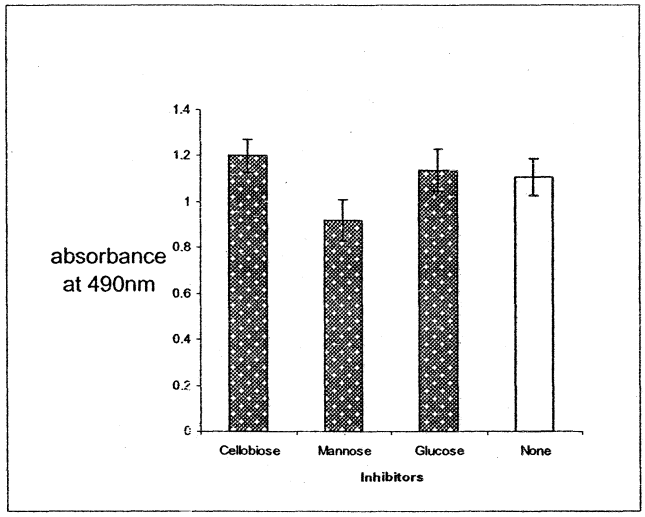

Fig.28. Sugar independent antibody binding to the 7th fraction. CCA was electrophoresed in 3.5% alkaline tube gel for 2 hours. The tube gels were cut into 8 sections and sections from the same regions were chopped and fungal polysaccharides were passively eluted to PBS pH 7.4.The CCA eluate (e.g., fraction 7) was coated on to microtitre wells and 20x dilution of the CCA–IC from normoglycemic serum which was incubated in the presence or absence of cellobiose, mannose and glucose (50mM each) were added to the wells. This was probed with HRP conjugates of anti-immunoglobulin mixtures of IgA, IgM and IgG. $P<0.05$

Result suggests that even though in normoglycemics the immune complex formation is mainly sugar dependent, antibodies that recognize the immunodominant region (7^{th} fraction) is sugar independent (Fig.28). This shows the non-carbohydrate nature of this epitope.

Sugar inhibition pattern shows that apart from fraction 7, binding to all other cut sections can be inhibited by cellobiose and mannose; where as binding to the 7th cut section was sugar independent. In diabetic CCA-IC also, binding of antibodies to the 7^{th} fraction is sugar independent (results not shown). This finding is in correlation with the previous observation that immunoglobulins that take part in IC formation with CCA in normoglycemics are more sugar reversible than the hyperglycemic cases.

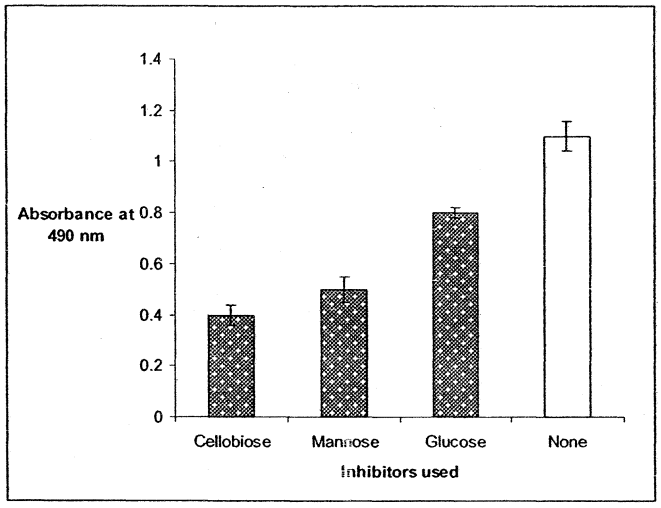

Fig.29. Sugar dependency of antibodies from CCA-IC of normal serum to fractions other than 7^{th}. CCA was electrophoresed in 3.5% alkaline tube gel for 2 hours. The tube gels were cut into 8 sections and sections from the same regions were chopped and CCA fractions were passively eluted to PBS pH 7.4.The CCA eluate (e.g., section 6) was coated on to microtitre wells and 20x dilution of the CCA –IC normal serum which was incubated in the presence or absence of cellobiose, mannose and glucose (50mM each) were added to the wells. This was probed with HRP conjugates of anti- immunoglobulin mixtures of IgA, IgM and IgG. $P<0.05$

Result suggests that the antibodies bind to all other sections apart from 7 is sugar dependent in nature (Fig.29). The molecular weight of the 7th fraction component was estimated to be 47 kDa by using ovalbumin as a marker (Results not shown).

Despite the considerable heterogeneity of the humoral responses to antigens of *C.albicans* in humans, several immunodominant antigens have been identified. Immunoblotting experiments with sera from patients suffering from systemic candidiasis had showed the presence of a 47kDa immunodominant antigen present in whole cell extracts of the fungus later identified as a heat-stable breakdown product of hsp90 with a cell wall location (Matthews, R. and Burnie, J. 1988). Patients who recover from invasive candidiasis generate a major antibody response to the 47-kDa component, whereas fatal cases seem to have little antibody or declining titres (Matthews, R. and Burnie, J. 1988). An immunodominant cytoplasmic 48kDa antigen (Candida enolase antigen) is present in many patients with invasive Candidiasis (Walsh, T.J., et al. 1991). Patients with disseminated Candidiasis had significantly higher levels of antibodies against 48kDa protein than did patients with non-invasive forms of Candidiasis, patients with other fungal infections, or normal healthy persons (Strockbine, N.A., et al. 1984). This 48kDa protein antigen was subsequently identified as enolase (Franklyn, K.M., et al. 1990).

Sugar inhibition studies done with cellobiose and mannose in diabetic sera have shown that in most of these patients, who supposedly have combated the *Candida* infection, there was significant reduction in response to all electrophoretically separated fractions other than the 47 kDa fraction which is sugar independently recognized. The former are rich in mannoproteins of varying molecular

weights to which β-glucan is covalently associated. But in few diabetics antibodies were found responsive to the non-47 kDa fractions as well.

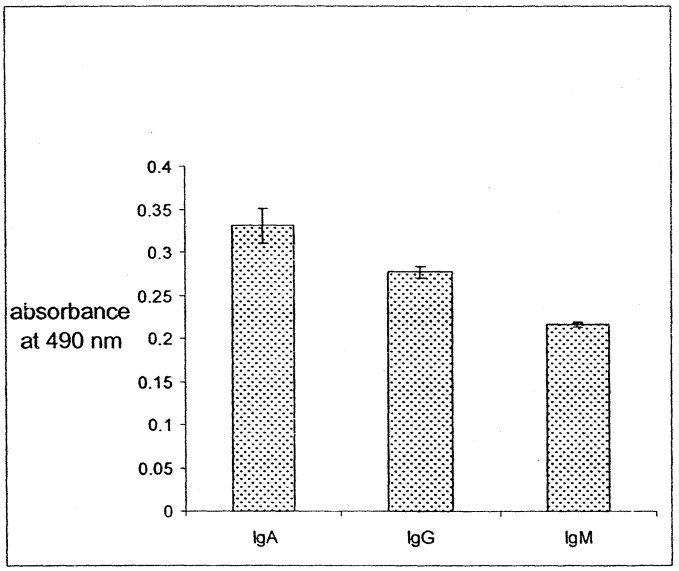

Fig.30. The major immunoglobulin type that binds to the immunodominant region of CCA- eluate. Fungal polysaccharide was electrophoresed in 3.5% alkaline tube gel for 2 hours. The tube gels were cut into 8 sections and sections from the same regions were chopped and CCA were passively eluted to PBS pH 7.4.The CCA eluate (e.g., section 7) was coated on to microtitre wells and 20x dilution of the CCA–IC was added to the wells. This was probed with HRP conjugates of anti-immunoglobulin IgA, IgM and IgG separately.

The immunoglobulin composition of the antibody population binding to the immunodominant antigen in diabetics was characterised. The significantly high concentration of IgA in this antibody population is noted. Result shows that IgA is the major immunoglobulin type that is involved in immune complex formation even in diabetics even though the role of anti-carbohydrate antibodies in immune response is minimal in them (Fig.30).

Type II Diabetes is marked by the high amounts of circulating IC rich in polymeric IgA. The relative contribution of monomeric and polymeric IgA was investigated in diabetic sera showing that 80-87% of IgA in diabetes are polymeric. A

defective mechanism of hepatobiliary transport of IgA is suggested in diabetes mellitus (Triolo, G., et al. 1984). Secretory IgA and immune complexes containing IgA and secretory component seem to participate in the hyper-IgA of patients with Type 2 (non-insulin-dependent) diabetes only, suggesting an altered hepatic clearance via secretory component receptors on hepatocytes. In Type 1 (insulin-dependent) diabetes, the high serum IgA levels might be explained by an increase in IgA production in response to antigenic stimuli. Evidence is also accumulated that immune complexes containing IgA of mucosal origin may be involved in microangiopathy production in Type 2 diabetes (Triolo, G., et al. 1984).

Immune complex formation by DIg.

Dextran is a normal contaminant in sugar produced by *L.mesenteroides*. Dextran from sugar is of average M_w 5×10^6 and 5×10^4 Da. Even macrocorpuscular food particles whose diameter is well within the micron range are regularly incorporated into the organism by persorption. This prompted investigations on the nature of dextran-induced immune complexes from human serum.

Hundred micrograms of dextran (Mw 4, 00,000-5, 00,000) was added to 1 ml of serum and incubated at 37^0 C for 3 hours. Polyethylene glycol precipitation of immune complexes was done as described in the methods. Immune complex formation against dextran was measured by ELISA using dextran coated wells. Naturally occurring circulating-immune complexes from normal young blood donors in our region, isolated by precipitation with 2 % PEG consisted also of those formed by DIg (Figure 31, 32). However addition of bacterial dextran resulted in a marked increase in DIg-containing IC. Even at high concentration (50 mM) 1-*O*-methyl α-Glc , the best inhibitor of DIg could only partially prevent the binding of

immunoglobulins from IC to dextran (Figure 29), indicating the high avidity of the antibody for the polymer. NDS, which consisted mostly of dextrans, behaved similar to dextran in IC formation (Fig.32) from serum and its inhibition by sugar.

Result shows that immune complexes are formed in serum upon the addition of dextran. The potential of non-dialysable sugar (NDS) to form immune complexes in serum was studied. The role of DIg in precipitating such immune responses was specially noted. This was compared with the IC formation mediated by dextran.

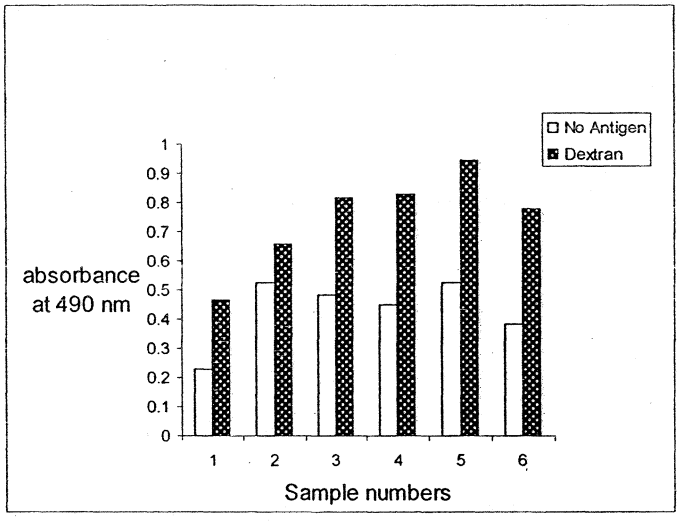

Fig.31. **Immune complex formation by DIg.** 100 μg dextran (400 KDa-500KDa) was added to 1 ml serum, incubated at 37°C for 3 h, PEG precipitation done, washed, 5x dilution of immune complexes were added to coated dextran 1μg/well and probed with a mixture of HRP conjugates of anti human Ig A, G, M .

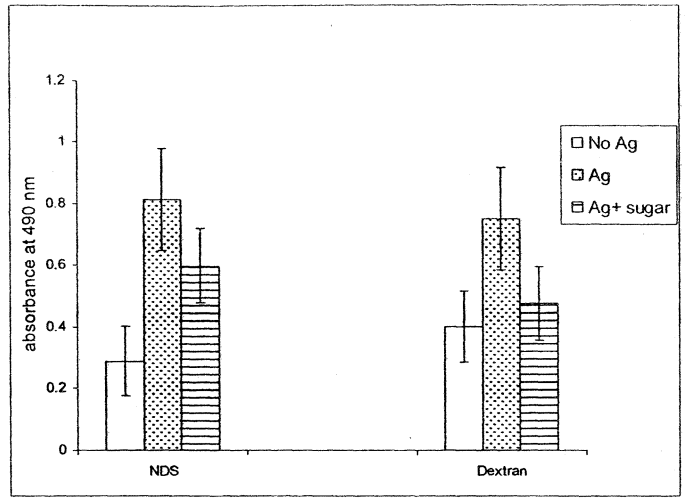

Fig.32. Formation of immune complexes in serum between DIg and polysaccharides. NDS (20 μg) or dextran (400 KDa-500KDa; 50 μg) were added to 500 μl serum and the mixture incubated at 37°C for 3 h. PEG precipitation of IC and isolation of proteins from IC were done as described in 'Methods'. IC proteins incubated at 4^0C with or without 50 mM αMG were added to microplate-coated dextran (1 μg per well) and probed with a mixture of HRP conjugates of goat antibodies to human IgA, IgG and IgM. Values are mean ± S.D of six trials.

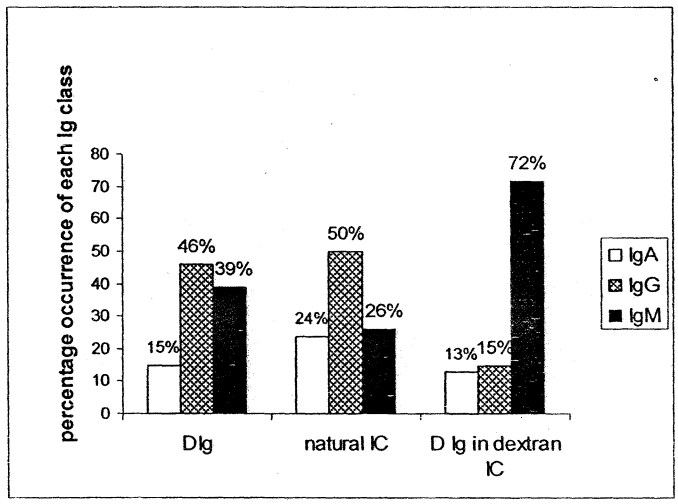

Fig.33. Comparison of immunoglobulin composition of DIg, natural IC and DIg in dextran-induced IC. One microgram of DIg, natural IC or DIg separated from IC which was isolated from serum after addition of dextran was coated on microtitre wells, blocked with 0.5% PBS-T and probed with HRP conjugates of anti-human IgA, IgG and IgM separately. Percentage of each immunoglobulin class was calculated assuming that optical density was proportional to the amount of respective Ig class. Mean of three trials shown; standard deviation was less than 10% of mean.

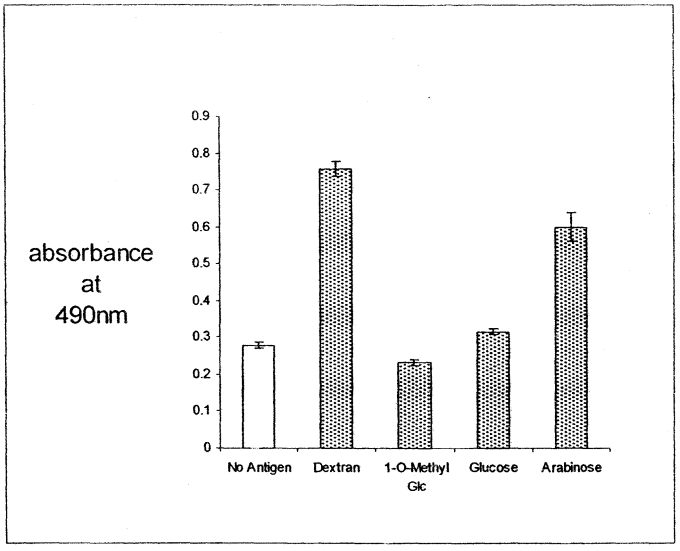

Fig.34. Effect of high glucose on DIg-IC formation. Shaded blocks represent dextran added samples whereas unshaded one is the control.) Hundred μg/ml dextran was added to sera, which was pre-treated with (3^{rd},4^{th} and 5th blocks) and without(2^{nd} block) sugar. [1) No antigen added, 2) serum glucose level; 80mg/dl), 3) serum glucose 80mg/dl + 1-O-methyl α-D glucoside,320mg/dl ,4) serum glucose level; 400mg/dl), 5) serum glucose 80mg/dl+ 320mg/dl arabinose]. Two micrograms of coated dextran was treated with 20 x dilutions of the immune complex pellet and the bound antibodies were probed with a 1:1:1 mixture of HRP conjugates of anti- human Ig. Values are mean ± S.D of six trials.

In total human serum, immunoglobulins are mostly IgG whereas IgA amounts to 20-25% of IgG in abundance and IgM is a still minor component.

In comparison DIg had IgM content nearly equal to its IgG content, while IgA content remained low (Figure 33). Notably DIg isolated from IC formed after addition of dextran to serum consisted overwhelmingly (about 72%) of IgM with both IgG and IgA contributing marginally (Figure 33). Role of high sugar in serum on DIg-IC formation was examined (Fig.34). As in the case of ABG, DIg also is likely to be inhibited by high sugar in serum. Natural IC prepared from normal healthy donors in this study consisted up to 50% of IgG and the rest equally of IgA and IgM.

Considerable amount of DIg exists in serum as immune complex (Figure 31, 32) in addition to the relatively high titre of free DIg that could be isolated by affinity chromatography (≈ 50μg/ml; see above). Such large turnover indicates heavy influx of corresponding antigens from the environment. The inability of 1-*O*-methyl α-Glc to fully reverse immune complex formation by serum DIg probably indicates the high affinity of these antigens for the antibody. The nearly consistent binding of DIg to each of the non-dialysable sugar fractions of differing electrophoretic mobility (Fig.15) suggests that such high molecular weight and indigestible biomolecules from diet may contribute substantially towards IC formation involving DIg. Dietary biomolecules have been shown to enter systemic circulation in normal individuals and more so in those with breaches in intestinal barriers (Husby, S., et al. 1985). NDS consisting of bacterial dextrans having been detected consistently in table sugar samples in this region, this indigestible high molecular weight biomolecule may enter systemic circulation as an antigen. The recognition by DIg of each of the eight fractions of NDS with varying size and charge points to the high potential of this dietary polysaccharide to act as a constant trigger for formation of IC with DIg. Results in figure 16 suggest that other environmental antigens from yeast and cereals that may enter the body in large quantities are also capable of IC formation with DIg.

The extremely high content of IgM in DIg-dextran ICs, far higher than expected from the relatively high IgM content of DIg as such may point to the biological fate of such ICs. IgM, being the most efficient immunoglobulin type in complement fixation (Davis, A.C. and Shulman, M.J. 1989), these ICs may be sequestered faster. It is interesting to examine if fast disposal of incoming antigens like dextrans and β-glucans

by the IgM-rich DIg precludes formation of secondary antibodies in which generally IgG dominates.

IgM- producing B cells class switch to IgA *in vivo* (Coutinho, A. and Forni, L. 1982). In fact, *in vivo* studies have shown that dextran immune response can result in formation of IgA-IC. In an active model of IgA nephropathy, induced in mice by repeated injections of dextran, serum total IgA and anti-dextran IgA antibody levels increased significantly over the period of immunization. Glomerular IgA deposition occurred as a result of circulating IgA complexes and/ or IgA polymers deposition (Gonzalez-Cabrero, J., et al. 1990). Also as stated by Gallo et al (Gallo, G.R., et al. 1981) cationic dextran complexes interact with anionic sites on endothelial cells and within the membrane proper. Such electrostatic interactions also may lead to the localization of Dextran-IC (Isaacs, K.L. and Miller, F. 1983). Although, the serum IgA anti-dextran antibody level is much less than that of IgM, IgA was detected in high density in glomerular deposits due to in situ complex formation (Bagheri, N., et al. 2005). Abnormally high humoral responses have been described in IgA nephropathy (IgAN) towards antigens commonly involved in infectious events or food intolerance. In a comparative analysis of humoral responses to a common environmental antigen, dextran B 512, present both in non-pathogenic microorganisms and normal diet, anti-dextran IgA and IgG were at significantly higher levels in IgAN patients than in controls (Kennel, A., et al. 1995). Considering all these factors DIg can also be a source of pIgA-IC in serum

PART III

Immobilisation of galectin-1 by biotinylation and demonstration of IgA binding to immobilised galectin-1 in immune complex and free form.

IgA in monomeric and polymeric forms are good ligands for galectin-1 in solution (Sangeetha and Appukuttan 2005). Since in vivo galectin-1 may be available on tissues in immobilized form direct coating of galectin-1 to NUNC polystyrene wells in order to study properties of immobilized galectin was not successful due to loss of sugar binding activity following immobilization. *In vitro* immobilization of galectin-1 was therefore done by adding biotinylated galectin-1 to NUNC-polystyrene well coated streptavidin.

Streptavidin is a 52,800 dalton tetrameric protein purified from *Streptomyces avidinii*. It finds wide use in molecular biology through its extraordinarily strong affinity for biotin (also known as vitamin H). This interaction is the strongest non-covalent binding known (Kd = 10^{-15} M) (Green 1990). Taking advantage of this property, the interaction biotin-(strept)avidin has been exploited for a large number of biotechnological applications (Parrott and Barry 2000; Cognet et al. 2005) Currently, biotinylation of proteins is achieved by different chemical reactions.

Biotin derivatives like NHS-biotin, Sulpho NHS-biotin, and NHS-LC Biotin are able to couple with particular functional groups in proteins. Biotin modification of secondary molecules, called "biotinylation" results in covalent derivatives containing one or more bicyclic biotin rings extending from the parent structure. Biotin moieties in these biotinylated molecules are still capable of binding avidin or streptavidin with the specificity and avidity of free biotin in solution. Since the biotin components are relatively small, macromolecules can be modified with these reagents without significantly affecting their physical or chemical properties. Common to all such modification reagents is the presence of the bicyclic biotin ring at one end of the structure and a reactive functional group at the other end that can be used to couple

with other molecules. As the binding sites for biotin on streptavidin are pockets buried about 9 A^0 beneath the surface of the proteins, spacers can affect the accessibility of biotinylated compounds for efficiently binding streptavidin (Green et al. 1971). The rate of binding of streptavidin to a biotinylated molecule is affected by the length of spacer in the biotinylation reagent used. When longer spacers are used to make biotinylated macromolecules, it potentially can result in a five-fold greater rate of streptavidin interaction.

The only disadvantage to the use of NHS-biotin or sulpho-NHS-biotin is the lack of a long spacer group off the valeric acid side chain. NHS-LC biotin is a derivative of D-biotin containing a spacer arm off the valeric acid side chain, terminating in an NHS ester. The compound is also known as succinimidyl-6-(biotinamido) hexanoate. The 6-aminocaproic acid spacer provides greater length between a covalently modified molecule and the bicyclic biotin rings. The total distance from an attached molecule to the biotin component is about 22.4 A^0 significantly greater than the 13.5 A^0 length of NHS-biotin without a spacer length. This increased distance can result in better binding potential for avidin or streptavidin probes, because the binding sites on these proteins are buried relatively deeper inside the surface plane.

The major disadvantage of this approach is the uncontrollable coupling of biotin to sites of the target protein that can be important for its biological activity. During biotinylation of galectin-1, this has been prevented by adding specific sugar lactose to the lectin during the time of biotinylation. Engagement of the carbohydrate binding site with the specific sugar moiety prevents biotinylation of the sites important for biological activity.

Galectin-1 obtained from bovine heart was used for the study. Both human and bovine heart galectin-1 contain 134 aminoacids but are different at 17 residues only resulting in 87% identity and several differences are non conservative (Abbott et al., 1989; Hirabayashi et al., 1989).

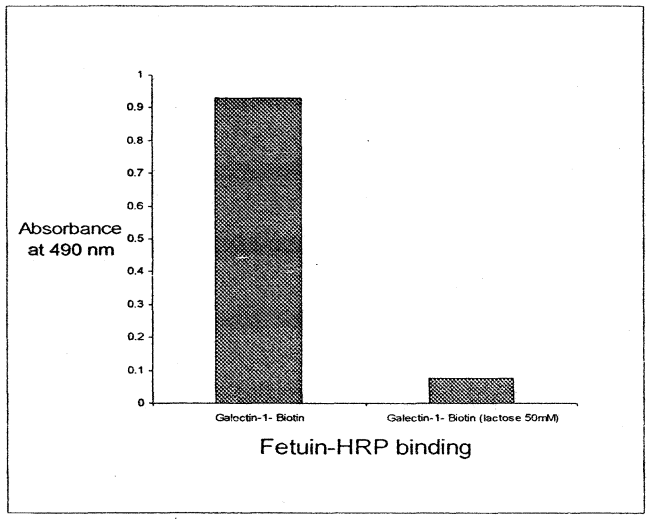

Fig 35. Immobilization of galectin-1 by biotinylation. Galectin was biotinylated using NHS-LC-Biotin (Methods). To streptavidin coated wells (two microgram/well) 10x dilution of the biotinylated lectin was added after blocking the wells with 0.5%T-20 PBS. Wells were kept at 4°C for 2 hours and probed with fetuin- HRP (100X) in the presence and absence of 50 mM lactose.

Fetuin, the major glycoprotein in fetal calf serum, consists of three alkali-labile, O-glycosidically linked moieties and three complex, asparagine-linked oligosaccharide moieties as established by (Spiro 1962; Spiro and Bhoyroo 1974). Bovine galectin-1 binds to fetuin through core-I-O glycan structure (Sangeetha and Appukuttan 2005). Sustenance of activity of galectin-1 after its immobilization by adding biotinylated lectin to plate-coated streptavidin was checked by addition of HRP-labelled fetuin (Fig.35).

Result shows that biotinylation of galectin-1 occurs effectively. Sugar reversibility of the fetuin-HRP binding by 50 mM lactose confirms that biotinylated

lectin retains carbohydrate binding activity. Thus biotinylated galectin-1 attached to plate coated streptavidin can be used for immobilization of galectin in an active form.

Efficiency of glycoconjugates as galectin-1 ligand was measured in terms of their ability to inhibit fetuin-HRP binding to immobilized galectin-1. When IgA and IgG were tried in this way there was 41% inhibition by IgA whereas IgG brings about no inhibition at all (Fig.36). Human serum IgG (Sigma chemicals, USA) contained traces of IgA and was purified by passing its solution in PBS, pH 6.5(2 mg/ml) through a 5ml jacalin-Sepharose 4B column in the same buffer at 4^0C. Unbound fraction (free from IgA) was used as IgG.

IgA is unique in having a hinge region with core I-O glycans that makes it a good ligand for galectin-1 (Sangeetha and Appukuttan 2005). IgG and IgM are devoid of any ligand for galectin-1.

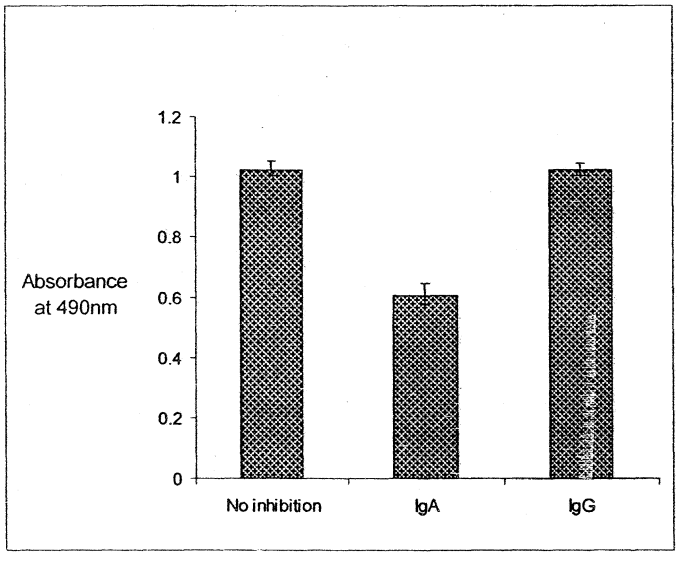

Fig.36. IgA binding to immobilized galectin-1. IgA (from Colostrum); 30 μg/100 μl and IgG (36 μg/100 μl) was added to immobilized galectin-1 by simultaneous addition along with 100 μl of fetuin-HRP (100X).

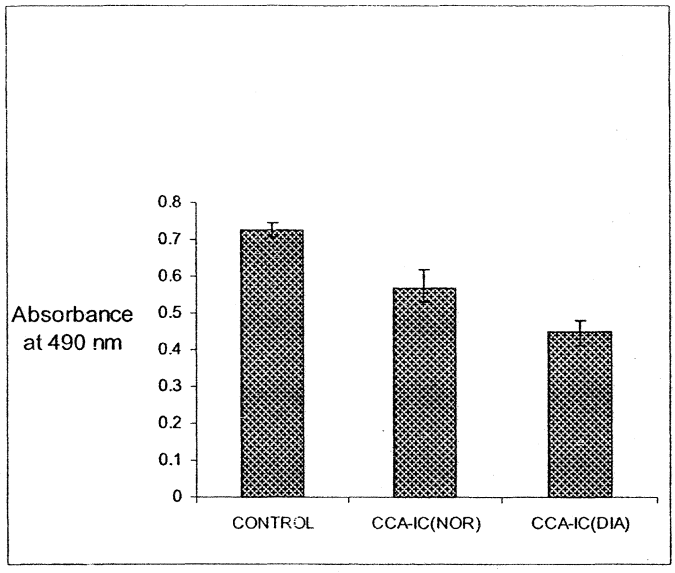

Fig.37. CCA-**IC from diabetic and normal sera binding to immobilized galectin-1.** Binding of 3 μg/ml of fetuin-HRP to immobilized galectin-1 was inhibited by simultaneous addition of dissolved CCA-IC; 10X dilution from both diabetic and normal sera.

To study the binding of IgA rich IC to immobilized galectin-1, inhibition of fetuin-HRP binding to immobilized galectin-1 by CCA-IC from normal and diabetic sera was checked. There is 21% inhibition in the binding of fetuin-HRP to immobilized galectin-1 by CCA-IC from normoglycemic cases whereas diabetic CCA-IC brings about 38% reduction in binding (Fig.37).

Studies on the relative contribution of monomeric and polymeric IgA in diabetic sera showed that 80-87% of IgA in diabetes are polymeric. (Triolo et al., 1984 a). ICs may play a role in developing pericarditis or they may be considered a marker of pericarditis by an immunological mechanism.(Triolo et al. 1984b). Though high amount of polymeric IgA1 in IC form is reported in type II diabetes, the etiology is unknown. The possibility that soluble immune complexes involving insulin and

anti-insulin, as well as other antigen-antibody systems play some role in the pathogenesis of the complications of diabetes mellitus was first postulated by Faulk et al (Faulk, Karam et al. 1971). Present study show that anti-β-glucan antibodies are kept in check by the high sugar concentrations attained in diabetics, but the antibodies that bind the immunodominant antigens mostly belong to IgA class.

Several studies, originating from the immunological implications of heterologous insulin administration suggested that immune mechanisms could be of importance in the etiology of diabetic vascular complications (Di Mario, Iavicoli et al. 1980). Various factors might influence the formation of high amounts circulating IC in diabetes, namely age and sex of the patient, type of treatment, insulin antibody levels, degree of metabolic control and the presence of complications (Di Mario, Iavicoli et al. 1980). An increase in soluble antigen-antibody complexes (due to impaired phagocytic clearance) in patients with severe microangiopathy may contribute to the vascular damage after passive binding or trapping of IC in the small vessel walls and in the perivascular tissues.

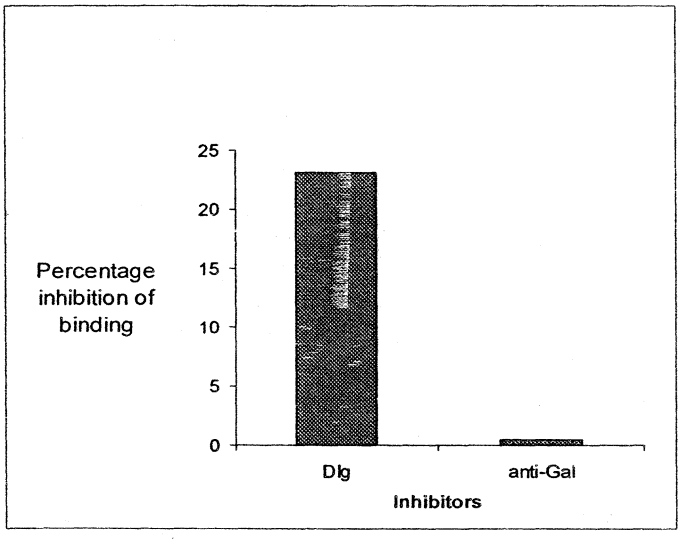

Fig.38.Percentage inhibition of binding of fetuin-HRP to immobilized galectin-1 by DIg and anti-Gal. DIg (20 μg) and anti-Gal (50 μg) was added simultaneously along with fetuin-

HRP (100X) to immobilized galectin-1. Percentage inhibition of binding of fetuin-HRP to immobilized galectin-1 by DIg and anti-Gal is calculated.

DIg and anti-Gal are two functional serum immunoglobulins; the former containing both polymeric and monomeric IgA while the latter was free from polymeric IgA (Fig.4 and Fig.5). Comparing the above antibodies as inhibitors of fetuin-HRP binding to immobilized galectin-1 revealed that DIg was a good inhibitor while anti-Gal was poor (Fig.38). Since the galectin-1 binding component in both these antibodies are IgA, it is reasonable to conclude that polymeric IgA is far superior to monomeric in attaching to galectin-1.

While galectin binding to a single saccharide ligand is typically a low-affinity interaction (association constants ~10^4 M−1), the multivalent nature of galectin-saccharide interactions results in high overall avidity (association constants ~10^6 M−1) (Vasta, Ahmed et al. 2004; Dam, Gabius et al. 2005). This multivalency also allows the formation of lectin-carbohydrate lattices. Both in solution and on the cell surface, multivalent galectins selectively cross-link a single species of glycoprotein to form homogeneous lectin-carbohydrate lattices (Brewer, Miceli et al. 2002). Prototype galectin-1 is a dimer in solution and crystallizes as a dimer in cross-linked complexes with a divalent oligosaccharide (Morris, Ahmad et al. 2004). Glycoproteins often bear multiple copies of the saccharide ligands that are recognized by galectins.

Discussion

IgA deposited in the glomeruli is composed of dimers and/or larger polymers of circulating IgA in some patients with IgA nephropathy (Tomino, Sakai et al. 1982).

The levels of serum IgA in patients with HSP nephritis increased as observed in IgA nephropathy. It is suggested that there are some immunopathological similarities between HSP nephritis and IgA nephropathy (Tomino, Endoh et al. 1982). Present results suggest that pIgA which has got multiple binding sites for the dimeric galectin-1 brings about the effective binding.

Galectin-1 is expressed on the surface of activated endothelium, stromal fibroblasts, tumor cells, and antigen presenting cells, with increased expression in inflamed and neoplastic tissues. Galectin-1 expression on the surface of endothelial cells is increased by endothelial cell activation in vitro with LPS or cytokines, and increased galectin-1 expression by endothelial cells has been observed in inflamed human lymph nodes (Baum, Seilhamer et al. 1995). In vitro, galectin-1 expression on HUVECs is increased by treatment with proinflammatory cytokines and by conditioned medium from prostate carcinoma cells (Clausse, van den Brule et al. 1999).

Even in atherosclerosis high concentration of IgA in circulation is noted even though few cite it as an event following the ischemic event. The infection etiology of atherosclerosis is well cited in literature. IgA response to infectious agents like *Cytomegalovirus, Chlamydia pneumoniae, Helicobacter pylori* etc which are associated with atherosclerosis has been noted very often. Cross sectional studies on atherosclerotic plaques have shown the presence of IgA in plaques.

Galectins form networks of glycoconjugates on the cell surface, where they may modulate various cell response pathways such as growth, activation and adhesion.

Galectin-1 is induced in the plasma of type 2 diabetic individuals, *in vivo*. (Liu, Feng et al. 2009). Galectin-1 is postulated to be an important regulator of the pathophysiology of type 2 diabetes. Galectin-1 is a novel plasma marker protein of this disease. IgA1 rich in polymeric IgA obtained from DIg is reported to be more efficient in its binding to human heart galectin-1 in solution than total serum IgA with proportionally less polymer content (Sangeetha, SR and Appukuttan, PS., 2005).

Lectin binding to cells often leads to cross-linking and aggregation of specific glycoprotein and glycolipid receptors. Structural studies of multivalent lectin-carbohydrate cross-linked complexes have provided new insights into the specificity of these interactions. In general, two types of multivalent complexes are observed, designated type 1 and type 2. In type 1 complexes, cross-linking occurs between a bivalent carbohydrate and bivalent lectin. Type 1 interaction, may result in the formation of linear (one-dimensional) cross-linked complexes. These complexes are often soluble (Bhattacharyya, Haraldsson et al. 1988; Mandal and Brewer 1993) and possess a high degree of polymorphism due to their flexibility. In theory, such complexes may accommodate more than one type of bivalent lectin or bivalent carbohydrate in the cross-linked complex.

In type 2 complexes, cross-linking occurs between lectins and carbohydrates in which one of the two molecules possesses a valency of >2. An example is a cross-linked complex between a tetravalent lectin and a bivalent carbohydrate. Such interactions lead to the formation of two- and three-dimensional cross-linked complexes, which can be insoluble and precipitate from solution (Bhattacharyya et al. 1990; Mandal and Brewer 1992; Mandal and Brewer 1992). Type 2 complexes may exist as ordered cross-linked lattices.

The X-ray crystal structures of a single tetravalent lectin cross-linked with four different bivalent carbohydrates have been reported (Dessen et al. 1995; Olsen et al. 1997). These results have provided insight into the molecular basis for the formation of type 2 homogeneous carbohydrate–lectin noncovalent cross-linked complexes.

IgA nephropathy is the most common form of glomerulonephritis worldwide. In this disease, IgA-containing immune complexes which are deposited in the mesangial area are supposed to stimulate the accumulation of matrix proteins and lead to glomerulosclerosis. These phenomena have been shown to be mediated by inflammatory cytokines and growth factors excreted on stimulation by immune complexes (Montinaro et al. 1999) . Immune complexes form and are deposited within the vessel walls leading to complement activation. Direct immunofluorescent tests for IgA deposits on biopsies of normal appearing skin from patients with proven renal disease have shown that patients with IgA nephropathy had high levels of IgA deposition, often accompanied by notable deposits of IgM, Clq and fibrin, and less frequently by C3 and IgG, in small vessels of the superficial dermis (Thompson et al. 1980).

Henoch-Schönlein purpura (HSP) is a systemic vasculitis of the small vessels of the skin, joints and kidney in which electron-dense deposits of pIgA is seen on the endothelium. In a variant of HSP, named as gastric enteropathy polymeric IgA1 deposits were ultrastructually seen along the plasma membranes of the endothelial cells (Kato et al. 2004). Mechanism of IgA-deposition in vascular endothelium as well as in mesangial tissues during IgA-deposition diseases is still an enigma since the receptors responsible for this pathogenic event still remain obscure.

The results presented here propose a possible role for galectin-1 in mediating diseases involving IgA deposition since a) galectin-1 binds only IgA1 among immunoglobulins; b) galectin-1 has remarkable preference for desialylated IgA1, over native IgA1 and the former only has been found to be involved in IgA nephropathy, for instance; c) galectin-1 has several fold affinity for polymeric compared to monomeric IgA and disease conditions that precipitate tissue deposition of IgA1 like diabetes are accompanied by spurt in circulating pIgA.

Chapter 5

SUMMARY, CONCLUSION AND FUTURE DIRECTIONS

SUMMARY AND CONCLUSION

Glycan antigens entering the human body through food, environment and microflora colonizing the skin, epithelia and gut, especially in humid tropics is enormous making it pertinent to characterize the anti-glycan antibodies and their immune complexes. Anti-glycan antibodies are rich in polymeric IgA content making it potential source of high quantities of pIgA-IC in serum. Different anti-glucan antibodies are used in this study to characterise their amount of polymeric IgA content. ABG was isolated at a yield of 4-7 mg/100 ml plasma. ABG has significantly higher IgA content in comparison with total serum Ig leading to a three times higher IgA to IgG ratio. Electrophoretic pattern showed that anti-β glucan consists of IgA in polymeric as well as monomeric form.

High circulating concentrations of dextran-binding immunoglobulin (3-6 mg/100 ml plasma) is seen in donors who had no history of exposure to dextran infusions. DIg consists of IgA in the polymeric and monomeric form eventhough IgM forms the major immunoglobulin component.

Anti α- galactoside was isolated at a yield of i.e., 0.8-1 mg/100 ml plasma. Anti α- galactoside is devoid of any polymeric IgA content. The above results indicate that presence of polymeric forms of IgA in the anti-glycan antibodies which make up a large portion of the natural antibody repertoire is observed. It is possible that anti-glycan antibodies that constitute such a high concentration is the major source of polymeric IgA in serum.

Before ascertaining the difference between polymeric IgA and monomeric IgA in their capacity as ligands for lectins, anti-IgA recognition of the two was studied. Treatment with 2ME (3mM) reduces the intersubunit disulphide bonds and cleaves apart polymeric IgA subunits. This treatment helps to quantitatively assess its unit monomer content. Results suggested that the response of polymeric IgA to anti-IgA HRP is 2.4 times more than that of the monomeric form.

Before polymeric IgA and monomeric IgA were compared in terms of their capacity to act as ligands for galectin-1, activity of another similar lectin i.e., peanut agglutinin (PNA) was checked. PNA-HRP response for unit monomeric IgA is 1.6 times more in the case of polymeric IgA compared to monomeric IgA fraction. Enhancement of the recognition by PNA-HRP upon neuraminidase treatment is 2.77 times more in the case of polymeric IgA than in the case of monomeric IgA. Desialylation increases PNA binding by 1520% for polymeric IgA and 750% for monomeric IgA.

Galectin-1 isolated from human placenta using lactose-Sepharose column was used for the study. Binding of HRP-conjugated galectin-1 to polystyrene plate-coated IgA was studied. Results showed that in native (non-desialylated) form, polymeric IgA was 1.71 times more efficient than monomeric as a ligand for galectin-1, when same protein contents of both were coated. Neuraminidase enzyme produced by various pathogens like influenza virus cleaves the α-linked N-acetyl neuraminic acid exposing the T antigen (Galβ-1,3GalNAc-α) which is an efficient ligand for galectin-1. Enhancement of the recognition by human placental lectin-HRP upon neuraminidase treatment is 2.66 times more in the case of polymeric IgA than for

monomeric IgA. HPL-HRP response for unit IgA after desialylation is 2.7 times more in the case of polymeric IgA compared to monomeric IgA. Together these results indicate that polymeric IgA compared to monomeric IgA and desialylated IgA compared to native IgA are far superior ligands for galectin-1.

Further studies involving polymeric IgA1 rich immune complexes are done to elucidate the role of galectin-1 in binding IgA1-IC. Anti-polysaccharide antibodies like ABG and DIg which are notably rich in polymeric IgA are used for the study. These anti-polysaccharide antibodies are present in very high concentrations in serum and hence make up a major portion of the natural antibody repertoire. Exposure to carbohydrate moieties like β-glucan and dextran from microbial, food and other environmental sources can lead to the formation of pIgA-rich immune complexes in circulation. Under situations of immune system overload, owing to increased formation or defective removal, ICs are likely to get deposited extrahepatically. Since high titres of anti-glycan antibodies are detected even in normal physiological situations, it is reasonable to assume that such antibodies and their ICs account for a major part of pIgA which is more pathogenic than mIgA, especially in desialylated state.

The presence of antigenic epitopes for ABG and DIg was checked in electrophoretically separated yeast glycoprotein fractions. Both of these anti-carbohydrate antibodies recognize yeast glycoproteins, even though the response was higher in the case of ABG-HRP. The recognition of the β-1,3 glucan containing YGP by DIg can be attributed to the extended specificity of this α (1,6) linked glucan specific antibody.

The carbohydrate dependency of the YBG recognition by ABG was confirmed by inhibiting the binding of ABG to coated Candida Cellwall Antigens (CCA) using different fractions of yeast glycoproteins.

Dextran is yet another polysaccharide that provides linear epitopes for natural antibody recognition. To obtain high molecular weight polysaccharides in the edible sugar that are larger than the 10,000 Da pore size of dialysis bags, non-dialysable part of commercial edible sugar was compared to standard high dextrans for presence of antigenic epitopes for ABG and DIg. Results suggest that non-dialysable portion of the edible sugar contains epitopes for ABG and to a lesser extent for DIg. Reversibility of the binding by inhibitory sugars confirms the sugar-specificity of binding. Even though these antibodies show anomer preference, their multiple specificity, which is characteristic of natural antibodies, enables them to bind to various epitopes present on microbial cell surfaces. All components of NDS separated by electrophoresis were recognized by DIg.

β-(1,3)Glucans from yeast and barley were significantly less effective in capturin DIg than dextran which consists almost exclusively of α(1→6)- linked glucose moieties Notably the reversibility of DIg binding with αMG also decreased from dextran to yeas and barley β –glucan.

In vitro studies were done to demonstrate the formation of anti-glycan antibody polysaccharide immune complexes between anti-β- glucan antibody on one hand and fungal polysaccharides on the other. In order to isolate serum antibodies specifically reacting with CCA, increasing amounts of CCA were added to fixed

volume of serum, immune complexes formed separated, and CCA-interacting antibodies thereof assayed by their capacity to bind to microplate coated CCA. Results show that as the concentration of CCA increases, there is a corresponding increase in immune complex formation till the zone of equivalence is reached. There is a minimal level of CCA-binding antibodies in ICs prepared without addition of CCA showing that a minimum amount of circulating IC contributed by CCA-reactive antibodies occurs in normal serum.

Sugar inhibition using 50 mM cellobiose as well as 50 mM mannose brings about remarkable reduction in the binding of CCA-IC immunoglobulins to the CCA-coated wells. This suggests that anti-β-glucan as well as anti-mannan antibodies which occur in the serum as natural antibodies are capable of forming immune complexes with the antigenic epitopes presented by *C.albicans*. Also both sugars together exerted a synergistic effect depicting the presence of two types of antibodies.

Results prove that ABG-mediated immune complexes are present in normal individuals and are markedly elevated during fungal infections. Immunoglobulin class prevalence in CCA immune complexes determined with coated antibody of CCA revealed that IgA is the most prevalent immunoglobulin class in CCA- ICs unlike the natural immune complexes where IgG forms the major portion. Since β-glucans and related antigens reside on cell surfaces and are released into host circulation, frequent exposure to these organisms as commensals or systemic infectants, experienced by even normal individuals leads to a considerable increase in IgA-containing IC, contributed by ABG and related antibodies.

Serum to which glucose was added to a final concentration of 400 mg/dl which is usually attained in diabetes showed marked reduction in IC formation with CCA compared to controls. Results point towards the possibility that high sugar in serum prevents the ABG population from forming IC. The marked reduction in IC formation in high serum sugar conditions indicates that glucose concentrations achieved in diabetic conditions can inhibit the ABG from taking part in IC formation. It is reasonable to postulate that inhibition of natural and front-line defence mechanism mediated by antibodies like ABG by high serum blood glucose may be the primary reason for the high susceptibility of diabetics to infections.

In diabetes there is a notable surge of IC formation following the addition of CCA in comparison to that in normal serum. Since the study showed that in normal sera artificially increased glucose concentrations keeps ABG from binding to added CCA, the pattern of CCA-IC formation in clinically confirmed diabetic patients was examined further.

Sugar inhibition pattern of CCA-precipitated antibodies varied widely among diabetic cases included for the study. In most cases there is only marginal inhibition by the sugars. Carbohydrate independent antibodies seem to bring about the elevated response in diabetic serum. Further characterization of the CCA-IC was done by studying the IC-interaction with electrophoretically separated CCA fractions. The mean percentage inhibition of CCA-IC from normal and diabetic serum samples using 50 mM cellobiose was 35% and 21% respectively.

Eventhough high glucose concentration was found to be inhibitory for ABG the results as a whole suggest that clinical status of the subject is relevant while studying the pattern of the antibody response. Out of the six diabetic cases

studied, having fasting blood glucose level above 300 mg/dl, binding of immunoglobulins from redissolved CCA-IC is restricted to a particular portion alone in four of the cases, i.e. the seventh fraction of the electrophoresed CCA eluate.

Results show that in all six normoglycemic cases studied, having fasting blood glucose level 80 mg/dl, binding of immunoglobulins from CCA-IC occurs to all regions, maximum being seen to the 7^{th} fraction. Result suggests that even though in normoglycemics the immune complex formation is mainly sugar-dependent, antibodies that recognize the immunodominant region (7^{th} fraction) is sugar independent. This shows the non-carbohydrate nature of this epitope.

Sugar inhibition pattern shows that apart from fraction 7, binding to all other cut sections can be inhibited by cellobiose and mannose; where as binding to the 7^{th} cut section was sugar independent. In diabetic CCA-IC also, binding of antibodies to the 7^{th} fraction is sugar independent. This finding is in correlation with the previous observation that immunoglobulins that take part in IC formation with CCA in normoglycemics are more sugar reversible than the hyperglycemic cases.

Result suggests that the antibodies that bind to all other sections than section 7 are sugar dependent in nature. The molecular weight of the 7^{th} fraction component was estimated to be 47 kDa by using ovalbumin as a marker.

Sugar inhibition studies done with cellobiose and mannose in diabetic sera have shown that in most of these patients, who supposedly have combated the *Candida* infection, there was significant reduction in response to all electrophoretically separated fractions other than the 47 kDa fraction which is sugar independently recognized. The former are rich in mannoproteins of varying molecular

weights to which β-glucan is covalently associated. But in few diabetics antibodies were found responsive to the non-47 kDa fractions as well.

The immunoglobulin composition of the antibody population binding to the immunodominant antigen in diabetics was characterized. The significantly high concentration of IgA in this antibody population is noted. Result shows that IgA is the major immunoglobulin type that is involved in immune complex formation even in diabetics even though the role of anti-carbohydrate antibodies in immune response is lower in them.

Immune complexes formation in serum upon the addition of dextran was studied. NDS consisting of bacterial dextrans having been detected consistently in table sugar samples in this region, this indigestible high molecular weight biomolecule may enter systemic circulation as an antigen. The recognition by DIg of each of the eight fractions of NDS with varying size and charge points to the high potential of this dietary polysaccharide to act as a constant trigger for formation of IC with DIg. Results also suggest the possibility that other environmental antigens from yeast and cereals that may enter the body in large quantities form IC with DIg.

The potential of non-dialysable sugar (NDS) to form immune complexes in serum was checked. The role of DIg in precipitating such immune responses was specially noted. This was compared with the IC formation mediated by dextran. In comparison DIg had IgM content nearly equal to its IgG content, while IgA content remained low. Notably DIg isolated from IC formed after addition of dextran to serum consisted overwhelmingly (about 72%) of IgM with both IgG and IgA contributing marginally. Role of high sugar in serum on DIg-IC formation was examined. As in the case of ABG, DIg also is likely to be inhibited by high sugar in serum. Natural IC

prepared from normal healthy donors in this study consisted up to 50% of IgG and the rest equally of IgA and IgM.

Immobilisation of galectin-1 on microtitre plate was done by adding biotinylated lectin to streptavidin coated plates. The binding of immobilized galectin-1 to IgA was demonstrated. DIg consisting of polymeric IgA binds to immobilised galectin-1 whereas Anti-Gal which lacks pIgA does not. This leads to the conclusion that the homogenous lattice formation that occurs by crosslinking of multivalent ligand i.e., pIgA and bivalent lectin galectin-1 leads to the effective binding of the former. Since monovalent ligand as occurs in monomeric IgA does not lead to such an efficient lattice formation with galectin-1 they do not bind. This demonstrates that when galectin-1 is in immobilised state as it occurs in endothelial or mesangial cell surfaces, its carbohydrate binding capacity is retained. High titre of pIgA in type II diabetes makes diabetic-IC more efficient in binding to galectin-1 since the glycosylated part of IgA in IC is free. Type II diabetes is associated with increased expression of galectin-1, polymeric forms of IgA as well as high concentrations of sialidases in circulation which altogether aggravates the scenario.

The results obtained from this thesis suggest that high titre anti-glycan antibodies in the circulation of tropical individuals meet their epitopes resulting in a surge of immune complexes. The heavy microbial load of the tropical environment enhances the phenomenon.

The binding of pIgA in free as well as complexed form to immobilised galectin-1 is demonstrated. This offers a likely explanation for the high turn over rate

of IgA1 in normal physiological situations as well as the pathogenicity owing to the deposition of pIgA1-IC. The correlation of place of IgA1 deposition with the occurrence of galectin-1 further validates this hypothesis. The reported occurrence of pIgA more in depositions as well as the asialo-nature of O-glycan in deposited IgA further supports this hypothesis.

Immune complexes that are known causes of vascular damages and inflammation have been shown to be formed between several carbohydrate binding serum antibodies in the one hand and carbohydrate antigens of dietary, environmental or microbial origin on the other. In particular, diabetic individuals have been shown to develop a quantitatively and qualitatively different antibody profile that exposes them to potential IC-induced damages. Polymeric IgA synthesized more in diabetes have been shown to be particularly susceptible to bind tissue galectin-1. Finally, in order to mimic the action of in vivo galectin-1 which is immobilized, a novel method to study the properties of immobilized tissue galectin-1 in active form has been developed.

An outcome of the study is the possible use of galectin-1 inhibitors in mitigating inflammatory immune damage to vessel walls in diabetes. It was demonstrated in this laboratory that human RBC glycopeptides can efficiently reverse the binding of galectin-1 to its ligands. Further studies in this topic may result in development of the therapeutics to block immune complex deposition mediated illnesses.

Future directions

1) Demonstration of uptake of IgA1-IC by galectin-1 in activated HUVEC cell line.

2) Kinetic studies to determine the dynamics of uptake of IgA1 in free as well as complexed form.

3) Studies on atherosclerotic patients to correlate the titre of anti-glycan antibodies with severity of illness.

4) Histochemical analyses to demonstrate *in situ* localization of pIgA on galectin-1 on human arterial sections.

5) Develop inhibitors of pIgA binding to galectin-1 to stall pIgA-IC deposition.

BIBLIOGRAPHY

Abbott, D., Bourne, E.J. and Weigel, H. (1966). J Chem Soc Perkin 1, 9, 827-831.

Abbott, W.M. and Feizi, T. (1989). Biochem J, 259, 291-294.

Acharya, S.K., Balwinder, S., Padhee, A.K., Nijhawan, S. and Tandon, B.N. (1992). J Clin Gastroenterol, 14, 31-35.

Adachi, Y., Ohno, N. and Yadomae, T. (1994). Biol Pharm Bull, 17, 1508-1512.

Adams, L., Scott, G.K. and Weinberg, C.S. (1996). Biochim Biophys Acta, 1312, 137-144.

Ahmad, N., Gabius, H.J., Sabesan, S., Oscarson, S. and Brewer, C.F. (2004). Glycobiology, 14, 817-825.

Ahren, I.L., Williams, D.L., Rice, P.J., Forsgren, A. and Riesbeck, K. (2001). J Infect Dis, 184, 150-158.

Akira, S. (2001). Adv Immunol, 78, 1-56.

Amano, M., Galvan, M., He, J. and Baum, L.G. (2003). J Biol Chem, 278, 7469-7475.

Anastase, S., Letourneur, D. and Jozefonvicz, J. (1996). J Chromatogr B Biomed Appl, 686, 141-150.

Andersen, H., Jensen, O.N., Moiseeva, E.P. and Eriksen, E.F. (2003). J Bone Miner Res, 18, 195-203.

Andre, S., Kojima, S., Yamazaki, N., Fink, C., Kaltner, H., Kayser, K. and Gabius, H.J. (1999). J Cancer Res Clin Oncol, 125, 461-474.

Andre, S., Sanchez-Ruderisch, H., Nakagawa, H., Buchholz, M., Kopitz, J., Forberich, P., Kemmner, W., Bock, C., Deguchi, K., Detjen, K.M., Wiedenmann, B., von Knebel Doeberitz, M., Gress, T.M., Nishimura, S., Rosewicz, S. and Gabius, H.J. (2007). Febs J, 274, 3233-3256.

Appukuttan, P.S. (2002). J Mol Recognit, 15, 180-187.

Appukuttan, P.S., Surolia, A. and Bachhawat, B.K. (1977). Indian J Biochem Biophys, 14, 382-384.

Ashwell, G. and Morell, A.G. (1974). Adv Enzymol Relat Areas Mol Biol, 41, 99-128.

Avrameas, S. (1991). Immunol Today, 12, 154-159.

Baenziger, J. and Kornfeld, S. (1974). J Biol Chem, 249, 7270-7281.

Baenziger, J.U. (1985). Am J Pathol, 121, 382-391.

Bagheri, N., Pepple, D.A., Hassan, M.O., Harding, C.V. and Emancipator, S.N. (2005). Lab Invest, 85, 354-363.

Bahl, O.P., Marz, L. and Moyle, W.R. (1974). Curr Top Mol Endocrinol, 1, 125-144.

Ballou,C.E (1982). The Molecular biology of the yeast Saccharomyces. Metabolism and gene expression. Cold Spring Harbor laboratory Press,NY.

Banas, J.A. and Vickerman, M.M. (2003). Crit Rev Oral Biol Med, 14, 89-99.

Bargellesi, A., Periman, P. and Scharff, M.D. (1972). J Immunol, 108, 126-134.

Barlow, A.J. and Chattaway, F.W. (1969). Br J Dermatol, 81, 103-106.

Barondes, S.H. (1984). Science, 223, 1259-1264.

Barondes, S.H., Castronovo, V., Cooper, D.N., Cummings, R.D., Drickamer, K., Feizi, T., Gitt, M.A., Hirabayashi, J., Hughes, C., Kasai, K. and et al. (1994). Cell, 76, 597-598.

Barondes, S.H., Cooper, D.N., Gitt, M.A. and Leffler, H. (1994). J Biol Chem, 269, 20807-20810.

Barrionuevo, P., Beigier-Bompadre, M., Ilarregui, J.M., Toscano, M.A., Bianco, G.A., Isturiz, M.A. and Rabinovich, G.A. (2007). J Immunol, 178, 436-445.

Basset, C., Devauchelle, V., Durand, V., Jamin, C., Pennec, Y.L., Youinou, P. and Dueymes, M. (1999). Scand J Immunol, 50, 572-579.

Basu, M. and Basu, S. (1973). J Biol Chem, 248, 1700-1706.

Baum, L. G., J. J. Seilhamer, et al. (1995). Glycoconj J 12(1): 63-8.

Baum, L.G., Pang, M., Perillo, N.L., Wu, T., Delegeane, A., Uittenbogaart, C.H., Fukuda, M. and Seilhamer, J.J. (1995). J Exp Med, 181, 877-887.

Berberat, P.O., Friess, H., Wang, L., Zhu, Z., Bley, T., Frigeri, L., Zimmermann, A. and Buchler, M.W. (2001). J Histochem Cytochem, 49, 539-549.

Berek, C. and Ziegner, M. (1993). Immunol Today, 14, 400-404.

Beyer, E.C., Zweig, S.E. and Barondes, S.H. (1980). J Biol Chem, 255, 4236-4239.

Bhatia, A. and Kast, R.E. (2007). Cell Mol Biol Lett, 12, 111-119.

Bhattacharyya, L., J. Fant, et al. (1990). Biochemistry 29(32): 7523-30.

Bhattacharyya, L., M. Haraldsson, et al. (1988). Biochemistry 27(3): 1034-41.

Bhavanandan, V.P. (1991). Glycobiology, 1, 493-503.

Blaser, C., Kaufmann, M., Muller, C., Zimmermann, C., Wells, V., Mallucci, L. and Pircher, H. (1998). Eur J Immunol, 28, 2311-2319.

Bleil, J.D. and Wassarman, P.M. (1988). Proc Natl Acad Sci U S A, 85, 6778-6782.

Blois, S.M., Ilarregui, J.M., Tometten, M., Garcia, M., Orsal, A.S., Cordo-Russo, R., Toscano, M.A., Bianco, G.A., Kobelt, P., Handjiski, B., Tirado, I., Markert, U.R., Klapp, B.F., Poirier, F., Szekeres-Bartho, J., Rabinovich, G.A. and Arck, P.C. (2007). Nat Med, 13, 1450-1457.

Bos, N.A., Bun, J.C., Popma, S.H., Cebra, E.R., Deenen, G.J., van der Cammen, M.J., Kroese, F.G. and Cebra, J.J. (1996). Infect Immun, 64, 616-623.

Bos, N.A., Kimura, H., Meeuwsen, C.G., De Visser, H., Hazenberg, M.P., Wostmann, B.S., Pleasants, J.R., Benner, R. and Marcus, D.M. (1989). Eur J Immunol, 19, 2335-2339.

Bradford, M.M. (1976). Anal Biochem, 72, 248-254.

Breuel, K.F., Kougias, P., Rice, P.J., Wei, D., De Ponti, K., Wang, J., Laffan, J.J., Li, C., Kalbfleisch, J. and Williams, D.L. (2004). Neuroimmunomodulation, 11, 1-9.

Brewer, C.F., Miceli, M.C. and Baum, L.G. (2002). Curr Opin Struct Biol, 12, 616-623.

Bromuro, C., Torosantucci, A., Chiani, P., Conti, S., Polonelli, L. and Cassone, A. (2002). Infect Immun, 70, 5462-5470.

Brown, G.D. and Gordon, S. (2001). Nature, 413, 36-37.

Brown, G.D., Herre, J., Williams, D.L., Willment, J.A., Marshall, A.S. and Gordon, S. (2003). J Exp Med, 197, 1119-1124.

Cabib, E., Bowers, B., Sburlati, A. and Silverman, S.J. (1988). Microbiol Sci, 5, 370-375.

Camby, I., Belot, N., Rorive, S., Lefranc, F., Maurage, C.A., Lahm, H., Kaltner, H., Hadari, Y., Ruchoux, M.M., Brotchi, J., Zick, Y., Salmon, I., Gabius, H.J. and Kiss, R. (2001). Brain Pathol, 11, 12-26.

Camby, I., Decaestecker, C., Lefranc, F., Kaltner, H., Gabius, H.J. and Kiss, R. (2005). Biochem Biophys Res Commun, 335, 27-35.

Camby, I., Le Mercier, M., Lefranc, F. and Kiss, R. (2006). Glycobiology, 16, 137R-157R.

Caron, M., Bladier, D. and Joubert, R. (1990). Int J Biochem, 22, 1379-1385.

Casanova, M., Lopez-Ribot, J.L., Martinez, J.P. and Sentandreu, R. (1992). Infect Immun, 60, 4898-4906.

Cassone, A. (1989). Curr Top Med Mycol, 3, 248-314.

Cassone, A., De Bernardis, F. and Torososantucci, A. (2005). Curr Mol Med, 5, 377-382.

Chacko, B.K. and Appukuttan, P.S. (2001). Int J Biol Macromol, 28, 365-371.

Chacko, B.K. and Appukuttan, P.S. (2003). Mol Immunol, 39, 933-939.

Chaffin, W.L. and Stocco, D.M. (1983). Can J Microbiol, 29, 1438-1444.

Chauhan, D., Li, G., Podar, K., Hideshima, T., Neri, P., He, D., Mitsiades, N., Richardson, P., Chang, Y., Schindler, J., Carver, B. and Anderson, K.C. (2005). Cancer Res, 65, 8350-8358.

Chellan, B., Narayani, J. and Appukuttan, P.S. (2007). Exp Mol Pathol, 83, 399-404.

Chen, Z.J., Wheeler, C.J., Shi, W., Wu, A.J., Yarboro, C.H., Gallagher, M. and Notkins, A.L. (1998). Eur J Immunol, 28, 989-994.

Cherayil, B.J., Weiner, S.J. and Pillai, S. (1989). J Exp Med, 170, 1959-1972.

Chevailler, A., R. C. Monteiro, et al. (1989). J Immunol 142(7): 2244-9.

Chiani, P., Bromuro, C., Cassone, A. and Torosantucci, A. (2009). Vaccine, 27, 513-519.

Chiariotti, L., Salvatore, P., Benvenuto, G. and Bruni, C.B. (1999). Biochimie, 81, 381-388.

Childs, R.A. and Feizi, T. (1979). Biochem J, 183, 755-758.

Cho, M. and Cummings, R.D. (1995). J Biol Chem, 270, 5207-5212.

Cho, Y., K. Usui, et al. (2006). Biochem Biophys Res Commun 345(1): 474-8.

Cisar, J., Kabat, E.A., Dorner, M.M. and Liao, J. (1975). J Exp Med, 142, 435-459.

Clausse, N., van den Brule, F., Waltregny, D., Garnier, F. and Castronovo, V. (1999). Angiogenesis, 3, 317-325.

Cleves, A.E., Cooper, D.N., Barondes, S.H. and Kelly, R.B. (1996). J Cell Biol, 133, 1017-1026.

Cochrane CG and Dixon FJ: Immune complex injury, Immunological Diseases. Vol I. Edited by M Samter. Boston, Little, Brown, 1978, pp 210-229.

Cognet, I., F. Guilhot, et al. (2005). J Immunol Methods 301(1-2): 53-65.

Colnot, C., Ripoche, M.A., Scaerou, F., Foulis, D. and Poirier, F. (1996). Biochem Soc Trans, 24, 141-146.

Cook, G.M. (1986). J Cell Sci Suppl, 4, 45-70.

Cooper, D.K. (1998). Xenotransplantation, 5, 6-17.

Cooper, D.N. and Barondes, S.H. (1990). J Cell Biol, 110, 1681-1691.

Cooper, D.N. and Barondes, S.H. (1999). Glycobiology, 9, 979-984.

Cooper, D.N., Boulianne, R.P., Charlton, S., Farrell, E.M., Sucher, A. and Lu, B.C. (1997). J Biol Chem, 272, 1514-1521.

Correa, S.G., Sotomayor, C.E., Aoki, M.P., Maldonado, C.A. and Rabinovich, G.A. (2003). Glycobiology, 13, 119-128.

Coutinho, A. and Forni, L. (1982). Embo J, 1, 1251-1257.

Cream,JJ ., Bryceson, ADM and Ryder , Br J Dermatol. 1971;84:106 –109

Cretin, N., Bracy, J., Hanson, K. and Iacomini, J. (2002). J Immunol, 168, 1479-1483.

Cuatrecasas, P. and Anfinsen, C.B. (1971). Methods Enzymol, 22, 345-378.

Czerkinsky, C., Prince, S.J., Michalek, S.M., Jackson, S., Moldoveanu, Z., Russell, M.W., McGhee, J.R. and Mestecky, J. (1987). Adv Exp Med Biol, 216B, 1709-1719.

Czop, J.K. and Austen, K.F. (1985). J Immunol, 134, 2588-2593.

Dam, T. K., H. J. Gabius, et al. (2005). Biochemistry 44(37): 12564-71.

Davin, J.C., Malaise, M., Foidart, J. and Mahieu, P. (1987). Kidney Int, 31, 1132-1139.

Davis, A.C. and Shulman, M.J. (1989). Immunol Today, 10, 118-122; 127-118.

Davis, B.J. (1964). Ann NY Acad Sci, 121, 404-427.

de Waard, A., Hickman, S. and Kornfeld, S. (1976). J Biol Chem, 251, 7581-7587.

Dean, P.D.G., Johnson, W.S. and Middle, F.A. (1985) Affinity Chroamtography: A Practical Approach. IRL Press, Oxford.

Del Prete, G., Maggi, E., Parronchi, P., Chretien, I., Tiri, A., Macchia, D., Ricci, M., Banchereau, J., De Vries, J. and Romagnani, S. (1988). J Immunol, 140, 4193-4198.

Den, H. and Malinzak, D.A. (1977). J Biol Chem, 252, 5444-5448.

Dessen, A., D. Gupta, et al. (1995). Biochemistry 34(15): 4933-42.

D'Haene, N., Maris, C., Sandras, F., Dehou, M.F., Remmelink, M., Decaestecker, C. and Salmon, I. (2005). Int J Immunopathol Pharmacol, 18, 431-443.

Di Mario, U., M. Iavicoli, et al. (1980). Diabetologia 19(2): 89-92.

Diamond, R.D. (1989). Rev Infect Dis, 11 Suppl 7, S1600-1604.

Dias-Baruffi, M., Zhu, H., Cho, M., Karmakar, S., McEver, R.P. and Cummings, R.D. (2003). J Biol Chem, 278, 41282-41293.

Dochez, A.R. and Avery, O.T. (1917). J Exp Med, 26, 477-493.

Domer, J.E. (1989). Crit Rev Microbiol, 17, 33-51.

Dotan, N., Altstock, R.T., Schwarz, M. and Dukler, A. (2006). Lupus, 15, 442-450.

Drickamer, K. (1988). J Biol Chem, 263, 9557-9560.

Dubois, M , Gills,KA, Hamilton JK, Rebers PA and Smith F (1956), Anal Biochem, 28, 350-356.

Dulley, J.R. and Grieve, P.A. (1975). Anal Biochem, 64, 136-141.

Durandy, A., Fischer, A. and Griscelli, C. (1983). J Clin Invest, 71, 1602-1613.

Edelman, G.M., Cunningham, B.A., Gall, W.E., Gottlieb, P.D., Rutishauser, U. and Waxdal, M.J. (1969). Proc Natl Acad Sci U S A, 63, 78-85.

Egnaczyk, G.F., Pomonis, J.D., Schmidt, J.A., Rogers, S.D., Peters, C., Ghilardi, J.R., Mantyh, P.W. and Maggio, J.E. (2003). Proteomics, 3, 689-698.

Eifuku, H., Yoshimitsu-Narita, A., Sato, S., Yakushiji, T. and Inoue, M. (1989). Carbohydr Res, 194, 247-260.

Ellerhorst, J., Nguyen, T., Cooper, D.N., Estrov, Y., Lotan, D. and Lotan, R. (1999). Int J Oncol, 14, 225-232.

Emancipator, S.N. and Lamm, M.E. (1989). Lab Invest, 60, 168-183.

Emsley, J., White, H.E., O'Hara, B.P., Oliva, G., Srinivasan, N., Tickle, I.J., Blundell, T.L., Pepys, M.B. and Wood, S.P. (1994). Nature, 367, 338-345.

Ensley, H.E., Tobias, B., Pretus, H.A., McNamee, R.B., Jones, E.L., Browder, I.W. and Williams, D.L. (1994). Carbohydr Res, 258, 307-311.

Erwig, L.P. and Henson, P.M. (2007). Am J Pathol, 171, 2-8.

Fagarasan, S. and Honjo, T. (2000). Science, 290, 89-92.

Farah, C.S., Elahi, S., Drysdale, K., Pang, G., Gotjamanos, T., Seymour, G.J., Clancy, R.L. and Ashman, R.B. (2002). Infect Immun, 70, 724-731.

Farah, C.S., Elahi, S., Pang, G., Gotjamanos, T., Seymour, G.J., Clancy, R.L. and Ashman, R.B. (2001). Infect Immun, 69, 6110-6118.

Finbloom, D.S., Abeles, D., Rifai, A. and Plotz, P.H. (1980). J Immunol, 125, 1060-1065.

Florman, H.M. and Wassarman, P.M. (1985). Cell, 41, 313-324.

Foy, T.M., Shepherd, D.M., Durie, F.H., Aruffo, A., Ledbetter, J.A. and Noelle, R.J. (1993). J Exp Med, 178, 1567-1575.

Frangione, B. and Wolfenstein-Todel, C. (1972). Proc Natl Acad Sci U S A, 69, 3673-3676.

Franklyn, K.M., Warmington, J.R., Ott, A.K. and Ashman, R.B. (1990). Immunol Cell Biol, 68 (Pt 3), 173-178.

Fransson, L.A., Silverberg, I. and Carlstedt, I. (1985). J Biol Chem, 260, 14722-14726.

Friedenreich V., 1930, The Thomsen Hemagglutination phenomenon. Levin and Munksgaard, Copenhagen, Denmark.

Frigeri, L.G., Robertson, M.W. and Liu, F.T. (1990). J Biol Chem, 265, 20763-20769.

Fukaya, K., Hasegawa, M., Mashitani, T., Kadoya, T., Horie, H., Hayashi, Y., Fujisawa, H., Tachibana, O., Kida, S. and Yamashita, J. (2003). J Neuropathol Exp Neurol, 62, 162-172.

Fulcher, J.A., Hashimi, S.T., Levroney, E.L., Pang, M., Gurney, K.B., Baum, L.G. and Lee, B. (2006). J Immunol, 177, 216-226.

Gabius, H.J. (1994). Int J Biochem, 26, 469-477.

Gala, F.A. and Morrison, S.L. (2002). J Biol Chem, 277, 29005-29011.

Galili, U. and Anaraki, F. (1995). Glycobiology, 5, 775-782.

Galili, U. and LaTemple, D.C. (1997). Immunol Today, 18, 281-285.

Galili, U., Clark, M.R., Shohet, S.B., Buehler, J. and Macher, B.A. (1987). Proc Natl Acad Sci U S A, 84, 1369-1373.

Galili, U., Buehler, J., Shohet, S.B. and Macher, B.A. (1987a). J Exp Med, 165, 693-704.

Galili, U., LaTemple, D.C., Walgenbach, A.W. and Stone, K.R. (1997). Transplantation, 63, 646-651.

Galili, U., Mandrell, R.E., Hamadeh, R.M., Shohet, S.B. and Griffiss, J.M. (1988). Infect Immun, 56, 1730-1737.

Galili, U., Rachmilewitz, E.A., Peleg, A. and Flechner, I. (1984). J Exp Med, 160, 1519-1531.

Gallo, G.R., Caulin-Glaser, T. and Lamm, M.E. (1981). J Clin Invest, 67, 1305-1313.

Garin, M.I., Chu, C.C., Golshayan, D., Cernuda-Morollon, E., Wait, R. and Lechler, R.I. (2007). Blood, 109, 2058-2065.

Geetha, M., Annamma, K.I., Mathai, J. and Appukuttan, P.S. (2007). Immunol Invest, 36, 73-83.

Gillenwater, A., Xu, X.C., el-Naggar, A.K., Clayman, G.L. and Lotan, R. (1996). Head Neck, 18, 422-432.

Giordanengo, L., Gea, S., Barbieri, G. and Rabinovich, G.A. (2001). Clin Exp Immunol, 124, 266-273.

Gitt, M.A. and Barondes, S.H. (1991). Biochemistry, 30, 82-89.

Gitt, M.A., Colnot, C., Poirier, F., Nani, K.J., Barondes, S.H. and Leffler, H. (1998). J Biol Chem, 273, 2954-2960.

Glinskii, O.V., Huxley, V.H., Glinsky, G.V., Pienta, K.J., Raz, A. and Glinsky, V.V. (2005). Neoplasia, 7, 522-527.

Gonzalez-Cabrero, J., Egido, J., Barat, A. and Gonzalez, E. (1990). Immunology, 70, 296-302.

Gordon, S. and Taylor, P.R. (2005). Nat Rev Immunol, 5, 953-964.

Gottschalk, A. and Thomas, M.A. (1961). Biochim Biophys Acta, 46, 91-98.

Green, N. M., L. Konieczny, et al. (1971). Biochem J 125(3): 781-91.

Greenfield, R.A., Bussey, M.J., Stephens, J.L. and Jones, J.M. (1983). J Infect Dis, 148, 275-283.

Greenwood, BM and Whittle, HC., Immunology of Medicine in the Tropics Edward Arnold, Publishers, Ltd., 1981

Gupta, D., Cho, M., Cummings, R.D. and Brewer, C.F. (1996). Biochemistry, 35, 15236-15243.

Haakenstad, A.O. and Mannik, M. (1974). J Immunol, 112, 1939-1948.

Hamadeh, R.M., Estabrook, M.M., Zhou, P., Jarvis, G.A. and Griffiss, J.M. (1995). Infect Immun, 63, 4900-4906.

Hammer, M., Zeidler, H., Klimsa, S. and Heesemann, J. (1990). Arthritis Rheum, 33, 1795-1800.

Hansson, G.K. (2005). N Engl J Med, 352, 1685-1695.

Harada, T., Nagi Miura, N., Adachi, Y., Nakajima, M., Yadomae, T. and Ohno, N. (2003). Biol Pharm Bull, 26, 1225-1228.

Hauptman, S.P. and Tomasi, T.B., Jr. (1975). J Biol Chem, 250, 3891-3896.

Hayes, B.K and Hart, G.W., 1994, Novel forms of protein glycosylation, Curr.Opin.Struct.Biol.4:692-696

He, J. and Baum, L.G. (2004). J Biol Chem, 279, 4705-4712.

He, J. and Baum, L.G. (2006). Methods Enzymol, 417, 247-256.

Heidelberger, M. and Avery, O.T. (1923). J Exp Med, 38, 73-79.

Heyderman, E., Strudley, K., and Richardson, T.C. (1989). Handbook of experimental immunology, Vol.129, Oxford, Blackwell.

Hernandez, J.D. and Baum, L.G. (2002). Glycobiology, 12, 127R-136R.

Hernandez, J.D., Nguyen, J.T., He, J., Wang, W., Ardman, B., Green, J.M., Fukuda, M. and Baum, L.G. (2006). J Immunol, 177, 5328-5336.

Hirabayashi, J. and Kasai, K. (1991). J Biol Chem, 266, 23648-23653.

Hirabayashi, J. and Kasai, K. (1993). Glycobiology, 3, 297-304.

Hirabayashi, J., Ayaki, H., Soma, G. and Kasai, K. (1989). FEBS Lett, 250, 161-165.

Hirabayashi, J., Hashidate, T., Arata, Y., Nishi, N., Nakamura, T., Hirashima, M., Urashima, T., Oka, T., Futai, M., Muller, W.E., Yagi, F. and Kasai, K. (2002). Biochim Biophys Acta, 1572, 232-254.

Hirabayashi, J., Ubukata, T. and Kasai, K. (1996). J Biol Chem, 271, 2497-2505.

Hollander, W., Colombo, M.A., Kirkpatrick, B. and Paddock, J. (1979). Atherosclerosis, 34, 391-405.

Hong, F., Hansen, R.D., Yan, J., Allendorf, D.J., Baran, J.T., Ostroff, G.R. and Ross, G.D. (2003). Cancer Res, 63, 9023-9031.

Hook, M., Couchman, J., Woods, A., Robinson, J. and Christner, J.E. (1984). Ciba Found Symp, 108, 44-59.

Horie, H., Inagaki, Y., Sohma, Y., Nozawa, R., Okawa, K., Hasegawa, M., Muramatsu, N., Kawano, H., Horie, M., Koyama, H., Sakai, I., Takeshita, K., Kowada, Y., Takano, M. and Kadoya, T. (1999). J Neurosci, 19, 9964-9974.

Horie, H., Kadoya, T., Inagaki, Y. and Sohma, Y. (2000). Seikagaku, 72, 1245-1249.

Huflejt, M.E., Vuskovic, M., Vasiliu, D., Xu, H., Obukhova, P., Shilova, N., Tuzikov, A., Galanina, O., Arun, B., Lu, K. and Bovin, N. (2009). Mol Immunol, 46, 3037-3049.

Humphrey, J.H., Parrott, D.M. and East, J. (1964). Immunology, 7, 419-439.

Hunt, L.A. (1983). Biochem J, 209, 659-667.

Husby, S., Jensenius, J.C. and Svehag, S.E. (1986). Scand J Immunol, 22, 83-92.

Iida, K., Nadler, L. and Nussenzweig, V. (1983). J Exp Med, 158, 1021-1033.

Ingrassia, L., Camby, I., Lefranc, F., Mathieu, V., Nshimyumukiza, P., Darro, F. and Kiss, R. (2006). Curr Med Chem, 13, 3513-3527.

Inohara, H. and Raz, A. (1994). Glycoconj J, 11, 527-532.

Isaacs, K.L. and Miller, F. (1983). Am J Pathol, 111, 298-306.

Ishibashi, K., Yoshida, M., Nakabayashi, I., Shinohara, H., Miura, N.N., Adachi, Y. and Ohno, N. (2005). FEMS Immunol Med Microbiol, 44, 99-109.

Iwase.H (1999), Trends in glycoscience and glycotechnology, 11, 113-118

Iwase, H., Katsumata, T., Itoh, A., Hiki, Y., Ikuko, N., Sano, T., Takatani, T. and Kobayashi, Y. (2002). J Nephrol, 15, 703-708.

Iwasaki, H., Zhang, Y., Tachibana, K., Gotoh, M., Kikuchi, N., Kwon, Y.D., Togayachi, A., Kudo, T., Kubota, T. and Narimatsu, H. (2003). J Biol Chem, 278, 5613-5621.

Iwase, H. (1988). Int J Biochem, 20, 479-491.

Iwase, H., Tanaka, A., Hiki, Y., Kokubo, T., Sano, T., Ishii-Karakasa, I., Toma, K., Kobayashi, Y. and Hotta, K. (1999). J Chromatogr B Biomed Sci Appl, 724, 1-7.

Jacobs, A.K., Lipka, V., Burton, R.A., Panstruga, R., Strizhov, N., Schulze-Lefert, P. and Fincher, G.B. (2003). Plant Cell, 15, 2503-2513.

Jaison P L and Appukuttan P S 1992; *Indian* J. *Biochem. Biophys.* **29** 266-270

Jancar S and Sanchez Crespo M. Trends Immunol. 2005;26:48 –55.

Jentoft, N. (1990). Trends Biochem Sci, 15, 291-294.

Jha, H.C., Srivastava, P., Sarkar, R., Prasad, J. and Mittal, A. (2008). J Cardiol, 52, 140-145.

Joubert, R., Caron, M., Avellana-Adalid, V., Mornet, D. and Bladier, D. (1992). J Neurochem, 58, 200-203.

Kabat, E.A. and Berg, D. (1953). J Immunol, 70, 514-532.

Kabat, E.A. and Bezer, A.E. (1958). Arch Biochem Biophys, 78, 306-318.

Kaminski, D.A. and Stavnezer, J. (2006). J Immunol, 177, 6025-6029.

Kano, K. and Milgrom, F. (1977). Curr Top Microbiol Immunol, 77, 43-69.

Kapteyn, J.C., Hoyer, L.L., Hecht, J.E., Muller, W.H., Andel, A., Verkleij, A.J., Makarow, M., Van Den Ende, H. and Klis, F.M. (2000). Mol Microbiol, 35, 601-611.

Kapteyn, J.C., Montijn, R.C., Dijkgraaf, G.J., Van den Ende, H. and Klis, F.M. (1995). J Bacteriol, 177, 3788-3792.

Karmakar, S., Cummings, R.D. and McEver, R.P. (2005). J Biol Chem, 280, 28623-28631.

Kato, I., Koenig, K.L., Watanabe-Meserve, H., Baptiste, M.S., Lillquist, P.P., Frizzera, G., Burke, J.S., Moseson, M. and Shore, R.E. (2005). Cancer Causes Control, 16, 1215-1224.

Kato, S., K. Ozawa, et al. (2004). Dig Dis Sci 49(11-12): 1777-81.

Kennel, A., Bene, M.C., Hurault de Ligny, B., Kessler, M. and Faure, G. (1995). Clin Nephrol, 43, 216-220.

Kerr, M.A. (1990). Biochem J, 271, 285-296.

Kim, D., Robyt, J.F., Lee, S.Y., Lee, J.H. and Kim, Y.M. (2003). Carbohydr Res, 338, 1183-1189.

Kim, Y.T., Kim, E.H., Cheong, C., Williams, D.L., Kim, C.W. and Lim, S.T. (2000). Carbohydr Res, 328, 331-341.

Kjellen, L. and Lindahl, U. (1991). Annu Rev Biochem, 60, 443-475.

Klaus, G.G. (1979). Immunology, 37, 345-351.

Klein, A., Carnoy, C., Wieruszeski, J.M., Strecker, G., Strang, A.M., van Halbeek, H., Roussel, P. and Lamblin, G. (1992). Biochemistry, 31, 6152-6165.

Knight, L. and Fletcher, J. (1971). J Infect Dis, 123, 371-377.

Kondori, N., Edebo, L. and Mattsby-Baltzer, I. (2003). Med Mycol, 41, 21-30.

Kopecka, M. and Kreger, D.R. (1986). Arch Microbiol, 143, 387-395.

Kopitz, J., von Reitzenstein, C., Andre, S., Kaltner, H., Uhl, J., Ehemann, V., Cantz, M. and Gabius, H.J. (2001). J Biol Chem, 276, 35917-35923.

Kornfeld, R. and Kornfeld, S. (1976). Annu Rev Biochem, 45, 217-237.

Kornfeld, R. and Kornfeld, S. (1985). Annu Rev Biochem, 54, 631-664.

Kovanen, P. T., M. Manttari, et al. (1998 Arch Intern Med 158(13): 1434-9.

Kougias, P., Wei, D., Rice, P.J., Ensley, H.E., Kalbfleisch, J., Williams, D.L. and Browder, I.W. (2001). Infect Immun, 69, 3933-3938.

Kraft, D., Hedin, H., Richter, W., Scheiner, O., Rumpold, H. and Devey, M.E. (1982). Allergy, 37, 481-489.

Krieg, A.M. (2002). Annu Rev Immunol, 20, 709-760.

Kroese, F.G., Timens, W. and Nieuwenhuis, P. (1990). Curr Top Pathol, 84 (Pt 1), 103-148.

Kutteh, W.H., Prince, S.J. and Mestecky, J. (1982). J Immunol, 128, 990-995.

Kyburz D and Corr M. Springer Semin Immunopathol. 2003;25:79 –90

La, M., Cao, T.V., Cerchiaro, G., Chilton, K., Hirabayashi, J., Kasai, K., Oliani, S.M., Chernajovsky, Y. and Perretti, M. (2003). Am J Pathol, 163, 1505-1515.

Lanteri, M., Giordanengo, V., Hiraoka, N., Fuzibet, J.G., Auberger, P., Fukuda, M., Baum, L.G. and Lefebvre, J.C. (2003). Glycobiology, 13, 909-918.

Lanteri, M., Giordanengo, V., Vidal, F., Gaudray, P. and Lefebvre, J.C. (2002). Glycobiology, 12, 785-792.

Leffler, H. and Barondes, S.H. (1986). J Biol Chem, 261, 10119-10126.

Lekakh, I.V., Bovin, N.V., Bezyaeva, G.P. and Poverenny, A.M. (2001). Biochemistry (Mosc), 66, 163-167.

Leppanen, A., Stowell, S., Blixt, O. and Cummings, R.D. (2005). J Biol Chem, 280, 5549-5562.

Leslie, R.G. (1980). Eur J Immunol, 10, 323-333.

Levi, G. and Teichberg, V.I. (1981). J Biol Chem, 256, 5735-5740.

Levi, G. and Teichberg, V.I. (1982). FEBS Lett, 148, 145-148.

Levroney, E.L., Aguilar, H.C., Fulcher, J.A., Kohatsu, L., Pace, K.E., Pang, M., Gurney, K.B., Baum, L.G. and Lee, B. (2005). J Immunol, 175, 413-420.

Lim, J.W., Kim, H. and Kim, K.H. (2003). Int J Biochem Cell Biol, 35, 1284-1296.

Lis, H. and Sharon, N. (1993). Eur J Biochem, 218, 1-27.

Lisowska E (1988), In A.M. Wu (ed.), The Molecular Immunology of Complex Carbohydrates, Plenum Press, New York, pp. 265-315.

Liu, F.T. (1993). Immunol Today, 14, 486-490.

Liu, F.T. (2002). Expert Opin Ther Targets, 6, 461-468.

Liu, F.T., Hsu, D.K., Zuberi, R.I., Kuwabara, I., Chi, E.Y. and Henderson, W.R., Jr. (1995). Am J Pathol, 147, 1016-1028.

Lloyd, K.O. (1991). Semin Cancer Biol, 2, 421-431.

Lobsanov, Y.D., Gitt, M.A., Leffler, H., Barondes, S. and Rini, J.M. (1993). J Mol Biol, 233, 553-555.

Lopez-Lucendo, M.F., Solis, D., Andre, S., Hirabayashi, J., Kasai, K., Kaltner, H., Gabius, H.J. and Romero, A. (2004). J Mol Biol, 343, 957-970.

Loris, R. (2002). Biochim Biophys Acta, 1572, 198-208.

Lotan, R., Belloni, P.N., Tressler, R.J., Lotan, D., Xu, X.C. and Nicolson, G.L. (1994). Glycoconj J, 11, 462-468.

Lowe, E.P., Wei, D., Rice, P.J., Li, C., Kalbfleisch, J., Browder, I.W. and Williams, D.L. (2002). Am Surg, 68, 508-517; discussion 517-508.

Lowe, J.B. (2001). Cell, 104, 809-812.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). J Biol Chem, 193, 265-275.

Lutomski, D., Joubert-Caron, R., Lefebure, C., Salama, J., Belin, C., Bladier, D. and Caron, M. (1997). Clin Chim Acta, 262, 131-138.

Luzzati, A.L. and Jacobson, E.B. (1972). Eur J Immunol, 2, 473-474.

Maemura K and Fukuda M (1994), Asymmetry, 5, 2093-2108.

Maina, N.H., Tenkanen, M., Maaheimo, H., Juvonen, R. and Virkki, L. (2008). Carbohydr Res, 343, 1446-1455.

Malhotra, R., Wormald, M.R., Rudd, P.M., Fischer, P.B., Dwek, R.A. and Sim, R.B. (1995). Nat Med, 1, 237-243.

Malik, R.K., Ghurye, R.R., Lawrence-Watt, D.J. and Stewart, H.J. (2009). Glycobiology, 19, 1402-1407.

Maliszewski, C. R., L. Shen, et al. (1985). J Immunol 135(6): 3878-81.

Mandal, D. K. and C. F. Brewer (1992). Biochemistry 31(36): 8465-72.

Mandal, D. K. and C. F. Brewer (1993). Biochemistry 32(19): 5116-20.

Mandal, D.K. and Brewer, C.F. (1992). Biochemistry, 31, 8465-8472.

Mannik, M. and Arend, W.P. (1971). J Exp Med, 134, 19-31.

Marshall, R.D. (1972). Annu Rev Biochem, 41, 673-702.

Martinez, J.P., Gil, M.L., Lopez-Ribot, J.L. and Chaffin, W.L. (1998). Clin Microbiol Rev, 11, 121-141.

Mascart-Lemone F, Duchateau J, Conley ME, Delacroix DL. Immunology. 1987 Aug;**61** (4):409–413.

Masuzawa,M , Yoshida,M , Ken-ichi Ishibashi , Nakaya Saito , Masakazu Akashi , Noriko Yoshikawa, Tatsuya Suzuki , Sachiko Nameda , Noriko N. Miura , Yoshiyuki Adachi , Naohito Ohno (2002) Drug development research 58; 2, 179-189

Matarrese, P., Tinari, A., Mormone, E., Bianco, G.A., Toscano, M.A., Ascione, B., Rabinovich, G.A. and Malorni, W. (2005). J Biol Chem, 280, 6969-6985.

Matsuda, S., Waldo, F.B., Czerkinsky, C., Moldoveanu, Z. and Mestecky, J. (1988). Clin Immunol Immunopathol, 48, 1-9.

Matsumoto, R., Matsumoto, H., Seki, M., Hata, M., Asano, Y., Kanegasaki, S., Stevens, R.L. and Hirashima, M. (1998). J Biol Chem, 273, 16976-16984.

Matthews, R. and Burnie, J. (1988). J Clin Microbiol, 26, 459-463.

Matthews, R. and Burnie, J. (1996). Trends Microbiol, 4, 354-358.

Mattu, T.S., Pleass, R.J., Willis, A.C., Kilian, M., Wormald, M.R., Lellouch, A.C., Rudd, P.M., Woof, J.M. and Dwek, R.A. (1998). J Biol Chem, 273, 2260-2272.

Mawhinney, T.P., Adelstein, E., Gayer, D.A., Landrum, D.C. and Barbero, G.J. (1992). Carbohydr Res, 223, 187-207.

Mayadas, TN., George C. Tsokos and Naotake Tsuboi (2009) Circulation, 120;2012-2024

Meddens, M.J., Thompson, J., Leijh, P.C. and van Furth, R. (1984). Br J Exp Pathol, 65, 257-265.

Medzhitov, R. and Janeway, C.A., Jr. (1997). Curr Opin Immunol, 9, 4-9.

Mehrabian, M., Gitt, M.A., Sparkes, R.S., Leffler, H., Barondes, S.H. and Lusis, A.J. (1993). Genomics, 15, 418-420.

Mehul, B. and Hughes, R.C. (1997). J Cell Sci, 110 (Pt 10), 1169-1178.

Mehvar, R. (2000). J Control Release, 69, 1-25.

Menon, R.P. and Hughes, R.C. (1999). Eur J Biochem, 264, 569-576.

Mestecky J,Russel MW, Jackson S and Brown,TA (1986), Clin.Immunol.Immunopathol, 40, 105

Mestecky, J. and J. R. McGhee (1987). Adv Immunol 40: 153-245.

Michalek, M., Melican, D., Brunke-Reese, D., Langevin, M., Lemerise, K., Galbraith, W., Patchen, M. and Mackin, W. (1998). J Leukoc Biol, 64, 337-344.

Michelin, MA., Crott, LSP., Assis- Pandochi,AI ., Coimbra, TM., Teixeira, JE and Barbosa JE. Int J Exp Pathol. 2002;83: 105–110).

Milland, J. and Sandrin, M.S. (2006). Tissue Antigens, 68, 459-466.

Mimura, Y., Church, S., Ghirlando, R., Ashton, P.R., Dong, S., Goodall, M., Lund, J. and Jefferis, R. (2000). Mol Immunol, 37, 697-706.

Mimura, Y., Sondermann, P., Ghirlando, R., Lund, J., Young, S.P., Goodall, M. and Jefferis, R. (2001). J Biol Chem, 276, 45539-45547.

Mitsutake, K., Miyazaki, T., Tashiro, T., Yamamoto, Y., Kakeya, H., Otsubo, T., Kawamura, S., Hossain, M.A., Noda, T., Hirakata, Y. and Kohno, S. (1996). J Clin Microbiol, 34, 1918-1921.

Miyanishi, N., Nishi, N., Abe, H., Kashio, Y., Shinonaga, R., Nakakita, S., Sumiyoshi, W., Yamauchi, A., Nakamura, T., Hirashima, M. and Hirabayashi, J. (2007). Glycobiology, 17, 423-432.

Moiseeva, E.P., Javed, Q., Spring, E.L. and de Bono, D.P. (2000). Cardiovasc Res, 45, 493-502.

Moiseeva, E.P., Spring, E.L., Baron, J.H. and de Bono, D.P. (1999). J Vasc Res, 36, 47-58.

Moiseeva, E.P., Williams, B. and Samani, N.J. (2003). Biochim Biophys Acta, 1619, 125-132.

Moiseeva, E.P., Williams, B., Goodall, A.H. and Samani, N.J. (2003). Biochem Biophys Res Commun, 310, 1010-1016.

Moldoveanu, Z., Egan, M.L. and Mestecky, J. (1984). J Immunol, 133, 3156- 3162.

Mond, J.J., Lees, A. and Snapper, C.M. (1995). Annu Rev Immunol, 13, 655-692.

Monteiro, R.C. and Van De Winkel, J.G. (2003). Annu Rev Immunol, 21, 177-204.

Montinaro, V., L. Gesualdo, et al. (1999). Ann Med Interne (Paris) 150(2): 99-107.

Montreuil, J. (1980). Adv Carbohydr Chem Biochem, 37, 157-223.

Morito, T., Nishimaki, T., Saito, K., Yoshida, H., Kasukawa, R. and Kano, K. (1985). Int Arch Allergy Appl Immunol, 78, 213-217.

Morris, S., N. Ahmad, et al. (2004). Glycobiology 14(3): 293-300.

Mosier, D.E., Zaldivar, N.M., Goldings, E., Mond, J., Scher, I. and Paul, W.E. (1977). J Infect Dis, 136 Suppl, S14-19.

Mosedale, D. E., A. Chauhan, et al. (2006 J Immunol Methods 309(1-2): 182-91.

Mota, G., M. Manciulea, et al. (2003). Eur J Immunol 33(8): 2197-205.

Moura, I. C., M. N. Centelles, et al. (2001) J Exp Med 194(4): 417-25.

Munoz, P., Bouza, E., Cuenca-Estrella, M., Eiros, J.M., Perez, M.J., Sanchez-Somolinos, M., Rincon, C., Hortal, J. and Pelaez, T. (2005). Clin Infect Dis, 40, 1625-1634.

Muscari, A., C. Bozzoli, et al. (1988). Atherosclerosis 74(1-2): 179-86.

Muscari, A., C. Bozzoli, et al. (1993). Cardiology 83(5-6): 383-9.

Mustafa, A., Nityanand, S., Berglund, L., Lithell, H. and Lefvert, A.K. (2000). Circulation, 102, 2576-2581.

Nangia-Makker, P., Hogan, V., Honjo, Y., Baccarini, S., Tait, L., Bresalier, R. and Raz, A. (2002). J Natl Cancer Inst, 94, 1854-1862.

Nieminen, J., Kuno, A., Hirabayashi, J. and Sato, S. (2007). J Biol Chem, 282, 1374-1383.

Nieminen, J., St-Pierre, C. and Sato, S. (2005). J Leukoc Biol, 78, 1127-1135.

Nishioka, T., Sakumi, K., Miura, T., Tahara, K., Horie, H., Kadoya, T. and Nakabeppu, Y. (2002). J Biochem, 131, 653-661.

Nono, I., Ohno, N., Masuda, A., Oikawa, S. and Yadomae, T. (1991). J Pharmacobiodyn, 14, 9-19.

Notkins, A.L. (2004). Trends Immunol, 25, 174-179.

Novak, J., H. L. Vu, et al. (2002). Kidney Int 62(2): 465-75.

Nowak, T.P. and Barondes, S.H. (1977). Prog Clin Biol Res, 15, 159-164.

Obayashi, T., Yoshida, M., Mori, T., Goto, H., Yasuoka, A., Iwasaki, H., Teshima, H., Kohno, S., Horiuchi, A., Ito, A. and et al. (1995). Lancet, 345, 17-20.

Ochsenbein, A.F. and Zinkernagel, R.M. (2000). Immunol Today, 21, 624-630.

Oda, Y., Herrmann, J., Gitt, M.A., Turck, C.W., Burlingame, A.L., Barondes, S.H. and Leffler, H. (1993). J Biol Chem, 268, 5929-5939.

Odds, F.C., Evans, E.G., Taylor, M.A. and Wales, J.K. (1978). J Clin Pathol, 31, 840-844.

Offner, H., Celnik, B., Bringman, T.S., Casentini-Borocz, D., Nedwin, G.E. and Vandenbark, A.A. (1990). J Neuroimmunol, 28, 177-184.

Ogden, A.T., Nunes, I., Ko, K., Wu, S., Hines, C.S., Wang, A.F., Hegde, R.S. and Lang, R.A. (1998). J Biol Chem, 273, 28889-28896.

Ogden, R.C. and Adams, D.A. (1987). Methods Enzymol, 152, 61-87.

Ohno, N., Suzuki, I. and Yadomae, T. (1986). Chem Pharm Bull (Tokyo), 34, 1362-1365.

Ohno, N., Uchiyama, M., Tsuzuki, A., Tokunaka, K., Miura, N.N., Adachi, Y., Aizawa, M.W., Tamura, H., Tanaka, S. and Yadomae, T. (1999). Carbohydr Res, 316, 161-172.

Olsen, L. R., A. Dessen, et al. (1997). Biochemistry 36(49): 15073-80.

Otten, M.A. and van Egmond, M. (2004). Immunol Lett, 92, 23-31.

Ouellet, M., Mercier, S., Pelletier, I., Bounou, S., Roy, J., Hirabayashi, J., Sato, S. and Tremblay, M.J. (2005). J Immunol, 174, 4120-4126.

Outenreath, R.L. and Jones, A.L. (1992). J Neurocytol, 21, 788-795.

Ozeki, Y., Matsui, T., Yamamoto, Y., Funahashi, M., Hamako, J. and Titani, K. (1995). Glycobiology, 5, 255-261.

Pace, K.E., Lee, C., Stewart, P.L. and Baum, L.G. (1999). J Immunol, 163, 3801-3811.

Palosuo, T. and Milgrom, F. (1981). Int Arch Allergy Appl Immunol, 65, 153-161.

Parkhouse, R.M., Virella, G. and Dourmashkin, R.R. (1971). Clin Exp Immunol, 8, 581-591.

Parrott, M. B. and M. A. Barry (2000). Mol Ther 1(1): 96-104.

Paz, A., Haklai, R., Elad-Sfadia, G., Ballan, E. and Kloog, Y. (2001). Oncogene, 20, 7486-7493.

Pecquet, S.S., Ehrat, C. and Ernst, P.B. (1992). Infect Immun, 60, 503-509.

Peppard, J.V. and Jackson, L.E. (1987). Adv Exp Med Biol, 216B, 1207-1213.

Perillo, N.L., Marcus, M.E. and Baum, L.G. (1998). J Mol Med, 76, 402-412.

Perillo, N.L., Pace, K.E., Seilhamer, J.J. and Baum, L.G. (1995). Nature, 378, 736-739.

Perone, M.J., Larregina, A.T., Shufesky, W.J., Papworth, G.D., Sullivan, M.L., Zahorchak, A.F., Stolz, D.B., Baum, L.G., Watkins, S.C., Thomson, A.W. and Morelli, A.E. (2006). J Immunol, 176, 7207-7220.

Petrescu, A.J., Milac, A.L., Petrescu, S.M., Dwek, R.A. and Wormald, M.R. (2004). Glycobiology, 14, 103-114.

Pfeifer, K., Haasemann, M., Gamulin, V., Bretting, H., Fahrenholz, F. and Muller, W.E. (1993). Glycobiology, 3, 179-184.

Pierce-Cretel A, Pamblanco M, Strecker G, Montreuil J, and Spik G (1981), Eur J Biochem, 114, 169-178.

Pierce-Cretel, A., Debray, H., Montreuil, J., Spik, G., Van Halbeek, H., Mutsaers, J.H. and Vliegenthart, J.F. (1984). Eur J Biochem, 139, 337-349.

Plotkin, B.J., Paulson, D., Chelich, A., Jurak, D., Cole, J., Kasimos, J., Burdick, J.R. and Casteel, N. (1996). J Med Microbiol, 44, 277-283.

Putnam, F.W., Florent, G., Paul, C., Shinoda, T. and Shimizu, A. (1973). Science, 182, 287-291.

Rabinovich, G.A., Ariel, A., Hershkoviz, R., Hirabayashi, J., Kasai, K.I. and Lider, O. (1999b). Immunology, 97, 100-106.

Rabinovich, G.A., Cumashi, A., Bianco, G.A., Ciavardelli, D., Iurisci, I., D'Egidio, M., Piccolo, E., Tinari, N., Nifantiev, N. and Iacobelli, S. (2006). Glycobiology, 16, 210-220.

Rabinovich, G.A., Daly, G., Dreja, H., Tailor, H., Riera, C.M., Hirabayashi, J. and Chernajovsky, Y. (1999a). J Exp Med, 190, 385-398.

Rabinovich, G.A., Iglesias, M.M., Modesti, N.M., Castagna, L.F., Wolfenstein-Todel, C., Riera, C.M. and Sotomayor, C.E. (1998). J Immunol, 160, 4831-4840.

Rabinovich, G.A., Riera, C.M. and Sotomayor, C.E. (1999). Braz J Med Biol Res, 32, 557-567.

Rabinovich, G.A., Sotomayor, C.E., Riera, C.M., Bianco, I. and Correa, S.G. (2000). Eur J Immunol, 30, 1331-1339.

Reisfeld, R.A., Lewis, U.J. and Williams, D.E. (1962). Nature, 195, 281-283.

Rice, P.J., Kelley, J.L., Kogan, G., Ensley, H.E., Kalbfleisch, J.H., Browder, I.W. and Williams, D.L. (2002). J Leukoc Biol, 72, 140-146.

Rifai, A. and Mannik, M. (1984). J Exp Med, 160, 125-137.

Rini, J.M. and Lobsanov, Y.D. (1999). Curr Opin Struct Biol, 9, 578-584.

Roff, C.F. and Wang, J.L. (1983). J Biol Chem, 258, 10657-10663.

Roff, C.F., Rosevear, P.R., Wang, J.L. and Barker, R. (1983). Biochem J, 211, 625-629.

Ross, G.D., Cain, J.A., Myones, B.L., Newman, S.L. and Lachmann, P.J. (1987). Complement, 4, 61-74.

Rowley, K., Walker, K.Z., Cohen, J., Jenkins, A.J., O'Neal, D., Su, Q., Best, J.D. and O'Dea, K. (2003). Med J Aust, 178, 495-500.

Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E. and Sitia, R. (1992). J Biol Chem, 267, 24161-24164.

Rubinstein, N., Alvarez, M., Zwirner, N.W., Toscano, M.A., Ilarregui, J.M., Bravo, A., Mordoh, J., Fainboim, L., Podhajcer, O.L. and Rabinovich, G.A. (2004). Cancer Cell, 5, 241-251.

Russell, M.W., Brown, T.A. and Mestecky, J. (1981). J Exp Med, 153, 968-976.

Sano, T., Hiki ,Y., Kokubo,T, Iwase,H., Shigematsu,H and Kobayashi, Y (2002), Nephrol Dial Transplant, 17, 50-56.

Saevarsdottir, S., Vikingsdottir, T. and Valdimarsson, H. (2004). Scand J Immunol, 60, 23-29.

Saito, H., Yoshioka, Y., Uehara, N., Aketagawa, J., Tanaka, S. and Shibata, Y. (1991). Carbohydr Res, 217, 181-190.

Saito, H., Yoshioka, Y., Yoloi, M. and Yamada, J. (1990). Biopolymers, 29, 1689-1698.

Sangeetha, S.R. and Appukuttan, P.S. (2005). Int J Biol Macromol, 35, 269-276.

Sanjuan, X., Fernandez, P.L., Castells, A., Castronovo, V., van den Brule, F., Liu, F.T., Cardesa, A. and Campo, E. (1997). Gastroenterology, 113, 1906-1915.

Sasaki, T., Hirabayashi, J., Manya, H., Kasai, K. and Endo, T. (2004). Glycobiology, 14, 357-363.

Sathisha, U.V., Jayaram, S., Harish Nayaka, M.A. and Dharmesh, S.M. (2007). Glycoconj J, 24, 497-507.

Sato, M., Nishi, N., Shoji, H., Seki, M., Hashidate, T., Hirabayashi, J., Kasai Ki, K., Hata, Y., Suzuki, S., Hirashima, M. and Nakamura, T. (2002). Glycobiology, 12, 191-197.

Sato, S. and Nieminen, J. (2004). Glycoconj J, 19, 583-591.

Schachter, H., and Brockhausen, I. (1992). in Glycoconjugates: Composition, Structure and Function (Allen, H. H., and Kisailus, E. C., Eds.), p 63, Marcel Dekker, New York.

Scher, I. (1982). Immunol Rev, 64, 117-136.

Schifferli, J.A. and Peters, D.K. (1982). Clin Exp Immunol, 47, 563-569.

Schifferli, J.A., Woo, P. and Peters, D.K. (1982). Clin Exp Immunol, 47, 555-562.

Schwarz, M., Spector, L., Gargir, A., Shtevi, A., Gortler, M., Altstock, R.T., Dukler, A.A. and Dotan, N. (2003). Glycobiology, 13, 749-754.

Schwarz, M., Spector, L., Gargir, A., Shtevi, A., Gortler, M., Altstock, R.T., Dukler, A.A. and Dotan, N. (2003). Glycobiology, 13, 749-754.

Sendid, B., Colombel, J.F., Jacquinot, P.M., Faille, C., Fruit, J., Cortot, A., Lucidarme, D., Camus, D. and Poulain, D. (1996). Clin Diagn Lab Immunol, 3, 219-226.

Sharon, N. (1986). Eur J Biochem, 159, 1-6.

Sharon, N. (1993). Trends Biochem Sci, 18, 221-226.

Sharon, N. and Lis, H. (1982). Mol Cell Biochem, 42, 167-187.

Shepherd, M.G. (1987). Crit Rev Microbiol, 15, 7-25.

Shimizu, A., Putnam, F.W., Paul, C., Clamp, J.R. and Johnson, I. (1971). Nat New Biol, 231, 73-76.

Shimizu, M., Khoshnoodi, J., Akimoto, Y., Kawakami, H., Hirano, H., Higashihara, E., Hosoyamada, M., Sekine, Y., Kurayama, R., Kurayama, H., Joh, K., Hirabayashi, J., Kasai, K., Tryggvason, K., Ito, N. and Yan, K. (2009). Lab Invest, 89, 178-195.

Shimonishi, T., Miyazaki, K., Kono, N., Sabit, H., Tuneyama, K., Harada, K., Hirabayashi, J., Kasai, K. and Nakanuma, Y. (2001). Hum Pathol, 32, 302-310.

Shoenfeld, Y. and Isenberg, D.A. (1989). Immunol Today, 10, 123-126.

Sidebotham, R.L. (1974). Adv Carbohydr Chem Biochem, 30, 371-444.

Skogh, T. (1982). Scand J Immunol, 16, 465-475.

Skogh, T. and Stendahl, O. (1983). Immunology, 49, 53-59.

Sparrow, C.P., Leffler, H. and Barondes, S.H. (1987). J Biol Chem, 262, 7383-7390.

Spicer, E.J., Goldenthal, E.I. and Ikeda, T. (1999). Food Chem Toxicol, 37, 455-479.

Spiro, R. G. (1962). J Biol Chem 237: 382-8.

Spiro, R. G. and V. D. Bhoyroo (1974). J Biol Chem 249(18): 5704-17.

Spiro, R.G. (1973). Adv Protein Chem, 27, 349-467.

Springer, G.F. (1984). Science, 224, 1198-1206.

Springer, G.F. and Desai, P.R. (1982). J Biol Chem, 257, 2744-2746.

Springer, G.F. and Horton, R.E. (1969). J Clin Invest, 48, 1280-1291.

Stanley, V.C. and Hurley, R. (1969). J Pathol, 97, 357-366.

Stockert, R.J. (1995). Physiol Rev, 75, 591-609.

Stowell, S.R., Dias-Baruffi, M., Penttila, L., Renkonen, O., Nyame, A.K. and Cummings, R.D. (2004). Glycobiology, 14, 157-167.

Stowell, S.R., Karmakar, S., Stowell, C.J., Dias-Baruffi, M., McEver, R.P. and Cummings, R.D. (2007). Blood, 109, 219-227.

Strockbine, N.A., Largen, M.T., Zweibel, S.M. and Buckley, H.R. (1984). Infect Immun, 43, 715-721.

Summerfield, J.A. and Taylor, M.E. (1986). Biochim Biophys Acta, 883, 197-206.

Sureshkumar G, Appukuttan PS and Basu D (1982), J Bioscie, 4, 257-261.

Sutherland, I.W. (1985). Annu Rev Microbiol, 39, 243-270.

Sverremark, E. and Fernandez, C. (1998). Int Immunol, 10, 851-859.

Symons, A., Cooper, D.N. and Barclay, A.N. (2000). Glycobiology, 10, 559-563.

Szondy, E., Mezey, Z., Fust, G., Szekely, J. and Gero, S. (1981). Br Heart J, 46, 93-98.

Tai, T., Yamashita, K., Ito, S. and Kobata, A. (1977). J Biol Chem, 252, 6687-6694.

Tarelli, E., Smith, A.C., Hendry, B.M., Challacombe, S.J. and Pouria, S. (2004). Carbohydr Res, 339, 2329-2335.

Tauber, A.I. and Podolsky, S.H. (1994). J Hist Biol, 27, 531-573.

Taylor, P.R., Brown, G.D., Reid, D.M., Willment, J.A., Martinez-Pomares, L., Gordon, S. and Wong, S.Y. (2002). J Immunol, 169, 3876-3882.

Teichberg, V.I., Silman, I., Beitsch, D.D. and Resheff, G. (1975). Proc Natl Acad Sci U S A, 72, 1383-1387.

Terg, R., Miguez, C.D., Castro, L., Araldi, H., Dominguez, S. and Rubio, M. (1996). J Hepatol, 25, 329-333.

Theofilopoulos, A.N. and Dixon, F.J. (1980). Am J Pathol, 100, 529-594.

Thijssen, V.L., Postel, R., Brandwijk, R.J., Dings, R.P., Nesmelova, I., Satijn, S., Verhofstad, N., Nakabeppu, Y., Baum, L.G., Bakkers, J., Mayo, K.H., Poirier, F. and Griffioen, A.W. (2006). Proc Natl Acad Sci U S A, 103, 15975-15980.

Thompson, A. J., Y. L. Chan, et al. (1980). Pathology 12(3): 407-13.

Thornton, B.P., Vetvicka, V., Pitman, M., Goldman, R.C. and Ross, G.D. (1996). J Immunol, 156, 1235-1246.

Timmons, P.M., Colnot, C., Cail, I., Poirier, F. and Magnaldo, T. (1999). Int J Dev Biol, 43, 229-235.

Tinari, N., Kuwabara, I., Huflejt, M.E., Shen, P.F., Iacobelli, S. and Liu, F.T. (2001). Int J Cancer, 91, 167-172.

Tomasi, T.B. and Czerwinski, D.S. (1976). Scand J Immunol, 5, 647-653.

Tomino, Y., H. Sakai, et al. (1982). Clin Exp Immunol 49(2): 419-25.

Tomino, Y., M. Endoh, et al. (1982). Tokai J Exp Clin Med 7(4): 527-32.

Torosantucci, A., Bromuro, C., Chiani, P., De Bernardis, F., Berti, F., Galli, C., Norelli, F., Bellucci, C., Polonelli, L., Costantino, P., Rappuoli, R. and Cassone, A. (2005). J Exp Med, 202, 597-606.

Torosantucci, A., Chiani, P., Bromuro, C., De Bernardis, F., Palma, A.S., Liu, Y., Mignogna, G., Maras, B., Colone, M., Stringaro, A., Zamboni, S., Feizi, T. and Cassone, A. (2009). PLoS One, 4, e5392.

Toscano, M.A., Bianco, G.A., Ilarregui, J.M., Croci, D.O., Correale, J., Hernandez, J.D., Zwirner, N.W., Poirier, F., Riley, E.M., Baum, L.G. and Rabinovich, G.A. (2007). Nat Immunol, 8, 825-834.

Towbin, H., Rosenfelder, G., Wieslander, J., Avila, J.L., Rojas, M., Szarfman, A., Esser, K., Nowack, H. and Timpl, R. (1987). J Exp Med, 166, 419-432.

Tracey, B.M., Feizi, T., Abbott, W.M., Carruthers, R.A., Green, B.N. and Lawson, A.M. (1992). J Biol Chem, 267, 10342-10347.

Triolo, G., Giardina, E., Rinaldi, A. and Bompiani, G. (1984). Boll Ist Sieroter Milan, 63, 173-174.

Triolo, G., Giardina, E., Zarcone, M.R., Giordano, C., Rinaldi, A. and Bompiani, G.D. (1984a). Diabetologia, 27 Suppl, 157-159.

Triolo, G., M. Traina, et al. (1984b). J Clin Lab Immunol 13(1): 35-9.

Uchiyama, M., Ohno, N., Miura, N.N., Adachi, Y., Tamura, H., Tanaka, S. and Yadomae, T. (2000). Biol Pharm Bull, 23, 672-676.

Usami, M., Ohata, A., Horiuchi, T., Nagasawa, K., Wakabayashi, T. and Tanaka, S. (2002). Transfusion, 42, 1189-1195.

van den Brule, F., Califice, S., Garnier, F., Fernandez, P.L., Berchuck, A. and Castronovo, V. (2003). Lab Invest, 83, 377-386.

van der Leij, J., van den Berg, A., Blokzijl, T., Harms, G., van Goor, H., Zwiers, P., van Weeghel, R., Poppema, S. and Visser, L. (2004). J Pathol, 204, 511-518.

Vandenheede, J.R., Ahmed, A.I. and Feeney, R.E. (1972). J Biol Chem, 247, 7885-7889.

Varki, A. (1993). Glycobiology, 3, 97-130.

Varki, A., Kannagi, R and Toole , BP (2009) In The Consortium of glycobiology (eds.) Essentials of Glycobiology, CSH Press, New York.

Vas, V., Fajka-Boja, R., Ion, G., Dudics, V., Monostori, E. and Uher, F. (2005). Stem Cells, 23, 279-287.

Vasta, G. R., H. Ahmed, et al. (2004). Curr Opin Struct Biol 14(5): 617-30.

Vetvicka, V., Thornton, B.P. and Ross, G.D. (1996). J Clin Invest, 98, 50-61.

von Bahr-Lindstrom, H. and Bennich, H. (1974). FEBS Lett, 40, 57-61.

von Wolff, M., Wang, X., Gabius, H.J. and Strowitzki, T. (2005). Mol Hum Reprod, 11, 189-194.

Vyakarnam, A., Lenneman, A.J., Lakkides, K.M., Patterson, R.J. and Wang, J.L. (1998). Exp Cell Res, 242, 419-428.

Wada, M., Ono, S., Kadoya, T., Kawanami, T., Kurita, K. and Kato, T. (2003). J Neurol Sci, 208, 67-70.

Walker, L.J., Aldhous, M.C., Drummond, H.E., Smith, B.R., Nimmo, E.R., Arnott, I.D. and Satsangi, J. (2004). Clin Exp Immunol, 135, 490-496.

Walsh, T.J., Hathorn, J.W., Sobel, J.D., Merz, W.G., Sanchez, V., Maret, S.M., Buckley, H.R., Pfaller, M.A., Schaufele, R., Sliva, C. and et al. (1991). N Engl J Med, 324, 1026-1031.

Wang, D., Wells, S.M., Stall, A.M. and Kabat, E.A. (1994). Proc Natl Acad Sci U S A, 91, 2502-2506.

Wang, J.L., Gray, R.M., Haudek, K.C. and Patterson, R.J. (2004). Biochim Biophys Acta, 1673, 75-93.

Watkins, W.M. and Morgan, W.T. (1952). Nature, 169, 825-826.

Wei, D., Williams, D. and Browder, W. (2002). Int Immunopharmacol, 2, 1163-1172.

Weissman, I.L., Gutman, G.A., Friedberg, S.H. and Jerabek, L. (1976). Adv Exp Med Biol, 66, 229-237.

Wells, V., Davies, D. and Mallucci, L. (1999). Eur J Cancer, 35, 978-983.

Whitney, P.L., Powell, J.T. and Sanford, G.L. (1986). Biochem J, 238, 683-689.

Williams, D.L., McNamee, R.B., Jones, E.L., Pretus, H.A., Ensley, H.E., Browder, I.W. and Di Luzio, N.R. (1991). Carbohydr Res, 219, 203-213.

Willment, J.A., Gordon, S. and Brown, G.D. (2001). J Biol Chem, 276, 43818-43823.

Wollina, U., Schreiber, G., Gornig, M., Feldrappe, S., Burchert, M. and Gabius, H.J. (1999). Histol Histopathol, 14, 779-784.

Yamaoka, A., Kuwabara, I., Frigeri, L.G. and Liu, F.T. (1995). J Immunol, 154, 3479-3487.

Yim, K.O. and Bradford, K.J. (1998). Plant Physiol, 118, 83-90.

Yoo, E.M. and Morrison, S.L. (2005). Clin Immunol, 116, 3-10.

Young, S.H., Robinson, V.A., Barger, M., Frazer, D.G., Castranova, V. and Jacobs, R.R. (2003). J Toxicol Environ Health A, 66, 551-563.

Young, W.W., Jr., Hakomori, S.I. and Levine, P. (1979). J Immunol, 123, 92-96.

Young, W.W., Jr., Hakomori, S.I. and Levine, P. (1979). J Immunol, 123, 92-96.

Zanetta, J.P., Bingen, A., Dontenwill, M. and Vincendon, G. (1984). Exp Cell Res, 155, 371-380.

Zhang, J., Liu, Y.J., MacLennan, I.C., Gray, D. and Lane, P.J. (1988). Eur J Immunol, 18, 1417-1424.

Zhou, Z.H., Zhang, Y., Hu, Y.F., Wahl, L.M., Cisar, J.O. and Notkins, A.L. (2007). Cell Host Microbe, 1, 51-61.

Zhu, J., F. J. Nieto, et al. (2001). Circulation 103(1): 45-51.

Zou, J., Glinsky, V.V., Landon, L.A., Matthews, L. and Deutscher, S.L. (2005). Carcinogenesis, 26, 309-318.

Zuniga, E., Gruppi, A., Hirabayashi, J., Kasai, K.I. and Rabinovich, G.A. (2001). Infect Immun, 69, 6804-6812.