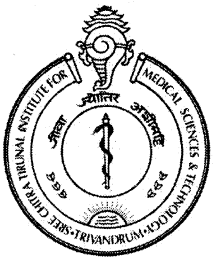
MICROBIAL ENZYME-MEDIATED CHANGES IN GLYCOSYLATED BIOMOLECULES LEADING TO THEIR RECOGNITION BY SUGAR-SPECIFIC SERUM AND TISSUE PROTEINS.

A THESIS PRESENTED BY

ANURADHA

TO

THE DEPARTMENT OF BIOCHEMISTRY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE SUBJECT OF BIOCHEMISTRY



SREE CHITRA TIRUNAL INSTITUTE FOR
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The thesis entitled

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Submitted by

ANURADHA

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(Name of the Guide)	(Names of Examiners)	

DECLARATION

I, **Anuradha**, hereby declare that I had personally carried out the work depicted in the thesis entitled **"Microbial enzyme-mediated changes in glycosylated biomolecules leading to their recognition by sugar-specific serum and tissue proteins"** 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.

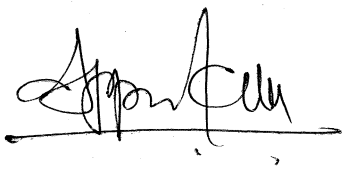
ANURADHA

Dr. P. S. Appukuttan
Professor and Head

Department of Biochemistry
Sree Chitra Tirunal Institute for Medical Sciences & Technology
Medical college P.O, Thiruvananthapuram

CERTIFICATE

This is to certify that **Ms. Anuradha**, 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 **"Microbial enzyme-mediated changes in glycosylated biomolecules leading to their recognition by sugar-specific serum and tissue proteins"** was carried out under my direct supervision.



Dr. P. S. Appukuttan (Guide)

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Anuradha

CONTENTS

ABBREVIATIONS

ABG	:	anti-β-glucoside antibody
Anti-T	:	Anti-T antibody
APC	:	Antigen presenting cells
Apo B	:	Apolipoprotein B
Apo(a)	:	Apolipoprotein(a)
BHL	:	Bovine heart lectin (galectin-1
BSA	:	Bovine serum albumin
CA	:	pancarcinoma
CAD	:	Coronary artery disease
CLGG	:	Cross-linked guar gum
CNBr	:	Cyanogen bromide
CRD	:	Carbohydrate Recognition Domain
d	:	Density
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme linked immunosorbent assay
Gal	:	Galactose
GalNAc	:	N-acetyl galactosamine
GlcNAc	:	N-acetyl glucosamine.
HDL	:	High density lipoprotein
HPL	:	Human placental lectin (galectin-1)
HRP	:	Horse radish peroxidase
IC	:	Immune complex

IDL	:	Intermediate density lipoproteins
IgA1	:	Immunoglobulin A1
IgA1P	:	IgA1 purified using polystyrene-immobilized jacalin
IgA2	:	Immunoglobulin A2
IgG	:	Immunoglobulin G
IgM	:	Immunoglobulin M
JSA	:	Jack seed agglutinin
KDa	:	Kilo Daltons
KSCN	:	Potassium thiocyanate
LDL	:	Low density lipoprotein
MAQ	:	Minimum agglutinating quantity
MC	:	Mesangial cell
MPS	:	Mononuclear phagocytic system
NANA	:	N-acetyl neuraminic acid
NHRBC	:	Neuraminidase treated human red blood cells
OPD	:	Ortho-phenylenediamine
PBS	:	Phosphate buffered saline
PBS-T	:	PBS containing 0.05% Tween 20
PNA	:	Peanut agglutinin
PVDF	:	Polyvinylenedifluoride
T / TF antigen	:	Thomsen-Friedenreich antigen (Galβ(1-3)GalNAc-)
TEMED	:	N,N,N',N'- tetramethyl ethylene diamine
Tn antigen	:	N-acetyl galactosamine-α-O-Ser/Thr

SYNOPSIS

Infection-mediated immune damage is increasingly recognized as cause for atherosclerosis and other vascular diseases. Molecular pathology of highly prevalent disorders such as IgA nephropathy, diabetes and atherosclerosis are marked by deposition of IgA-containing immune complexes on vessel walls and tissues, by mechanisms yet unknown. IgA1 is a prominent serum O-glycosylated protein. Incidentally IgA1, the only O-glycosylated immunoglobulin deposited in glomeruli in IgAN is also largely desialylated. Lp(a) is unique in being rich in O-glycosylated sugar side chains that are either absent or sparse in other serum lipoproteins in man. It has been recently reported to be an independent risk factor for coronary artery disease and stroke. Also Lp(a) from CAD patients was reported to be sialic acid-poor (2.5 fold) and atherogenic in cell culture compared with that of healthy subjects. Hence, the most prominent T antigen-bearing glycoconjugates in serum, namely IgA1 and Lp (a), seemed to be involved in vascular and perivascular inflammations. Also humans have naturally occurring serum anti-T antibodies induced predominantly by the intestinal flora so that its titre goes up with pathogen burden. Interestingly enough, the T-antigens in both IgA1 and Lp (a) as they occur in serum are masked (from recognition by anti-T antibody) by a terminally attached sialic acid moiety. Removal of this sialic acid moiety by neuraminidase secreted by pathogenic bacteria such as *Vibrio cholerae, Streptococcus pneumoniae* and viruses such as *influenza* A and B, *parainfluenza*, measles etc during their infection cycle, renders IgA1 and Lp (a) susceptible to anti-T recognition. It remains a lacuna that interaction between IgA1 and Lp(a) on one hand and anti-T on the other, leading to immune complex formation

and tissue deposition as cause for inflammation has not been investigated. The mechanism of IgA- or Lp(a)-mediated pathology being hardly known, this thesis work elucidates recognition by human serum antibody (anti-T) of the disaccharide T antigen that gets exposed when IgA1or Lp(a) is desialylated to form immune complexes. Possible pathophysiological consequences of the resulting immune complexes are discussed in view of the affinity for IgA1 and Lp(a) of a human cell surface T-antigen-specific lectin, galectin-1 that is expressed in several tissues including blood vessel walls.

Objectives of the study

1) Identification of the most prominent serum O-glycosylated protein.
2) Demonstration of recognition of serum O-glycosylated proteins after their desialylation (and exposure of T-antigenic groups) by serum anti-T leading to formation of immune complexes.
3) Demonstration of carbohydrate structure and T-antigen prevalence in serum lipoproteins.
4) To develop a faster and inexpensive method for Lp(a) assay.
5) Desialylation-dependent recognition of principal O-glycosylated lipoprotein [Lp(a)] by anti-T leading to formation of immune complexes.
6) Desialylation-dependent recognition of serum O-glycosylated protein [IgA1] by anti-T leading to formation of immune complexes in diabetic patients.

Results

Prevalence of IgA1 among O-glycosylated human serum proteins has been examined in two ways employing the lectin jacalin which recognizes exclusively the O-linked oligosaccharides of glycoproteins regardless of their sialylation status. Firstly in SDS-polyacrylamide gel electrophoresis of all the O-glycosylated serum glycoproteins isolated by precipitation with jacalin the most prominent subunit was identical in mobility with the major subunit of standard IgA (mainly IgA1). Further the most prominent subunit was singularly reduced in intensity when solubilized jacalin-precipitated glycoproteins were passed through anti-human IgA-Sepharose before electrophoresis. Secondly, among the O-glycosylated proteins of serum prepared by affinity binding to jacalin-Sepharose (JSSP), the most prominent protein band is also the one recognized by anti-human IgA. These results together indicate that IgA1 (that makes up most of serum IgA) is the most prominent serum O-glycosylated protein.

Human anti-β-glucoside antibody (ABG) which is richer in IgA than total serum immunoglobulins was used to prepare pure IgA1 employing selective binding to polystyrene-coated jacalin. Yield of this IgA1 (IgA1P) was 500 ng per well on average. Dot-blotted IgA1P was tested as a ligand for anti-T. Results show that unlike native IgA1P, desialylated IgA1P was a very efficient ligand for anti-T.

Anti-T-mediated agglutination of desialylated human RBC was inhibited by desialylated IgA1P, the minimum inhibitory quantity under conditions of assay being 146 ± 57.6 ng/ ml while several times more of native IgA1P was not inhibitory. T antigen specificity of this inhibition was further established by the inability of

desialylated forms of IgA1-free ABG or of the non-O-glycosylated glycoprotein bovine thyroglobulin to inhibit anti-T.

Since desialylation exposes free T antigen in most O-glycosylated proteins anti-T was expected to form immune complex (IC) with such glycoproteins in serum desialylated by microbial neuraminidase. This was examined by assaying those immune complexes that contained both IgM and O-glycosylated proteins since anti-T is mostly IgM. After desialylation of serum using *Clostridium perfringens* neuraminidase (50mU/ ml) immune complexes were captured on anti-human IgM-coated plastic plates and O-glycosylated proteins in the bound immune complexes assayed using jacalin-HRP. Results indicate a significant increase in IgM-O-glycosylated protein immune complexes in serum desialylated by neuraminidase (N+) compared to serum treated with inactivated enzyme (NH) (P value < 0.02; $n = 6$).

Involvement of the premier serum O-glycosylated protein IgA1 in IC formation with anti-T was then examined by directly assaying IgA-IgM immune complexes. From among IgA-containing IC that bound to anti-IgA immobilized on plastic wells, those that contained IgM as well were assayed by probing with anti-human IgM-HRP. Results suggest a significant increase in IgA-IgM IC following desialylation of serum (P value $= 0.005$; $n = 6$).

Since IgA1, the sole O-glycosylated immunoglobulin far exceeds anti-T in serum concentration, desialylation of serum would leave most of the asialo-IgA1 unoccupied even after formation of IgA1-anti-T complexes using all of serum anti-T available. This was also verified by addition of purified anti-T to desialylated serum from which IC had been removed. Results showed a significant increase in IgM-IgA

immune complexes in desialylated serum, compared to non-desialylated serum, upon addition of purified anti-T (P value = 0.003; n = 10).

Involvement of anti-T in shifting desialylated IgA1 to the IC phase was examined alternatively by withdrawing anti-T from plasma. Plasma depleted of anti-T by treatment with desialylated RBC of the same group and control treated with unmodified RBC were both desialylated, IC separated and IgA1 in IC assayed after capture on microwell-coated jacalin and probing with HRP conjugate of anti-human IgA. Results of six consecutive trials show very significant decrease in transfer of IgA1 to IC in serum in which anti-T was absent, compared to control serum (P value = 0; n = 6).

Decrease in free anti-T titre in serum following desialylation, expected in the event of anti-T forming IC with IgA1 and other O-glycosylated proteins in desialylated serum, was verified by agglutination assay using RBC of the same group. Serum treated with neuraminidase (N+) or with inactivated enzyme (NH), and dialysed after removal of IC as described above was used. Limitations of agglutination assay using serial 2-fold dilutions not-withstanding, results revealed a 2- to 8-fold lower free anti-T concentration in desialylated serum than in non-desialylated serum.

Desialylation-dependent entry of IgA1 into IC was even more explicitly demonstrated in serum from which natural IC had been removed by PEG precipitation. After dialysis to remove PEG and incubation with active (N+) or inactive (NH) neuraminidase, secondary IC formed were collected by precipitation with 2% PEG. Secondary IC from N+ serum contained substantially higher IgA1 than

did secondary IC from NH serum (P value < 0.01; n = 6). Desialylation per se did not enhance binding of IgA to jacalin.

IgA1P and JSSP used in experiments described earlier were prepared by sugar-specific elution from immobilized jacalin. Lest any serum glycoprotein fails to bind or to get eluted from immobilized jacalin, a range of O-glycosylated proteins from serum was precipitated by addition of jacalin which is a wide-spectrum O-glycosylated protein-specific lectin. The precipitate collected by centrifugation was redissolved by addition of jacalin-specific sugar (1-O-methyl-α-D-galactoside). Western blot of these glycoproteins probed with anti-T confirmed the desialylation-dependent recognition by anti-T of almost all O-glycosylated proteins of serum, chiefly IgA1. Most of these bands were also recognized by the T antigen-specific animal lectin galectin-1 though the relative intensities of bands were different.

Polystyrene microplate-coated purified Lp(a) was recognized by serum anti-T, only after desialylation of the former. O-glycosylated serum lipoproteins purified by jacalin-mediated precipitation followed by ultracentrifugation were separated by alkaline pH electrophoresis. On Western blot of these lipoproteins, Lp(a) and LDL were identified by antibodies against apo (a) and apo B chains. Binding of anti-T to Lp(a), though negligible to the native lipoprotein, was significant following its desialylation, while LDL was not recognized even after desialylation.

A faster and inexpensive method was developed for Lp(a) assay, which involves microplate-coating of jacalin, incubation with Lp(a) dilutions and probing with anti-apo (a)-HRP. The new jacalin-based assay method is more sensitive than commercial ELISA Lp(a) kit method (the limit of detection of Lp(a) being as

low as 5 ng). The method is linear over a wide range of Lp(a) concentrations and showed high percentage cumulation in response and strong correlation with standard Lp(a) assays.

Desialylation of fresh serum by *Clostridium perfringens* neuraminidase gave rise to far more Lp(a)-containing immune complexes than did native serum Lp(a) or serum Lp(a) treated with heat-inactivated neuraminidase (P value=0; n = 6). Desialylation-dependent entry of Lp(a) into IC was even more explicitly demonstrated in serum from which natural IC had been removed by PEG precipitation. Secondary IC from N+ serum contained substantially higher Lp(a) than did secondary IC from NH serum (P value=0; n = 12). Depletion of anti-T from serum by treating with desialylated human RBC led to decreased formation of immune complexes with Lp(a), following desialylation (P value=0.001; n = 6).

Desialylation of fresh diabetic serum by *Clostridium perfringens* neuraminidase led to increased formation of immune complexes containing IgM, the major immunoglobulin type in anti-T on one hand and IgA1 on the other, than desialylated normal serum. The percentage increase in entry of IgA1 to IC formed from neuraminidase treated serum (N+) over non- desialylated control (NH) is enormously higher (112% ± 42.6) in the case of diabetic sera, than in the case of normal sera (33% ± 9). Similarly, the percentage increase in IgA-IgM content in IC formed from neuraminidase treated serum (N+) over non-desialylated control (NH) is much higher (116% ± 29.3) in the case of diabetic sera, than in the case of normal sera (28% ± 9.5).

Discussion

Exclusive specifity for O-linked oligosaccharides of glycoproteins regardless of sialylation and sugar-binding activity that is unaffected by immobilization on Sepharose or coating on polystyrene make jacalin an excellent tool for isolation and analysis of O-glycosylated proteins. In this study O-glycosylated proteins recognized by jacalin in solution (JPSP) as well as those that bind to Sepharose-immobilized jacalin (JSSP) from human serum were separately used to demonstrate the dominance of IgA1 in both populations.

Recognition of serum O-glycosylated proteins including IgA1 on dot blots and Western blots by anti-T akin to known T antigen-specific lectins as well as inhibition of anti-T-mediated hemagglutination by IgA1 suggested that *in vivo* this antibody may bind to serum O-glycosylated proteins, chiefly IgA1, consequent to their desialylation. This was confirmed by demonstration of increased formation of immune complexes containing both IgM and O-glycosylated serum proteins including IgA1 following desialylation of serum. Sialylation-dependent formation of IC between IgA1 and IgM was even more decidedly demonstrated in serum from which natural IC had been removed prior to desialylation. Since hardly any serum immunoglobulin other than anti-T is known that recognizes desialylated O-linked oligosaccharides of serum in contrast to their native forms and since anti-T is largely IgM the amount of IgM that is in combination with O-glycosylated proteins or IgA1 was taken as a measure of IC formed between anti-T and the glycoproteins.

Formation of fresh IgM-desialylated IgA1 complexes upon addition of anti-T to desialylated serum from which IC had been removed, decrease in IgA1-

containing IC following removal of anti-T prior to desialylation and the marked decrease in free anti-T titre in serum following desialylation offered further proof for formation of immune complexes between anti-T and O-glycosylated proteins including IgA1.

The potential of IC to cause vascular lesion by recruitment of PMN, leukocytes and macrophages through complement-dependent chemotaxis is well established and underlined by the prevention of vascular injury following complement depletion. Circulating IC had been shown to be strong and independent risk factor for myocardial infarction in many studies. Molecular pathology of several diseases has been suggested to involve IgA-containing IC. In IgA nephropathy and its systemic analogue Henoch-Schonlein purpura, both suggested to be mediated by IgA-containing IC, the IgA deposited in glomerular and other lesion sites was found to be exclusively of IgA1 class. When injected into experimental rats desialylated IgA1 in contrast to native IgA1 accumulated in glomeruli. Reduced sialylation of IgA1 in polyethylene glycol-precipitated macromolecules of serum was observed in focal proliferative sclerosing IgA nephropathy patients, compared to controls. Particularly, glomerular IgA in IgA nephropathy was enriched in T antigen-rich (ie, desialylated) IgA1. Increased serum IgA against several periodontic pathogens and *Chlamydia pneumoniae* are risk factors for cardiovascular diseases. Influenza virus noted for secretion of neuraminidase as well as for induction of serum IgA is strongly associated with atherosclerosis. At the same time infectious etiology is now being increasingly attributed to cardiovascular diseases. Also, IgA nephropathy has been reported to be associated with infections of the upper respiratory tract.

Pathogen-derived antigens are often rich in polysaccharide structures that elicit predominantly IgA antibodies (most of which is IgA1). Also many bacteria and viruses including those that are pro-atherogenic and pro-nephropathic are known to secrete the enzyme neuraminidase that can desialylate IgA1. Further investigations to assess the level of IgA1-containing IC in patients infected with such microbes are therefore relevant.

Immune complex between anti-T and desialylated IgA1 *in vivo,* if formed may be aided by its unique composition in bringing about vascular injury. Firstly, as is evident from present results anti-T-bound desialylated IgA1 still has binding sites left for galactose-binding lectins. For the most abundant human galactose-binding lectin, galectin-1 which is expressed on endothelial cell surface as well, IgA1 has been reported to be the most prominent serum ligand and more so after desialylation. Present results suggest the possibility of galectin-1-dependent anchoring of anti-T-desialylated IgA1 IC on vessel walls and tissues during infections and diabetes, both known to induce vascular damage. Secondly, IgM as well as IgG of anti-T-IgA1 immune complexes have their binding sites engaged so as to enable the respective Fc portions to attract complement and initiate an inflammatory cascade.

The results suggest that immune complexes involving IgA1 and anti-T could be a key component in diabetic vascular immune inflammations, especially since high serum IgA concentration as well as increased serum neuraminidase titre has been shown to accompany diabetes.

Lp(a) from CAD patients was reported to be sialic acid-poor (2.5 fold) and atherogenic in cell culture compared with that of healthy subjects. Also

neuraminidase concentration in serum has been found to be higher in CAD+ than CAD- subjects. Present results suggest that increased desialylation of Lp(a) followed by its recognition by anti-T leading to immune complexes may be a major mechanism by which the lipoprotein gets anchored on vessel walls, especially as Lp(a) is the best lipoprotein ligand for tissue galectin-1.

Chapter 1

INTRODUCTION

INTRODUCTION

Cell-surface carbohydrates possess the ideal attributes of a cell-surface code system for intercellular interactions. First, they are in the right place. Most cells are completely covered with a carbohydrate layer termed the glycocalyx, which consists of glycoproteins and glycolipids inserted in the cell membrane, and proteoglycans, which may be more loosely associated with the cell surface. Secondly, carbohydrates have the structural diversity to act as informational molecules. Complex carbohydrates of the glycocalyx are made up of a small variety of monosaccharide building blocks, primarily the hexoses (glucose, galactose, mannose, and fucose), the N-acetyl aminosugars (N-acetylglucosamine and N-acetylgalactosamine), and the negatively charged glucuronic acid and sialic acids. However, the ability of these sugars to link to one another in any of several positions for each sugar ring allows the formation of a very large number of oligosaccharide structures [Hakomori, 1983; Hook et al., 1984; Kobata, 1984]. In addition, sulfates, phosphates, and acetyl groups can be added after formation of the glycosides, to further differentiate their structures The third reason for implicating carbohydrates in the control of cell-cell interactions is by far the most compelling: across a broad phylogenetic spectrum, many investigators have demonstrated that cell-surface carbohydrates and complementary lectins on apposing cell surfaces mediate cell-cell adhesion in vitro [Frazier and Glaser, 1979; Monsigny, 1984]. Such carbohydrate-directed cell adhesion appears to be important in many intercellular activities including infection by bacteria and viruses, communication among cells of lower eukaryotes, specific binding of sperm to egg and recirculation of lymphocytes, among

others [Monsigny , 1984]. In many systems where cell adhesion plays a critical role, carbohydrate binding proteins have been shown to bind to cell surface carbohydrates and participate in cell-cell interactions. Such systems include fertilization, development, pathogen-host recognition, in cancer, immunological processes and inflammation [Brandley, 1991]. Lectins, carbohydrate recognizing antibodies and enzymes with carbohydrate substrates are three different classes of carbohydrate binding proteins widely occurring in most life forms. The present study demonstrates interaction between Thomsen-Friedenreich (T) antigen [Gal β1→3 GalNAc-α-]-containing serum O-glycosylated proteins [IgA1 and Lp(a)] after their desialylation (and exposure of T-antigenic groups) with anti-T antigen-specific antibody leading to formation of immune complexes.

Recent investigations worldwide have identified immune mediated inflammations as major contributor towards atherosclerosis [Lefvert et al., 1995; Mustafa et al., 2000] as much as towards nephritis [Mestecky et al., 1986] and autoimmune neuropathy. Molecular pathology of highly prevalent disorders such as IgA nephropathy, diabetes and atherosclerosis are marked by deposition of IgA-containing immune complexes on vessel walls and tissues, by mechanisms yet unknown. High serum IgA (mostly IgA1) is unique to man; [Vaerman, 1973]. High serum IgA concentrations (as in diabetes) correlate with chronic vascular complications. [Rodriguez-Segade et al., 1996].High serum IgA1 turnover (5-6 times faster than IgG) is poorly accounted for by Fcα receptors or liver asialoglycoprotein receptors.

In tropical populations, high pathogen burden and serum immunoglobulin titre may aggravate immune inflammatory events. Sialylated or cryptic T-antigens (Gal β1→3 GalNAc) are present in normal cells and tissues [Springer, 1984]. Also humans have naturally occurring serum anti-T antibodies induced predominantly by the intestinal flora so that its titre goes up with pathogen burden [Boccardi et al., 1974; Springer and Tegtmeyer, 1981]. Tissue deposition of IgA1 has been observed, in inflammations ranging from nephropathies [Mestecky et al., 1986], atherosclerosis [Pussinen et al., 2005; Saiku et al., 1992] to neuropathies of infectious etiology .On the other hand, atherosclerotic plaques have been repeatedly shown to be rich in Lp(a) [Smith and Cochran, 1990]. Lp(a), which is the most O-glycosylated lipoprotein has been recently reported to be an independent risk factor for coronary artery disease and stroke [Koschinsky, 2005]. Incidentally IgA1, the only O-glycosylated immunoglobulin type is as well the sole immunoglobulin type which gets deposited in the glomeruli in IgA nephropathy. Also glomerular IgA in IgA nephropathy was enriched in T antigen-rich (ie, desialylated) IgA1 [Iwase et al., 2002]. Evidences are emerging for a physiological role for T antigen as a natural ligand for the most prevalent mammalian tissue lectin, galectin-1 whose expression increases in tumor tissues [Raz et al., 1986] and whose sugar inhibitors retard tumor colonization [Koshik et al., 1997]. Also, primary cultures of human aortic endothelial cells express galectin-1 [Baum et al.,1995].Thus between the two most common animal tissue galactosides viz. T antigen of O-linked oligosaccharides and N-acetyl lactosamine of N-linked oligosaccharides, the former has been reported to be a far stronger ligand for human galectin-1 in *in vitro* studies [Sangeetha and Appukuttan,

2005]. Also, the best serum glycoprotein inhibitor for human heart galectin-1 was IgA1, the lone O-glycosylated (T antigen-bearing) immunoglobulin while enzymatic removal of sialic acid moiety from IgA1 to expose free T antigen substantially increased its efficiency as a galectin-1 ligand [Sangeetha and Appukuttan, 2005]. Among human brain gangliosides, GM1 which alone contains T antigen was the best ligand for galectin-1 [Kannan and Appukuttan, 1997]. Among human serum lipoproteins, Lp(a) which is unique in being profusely O-glycosylated, was the best ligand for human galectin-1 [Chellan et al., 2007].

Desialylation of human erythrocytes by microbial neuraminidase renders them agglutinable by serum anti-T due to exposure of free T antigen on these cell surfaces [Burnet and Anderson, 1947]. Localized or systemic release of neuraminidase is a characteristic of many bacterial and viral infections [Soong et al., 2006; Gimsa et al., 1996]. Interestingly enough, the T-antigens in both IgA1 and Lp (a) as they occur in serum are masked (from recognition by anti-T antibody) by a terminally attached sialic acid moiety. Removal of this sialic acid moiety is easily achieved at physiological pH and ionic strength by sialidase enzymes. These enzymes are secreted in serum and tissues by pathogenic bacteria such as *Vibrio cholerae*, *Streptococcus pneumonia* and viruses such as *influenza* A and B, *parainfluenza*, measles etc. during their infection cycle [Yarnell, 2001].

Anti-T titre goes down substantially during malignancy due to cancer cells possessing exposed surface T antigen and is recovered consequent to surgical / otherwise removal of malignant tumors [Springer, 1976]. This shows the surveillance of exposed T antigens by serum anti-T. On the other hand, the most prominent T

antigen bearing glycoproteins in serum, IgA1 and Lp(a) are involved in vascular and perivascular inflammations. It remains a lacuna that their mutual interactions leading to immune complex formation and tissue deposition as cause for inflammation has not been investigated. In this study we demonstrate the interaction of human serum anti-T with the premier T antigen-containing serum glycoproteins, IgA1 and Lp(a) following desialylation of the latter by bacterial neuraminidase. Possible pathophysiological consequences of the resulting immune complexes are discussed in view of the affinity for IgA1 and Lp(a) of galectin-1 that is expressed on several tissues including blood vessel walls. The mechanism of IgA- or Lp(a)-mediated pathology being hardly known, this work elucidates the possible primary event in the process, namely recognition by human serum antibody (anti-T) of the disaccharide T antigen that gets exposed when IgA1or Lp(a) is desialylated to form immune complexes.

Further investigations carried out in this direction may explain the observed larger contribution of infection prevalence and pathogen burden compared to hyperlipidemia as predisposing factor towards atherosclerosis, diabetic vascular inflammations and nephritis.

OBJECTIVES OF THE STUDY

1) Identification of the most prominent serum O-glycosylated protein.

2) Demonstration of recognition of serum O-glycosylated proteins after their desialylation (and exposure of T-antigenic groups) by serum anti-T leading to formation of immune complexes.

3) Demonstration of carbohydrate structure and T-antigen prevalence in serum lipoproteins.

4) To develop a faster and inexpensive method for Lp(a) assay.

5) Desialylation-dependent recognition of principal O-glycosylated lipoprotein [Lp(a)] by anti-T leading to formation of immune complexes.

6) Desialylation-dependent recognition of serum O-glycosylated protein [IgA1] by anti-T leading to formation of immune complexes in diabetic patients.

Chapter 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Glycoproteins

A glycoprotein is a compound containing carbohydrate (glycan) covalently linked to protein. 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). One, a few or many carbohydrate units may be present on each protein molecule. The carbohydrate content of glycoproteins varies from less than 1% to over 90% of the total weight [Sharon, 1986]. This class of compounds includes enzymes, hormones, immunoglobulins, lectins, toxins, carrier and structural proteins. Although nearly 200 different monosaccharides are found in nature only 11 are known to occur in glycoproteins [Sharon and Lis, 1981]. Proteoglycans are a subclass of heavily glycosylated glycoproteins in which the carbohydrate units are polysaccharides that contain amino sugars. Such polysaccharides are also known as glycosaminoglycans. Glycoproteins are widely distributed in all forms of life, with the possible exception of the eubacteria. They occur in cells, both in soluble and membrane-bound forms, as well as in the extracellular matrix and in extracellular fluids. Glycosylation is the most common co-translational and post-translational modifications of proteins [Sharon, 1986]. More than half of known protein sequences can potentially be glycosylated.

Glycosylation types in glycoproteins

Glycosylation can be mainly divided into two major types viz O-glycosylation, where the sugar is bound to the hydroxyl of a serine (Ser) or a

threonine (Thr) residue and N-glycosylation where the sugar is attached to the amide group of an asparagine (Asn) in the consensus sequence Asn-X-Ser/Thr where X is any residue but a proline [Petrescu et al., 2004].

N-glycosidic linkages

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 and Kornfeld, 1976]. The oligosaccharide chain is attached to the amide group of an asparagine (Asn) in the consensus sequence Asn-X-Ser/Thr, where X is any residue but a praline and aspartic acid [Petrescu et al., 2004; Marshall, 1972]. The β-glycosylamine linkage of GlcNAc to Asn represents the most widely distributed carbohydrate-peptide bond and is the site of attachment for a large variety of complex and polymannose oligosaccharides [Spiro, 1973; Montreuil, 1980] in proteins with demonstrated biological importance [Varki, 1993]. 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, 1980]. Three types of *N*-linked oligosaccharide chains are observed [Montreuil et al., 1986; Pan and Elbein, 1995]: (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 dissacharide N-acetyl lactosamine (Gal β1→4GlcNAc) attached to

the core [Tai 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 et al., 1983].

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. Since glycosylation occurs co-translationally, once the protein has folded potential glycosylation sites are no longer accessible to the glycosyltransferases [Marshall, 1972].

O-glycosidic linkages

The other major type of saccharide-peptide linkage occurring in glycoproteins is the O-glycosidic linkage. In O-glycans, carbohydrate is attached to hydroxyl groups of amino acids, serine, threonine, hydroxyl proline and hydroxyl lysine. Many linkage types appear among the O-glycosylproteins, which are distributed in a variety of anatomical locations [Vliegenthart and Montreuil, 1995]. These include (i) mucins [Klein et al., 1992; Brockhausen, 2004] containing D-GalNAc α-1,3-linked to Ser or Thr, (ii) proteoglycans, acidic mucopolysaccharides joined though D-Xyl in β-1,3-linkages to L-Ser [Kjellén and Lindahl, 1991], (iii) collagens, including D-Gal β-1,5-linked to 5-hydroxy-D-Lys [Noelken and Hudson, 1995], and (iv) the plant glycoproteins which include extensin and AGPs (L-Arab to 4-hydroxy-L-Pro) [Klis, 1995]. Examples of (i) are submaxillary mucins, human erythrocytes and ovarian cysts [Wu, 1988], serum immunoglobulin IgA [Iwase et al., 1999], glycophorin [Lisowska 1988] and bronchial mucin [Mawhinney *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, 1985].

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]. Other modifications to the core GalNAc structure have also been found but appear to be uncommon. 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 he digestive tract. Commonly the core 2 and the core 4 branches are elongated with one or multiple lactosamine structures (Gal β1→4GlcNAc) [Lowe, 2001].

Core 1, O-glycan or T-antigen (Gal β1→3GalNAc) (Figure 1) 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. The immunodeterminant group GalNAc, linked to the hydroxyl group of Serine or Threonine in the amino terminal region of glycoproteins is called Tn-antigen. T and Tn are usually covered by covalently linked carbohydrates holding high negative charge density in the case of sialic acid substitution [Springer and Desai, 1982] or are physically separated from the immune system [Pierce-Cretel et al., 1981]. Unmasked T and Tn specific epitopes are unique carcinoma markers.

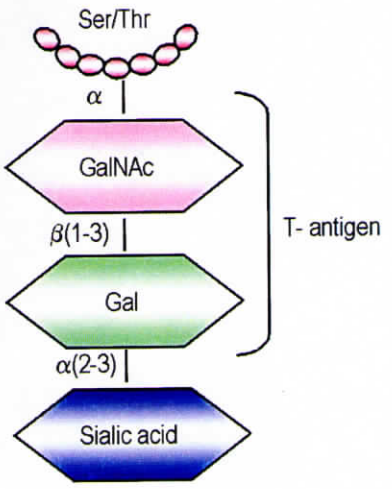

Figure.1. Structure of T-antigen, Core 1, O-glycan

Glycosylation pattern of Immunoglobulin A1

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 [Shimizu et al., 1971; Putnam et al., 1973]. In contrast, the γ chain of human IgG has a single oligosaccharide [Edelman et al., 1969], and the ε chain of human IgE is reported to have six [Bennich and Bahr-Lindstrom, 1974]. In all these cases the oligosaccharide contains glucosamine (GlcN), which is attached to asparagine by an N-glycosidic linkage. The IgAl 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 and Kornfeld, 1974]. IgA1 is relatively heavily glycosylated (8% carbohydrate).

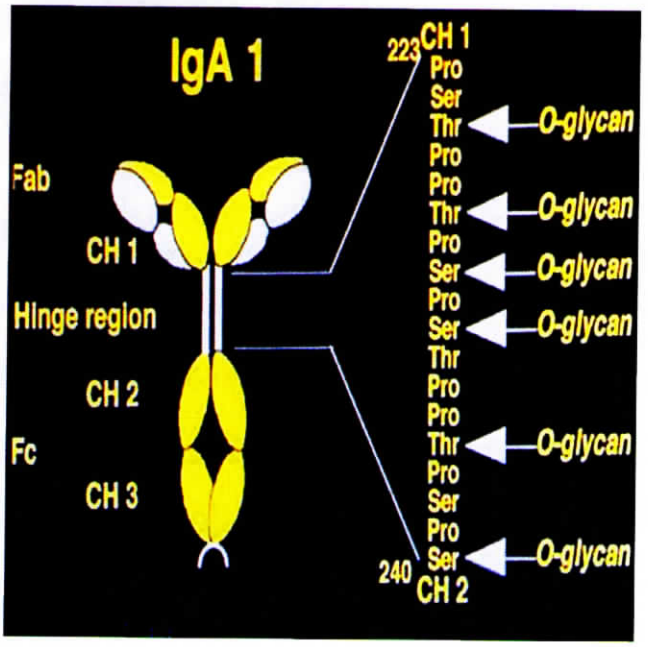

Figure 2. Position of IgA1 hinge region O-glycosylation sites. On the left is the human IgA1 molecule, showing the location of the hinge region between the CH1 and CH2 domains. On the right is the amino acid sequence of the hinge region. All of the serine and threonine residues are potential O-glycosylation sites, but in a recent study, only those marked were found to be occupied.

Human IgA1 contains two conserved *N*-glycosylation sites in each α-chain (Asn 263 and Asn 459), while the IgA2 subclass contains an additional two (IgA2m(1)) or three (IgA2m(2)) conserved *N*-glycans. The *N*-glycans of IgA1 have been reported as biantennary complex-type structures with sialic acids attached exclusively in the α2–6 linkage. In addition, approximately 13.6% of tri- and tetraantennary structures were reported [Baenziger and Kornfeld, 1974b]. IgA1 contains a proline-rich hinge sequence (17 aa sequence) between the Fab and Fc regions of the glycoprotein (Figure 2).

Within this sequence, there are nine potential *O*-glycosylation sites/α-chain (18 sites/molecule). In serum IgA1, the sites of *O*-glycosylation were located at the five serine residues (3 linked to threonine and 2 to serine) [Baenziger and Kornfeld, 1974a]. IgA1 *O*-linked glycans consist of GalNAc with a β1,3-linked Gal.

Sialic acid (NeuAc) may be attached to GalNAc by an α2,6-linkage or to Gal by an α2,3-linkage [Baenziger and Kornfeld, 1974a; Mattu et al., 1998]. 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 [Franqione and Wolfenstein-Todel, 1972].

Several possible roles have been proposed for the carbohydrate in immunoglobulins, but none has been established. It has been suggested [Marshall, 1972; Clamp, 1975] that carbohydrates increase solubility, facilitate secretion, and act as spacers between the domains.

Biological roles of oligosaccharides

The oligosaccharide chains (glycans) attached to cell surface and extracellular proteins and lipids are known to 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, 1993].

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. It is also clear that the 'coating' of oligosaccharides on many glycoproteins can serve to protect the polypeptide chain from recognition by proteases or antibodies [Gottschalk and Thomas, 1961] and that the coating of glycoconjugates covering a whole cell can present a 'glycocalyx' of substantial proportions [Cook, 1986]. These regions of sugar chains are best positioned to mediate recognition by carbohydrate-binding proteins [Baenziger, 1985] 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 consequently degraded [Varki, 1993]. The antifreeze glycoprotein of antarctic fish depends on the integrity of the disaccharide (Gal β1→3GalNAc) units for their activity.

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. Recently, the role of such oligosaccharide-binding domains in the organization of the matrix has been clearly demonstrated *in vitro.* 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 and Morgan, 1952].

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

Just as certain oligosaccharides act as 'traitorous' signposts for microbial and immune attack, others can serve to abrogate these detrimental reactions. In these cases, the addition of specific monosaccharides or modifications masks the sequences recognized by microorganisms, toxins or autoimmune antibodies. Thus, for example, the addition of a single O-acetyl ester to the 9-position of terminal sialic acid residues abrogates binding of the highly pathogenic influenza A viruses, and the extension of the oligosaccharide chain of GM1 would prevent binding of cholera toxin [Schauer, 1985]. Likewise, the addition of galactose and sialic acid to the Tn antigen would abolish its autoimmune reactivity.

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.

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'. 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). In each case, the minimal carbohydrate ligands involved in recognition appear to be sialylated fucosylated sugar chains, such as sialyl Lewis^x and sialyl Lewis^a [Varki, 1993].

Many pathological states are characterized by changes in the carbohydrate structure of cellular glycoproteins. 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, 1991].

T antigen and anti-T antibody

Nature of T and Tn Antigens

T antigen, the immediate precursor of the human blood group MN antigens [Springer and Ansell, 1958] and a distant precursor in other complex carbohydrate chains [Lloyd and Kabat, 1968], was discovered by Thomsen and Friedenreich about 75 years ago as a laboratory curiosity. Sialidase-producing microbes unmasked the cryptic T antigen on stored human red blood cells, and rendered them panagglutinable by complementary anti-T present in all human sera [Friedenreich, 1930]. T antigen or Thomsen-Friedenreich disaccharide is chemically defined as α-anomeric Gal β (1-3) GalNAc and described as type I core of O-linked oligosaccharide constituents of mucins [Hounsell et al., 1996]. Tn antigen expression, in contrast, is caused by a somatic mutation in adulthood at the pluripotent stem-cell level [Myllyla et al., 1973]. Tn antigen was discovered by Dausset et al. in a patient with hemolytic anemia and polyagglutinability due to anti-Tn (Tn syndrome) [Dausset et al., 1959]. Tn antigen on red blood cells is the result of a genetic block of a single biosynthetic step: the transfer of D-galactose (Gal) to N-acetyl-D-galactosamine (GalNAc) [Springer et al., 1976; Cartron et al., 1978]. Biosynthetic pathways of T and Tn antigens have been established in two ways: (i) by stepwise degradation [Springer and Desai, 1974] and (ii) by biosynthesis [Springer et al., 1976; Cartron et al., 1978]; the Tn immunodeterminant group is Gal-NAc linked to the hydroxyl group of serine (Ser) or threonine (Thr) in the amino-terminal region of the active glycoprotein. Subsequent action of, β-galactosyltransferase adds Gal from Gal-nucleotide (UDP-Gal), and this results in: Gal-β1→3-GalNAc-α-O-Ser/Thr

(T immunodeterminant). The T and Tn immunodeterminant structures have been synthesized chemically in different laboratories [Kaifu and Osawa, 1977]. The high specific activity of these epitopes at least in vitro depends on their clustered occurrence in the amino-terminal region of erythrocyte-derived T antigen [Springer et al., 1983]. T and Tn are usually covered by covalently linked carbohydrates [Springer and Ansell, 1958; Lloyd and Kabat, 1968], occasionally by tertiary structures [Jirgensons and Springer, 1968], by high negative-charge density due to sialic acid [Springer and Desai, 1982], or are physically separated from the immune system [Pierce-Cretel et al., 1981]. But traces of unmasked T antigen in healthy persons' nerve tissues is also reported [Springer et al., 1979]. At the time of their discovery no association of T and Tn antigens with malignant tumors was suspected.

T and Tn Antigens are Carcinoma-Associated

Changes in glycoconjugate metabolism (incomplete or abnormal glycosylation) is one of the features accompanying malignant cell transformation. The expression of glycoproteins is modified because of depression of genes encoding for glycosyltransferases, and this leads to decreased cell–cell adhesiveness, conferring to cancer cells an advantage for metastatic spread [Dall'Olio, 1996; Kemmer et al., 1992; Gessner et al., 1993]. Two such carbohydrate antigens are the Thomsen-Friedenreich (T) antigen (Galβ(1-3) GalNAcα(1-O)-Ser/Thr) and its immediate precursor, the Tn antigen (GalNAcα(1-O)-Ser/Thr). These are core disaccharides of O-glycosylated complex carbohydrates, thought to be cryptic in normal post-fetal tissues but expressed in carcinomas of several organs, thereby being considered as "general pancarcinoma" antigens [Springer and Desai, 1990; Springer et al., 1995;

Hanish and Baldus, 1997]. The unmasked T- and Tn-specific epitopes are unique carcinoma markers. T and Tn antigens were shown to be abundantly expressed in immunoreactive form in about 90 percent of carcinoma tissues [Springer, 1984]. Interestingly, the expression of the T or Tn antigens has been found to correlate with tumour aggressiveness in terms of local invasion, metastasis, and poor survival in carcinomas of the pancreas [Schuessler et al., 1991], colon [Campbell et al., 1995; Itzkowitz et al., 1989; Orntoft et al., 1990], breast [Springer, 1989], uterine cervix [Hamada et al., 1993; Hirao et al., 1993], stomach [Chung et al., 1996], urinary bladder [Blasco et al., 1988; Langkilde, 1995] and ovary [Ghazizadeh et al., 1990]. The high specific inhibitory activity of tumor cell adhesion to hepatocytes by T and Tn-specific glycoconjugates suggests that they may be involved in specific cell-cell adhesions required for invasion and metastasis by cancer cells [Springer, 1984]. Coon et al. [1983] on the basis of work by Limas and Lange [Limas and Lange, 1980], demonstrated the predictive value of the T antigen status for future invasiveness of grade I and II papillomatous transitional-cell urinary bladder carcinoma. A similar predictive value of T antigen expression and prostatic carcinoma invasiveness and metastasis has been reported [Springer, 1984]. The absolute densities of T and Tn epitopes on CA cell membranes, as well as the relative densities of T compared to Tn, have been shown to correlate with histologic grade, invasive potential, and hence with probability of early recurrence in breast [Springer, 1984; Springer et al., 1985] urinary bladder [Ohoka et al., 1985; Nishiyama et al., 1987; Blasco et al., 1988] and prostatic CAs [Ghazizadeh et al., 1984]. Therefore these antigens are not only of diagnostic but also of prognostic significance.

Anti-T antibody

All humans have preexisting antibodies against T (anti-T) and Tn (anti-Tn) [Burnet and Anderson , 1947; Dausset et al., 1959]. Boccardi et al. have demonstrated that these are elicited predominantly by the intestinal flora [Boccardi et al., 1974; Springer et al., 1981]. By far the most prevalent anti-T fraction is immunoglobulin M (IgM), which does not readily permeate blood vessels and has no access to extravascular structures. Anti-T agglutinin levels have been found by others to remain rather steady in a given healthy adult (Burnet and Anderson, 1947; Boccardi et al., 1974; Bray et al., 1982]. Presence of anti-T at rather constant levels like the anti-A and anti-B blood group Abs, from 3 years of age throughout adulthood has been reported [Lind and McArthur, 1947]. IgM constitutes most of anti-T in human serum, followed by IgA (30%) and IgG in that order [Springer et al., 1979a, b]. T and anti-T promise to elucidate important aspects of carcinoma pathogenesis; also, they may permit truly early diagnosis. Anti-T titre in serum decreased with tumor proliferation and was restored rapidly following tumor removal [Springer et al., 1976]. Bray and her colleagues found cytotoxic anti-T in the sera of all healthy persons, and a specific, quantitative, inverse correlation between hemolytic anti-T and tumor burden in patients with gastrointestinal carcinoma [Bray et al., 1982]. Thatcher et al. reported a direct relation between severely depressed anti-T, poor response to chemotherapy, and decreased survival in patients with melanocarcinoma [Thatcher et al., 1980]. The level of circulating anti-T as measured by agglutination of N'RBC was also depressed in certain breast and gastrointestinal cancer patients when compared to controls, although there was no correlation with anti-T titre and extent of

disease [Springer et al., 1979a]. Depression of circulating anti-T in certain cancer patients could be explained by the absorption of antibodies by T antigen expressed or shed from the tumour [Anglin et al., 1977; Springer et al., 1979a]. Measurement of circulating anti-T may be indicative of tumour burden or disease progression in some malignancies and also radiolabelled anti-T antibodies may prove useful in tumour localization studies [Bray et al., 198l b]. Recently, a novel immunosorbent consisting of synthetic TF alpha disaccharides (Galbeta1-3GalNAc alpha-) coupled to polyacrylamide (PAA), which itself was covalently bound to cross-linked Sepharose was used to obtain purified anti-T from plasma. The affinity-purified anti-T antibodies were of the IgM (> or = 0.5 mg/100 ml of serum) and IgG (approximately 0.05 mg/100 ml of serum) classes [Butschak and Karsten, 2002].

Lipoproteins- emphasis on Lp(a).

In human plasma, lipids are transported as lipoproteins which consist of cholesterol esters and triglycerides in the hydrophobic core, and of free cholesterol, phospholipids and apolipoproteins on the hydrophilic surface. Plasma lipoproteins are separated into five major classes on the basis of their density: chylomicrons (d < 0.94 g/ml), very low density lipoproteins (VLDL, d = 0.94-1.006 g/ml), intermediate density lipoproteins (IDL, d = 1.006-1.019 g/ml), low density lipoproteins (LDL, d = 1.019-1.063 g/ml), and high density lipoproteins (HDL, d = 1.063-1.210 g/ml) (Havel and Kane 1995).

A sixth lipoprotein class, lipoprotein (a) (Lp(a)), consists of one LDL particle associated with one molecule of a glycoprotein called apo(a) (Utermann, 1989, Utermann, 1995). Its density range is 1.04-1.125 g/ml, overlapping

those of LDL and HDL. Apo(a) is secreted by the liver but the site of assembly and that of catabolism of Lp(a) are unclear. Its plasma concentration is genetically determined and there are large inter individual differences. A physiological role for Lp(a) has not been found, but apo(a) has a close structural resemblance with plasminogen, and it has been shown to be able to interfere with fibrinolysis (Utermann 1995).

Table 1. VLDL, very-low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Lp(a), lipoprotein (a). Data from [Deckelbaum, 1987; Mancini, 1991].

Characteristics and composition of plasma lipoproteins

				% Composition				
	Diameter (nm)	Density (kg/L)	Electrophoretic mobility	Cholesteryl ester	Free cholesterol	Trigly-ceride	Phospho-lipid	Protein
Chylomicrons	80-500	<0.95	Null	1-3	1	86-94	3-8	1-2
VLDL	30-80	0.95-1.006	Pre-beta	12-14	6-8	55-65	12-18	8-15
IDL	25-30	1.006-1.019	Slow pre-beta	20-35	7-11	25-40	15-22	12-19
LDL	19-25	1.019-1.063	Beta	35-45	6-10	6-12	20-25	20-25
HDL_2	8-11	1.063-1.125	Alpha	15-20	4-6	3-8	30-40	35-40
HDL_3	6-9	1.125-1.210	Alpha	10-18	1-4	3-6	25-35	45-55
Lp(a)	25-30	1.055-1.085	Pre-beta	30-36	8-10	3-4	20-25	30-35

LDL consists of one single copy of apo B-100, and of large amounts of cholesterol esters and smaller amounts of free cholesterol, triglycerides and phospholipids. LDL is the main carrier of cholesterol in blood, and it is

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HDL_2	8-11	1.063-1.125	Alpha	15-20	4-6	3-8	30-40	35-40
HDL_3	6-9	1.125-1.210	Alpha	10-18	1-4	3-6	25-35	45-55
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LDL consists of one single copy of apo B-100, and of large amounts of cholesterol esters and smaller amounts of free cholesterol, triglycerides and phospholipids. LDL is the main carrier of cholesterol in blood, and it is

responsible for transporting cholesterol to peripheral cells. Apo B interacts with specific LDL receptors located on cell surfaces in many tissues, including the liver, facilitating removal of LDL particles from the circulation.

Structure of Lipoprotein (a)

Lipoprotein(a) [Lp(a)] was first described ≈ 44 years ago by Berg in 1963, and interest in this entity is largely derived from its putative role as a cardiovascular risk factor [Berg, 1963; Albers et al., 1975; Utermann, 1989]. Lipoprotein (a) (Lp(a)) is a LDL-like lipoprotein that has been associated with increased risk of coronary heart disease, stroke and restenosis [Scanu, 2001]. Lp(a) is one of the most atherogenic lipoproteins in human plasma [Koschinsky, 2005]. Lp(a), which closely resembles LDL in lipid composition (Table 1), contains a single apolipoprotein B100 (apoB100) molecule and an additional apolipoprotein, called apolipoprotein(a) (apo(a)) which is connected via a disulfide linkage to apoB100 [Scanu, 2001].Apo B, the characteristic glycoprotein in LDL has a carbohydrate composition varying from 4 to 10 % by mass [La Belle and Krauss, 1990], while the apo (a) of Lp(a) contains 28.1 % by mass of carbohydrates [Fless et al., 1986]. Apo(a) is a polymorphic glycoprotein that contains repeating domains of varying length that are homologous to kringle IV of plasminogen. Each kringle IV domain contain N and O-linked glycosylation sites. Apo (a) contained two major N-glycans that accounted for 17 % of the total oligosaccharide structures. The N-glycans were complex biantennary structures present in either a mono or disialylated state within the core of each apo (a) K4 motif [McLean et al., 1987; Sangrar et al., 1994]. There are a minimum of six O-linked glycosylation sites present in the linker sequences that join

individual kringles [Kratzin et al., 1987]. The O-glycans were mostly (80%) represented by monosialylated core type I structure, NeuNAcα2-3Galβ1-3GalNAc and the remaining 20% consist of disialylated and non-sialylated O-glycans [Garner et al., 2001]. Lp(a), which consists of LDL and apo(a), contains six times as much sialic acid as LDL [Ehnholm et al 1972, Utermann, 1989]. The structure of apo(a) exhibits 80 % homology with plasminogen [McLean et al., 1987]. More than 30 different isoforms of apo[a] have been described in humans ranging in size from less than 300 kDa to greater than 800 kDa [Hobbs and White, 1999]

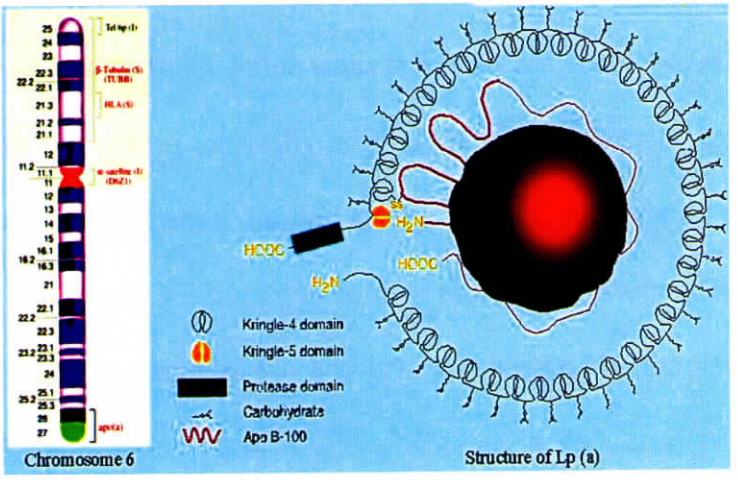


Figure 3. Model of Lp(a). The LDL-like moiety consists of a lipid core of cholesteryl esters and triglycerides surrounded by a surface layer of phospholipid and free cholesterol. In addition to lipids, it also contains one molecule of apolipoprotein B, which is linked to apolipoprotein(a) through a single disulfide bond. The apo(a) moiety consists of a single copy of kringles KIV, types 1 and 3 to 10, kringle V, and a protease domain analogous to plasminogen. In addition, it contains multiple copies of kringle IV, type 2.

Lp(a) is very heterogeneous and the underlying reasons for this heterogeneity were uncovered by the elegant work on the gene structure of apo(a) by Lawn, Scanu, and their collaborators [McLean et al., 1987]. They reported an analogy between the apo(a) and plasminogen genes; both genes have coding sequences for

loop structures stabilized by intrachain disulfide bonds, so-called kringle (K) domains. The plasminogen gene contains coding sequences for 5 different K domains (K1 to K5), and 2 of these are present in the apo(a) gene, K4 and K5. Interestingly, the sequence coding for one of these K domains, K4, is repeated many-fold in the apo(a) gene [Utermann, 1989; McLean et al., 1987; Lackner et al.,1993; van der Hoek et al., 1993 ; Koschinsky et al., 1990]. Altogether, the apo(a) gene has 10 different types of plasminogen- like K4 domains, referred to as K4 type 1 through 10. K4 types 1 and 3 to 10 are present as single copies, whereas K4 type 2 is present as multiple copies, varying in number from 3 to > 40 copies. [Hobbs and White,1999; McLean et al., 1987; Lackner et al.,1993; van der Hoek et al., 1993; Koschinsky et al., 1990]. Each kringle contains ≈ 80 to 85 amino acids and has a molecular weight of ≈10 kDa, and the K4 repeat unit is thus unusually large. This heterogeneity in apo(a) gene size corresponds to a size variation in the apo(a) protein and apo(a) size isoforms containing from 12 to ≈ 50 K4 motifs have been reported, corresponding to a protein molecular weight ranging from 300 to 800 kDa. [Hobbs and White, 1999; Gavish et al., 1989].

Structure of LDL

LDL has sialic acid residues both on its lipid and protein parts. The largest part of the sialic acid has been reported to be in the protein part of LDL, that is, in apo B (Tertov et al 1993). Apo B is a glycoprotein with 20 potential N-glycosylation sites, out of which up to 16 asparagines (Asn) residues are glycosylated (Yang et al 1986, Taniguchi et al 1989) (Figure 4).

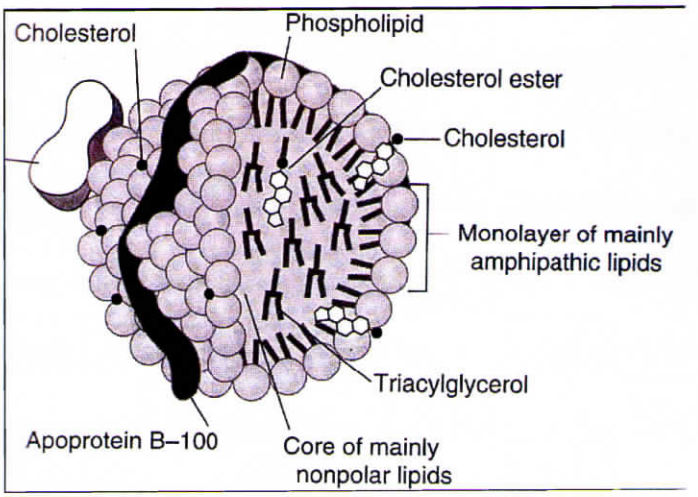
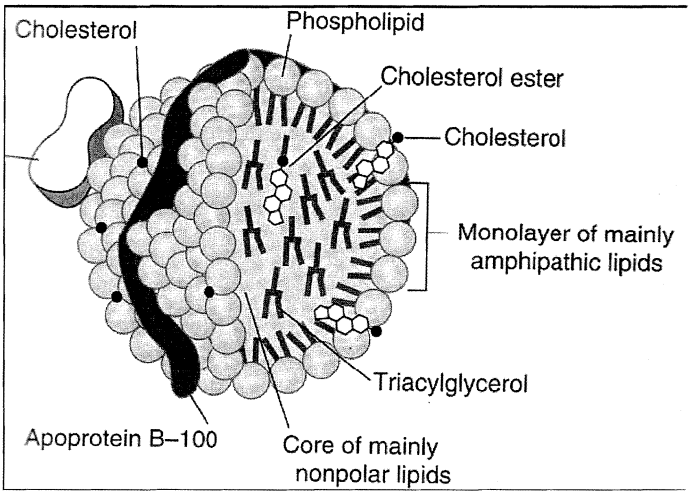


Figure 4. Structure of Low density lipoprotein.

About 4-10 mass-% of apo B is carbohydrate, and there are two major types of carbohydrate chains, one of which is neutral and the other acidic [Swaminathan and Aladjem 1976, Taniguchi et al 1989]. They consist of the monosaccharides mannose, galactose, N-acetylglucosamine and sialic acid. The acidic chain has one or two terminal sialic acid residues followed by galactose molecules. Sialic acid constitutes 10% of the total carbohydrate in apo B. Some O-glycosylation could also be detected in apo B or LDL [Chellan et al., 2006].

Plasma levels of Lp(a)

Lp(a) is found in humans and also old world non-human primates such as rhesus monkeys, baboons, chimpanzees as well as in the insectivore, the hedgehog. Plasma levels of Lp(a) vary greatly between individuals, from less than 1 mg/dl to greater than 100 mg/dl and there is general agreement that plasma levels of > 30 mg/dl are causally linked to atherosclerosis, coronary heart disease and stroke [Hobbs and White,1999]. The size of the apo(a) gene is highly variable, resulting in the protein molecular weight ranging from 300 to 800 kDa; this large variation may



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be caused by neutral evolution in the absence of any selection advantage. Apo(a) influences to a major extent metabolic and physicochemical properties of Lp(a), and the size polymorphism of the apo(a) gene contributes to the pronounced heterogeneity of Lp(a). There is an inverse relationship between apo(a) size and Lp(a) levels. Further, Lp(a) levels differ between populations, and blacks have generally higher levels than Asians and whites, adjusting for apo(a) sizes [Berglund and Ramakrishnan, 2004]. According to recent studies, small-size apolipoprotein(a) isoforms may represent a cardiovascular risk factor either by themselves or synergistically with plasma Lp(a) concentration [Scanu, 2003].

Biosynthesis and catabolism of Lp(a)

The primary site of synthesis of Lp(a) appears to be the hepatocyte, although apo(a) mRNA has also been identified in testes and brain [Tomllnson et al., 1989]. The site of assembly of the Lp(a) particle, by covalent linkage of apo B to apo(a) is so far unknown. Lp(a) is not the result of VLDL catabolism, nor does Lp(a) assembly take place by linkage of apo(a) to circulating LDL [Fless et al., 1989].

LDL-receptor activity does not fully account for the main catabolic pathway of Lp(a) as Lp(a) is not quite as good a ligand for the LDL receptor as LDL itself. The cysteine residue in apoB involved in the covalent bond between apoB and apo(a) is close to the postulated LDL receptor-binding region of apoB, suggesting that the large, carbohydrate-rich apo(a) protein introduces a charge and/or steric interaction affecting the binding potential of apoB in Lp(a) for the LDL receptor [McCormick et al., 1995; Callow and Rubin, 1995]. So Lp(a) may be preferentially taken up by the scavenger receptors on macrophages, suggesting a mechanism by

which Lp(a) may contribute to atherogenic foam cell formation [Kostner et al., 1989]. One additional possible mechanism by which Lp(a) might facilitate atherogenic processes is through non-receptor uptake of the particle by the endothelium [Cushing et al., 1989; Rath et al., 1989].

The pathogenicity of Lp(a): a complex cardiovascular pathogen.

Lp(a) and atherosclerosis: Mechanistic Insights and Clinical Implications

Serum levels of Lp(a) have been widely reported to be higher in CAD patients than in healthy controls [Genest et al., 1991, Sandholzer et al., 1992, Kario et al., 1994; Bostom et al., 1996], and thus Lp(a) has been suggested to be a CAD risk factor [Utermann, 1989, Loscalzo, 1990, Stein and Rosenson, 1997]. The physiological role and metabolism of Lp(a) [lipoprotein (a)] remain enigmatic [Kostner and Kostner, 2002]. On the other hand, there is compelling evidence from numerous studies that individuals with high plasma Lp(a) levels (>30 mg/dl) are at a significantly increased risk of atherosclerosis, coronary heart disease and ischemic stroke [Craig et al., 1998; Kronenberg et al., 1999; Evans, 2002; Von Eckhardstein et al., 2002]. Lp(a) was shown to be an independent risk factor for coronary atherosclerosis, conferring a relative risk ranging from 1.6 to 3.6 [Dahlen et al., 1986]. Increased Lp(a) is also a risk factor, with similar magnitudes of effect, for Ischemic peripheral vascular disease, Ischemic stroke and abdominal aortic aneurysm [Jones et al., 2007].

The mechanism of action of Lp(a) in atherosclerosis is still not clear, but Lp(a) has been shown to be able to interfere with various factors involved in thrombogenesis and thrombolysis [Scanu, 1992; Stein and Rosenson 1997].

Lp(a) shows both atherogenic and thrombogenic properties. Atherogenic properties include a high affinity for extracellular matrix proteins [Marcovina and Koschinsky, 1998] , an ability to accumulate oxidized phospholipids [Tsimikas et al., 2005], which promote inflammation and preferential uptake by macrophage scavenger receptors [Kostner et al., 1989]. Thrombogenic properties center around the ability of the apo(a) protein to inhibit plasminogen activation [Marcovina and Koschinsky, 2003]. It has also become apparent that Lp(a) can be modified by oxidative events and by the action of lipolytic and proteolytic enzymes with the generation of products that exhibit atherothrombogenic potential. The role of the O-glycans linked to the inter-kringle linkers of apolipoprotein(a) is also emerging. This information is raising the awareness of the pleiotropic functions of Lp(a) and is opening new vistas on pathogenetic mechanisms whose knowledge is essential for developing rational therapies against this complex cardiovascular pathogen [Scanu, 2003]. Lp(a), which consists of LDL and apo(a), contains six times as much sialic acid as LDL [Ehnholm et al 1972; Utermann 1989]. Lp(a) from CAD patients was reported to be sialic acid-poor and atherogenic in cell culture compared with Lp(a) from healthy controls; and their formation was strongly correlated with the extent of intracellular cholesterol deposition [Tertov and Orekhov 1994].

Interestingly, hypercholesterolemic subjects had the higher plasma Lp(a)-IC levels. The circulating levels of these immune complexes are likely to vary with plasma concentrations of Lp(a) [Wang et al., 2004]. Higher and significant levels of immune complexes were found in the patients with atherosclerotic lesions [Romano et al., 1984]. Substantial evidence suggests that complement activation may

be a link between lipoprotein deposition and subsequent lesion development in atherosclerosis [Torzewski et al., 1997] It is suggested that excessive uptake of lipoprotein-antibody complexes by macrophages leading to formation of foam cells may play an important role in atherogenesis [Klimov et al., 1985].

Mechanisms of vascular damage in atherosclerosis.

Atherosclerosis is the major source of morbidity and mortality in the developed world. Atherosclerosis is characterized by the accumulation of cholesterol deposits in macrophages in large- and medium-sized arteries. This deposition leads to a proliferation of certain cell types within the arterial wall that gradually impinge on the vessel lumen and impede blood flow. This process may be quite insidious lasting for decades until an atherosclerotic lesion, through physical forces from blood flow, becomes disrupted and deep arterial wall components are exposed to flowing blood, leading to thrombosis and compromised oxygen supply to target organs such as the heart and brain. The loss of heart and brain function as a result of reduced blood flow is termed heart attack and stroke, respectively, and these two clinical manifestations of atherosclerosis are often referred to as coronary artery disease and cerebrovascular disease [Stocker and Keaney, 2004].

Hypotheses of Atherogenesis

Over the past 150 years, there have been numerous efforts to explain the complex events associated with the development of atherosclerosis. In this endeavor, three distinct hypotheses have emerged that are currently under active investigation. These hypotheses of atherosclerosis are not mutually exclusive but rather emphasize

different concepts as the necessary and sufficient events to support the development of atherosclerotic lesions. These three general hypotheses are: 1) the response-to-injury, 2) the response-to-retention, and 3) oxidative modification.

Response-to-injury

This hypothesis, Figure 5, proposes that the first step in atherosclerosis is "endothelial denudation". Endothelial denudation refers to the loss of endothelial cells along the intima. This loss leads to a slue of compensatory responses that alter the normal vascular homeostatic properties of the artery [Ross and Glomset, 1973]. An injury would consequently lead to increased endothelial permeability and deposition of leukocyte and LDL/ apolipoprotein B-containing lipoproteins into the sub-endothelial space. Recruited leukocytes release cytokines, vasoactive agents [such as nitric oxide ($NO^{\bullet}$)], and growth factors that further promote a proinflammatory response that is depicted by recruitment of macrophages into the arterial wall. The macrophages subsequently pick up LDL/ apolipoprotein B-containing lipoproteins to form "foam cells", which is considered to be the hallmark of early atherosclerotic lesions. Recently, Ross (1999) has demonstrated that endothelial "desquamation" is not common and that an intact endothelial cell layer covers the atherosclerotic lesion [Ross, 1999]. It was further shown that even normal segments of artery have higher entry rates of LDL/ apolipoprotein B-containing lipoproteins than the rate of their accumulation; suggesting that the atherogenic lipoprotein entry into the arterial wall is not dependent on endothelial dysfunction/ injury [Carew et al., 1984].

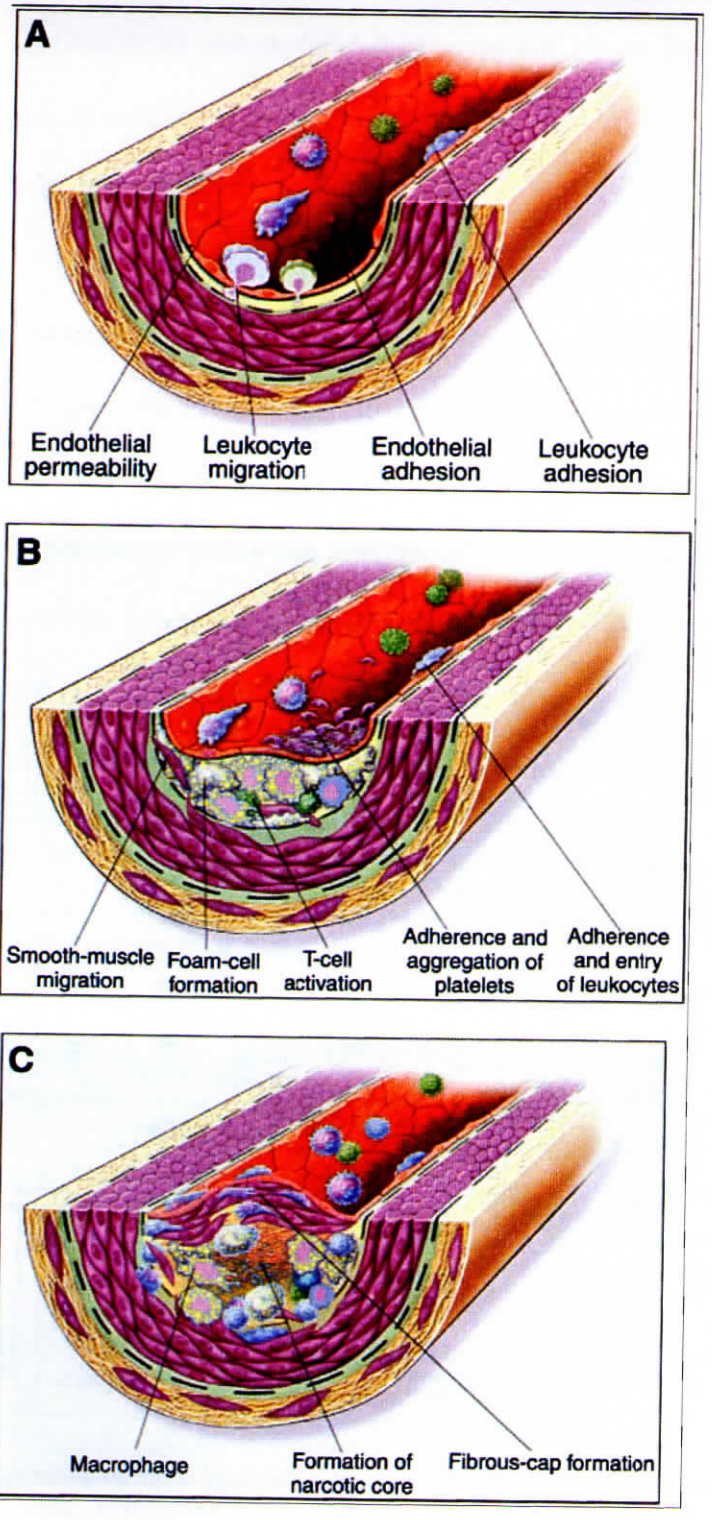

Figure 5. Response-to-injury hypothesis of atherosclerosis [4]. A: Endothelial injury is characterized by enhanced permeability and LDL deposition. Followed by leukocyte adhesion and transmigration. B: Intermediate stages characterized by foam cell development. C: Advanced atherosclerosis shows a fibrous cap and necrosis of the lesion core.

Rather an endothelial injury is capable of inducing atherogenesis but atherosclerosis in not dependent upon an injury.

Response-to-retention

This hypothesis, Figure 6, is based upon the supposition that lipoprotein retention is the "inticing event" for atherosclerosis [Williams and Tabas, 1995, 1998; Nievelstein et al., 1991]. It is believed that lipoprotein lipase activity is required for lipoproteins to reach the subendothelial space [Zilversmit, 1973]. The retention of lipoproteins within the arterial wall, however, appears tightly linked to components of the extracellular matrix. Apolipoprotein B-100, the single protein associated with LDL, is retained within the arterial wall in close association with arterial proteoglycans [Camejo et al., 1993; Yla¨-Herttuala et al., 1987].

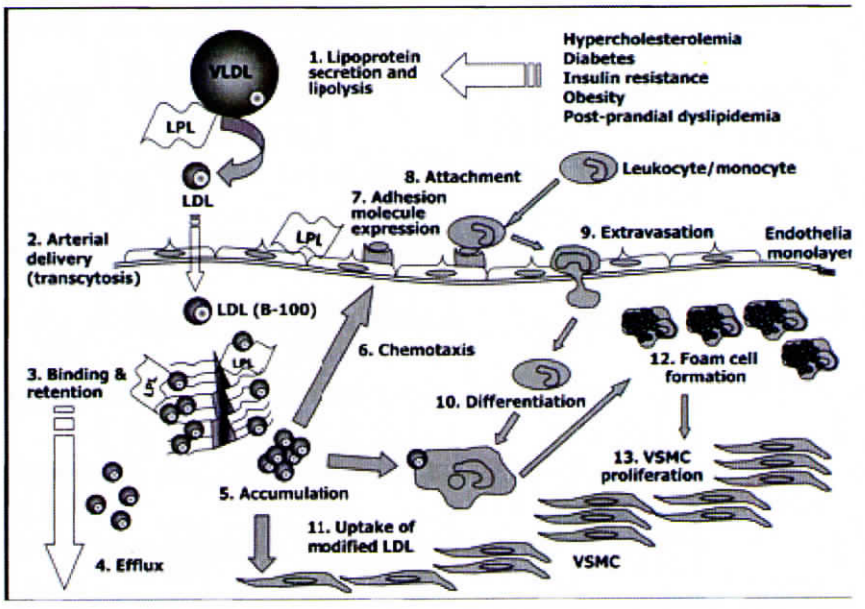

Figure. 6. Response-to-retention hypothesis of atherosclerosis. According to the original hypothesis [Williams and Tabas, 1995], mild to moderate hyperlipidemia causes lesion development only in specific sites within the arterial tree characterized by local synthesis of apolipoprotein B-retentive molecules such as biglycan and decorin. The cartoon shows the initial stages of arterial lipoprotein delivery, retention, and efflux (*1–5*). Accumulation (*5*) is thought to result from apolipoprotein B-100 motifs that mediate proteoglycan binding. The accumulation of apolipoprotein B-100-containing lipoproteins within the arterial wall is thought to further trigger a proinflammatory cascade (*6–13*).

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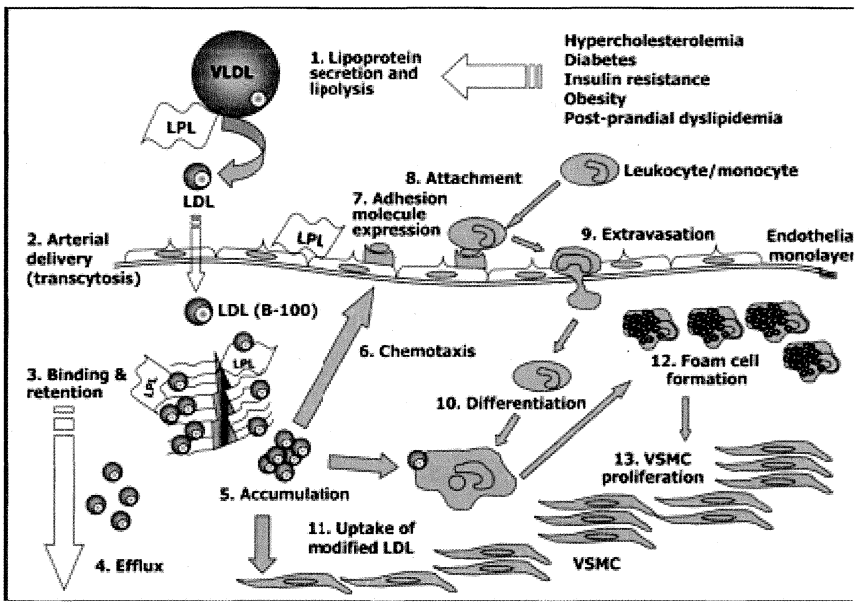

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Thus these data support an important role for proteoglycan binding in the retention of apolipoprotein B-containing lipoproteins in the early stages of atherosclerosis; however it fails to demonstrate how the preservation of LDL or Lp(a) along the vessel induces atherogenesis.

The oxidative modification hypothesis

The oxidative modification hypothesis (Figure 7) focuses on the concept that LDL in its native state is not atherogenic. However, LDL modified chemically is readily internalized by macrophages through a so-called "scavenger receptor" pathway [Goldstein et al., 1979]. Exposure to vascular cells in medium that contains transition metals also results in modification of LDL such that it serves as a ligand for the scavenger receptor pathway [Henriksen et al., 1981]. It is now clear that one mechanism whereby cells in vitro render LDL a substrate for the scavenger receptor pathway is via oxidation of LDL lipids and the resulting modification of apolipoprotein B-100 [Steinbrecher et al., 1984]. These observations form the basis for the oxidative modification hypothesis of atherosclerosis (Fig. 7), in which LDL traverses the subendothelial space of lesion-prone arterial sites. During this process, LDL lipids are subject to oxidation and, as a consequence, apolipoprotein B-100 lysine groups are modified so that the net negative charge of the lipoprotein particle increases [Haberland et al, 1982]. This modification of apolipoprotein B-100 renders LDL susceptible to macrophage uptake via a number of scavenger receptor pathways producing cholesterol ester-laden foam cells [Haberland et al., 1984]. It is this accumulation of foam cells that forms the nidus of a developing atherosclerotic lesion.

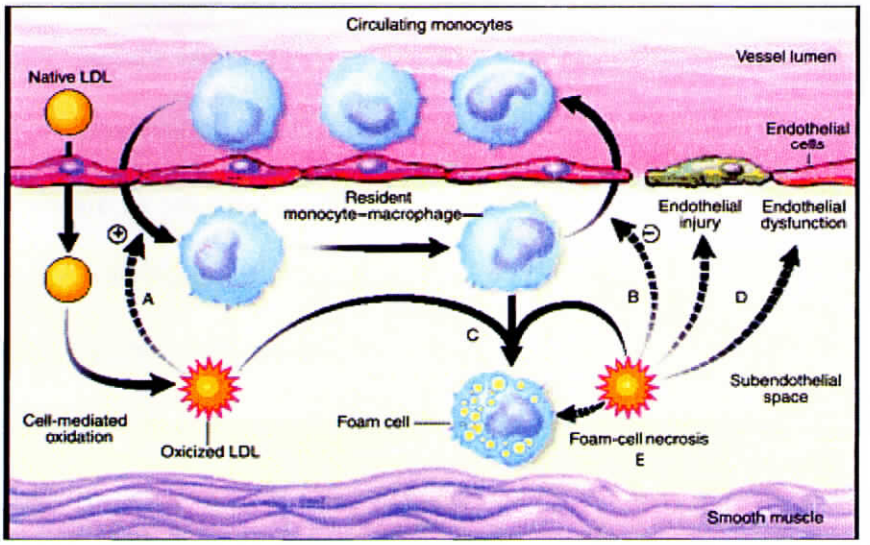


Figure 7. Oxidative modification hypothesis for atherosclerosis [Diaz et al., 1997]. LDL is entrapped in the subendothelial space where it is subject to oxidative modifications. This modification stimulates monocyte chemotaxis (A), prevents monocyte departure (B), and encourages foam cell formation (C). This process encourages endothelial injury (D), which leads to foam cell necrosis (E).

Oxidized LDL has been shown to stimulate the proliferation of smooth muscle cells [Stiko-Rahm et al., 1992]. Other proatherogenic events include: aiding the recruitment of circulating monocytes to the intimal space, inhibiting macrophages from leaving the intima, enhancing LDL uptake, and cytotoxicity that leads to loss of endothelial integrity [Quinn et al., 1985].

As previously mentioned each hypothesis is distinct, particularly in their initiating events, however all three possess common features such as a component of inflammation, a known feature of atherosclerosis [Libby, 2002] and an important role for LDL.

Infection, Inflammation and Atherosclerosis

As many as 50% of patients with atherosclerosis lack currently identified risk factors (such as hypertension, smoking, hypercholesterolemia, and diabetes), an observation indicating that additional factors predisposing to atherosclerosis are as yet undetected. A candidate trigger of inflammatory responses in atherosclerosis is infection [Epstein et al., 1999]. The role of infectious agents in atherosclerosis has been recognized for more than a century. William Osler was one of the first to propose a major role for acute infection in the pathogenesis of atherosclerosis [Osler, 1985]. In the early 20th century, a few pioneer scientists used several infectious agents (*Salmonella typhi,* streptococci, etc.) to induce atherosclerosis in animal models. By the late 1970s, scientists began to study the role of herpes viruses and *Chlamydia pneumoniae* and, later, of *Helicobacter pylori, Mycoplasma pneumoniae, Porphyromonas gingivalis,* enterovirus, and a growing list of other agents in atherogenesis (Table 2) [Higuchi et al., 2000; Haraszthy et al., 2000; Alber et al., 2000; Epstein et al., 1999; Li et al., 2002; Muhlestein et al., 1998]. This effort coincided with the emergence of new evidence pointing to atherosclerosis as an inflammatory disease [Ross and Glomset, 1976].

TABLE I. Infectious Agents Implicated in Atherosclerosis

Chlamydia pneumoniae
Cytomegalovirus
Herpes simplex viruses 1 and 2 (HSV-1, HSV-2
Helicobacter pylori
Mycoplasma pneumoniae
Porphyromonas gingivalis
Enterovirus species
Salmonella typhi
Streptococcus sanguis
Coxsackie B virus
Adenovirus species
Mycoplasma gallisepticum
Marek's disease virus
Measles virus
Epstein-Barr virus
Human immunodeficiency virus
Mycoplasma fermentans
Coxiella burnetti
Actinobacillus actinomycetemcomitans
Bacteroides forsythus
Hepatitis A virus
Prevotella intermedia
Influenza virus

Circulating pathogen-derived or pathogen-stimulated factors could induce changes in the vascular wall. Acute respiratory-tract infections are associated with an increased risk of acute myocardial infarction. The data revealed a relative risk of 2.7 for acute myocardial infarction occurring in relation to an acute respiratory-tract infection in the preceding 10 days [Epstein et al., 1999]. Finally, some studies relate increased prevalence of periodontal disease to increased prevalence of CAD [Loesche et al., 1998; Beck et al., 1996; Destefano et al., 1993; Mattila et al., 1996; Joshipura et al., 1996]. One hypothesis driving these studies is that chronic periodontal infection leads to CAD through the production of a long-term inflammatory response. In this regard, elevated levels of specific salivary IgA antibodies against mycobacterial HSP65 were found to be significantly increased in patients with gingivitis [Schett et al., 1997]. Influenza virus noted for secretion of neuraminidase as well as for induction of serum IgA [Janeway et al., 2001] is strongly associated with atherosclerosis [Madjid et al., 2003]. At the same time infectious etiology is now being increasingly attributed to cardiovascular diseases [Libby et al., 1997; Madjid et al., 2003].

Elevated total serum sialic acid (SA) concentration is a risk factor for cardiovascular mortality in humans [Taniuchi et al., 1981]. Results from prospective studies suggest that inflammation is involved in the pathogenesis of atherosclerosis [Ross, 1999].

Role of Immune complexes in infection-mediated pathology

Immune complexes- the saga of the close encounter of antigen and antibody.

Circulating immune complexes (ICs) are produced continuously in response to infection, tissue injury, and immune reactions to foreign antigens (Ags). Most ICs are of little pathologic significance because they are rapidly cleared by hepatic and splenic phagocytes [Schifferli and Taylor, 1989; Abrass, 2001]. This cycle of IC formation and clearance is an important component of acquired immunity, ensuring removal or processing of Ags. However, excessive IC accumulation occurs in several immune-mediated diseases in human patients [Firestein, 2003; Lamprecht et al., 1999]. The factors influencing IC deposition have mostly been determined for the glomerulus, which has a unique fenestrated vascular bed that passively traps circulating ICs [Abrass, 2001]. Tissue injury of many types may be caused by deposited complexes of antigen and antibody. The circumstances under which the complexes form and deposit often determine the location and type of injury observed: if the complex forms in circulation, deposition may occur in arterial walls and glomeruli, initiating lesions in those tissues. If the complex forms in the synovial tissues or spaces, then the reaction will develop at that point [Cochrane, 1977].

In 1903 Maurice Arthus published his observations on the induction of a localized inflammatory reaction at the site of repeated immunizations with a foreign antigen [Arthus, 1903]. The Arthus reaction, as it has become known, results from the deposition of ICs in specific anatomic sites and the subsequent activation of inflammatory responses to these complexes. This reaction has served as the basis for

dissecting the cellular and molecular events that are triggered by IC deposition and serves as the basis for our understanding of the pathophysiology of IC-mediated diseases, such as lupus and rheumatoid arthritis.

The authors speculate that the Arthus reaction may involve binding of the immune complexes to the Fc receptors on mast cells, with subsequent release of preformed mast cell mediators that increase vascular permeability, activate complement, and stimulate the adhesion and migration of neutrophils. Similarly, tissue macrophages and neutrophils may bind immune complexes and further stimulate neutrophil chemotaxis [Sylvestre and Ravetch, 1994]. IC activate the classical and alternative pathways [Ji et al., 2002] of the complement system and thus interact with FcγR and a variety of complement receptors. Both classes of receptors have been implicated in immune adherence of opsonized particles, phagocytosis, IC clearance, and signal transduction. When clearance mechanisms are overwhelmed, IC can become an important cause of tissue damage [Davies and Walport, 1998]. In such settings, IC can lead to the generation of destructive proinflammatory processes marked by the chemotaxis and activation of myeloid cells at sites of IC deposition [Godau et al., 2004]. Complement participates in the elimination of IC in many circumstances. When antigen/antibody IC first form in the circulation, complement inhibits their aggregation because the covalent binding of C3b to the IC modifies their biophysical properties and they remain soluble. Such opsonized (C3b coated) IC attach to cells bearing C3b receptors (CR1) in the circulation, in particular to erythrocytes, since in humans 85 to 90% of CR1 in the blood is located on these cells. This immune adherence binding reaction appears to be a physiological system that

allows IC to be transported through the circulation to the fixed macrophages of the MPS (Mononuclear phagocytic system) where they are safely eliminated. The deposition of circulating complement-fixing IC in various organs such as the kidney may be considered as a failure of this transport system. This is apparent in complement deficient and depleted states, and also for non-complement-fixing IC (IgA IC). The formation of insoluble IC (by definition immune deposits found in human pathology are insoluble) produces complement activation and inflammation at the site of the immune aggregate [Schifferli and Taylor, 1989].

IgA nephropathy, the most common primary glomerulonephritis worldwide [Levy and Berger, 1988], accounts for 5–20% of renal biopsies in some countries, and 20–40% of patients progress to end-stage renal failure after 20–25 years [D'Amico, 2000]. Several pieces of evidence suggest that circulating immune complexes (IC) may play a role in the deposition of IgA in patients with IgA nephropathy. These include the mesangial localization of IgA in a granular pattern, the presence of electrondense deposits in the mesangium and the recurrence of IgA-dense deposits in renal allografts [Gonzalez-Cabrero et al., 1990]. This aberrant IgA1 probably escapes hepatic catabolism because the CICs are too large to pass through endothelial fenestrae to enter the space of Disse to reach the hepatocytes [Novak et al., 2001]. Instead, it is shunted to the renal circulation where endothelial fenestrae overlying the glomerular mesangia are larger. The mesangial cells (MCs) bind high molecular weight IgA1 with high affinity [Leung et al., 2002]. The activation of MCs by IgA1 immune complexes is considered the initiating event in the pathogenesis of IgA nephropathy. In vitro, IgA1 immune complexes stimulate the pro-inflammatory

mediators, such as cytokines, chemokines, and growth factors [Chen et al., 1994], which may, in turn, induce MC proliferation or the production of extracellular matrix [Schocklmann et al., 1999]. Although many studies have reported that O-glycosylation of serum IgA1 of patients with IgAN is different from that of controls, the exact structural nature of the IgA1 O-glycosylation defect in IgAN and related diseases has yet to be fully elucidated, and has proved technically challenging to investigators in the field. Incidentally in IgAN, the IgA molecules found in the glomerular mesangium are polymeric (pIgA1) [Tomino et al., 1982]. Particularly, IgA1 from patients was found to be relatively abundant in asialo type sugar chains [Iwase and Hiki, 1999]. In an animal model experiment different IgA subfractions was injected into the aorta of a rat kidney to confirm the presence of deposits in the glomeruli. Among those IgA1 subfractions, only one subfraction containing abundant asialo sugar chains (as detected by gas-phase hydrazinolysis) exhibited deposition. Structural characteristics of deposited IgA1 from IgA nephropathy patients having asialo mucin-type sugar chains thus became clear. Although an animal model experiment, this study demonstrates for the first time the involvement of the incomplete sugar chain in deposits in the rat glomeruli [Hiki et al., 1998]

What are the biological consequences of desialylated IgA1 and the formation of CICs? Circulating IgA1 has a relatively short half-life of 5–6 days [Mestecky and Russell, 1986]. Approximately 90% of circulatory IgA is catabolized primarily in the liver by hepatocytes [Tomana et al., 1985; Moldoveanu et al., 1988; Moldoveanu et al., 1990; Mestecky and McGhee, 1987] that express the asialoglycoprotein receptor (ASGP-R) [Tomana et al., 1985; Moldoveanu et al., 1988;

Moldoveanu et al., 1990; Tomana et al., 1988; Stockert et al., 1980; Stockert et al., 1982]. Before reaching the ASGP-R, molecules of glycoproteins must exit the circulation to enter the space of Disse through fenestrae found in the vascular endothelial cells. These fenestrae have effective pore sizes of 180–250 Å [Granger et al., 1979]; larger molecules, such as IgM and its complexes, remain in the circulation and may be catabolized or deposited elsewhere [Phillips et al., 1986]. Consequently, large CICs do not penetrate this molecular sieve because of their size. Glycoproteins with terminal Gal and GalNAc that enter the space of Disse and interact with ASGP-R in the presence of calcium are readily internalized and degraded [Ashwell and Harford, 1982; Baenziger and Maynard, 1980; Baenziger, J.U., and Fiete; 1980]. However, the presence of IgM antibodies bound to Gal NAc residues in the hinge region likely prevents the interaction of GalNAc with ASGP-R. In addition, an increased molecular mass of CICs comprising pIgA and IgM may hinder penetration through endothelial fenestrae, resulting in the diversion of such CICs from the catabolic pathway. Whether CICs that escape the liver-mediated clearance are identical to those in the mesangium is at present uncertain. Nevertheless, CICs and mesangial deposits share many common features, including the exclusive presence of IgA1 (partially in the polymeric form) [Czerkinsky et al., 1986; Valentijn et al., 1984] and IgM [Russell et al., 1986]. The antigenic specificity of IgM, which is also frequently detected in mesangial deposits in IgAN ([Russell et al., 1986], is not known. The relative proportion of such antibodies, as well as their affinity and, especially, avidity (higher in immunoglobulin with multiple binding sites), likely play important roles in the formation of CICs and their probable mesangial deposition.

Diabetes mellitus is a group of metabolic disorders characterized by elevation of blood glucose concentration and is associated with increased prevalence of microvascular complications. Type 1 diabetes mellitus results from cellular mediated autoimmune destruction of pancreatic β-cells of islets of langerhans and results in loss of insulin production. Type 2 diabetes mellitus is the most common form of diabetes accounting for 90% of cases. Type 2 diabetes is characterized by insulin resistance or abnormal insulin secretion. One of the more debilitating aspects of diabetes is the numerous complications that can arise from the disease. These complications include diabetic retinopathy, kidney nephropathy and peripheral neuropathy. The development and severity of these complications are dependent on the duration of the disease and how well it is managed. Prospective studies have reported associations among various markers of inflammation and incidence of diabetes [Pradhan et al., 2001], and it has been proposed that inflammation has a causal role in the development of diabetes [Pickup and Crook, 1998]. Diabetes is another risk factor for myocardial infarction and stroke [Vilbergsson et al., 1998; Kannel et al., 1990]. Elevated levels of immune complexes (CIC) have been detected in the circulation of all types of diabetic humans [Irvine et al., 1977; Iavicoli et al., 1982; Abrass et al., 1983] and have been postulated to play a role in the development of diabetic complications [Minick & Murphy, 1973; Irvine et al., 1978]. The precise relationship of immune-mediated injury to the pathogenesis of diabetic microangiopathy remains to be determined. The decreased rate of plasma clearance of model immune complexes in diabetic rats is consistent with previous reports of elevated levels of circulating immune complexes in both diabetic humans and animals

[Abrass et al., 1983; Abrass, 1984]. An increase in circulating IgA concentrations is a generalized phenomenon among diabetic patients; IgA concentrations above the reference range are more common among male than female diabetics; and diabetic complications are associated with a significant increase in serum IgA concentration [Rodriguez-Segade et al., 1996; Singh and Kulig, 1992]. Also, 80-87% of IgA in diabetes are polymeric [Triola et al., 1984]. All the type 1 and type 2 diabetic subgroups defined on the basis of their micro- or macrovascular diabetic complications (nephropathy, retinopathy etc.) had higher serum IgA concentrations than the corresponding groups of patients without complications. This suggests that monitoring IgA may provide early warning of the possible presence of complications [Rodriguez-Segade et al., 1996; Cheta et al., 1982].A role of IgA system abnormality has been suggested in the pathogenesis of diabetic vascular complications [Casiglia et al., 1990] Abnormal immunoreactive IgA-containing renal glomerular deposits are found in diabetic nephropathy patients [Miller et al., 1988]. Accumulative evidences also suggest that immune complexes containing IgA of mucosal origin may be involved in microangiopathy production in Type 2 diabetes [Triola et al., 1984]. CIC are known to persist in the blood for long periods of time. Such CIC following deposition in the small blood vessels have the potential to lead to microangiopathy with debilitating clinical consequences [Nicoloff et al., 2004]. It was postulated that the elevations of serum IgA and IgA-CIC were based on subclinical infection of the mucosa and/or deterioration of IgA clearance in patients with NIDDM [Eguchi et al., 1995]. Sialic acid is a terminal component of the non-reducing end of carbohydrate chains of glycoproteins and glycolipids [Ng and Dain, 1976]. It has been reported

earlier that total serum sialic acid concentration increase in type 2 diabetes mellitus associated with microvascular complications [Crook et al., 1993; Crook et al., 1994]. Increased total serum sialic acid leads to increased excretion of sialic acid in urine of the patient presented with high urinary microalbumin (Table 3). In type-2 diabetes, the circulating sialic acid concentration is elevated in comparison with non-diabetic subjects [Crook et al., 1993]. Crook M et.al found that serum sialic acid was significantly higher in men with diabetic complications than in those without any of the complications [Crook et al., 2001]. Incidentally, diabetes which causes pervasive vascular damage is often accompanied by high serum levels of neuraminidase [Table 4; Merat et.al, 2003].

Table 3. Serum and urinary sialic acid and microalbumin levels in Type 2 diabetes with nephropathy and retinopathy (Nayak and Bhaktha, 2005).

Parameters	Diabetes without any complications	Diabetic nephropathy	Non-diabetic subjects	p value
rum Sialic acid (mg%)	55.05 ± 2.9*	85.05 ± 2.7***	46.6 ± 2.08	< 0.001
ine sialic acid (mg%)	6.02 ± 2.58**	13.06 ± 1.58***	3.2 ± 0.65	< 0.001
croalbumin (mg %)	8.2 ± 3.24	132.2 ± 35.24***	7.67 ± 3.28	< 0.001
S (mg%)	140.02 ± 70.08	155.6 ± 50.7	90.02 ± 80.08	< 0.01
BS (mg%)	150.02 ± 102.10	207.3 ± 57.6	120.02 ± 102.10	< 0.01
A_{1C} (%)	9.10 ± 5.20	11.1 ± 2.3	6.10 ± 5.20	< 0.05
iglyceride (mg%)	122.04 ± 75.01	178.02 ± 78.01	120.04 ± 76.01	< 0.05
olesterol(mg%)	148.04 ± 120.01	256.03 ± 134.01	140.04 ± 119.01	NS
DL (mg %)	35.01 ± 20.04	38.01 ± 26.02	36.01 ± 19.04	NS
L (mg %)	90.00 ± 76.06	165.00 ± 97.01	87.00 ± 76.05	< 0.05
eatinine (mg %)	2.00 ± 1.6.06	10.05 ± 2.03	1.40 ± 1.20	< 0.001
ine creatinine (mg%)	145.00 ± 102.6	155.03 ± 65.02	146.00 ± 113.06	< 0.05

Mean ± SD *** $p < 0.001$, NS = not significant, n = 50

Table 4. Concentration of SSA and serum neuraminidase activities of 5 patient groups compared with normal cases adjusted for age and sex. (Merat et.al, 2003)

Groups	No. of subjects	SSA (mg/100ml)	Neuraminidase Activity (mU/ml)
Normal	59	61.20 ± 3.01	52.25 ± 2.70
Group I (D +CVD)	45	104.35 ± 4.81	65.49 ± 2.69
Group II (CVD)	45	69.66 ± 2.66	64.60 ± 2.96
Group III (D)	62	82.98 ± 3.68	64.71 ± 2.69
Group IV (D + R)	62	91.80 ± 4.90	64.61 ± 2.94

Values are mean±SEM. $p<0.05$ (according to the one-way ANOVA and Duncan's multiple range as *post hoc* test). D = Diabetes, CVD = Cardiovascular disease, SSA= Serum sialic acid.

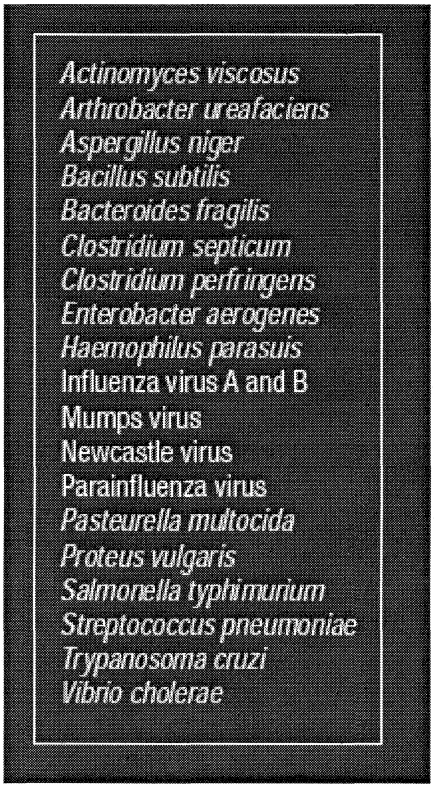
Role of Neuraminidase in infection-mediated pathology

Neuraminidase (sialidase, N-acylneuraminosyl glycohydrolase, EC 3.2.1.18) is an exo-glucosidase which hydrolyses α-glycosidically bound sialic acids which are mostly found as terminal constituents of glycoproteins, glycolipids and oligosaccharides in higher animals and some microorganisms [C o r f i e l d and S c h a u e r, 1982]. Whereas the different kinds of sialidases in animals play an important role in the turnover of sialoglycoconjugates, thereby influencing the development of cells [S c h a u e r, 1983], the origin and biological function of the microbial enzymes have not been finally clarified.

The enzyme neuraminidase (sialidase) has been implicated as a virulence factor in several species of bacteria and viruses, particularly those possessing the ability to survive on mucosal surfaces, examples of which include Corynebacterium diphtheriae, Vibrio cholerae, Streptococcus pyogenes and viruses such as influenza virus [Pardoe, 1974]. Gottschalk [1960] originally demonstrated that the removal of terminal sialic acid residues from salivary glycoproteins caused a loss in the viscosity and adhesiveness of these secretions, hindering the normal protective function of the epithelial mucous lining (e.g., the entrapment and removal of invading microorganisms). Thus, the enzyme appeared to have an adaptive function in enhancing survival of these microorganisms in the respiratory, intestinal, and urogenital tracts. In addition to this localized effect, Muller [1974] has demonstrated altered electrophoretic patterns of serum glycoproteins in patients suffering from invasive pneumococcal disease. Many human pathogens (Table 5), including influenza viruses, possess neuraminidase enzymes (also known as sialidases) [Freed, 1987]. These enzymes remove sialic acid molecules from the cell surface. Normally, sialic acid acts as a physical barrier against invasion by microbes by decreasing access to cell membrane receptors. Influenza's hemagglutinin molecule actually binds to sialylated lectins, as well as sialylated glycolipids known as gangliosides. For influenza to migrate into the cell, the virus uses neuraminidase to cleave the sialic acids and release itself from the membrane. Removal of sialic acid enables a virus to enter a host cell to replicate as well as to exit the host cell [Gubareva et al., 2000] Influenza virus noted for secretion of neuraminidase as well

as for induction of serum IgA [Janeway et al., 2001] is strongly associated with atherosclerosis [Madjid et al., 2003].

Table 5. List of Microorganisms Producing Neuraminidase



IgAN is the most common primary cause of glomerulonephritis in the world today. Classically, it presents with fatigue, rising creatinine, microscopic hematuria, and a history of upper respiratory infection (URI) [Wakai et al., 2002]. The URI is a result of influenza or other virus whose neuraminidase hastens the removal of IgA by ASGPR but when overburdened expose excess amounts of desialylated IgA to circulation, resulting in formation of circulating immune complexes. Excess IgA ultimately deposits in/on glomerular mesangial cells in the kidney causing inflammation, scarring, and decreased renal function. IgA

nephropathy (IgAN) in general, and certain histological subtypes in particular show abnormally increased desialylated IgA species in circulation, believed to be of pathological significance to its glomerular deposition [Xu and Zhao, 2005].

Specifically, the spectrum of IgA nephropathy biopsies range from mild or no visible changes to extensive inflammation, mesangial expansion, and glomerularsclerosis. And the degree of pathological abnormalities seen is proportional to desialylated IgA, implicating the degree of desialylation of IgA to inflammation and glomerular injury. By treating IgAN with neuraminidase inhibitors [Altschuler et al., 2005], it may be possible to prevent the abnormal IgA from ever forming in the first place. Theoretically, this would arrest the illness before any deposition in the mesangium ever occurred. And for the millions worldwide who suffer from IgAN, neuraminidase inhibitors with its high threshold of tolerability and low side-effect profile could represent an effective treatment [Bhatia and Kast, 2007]

Chapter 3

MATERIALS AND METHODS

MATERIALS AND METHODS

Materials

Neuraminidase from *Clostridium perfringens,* human IgA, IgG, horse radish peroxidase (HRP), orthophenylene diamine (OPD), PEG 6000 ,1-O-methyl-α-D-galactoside, Soluble guar gum, myosin, β-galactosidase, bovine serum albumin, ovalbumin, trypsin, amido black, Sudan black B, Coomassie brilliant blue G, divinyl sulphone, cyanogen bromide, Tween-20, 4-chloro-1-naphthol, diaminobenzidine, sodium dodecyl sulphate, acrylamide, N, N`-methylene bisacrylamide, TEMED, agarose, glucose, galactose, lactose, melibiose , 2-mercaptoethanol, Iodoacetamide, potassium thiocyanate, L-proline were obtained from Sigma Chemical Company, St. Louis, USA. Polystyrene 96 well microplates (MAXISORB) were purchased from Nunc, Denmark. Goat anti-human IgA Antibodies to human IgA, IgM raised in goat and Rabbit anti-human Lp(a) were purchased from Dako, Denmark. Lp(a) assay kit [ELITEST-Lp(a)] was from Hyphen Biomed (France) and Lp(a) assay kit [Turbidimetric method] used in Dade dimension AR (autoanalyzer) was from Wako Pure Chemical Industries, Ltd., Japan. Sepharose 4B and 6B were the products of Pharmacia Fine Chemicals, Uppsala, Sweden. Nitrocellulose filters (0.45 μm) were from Millipore, USA. PVDF and Coomassie brilliant blue R-250 were purchased from Pierce Chemical Co. USA. Triton X-100, 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 hypogaea* and *Artocarpus integrifolia* were obtained locally.

Methods

Tissue Collection

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 -20^0 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. Fasting serum and blood samples were collected from the Central Clinical Laboratory and Blood Bank of this institute.

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, 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 and Grieve, 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.

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.* [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. [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

Sepharose 4B (40 ml) (Pharmacia Biotech, Sweden) was activated using cyanogen bromide by the method of [Cuatrecasas and Anfinsen, 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.

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 was stored in ice [Heyderman et al., 1989].

Electrophoresis

Alkaline-PAGE

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

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight range of glycoproteins were determined by SDS-PAGE on 7.5% slab gels according to the method of Laemmli [1970].

Reagents

a. Gel Solution: 15 g of acrylamide and 0.4 g of bisacrylamide were dissolved in distilled water and the volume made up to 50 ml. The solution was filtered and stored at 4 °C in amber colored bottle.

b. Buffer I: 0.614 M Tris/HCl, pH 8.8, containing 164 mg of SDS per 100 ml of the buffer.

c. Buffer II: 0.147 M Tris/HCl, pH 6.8, containing 108 mg of SDS per 100 ml of the buffer.

d. Chamber buffer: 0.25 M Tris/0.192 M glycine, pH 8.3, containing 0.1% SDS.

e. Ammonium per sulfate (15 mg/ml) in distilled water.

The preparation of 7.5% separating gel involved the mixing of 2.25 ml of 'a,' 0.75 ml of glass-distilled water, 4.25 ml of buffer I, 0.45 ml of ammonium per sulfate and 10 μl of TEMED at the time of polymerization. For spacer gel, 0.5 ml of 'a', 4.25 ml of buffer II, 0.25 ml of ammonium per sulfate and 5 μl of TEMED were mixed at the time of polymerization. Myosin (205 kDa), β-galactosidase (116 kDa) bovine serum albumin (67 kDa) ovalbumin (45 kDa), trypsinogen (24kDa) and cytochrome C (14 kDa) were used as molecular weight marker proteins. The mini slab gel (Hoefer Scientific 7.3 x 8.3 cm) was run at 15 mA till the bromophenol blue dye had reached the bottom of the gel (app. 6 cm). The proteins in the gels were fixed using 50% methanol, stained with Coomassie brilliant blue R-250 and destained with methanol: acetic acid: water (1:1.5:17.5, v/v).

Western blot

Glycoproteins (1 mg/ml) were separated by SDS- polyacrylamide slab gel electrophoresis (7.5%) as described by Laemmli [1970]. Separated glycoproteins were transferred to polyvinyl difluoride (PVDF) membrane using transfer buffer consisting of 25mM Tris, 192 mM glycine and 15% methanol, pH 8.3 and applying a constant current of 0.8 mA/cm^2 membrane as described by Towbin et al. [1979]. The

strips (3-4 mm wide) were cut out from the transferred membrane, blocked with PBS containing 0.2 % Tween 20 and probed with antibody/lectin or their conjugates with enzyme (horse radish peroxidase) for 2 h at 4 °C. The strips were then washed twice with 0.05% PBS-T and once with PBS alone and stained using freshly prepared 4-chloronaphthol substrate solution (1ml 0.3% 4-chloronaphthol) in anhydrous methanol mixed with 5 ml PBS and 3 µl 30% H_2O_2). After staining for 2-5 minutes in 4-chloronaphthol solution, strips were washed again in PBS and photographed.

Enzyme-labeled antibody or lectin binding to micro well-coated or dot-blotted proteins and glycoproteins.

Polystyrene micro well coating of antibodies and proteins followed by blocking of wells and washing with solutions of Tween 20 in PBS was done as described earlier [Chacko and Appukuttan, 2003]. Where specified, polystyrene well-coated glycoproteins were treated at 37°C with solutions of neuraminidase (50 mU/ml) for 1 h. Coated proteins were then treated with the specified solutions (200 µl) and washed after each step with PBS containing 0.05% Tween 20 (PBS-T) thrice at 4°C. After final incubation with 200 µl HRP conjugate and washing, wells were incubated at 25°C for 15 min with 200 µl OPD (0.5 mg/ml) in 0.1M citrate-phosphate buffer, pH 5.0 containing 0.03% H_2O_2, followed by addition of 50 µl 12.5% H_2SO_4. Absorbance at 490 nm was measured in a BIOTECH (USA) microplate reader. Polyvinyl difluoride membrane strips (Millipore, USA) containing dot blots or Western blots of glycoproteins were blocked with 0.2% Tween 20 in PBS for 2h and incubated where indicated at 37°C in neuraminidase solution (50 mU/ml) in PBS-T before successive treatments with other reagents. After incubation with HRP

conjugate and three washings with PBS-T at 4°C bound conjugate was detected by dipping the dots for 5 min in HRP substrate (1ml 0.3% 4-chloronaphthol in anhydrous methanol mixed with 5 ml PBS and 3 µl 30% H_2O_2) at 25°C, followed by washing with PBS.

Preparation of anti-T antibody from human plasma.

A procedure developed in this laboratory was used [Chacko, 2001]. Briefly human RBC glycoproteins containing T antigen were isolated by affinity chromatography of glycoproteins of lysed human RBC on jacalin-Sepharose 4B. These glycoproteins were desialylated by treatment with 0.1N H_2SO_4 at 80°C for 1h, dialysed and immobilized on Sepharose-4B using cyanogen bromide [Lowe and Dean, 1974]. Human plasma (50 ml) dialysed against 20 mM potassium phosphate buffer containing 150 mM NaCl (PBS) was passed successively through cross-linked guar galactomannan (50 ml) and lactose-Sepharose (40 ml) affinity chromatography columns at 4°C to remove naturally occurring serum anti-α-galactoside antibody [Jaison and Appukuttan, 1992] and lactose-binding IgG [Chacko, 2001] respectively. Unbound proteins were passed through the desialylated RBC glycoprotein-Sepharose column at 4°C in PBS and column washed to remove unbound proteins. Bound anti-T was eluted using 3.5 M potassium thiocyanate in PBS and dialysed against PBS.

Desialylation of human erythrocytes for hemagglutination assays and removal of anti-T from serum.

Human RBC from non-coagulated blood were washed thrice with PBS by centrifugation. Cell suspension (5% in PBS) was treated at 37°C with neuraminidase (50 mU/ml) for 1 h before washing thrice with PBS by centrifugation. For hemagglutination assay a suspension of these cells (1%) was incubated for 1 h

with dilutions of serum sample of same blood group in U-bottom wells of 96 well polystyrene plates in a final volume of 125 µl PBS-T at 25°C for 1 h. Afterwards contents of wells were mixed with a stainless steel wire and clumping of cells within two minutes was taken as positive agglutination. For inhibition studies serial dilutions of the inhibitor was incubated with anti-T at 4°C for 1 h in a total volume of 75 µl before addition of erythrocyte (25 µl 5% suspension). For removal of anti-T, serum (2 ml) was treated for 2 h with 3 ml packed desialylated erythrocytes at 4°C with occasional mixing. The supernatant was collected by centrifugation.

Hemagglutination assay of Anti-T antibody.

Hemagglutination assay to determine the activity of purified anti-T was done using 'U' bottom 96-well microtitre plates (Laxbro). The required number of wells were blocked with 0.05% Tween 20 in PBS 7.4 (PBS-T) for 30 min. prior to the assay. After the blocking solution was drained out completely, serial double dilutions of anti-T antibody which was thoroughly dialysed were made in the blocked wells in a volume of 100 µl using 0.05% PBS-T. Neuraminidase treated human RBC (25 µl, 5% v/v suspension) was added to the diluted anti-T antibody so that the final cell suspension is 1%. The contents in each well were mixed gently and thoroughly with a steel wire. Agglutination titer was scored after 1 h with mixing. Clumping and settling of RBCs within 2 minutes after the final mixing is taken as positive agglutination.

Hemagglutination Inhibition Assay

Neuraminidase treated human erythrocytes (NHRBC) was used for inhibition assay. The minimum agglutinating quantity (MAQ) of anti-T antibody

using NHRBC was determined using serial double dilution method in a 96-well microtitre plate (Laxbro) blocked with PBS-T.

Serial 2 fold dilutions of glycoproteins were done in a fixed volume of 75 µl in different rows of wells of a PBS-T blocked microtitre plate. To each well, 25 µl PBS-T containing twice the MAQ of anti-T antibody was added. The well contents were mixed and incubated at 4°C for 45 min. Twenty five microlitre of 5% suspension of NHRBC was added and the well contents mixed well. Two controls one without inhibitor (positive control) and the other without antibody (negative control) were also done. Thirty minutes later, well contents were mixed and agglutination noted as done for hemagglutination assay to obtain the concentration required for 50% inhibition.

Preparation of immune complex (IC) from serum.

IC was precipitated with 2% polyethylene glycol (PEG) 6000 in veronal buffer (2 mM sodium barbitone, 3 mM barbitone and 140 mM sodium chloride) pH 7.4 by a modification of the procedure by Hudson and Hay [1980]. Briefly, 450 µl serum was treated at 37 °C with 30 µl PBS containing 25 mU active neuraminidase (for N+ IC) or 25 mU heat killed neuraminidase (for NH IC) or no enzyme (for naturally occurring IC). PEG 6000 (96 µl 12% solution in veronal buffer) was then added and solutions kept at 4°C overnight. Precipitated IC was collected by centrifugation at 2000 g, washed once with 2% PEG 6000 in veronal buffer by centrifugation and redissolved in 450 µl PBS.

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.

Isolation of jacalin-binding serum glycoproteins and IgA1.

All steps were at 4°C unless indicated otherwise. Jacalin-precipitated serum O-glycosylated proteins (JPSP) were prepared by keeping a mixture of 1ml human serum and 1ml jacalin solution (2 mg/ml in PBS) overnight. The mixture was diluted with an equal volume of PBS and precipitate collected by centrifugation at 248,000 g for 30 min. After washing with jacalin solution (0.5 mg per ml) by a similar centrifugation the precipitate was dissolved in 50 mM 1-O-methyl-α-D-galactoside in PBS. JPSP (1.2 mg) was treated with 1 ml anti-human IgA-Sepharose or control Sepharose in PBS at 4°C and supernatant used for electrophoresis.

IgA1 that binds to polystyrene immobilized jacalin (IgA1P) was isolated from the naturally occurring human plasma anti-β-glucoside antibody (ABG) [Geetha et al., 2007] essentially as described earlier [Sangeetha and Appukuttan, 2005]. Wells of polystyrene 96 well U- bottom microplates were coated with jacalin by 24 h incubation with 300 µl of 50 µg/ml solution of the lectin. Wells were then blocked by 30 min incubation at 37 ^{0}C with PBS containing 0.5% Tween 20 and washed with PBS-T. Fifty microgram ABG in 300 µl PBS-T was added to each well. After incubation for 2 h, unbound proteins were removed by washing with PBS-T twice and finally with PBS. Bound IgA1P was eluted by incubation with 50 mM 1-O-methyl-α-D-galactoside in PBS (200 µl per well), dialysed and concentrated by ultrafiltration.

To prepare jacalin-Sepharose-binding serum O-glycosylated proteins (JSSP) serum (10ml) diluted twice with PBS was passed through a 10 ml column of jacalin-Sepharose 4B at 4°C. After washing with 100 ml PBS, bound proteins were extracted by heating this gel with 10 ml 4% SDS in PBS in a boiling water bath for 5 min. The extract contained JSSP along with extracted jacalin.

IgA1 assay using microwell-immobilized jacalin.

An assay procedure recently developed in this laboratory was used (Appukuttan et al.; manuscript in preparation). Wells of polystyrene 96-well plates were each coated with 1µg jacalin in 200 µl PBS by overnight incubation at 4°C. After blocking with PBS containing 0.5% Tween 20 for 30 min at 37 °C and washing with PBS-T at 25°C wells were treated with dilutions of samples containing IgA1 in PBS-T (200 µl) for 2 h at 4°C, followed by three washings with PBS-T. After further treatment with 200 µl solution of HRP conjugate of anti-human IgA (containing 1.5

μg antibody per ml) at 4°C for 2 h and washing with PBS-T, bound HRP was assayed using OPD as substrate as described earlier.

Assay of anti-T-O-glycosylated serum protein immune complex

Immune complex from 450 μl serum prepared as described above (N+ or NH) was redissolved in the same volume of PBS and its 20 times dilutions in PBS-T (200 μl) incubated with polystyrene ELISA well-coated anti-human IgM (10 μg/well) for 2 h at 4°C. After washing with PBS-T and further incubation with 200 μl HRP conjugate of jacalin (35 μg lectin per ml), bound HRP activity was measured using ELISA reader.

Assay of IgA-IgM immune complex

Anti-human IgA (10 μg/well) coated on polystyrene wells of 96 well ELISA plate was incubated with 200 μl 20 times dilution in PBS of IC samples (N+ or NH) for 2 h at 4°C. After washing followed by 2 h incubation at 4°C with 200 μl HRP conjugate of anti-human IgM (3 μg/ml antibody in PBS-T) and washing, bound HRP was measured as described above.

Assay of IgA1 in secondary IC upon desialylation of IC-free serum.

To serum from which natural IC had been removed by PEG precipitation and PEG removed by dialysis against PBS, neuraminidase (N+ or NH) was added followed by incubation at 37°C for 2 h and secondary IC precipitated using 2% PEG as described earlier. IC was redissolved in original serum volume of PBS. Diluted IC (800 times) in PBS-T was incubated with ELISA plate-coated jacalin (1μg per well). In controls, instead of IC, purified IgA (Sigma) and IgA1P both treated with neuraminidase as for serum (N+ and NH) were used at this stage (200 ng protein

in 200 µl PBS-T per well). Bound IgA was assayed using HRP conjugate of anti-human IgA (1.5 µg antibody per ml).

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 Suresh kumar et al. [1982]. 30 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 4^0C. 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 7.4.

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 and purification of Lp(a)

Isolation of serum Lp(a) fraction (density I.O5-I.12g/ml) by ultra centrifugation

Lp(a) was isolated as described by Chellan et al. [2006]. Approximately 10 ml serum was collected from each volunteer after a 12h overnight fast. EDTA and sodium azide at final concentrations of 0.1% and 0.01% w/v, respectively, in addition to 0.001M of phenylmethanesulfonyl fluoride (PMSF) and benzamidine hydrochloride were immediately added. Ultra centrifugation was carried out in a Hitachi CS 150GXL preparative ultracentrifuge with fixed angle, type S 150AT rotor.

Lp(a) was isolated from serum between density (d) 1.05 - 1.12 g/ml on the basis of the method described by Kostner et al.(1999) with some modifications. Briefly, the density of 1 ml of serum was adjusted to 1.05 g/ml by adding solid sodium bromide (NaBr). After centrifugation at 435000 x g for 90 min at 15°C, the top layer (200 µl) containing VLDL, IDL and LDL *(d* <1.05 g/ml) was discarded. L-proline was then added to the bottom fraction to a final concentration of 0.2 M and the density adjusted to 1.12 g/ml with solid NaBr followed by ultracentrifugation (435000 x g, 150 min at 15°C). The floating Lp(a) fraction (top 200 µ1; *d* 1.05-1.12 g/ml) consisted of Lp(a), LDL *(d* >1.05 g/ml) and HDL_2.

Preparation of native 3.75% polyacrylamide disc gels

Native 3.75% polyacrylamide disc gels were cast in glass tubes (5mm ID, 9 cm *1).* Ten ml of the gel solution contained 2 ml of TBE buffer, pH 8.7 (0.05 M Tris, 0.025 M boric acid, 0.003 M EDTA), 2.5 ml of 15% acrylamide / bisacrylamide (19:1), 5.5 ml of 0.2% ammonium persulfate and 10 µL TEMED.

PAGE of the isolated Lp(a)fraction and the electroelution of lipoproteins

PAGE of isolated Lp(a) fraction

The isolated Lp(a) fraction was dialyzed against the sample buffer (1: 9 diluted chamber buffer). TBE buffer, pH 8.7 was used as the chamber buffer. The sample contained 180 µl of the dialyzed Lp(a) fraction from serum *(d* 1.05-1.12 g/ml). L-Proline was added to each sample (0.1 M final concentration) and incubated for 15 min at room temperature before adding ethylene glycol (10% v/v). One Lp(a) sample was then prestained for lipids with 20 µl of Sudan black saturated ethylene glycol and incubated for 15 min. The lipoprotein load was approximately 300

μg/tube. Each sample was then loaded over individual disc gels, layered with chamber buffer (TBE) and subjected to electrophoresis for 2h at a constant current of 2 mA/tube. The chamber buffer was pre-cooled to 15°C prior to electrophoresis. Both PAGE and the subsequent electroelution of the Lp(a) were done at room temperature (25°C) to avoid cryoprecipitation of Lp(a).

Electroelution of lipoproteins

Proceeding PAGE, the gels were taken out of the glass tubes and washed with distilled water. One of the gels was then stained for protein with 0.4 g/L Coomassie Brilliant Blue R-250 in 35 ml/L perchloric acid and the bands appeared in about 30 min. Three bands were visible: the far migrating HDL_2, the β-lipoprotein (LDL) and the pre-β lipoprotein [Lp(a)]. Using this gel as a marker, individual bands were sliced from the non stained gels and equilibrated in 2 to 4 ml Tris-acetate buffer (0.05 M, pH.8) for 15 min. Pre-staining the marker gel with Sudan black was avoided as the dye may interfere with the actual mobility of the lipoproteins. The gel suspensions were then taken into individual dialysis bags and placed immersed in between opposite electrodes in a rectangular reservoir 20x20x7 (cm) with 2 L of cold (15°C) Tris- acetate buffer (0.05 M, pH 8). A constant voltage of 50 V was applied across the electrodes for 6 h with a buffer change after 3 h. The lipoproteins were then dialyzed against 0.01 M phosphate buffer, pH 7, containing 0.1% EDTA and 0.01% sodium azide and the gel pieces were pelleted by brief centrifugation at 3000 x g. The protein concentration of the supernatant lipoprotein was estimated according to Lowry et al. (1951) using BSA as standard and stored at -20°C with 50% v/v ethylene

glycol. Alternatively, the lipoproteins may be eluted in a single step with the same native electrophoretic buffer using any commercial electroeluter.

Lp(a) assay using microwell-immobilized jacalin

An assay procedure recently developed in this laboratory was used. Wells of polystyrene 96-well plates were each coated with 1μg jacalin in 200 μl PBS by overnight incubation at 4°C. After blocking with PBS containing 0.5% Tween 20 for 30 min at 37 °C and washing with PBS-T at 25°C wells were treated with dilutions of samples containing Lp(a) in PBS-T (200 μl) for 2 h at 4°C, followed by three washings with PBS-T. After further treatment with 200 μl solution of HRP conjugate of anti-apo (a) (containing 1.5 μg antibody per ml) at 4°C for 2 h and washing with PBS-T, bound HRP was assayed using OPD as substrate as described earlier.

Assay of Lp(a)- containing immune complexes

Jacalin (1 μg/well) coated on polystyrene wells of 96 well ELISA plate was incubated with 200 μl 80 times dilution in PBS of IC samples (N+ or NH) for 2 h at 4°C. After washing followed by 2 h incubation at 4°C with 200 μl HRP conjugate of anti-apo (a) (1.5 μg/ml antibody in PBS-T) and washing, bound HRP was measured as described earlier.

Assay of Lp(a) in secondary IC upon desialylation of IC-free serum.

To serum from which natural IC had been removed by PEG precipitation and PEG removed by dialysis against PBS, neuraminidase (N+ or NH) was added followed by incubation at 37°C for 2 h and secondary IC precipitated using 2% PEG as described earlier. IC was redissolved in original serum volume of PBS.

Diluted IC (80 times) in PBS-T was incubated with ELISA plate-coated jacalin (1μg per well). Bound Lp(a) was assayed using HRP conjugate of anti-apo (a) (1.5 μg antibody per ml).

Lp(a)-containing immune complexes formed on depletion of anti-T

Human RBC from non-coagulated blood were washed thrice with PBS by centrifugation. Cell suspension (5% in PBS) was treated at 37°C with neuraminidase (50 mU/ml) for 1 h before washing thrice with PBS by centrifugation. For removal of anti-T, serum (2 ml) was treated for 2 h with 3 ml packed desialylated erythrocytes at 4°C with occasional mixing. Control serum was treated with native red blood cells; the respective supernatants were collected by centrifugation and desialylated with neuraminidase (25 μg/ml). Immune complex were precipitated using 2% PEG. The Lp(a) content of the immune complex was assessed by jacalin-based assay as described earlier.

Isolation of serum O-glycosylated lipoproteins

O-glycosylated serum lipoproteins were purified by jacalin-mediated precipitation The redissolved jacalin precipitate was subjected to density gradient ultra centrifugation. Ultra centrifugation was carried out in a Hitachi CS 150GXL preparative ultracentrifuge with fixed angle, type S 150AT rotor. Briefly, the density of 1 ml of serum was adjusted to 1.24 g/ml by adding solid potassium bromide (KBr). After centrifugation at 435000 x g for 240 min at 15°C, the top layer (200 μl) containing the protein-free lipid was collected. Finally, the lipid sample was resolved by alkaline pH electrophoresis as described earlier and Western blot analysis of these lipoproteins done.

Statistics

For statistical analysis, statistical software SPSS 11·0 (SPSS, Chicago, IL, USA) was employed. 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

Microbial neuraminidase-mediated desialylation exposes T antigen in the most prominent human serum O-glycosylated protein IgA1 leading to formation of immune complexes with anti-T antibodies in serum.

RESULTS AND DISCUSSIONS

Identification of the most prominent serum O-glycosylated protein

Glycosylation of extracellular proteins occurs in two major forms [Lis and Sharon, 1993]. Carbohydrate moieties may be N-linked to asparagine residues or O-linked to serine or threonine residues. Complex N-glycan chains are a common feature of serum proteins including all immunoglobulin isotypes. Though several membrane bound proteins are heavily O-glycosylated, this is a feature displayed by very few serum proteins [Allen, 1999]. Serum IgA is predominantly IgA1, and this antibody subclass also predominates in mucosal effector sites such as the mammary gland, salivary glands, nasal mucosa, bronchial mucosa and the upper digestive tract. The IgA2 subclass is, however, more abundant than IgA1 in the colon [Brandtzaeg and Johansen, 2005]. These antibody subclasses are highly homologous except for a short mucin like hinge region which is unique to primate IgA1 and is responsible for some of the unique receptor and lectin-binding properties of this molecule [Wines and Hogarth, 2006]. In addition to two N-glycosylation sites, human IgA1 has five O-glycans linked to serine or threonine residues in the hinge region of the molecule, a short repeating sequence of amino acids lying between the CH1 and CH2 domains of the heavy chain while IgA2 lacks O-linked sugars [Putnam et al., 1979].

Prevalence of IgA1 among O-glycosylated human serum proteins has been examined in two ways employing the lectin jacalin which recognizes exclusively the O-linked oligosaccharides of glycoproteins regardless of their sialylation status. Firstly in SDS-polyacrylamide gel electrophoresis the most prominent subunit from all the O- glycosylated serum glycoproteins isolated by

precipitation with jacalin was identical in mobility with the major subunit of standard IgA (mainly IgA1) (Fig.8A). Further the most prominent subunit was singularly reduced in intensity when solubilized jacalin-precipitated glycoproteins were passed through anti-human IgA-Sepharose before electrophoresis. Secondly, among the O-glycosylated proteins of serum prepared by affinity binding to jacalin-Sepharose (JSSP), the most prominent protein band is also the one recognized by anti-human IgA (Fig 8B). These results together indicate that IgA1 (that makes up most of serum IgA) is the most prominent serum O-glycosylated protein.

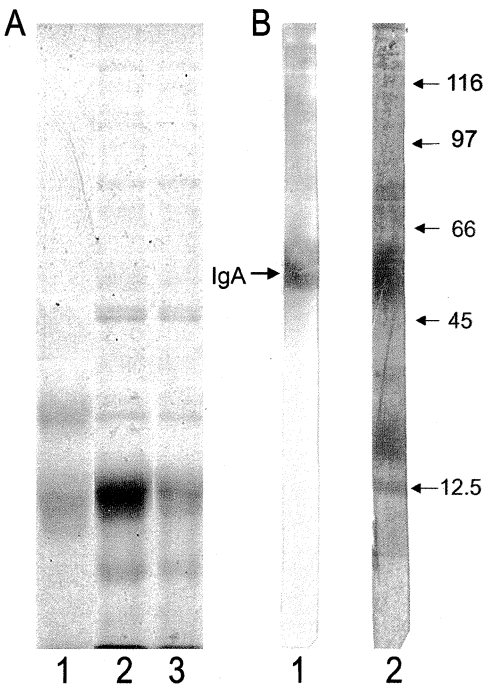


Figure 8. IgA1 content of serum O-glycosylated proteins. A. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 7.5% gel of 1, Standard human IgA (Sigma Chemicals, USA), 20 μg; 2, jacalin-precipitated serum glycoproteins (JPSP) treated with control Sepharose 4B, 50 μg; 3, JPSP treated with anti-IgA-Sepharose 4B, 50 μg. B. Western blot of jacalin-Sepharose-binding serum glycoproteins (JSSP) after electrophoresis as for A, probed with anti-IgA-HRP (1.5 μg antibody per ml) (1) or amidoblack (2).

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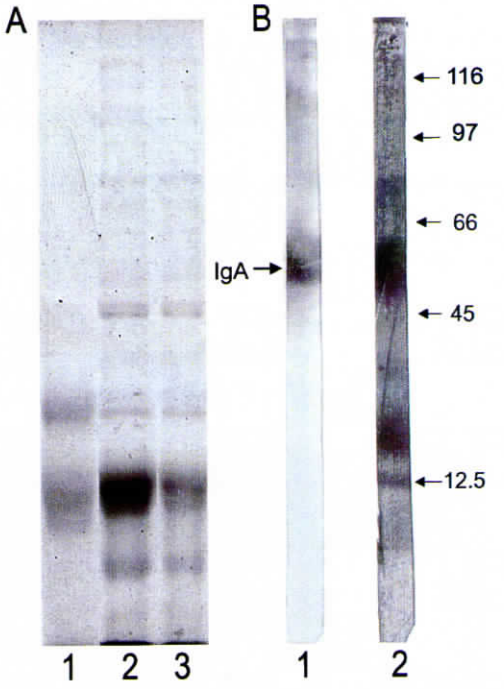

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Demonstration of recognition of serum O-glycosylated proteins by the principal T antigen binding antibody of serum.

T antigens (Gal β(1→3)GalNAc-) are expressed on many glycoproteins as part of O-linked oligosaccharides. In normal adult human tissue, the antigen is substituted by sialic acid residues, but is expressed in unsubstituted form on certain human tumours [Springer et al., 1975]. All humans have preexisting anti-T antibodies, predominantly elicited by their own intestinal flora [Springer and Tegtmeyer, 1981]. Identification of glycoproteins having complementary structures for anti-T may be helpful in elucidating its physiological role.

Human anti-β-glucoside antibody (ABG) which has a higher IgA content than total serum immunoglobulins [Geetha et al., 2007] was used to prepare pure IgA1. The fraction of ABG that binds to polystyrene-coated jacalin was termed IgA1 (P). Yield of IgA1P was 500 ng per well on average. Dot-blotted IgA1P was tested as a ligand for anti-T. Results show that unlike native IgA1P, desialylated IgA1P was a very efficient ligand for anti-T (Figure 9).

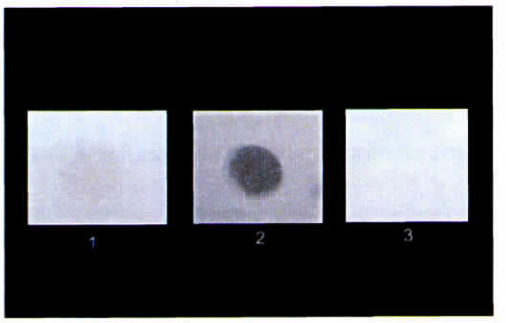

Figure 9. Anti-T recognition of native and desialylated human serum IgA1. Dot blotted IgA1P (2 μg) treated with (2 & 3) or without (1) neuraminidase was probed with anti-T, followed by anti-human IgM-HRP(1.5 μg antibody per ml) (1 and 2) or anti-human IgM-HRP alone (3).

Demonstration of recognition of serum O-glycosylated proteins by the principal T antigen binding antibody of serum.

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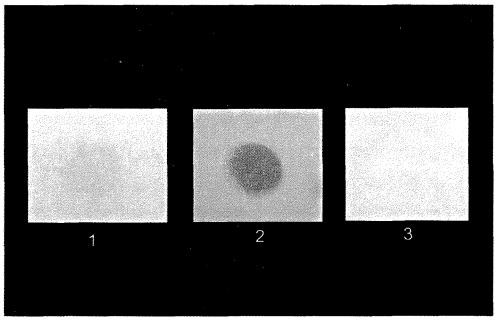

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Anti-T-mediated agglutination of desialylated human RBC was inhibited by desialylated IgA1P, the minimum inhibitory quantity under conditions of assay being 146 ± 57.6 ng while several times more of native IgA1P was not inhibitory (Table 6). T antigen specificity of this inhibition was further established by the inability of desialylated forms of IgA1-free ABG and the non-O-glycosylated glycoprotein bovine thyroglobulin to inhibit anti-T.

Table 6. Inhibition of anti-T hemagglutination by native and desialylated IgA1

Glycoprotein	Concentration for 50% inhibition* (ng/ml)
Desialylated IgA1P	146 ± 57.6
Native IgA1P	> 800[a]
IgA1-free ABG (desialylated)	> 20,000[a]
Bovine thyroglobulin (desialylated)	> 6000[a]
Neuraminidase	> 15,000[a]

Glycoproteins were desialylated by 1h incubation at 37°C with 50 mU/ml neuraminidase prior to serial dilution.

* : Mean ± SD of 4 consecutive trials; a: maximum concentration tried.

IgA1P and JSSP used in experiments described earlier (Fig.8 and 9; Table 6) were prepared by sugar-specific elution from immobilized jacalin. Lest any serum glycoprotein fails to bind or to get eluted from immobilized jacalin, a range of O-glycosylated proteins from serum was precipitated by addition of jacalin which is a

wide-spectrum O-glycosylated protein-specific lectin. The precipitate collected by centrifugation was redissolved by addition of jacalin-specific sugar (1-O-methyl-α-D-galactoside). Western blot of these glycoproteins probed with anti-T confirmed the desialylation-dependent recognition by anti-T of almost all O-glycosylated proteins of serum, chiefly IgA1. Most of these bands were also recognized by the T antigen-specific animal lectin galectin-1 (Figure 10) though the relative intensities of bands were different.

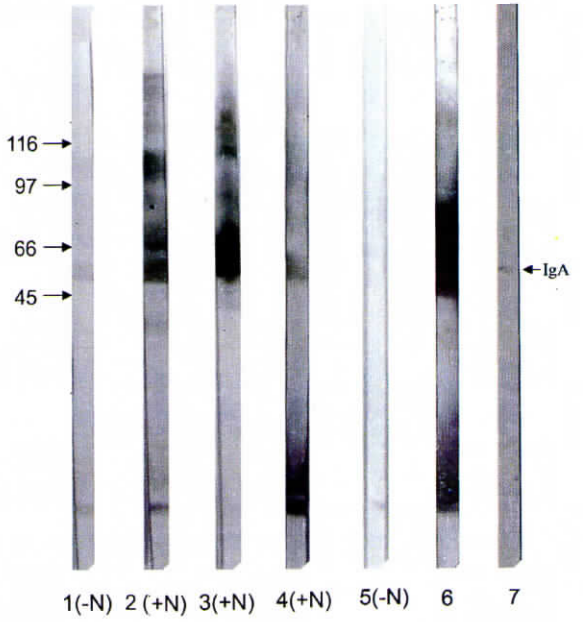

Figure 10. Recognition of Western blotted desialylated and native serum O-glycosylated proteins by T antigen-specific lectins and anti-T. Western blotted jacalin precipitate of serum glycoproteins (JPSP) were probed with HRP conjugate of bovine heart galectin-1 without (-N) or with (+N) prior neuraminidase treatment (1&2). Strip 3 was probed with PNA-HRP (+N); strips 4&5 probed with anti-T-HRP with (+N) or without (-N) prior neuraminidase treatment and strip 6 stained with amidoblack. Strip 7 shows the position of IgA1 identified using anti-IgA HRP.

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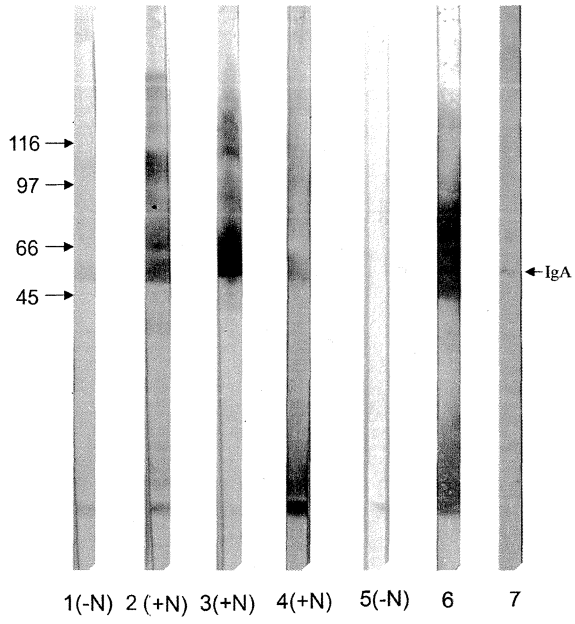

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Demonstration of recognition of serum O-glycosylated proteins after their desialylation by serum anti-T leading to formation of immune complexes.

Since desialylation exposes free T antigen in most O-glycosylated proteins anti-T was expected to form IC with such glycoproteins in desialylated serum. This was examined by assaying those immune complexes that contained both IgM and O-glycosylated proteins since anti-T is mostly IgM. Such immune complexes were captured on anti-human IgM-coated plastic plates and O-glycosylated proteins in the bound immune complexes assayed using jacalin-HRP.

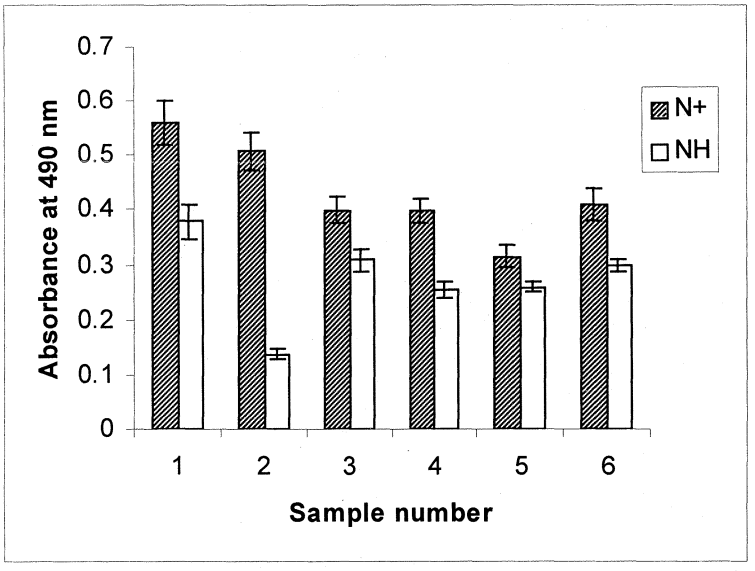

Figure 11A. Increase in anti-T-O-glycosylated protein immune complex following desialylation of serum. Details are under 'Methods'. Results of six consecutive trials given. Mean ± S.D. of triplicate trials shown. P value < 0.02.

Results (Figure 11A) indicate a significant increase in IgM-O-glycosylated protein immune complexes in serum desialylated by neuraminidase (N+) compared to serum treated with inactivated enzyme (NH) (P value < 0.02; n = 6).

Involvement of the premier serum O-glycosylated protein IgA1 in IC formation with anti-T was then examined by directly assaying IgA-IgM immune

complexes. From among IgA-containing IC that bound to anti-IgA immobilized on plastic wells, those that contained IgM as well were assayed by probing with anti-human IgM-HRP. Results (Figure 11B) suggest a significant increase in IgA-IgM IC following desialylation of serum (P value = 0.005; n = 6).

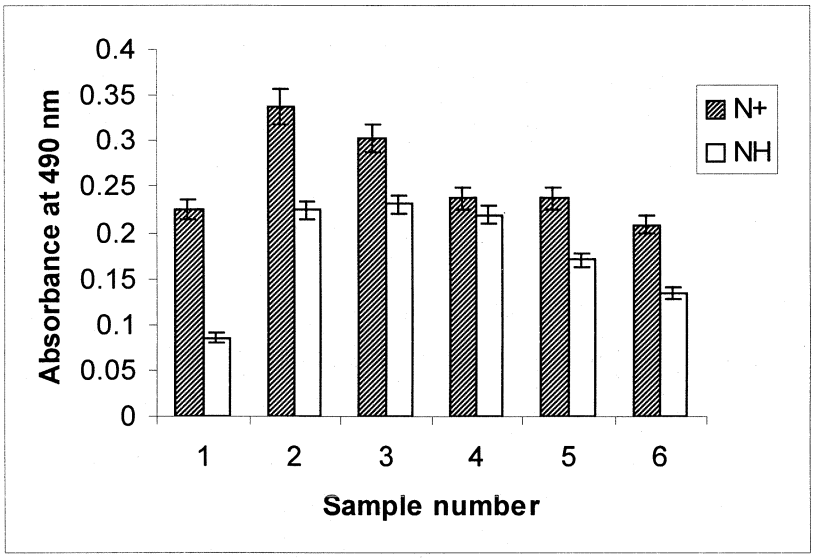

Figure 11B. Increase in IgA-IgM immune complex following desialylation of serum. Details are under 'Methods'. Results of six consecutive trials given. Mean ± S.D. of triplicate trials shown. P value = 0.005.

Since IgA1, the sole O-glycosylated immunoglobulin far exceeds anti-T in serum concentration, desialylation of serum would leave most of the asialo-IgA1 unoccupied even after formation of IgA1-anti-T complexes using all of serum anti-T available. This was also verified by addition of purified anti-T to desialylated serum from which IC had been removed. Results (Figure 12) showed a significant increase in IgM-IgA immune complexes in desialylated serum, compared to non-desialylated serum, upon addition of purified anti-T (P value = 0.003; n = 10).

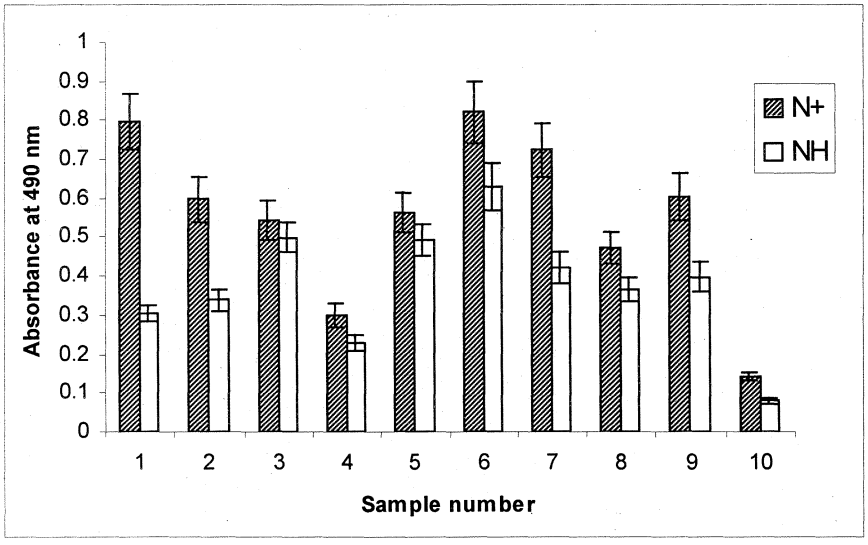

Figure 12. Increased formation of IgA-IgM IC following addition of anti-T to IC-free desialylated serum. After removal of primary immune complex formed on neuraminidase treatment (N+ and NH; 'Methods') by centrifugation, anti-T (42 µg in100 µl PBS) was added to 500 µl of supernatant. After 4 h incubation at 4°C, 2 mg PEG-6000 was added. Following overnight incubation at 4°C and centrifugation, the resulting IC was dissolved in 500 µl PBS and 200 µl added to polystyrene ELISA well-coated anti-human IgA. Bound IgM was measured as under Fig. 3B. Results of ten consecutive trials are given. Mean ± S.D. of triplicate trials shown. P value = 0.003.

Similar to previous experiment, but instead of adding purified anti-T fresh serum was added as a source of anti-T to desialylated serum from which IC had been removed. Results (Figure 13) showed a significant increase in IgM-IgA immune complexes in desialylated serum, compared to non-desialylated serum, upon addition of purified anti-T (P value = 0.01; n = 5).

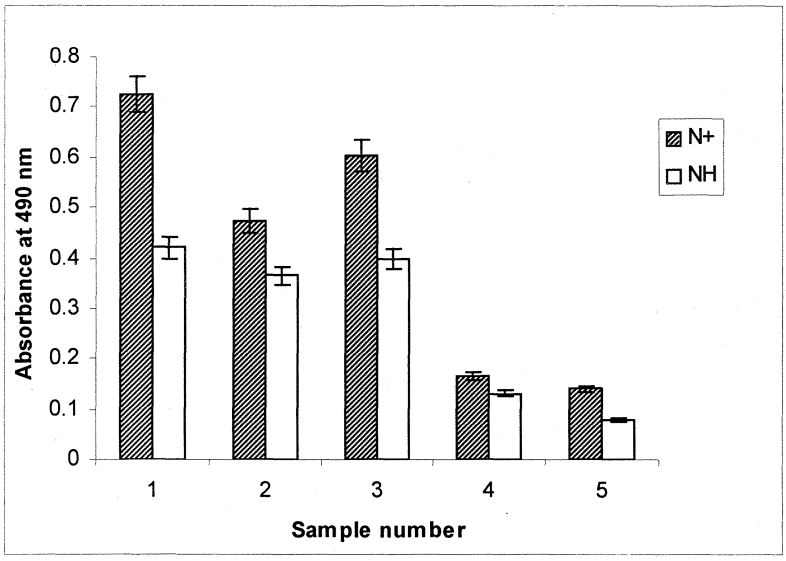

Figure 13. Increased formation of IgA-IgM IC following addition of anti-T to IC-free desialylated serum. After removal of primary immune complex formed on neuraminidase treatment (N+ and NH; 'Methods') by centrifugation, fresh serum (400 µl) was added to 200 µl of supernatant. After 4 h incubation at 4°C, 2 mg PEG-6000 was added. Following overnight incubation at 4°C and centrifugation, the resulting IC was dissolved in 500 µl PBS and 200 µl added to polystyrene ELISA well-coated anti-human IgA. Bound IgM was measured as under Fig. 3B. Results of five consecutive trials are given. Mean ± S.D. of triplicate trials shown. P value = 0.01.

Involvement of anti-T in shifting desialylated IgA1 to the IC phase was examined alternatively by withdrawing anti-T from plasma. Plasma depleted of anti-T by treatment with desialylated RBC of the same group and control treated with unmodified RBC were both desialylated, IC separated and IgA1 in IC assayed after capture on microwell-coated jacalin and probing with HRP conjugate of anti-human IgA. Results of six consecutive trials (Table 7) show very significant decrease in transfer of IgA1 to IC in serum in which anti-T was absent, compared to control serum (P value = 0; n = 6).

Table 7. Involvement of anti-T in transferring IgA1 of desialylated serum to IC.

Trial No.	IgA1 in IC* (absorbance at 490 nm)		Decrease of IgA in IC following depletion of anti-T (%)
	Normal serum	Anti-T-depleted serum	
1	0.435(± 0.03)	0.270(± 0.02)	38.0
2	0.721(± 0.06)	0.509(± 0.05)	29.4
3	0.692(± 0.04)	0.440(± 0.03)	36.4
4	0.629(± 0.06)	0.421(± 0.02)	33.0
5	0.717(± 0.05)	0.474(± 0.04)	34.0
6	0.321(± 0.02)	0.230(± 0.02)	28.3

Immune complex dissolved in PBS of original serum volume was diluted 800 times in PBS-T before addition to wells coated with jacalin (1µg/well). After 2 h incubation, bound IgA1 was assayed using HRP conjugate of anti-human IgA (1.5 µg antibody per ml) and OPD as substrate. Results of six consecutive trials given. * : Mean ± S.D. of triplicate trials shown. P value = 0

Decrease in free anti-T titre in serum following desialylation, expected in the event of anti-T forming IC with IgA1 and other O-glycosylated proteins in desialylated serum, was verified by agglutination assay using RBC of the same group. Serum treated with neuraminidase (N+) or with inactivated enzyme (NH), and dialysed after removal of IC as described above was used. Limitations of agglutination assay using serial 2-fold dilutions not-withstanding, results in Figure 14 revealed a 2- to 8-fold lower free anti-T concentration in desialylated serum than in non-desialylated serum.

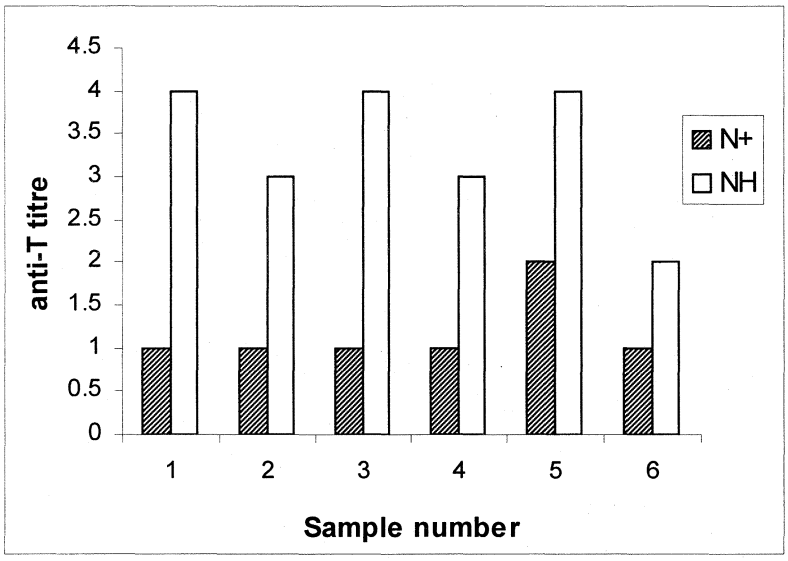

Figure 14. Decrease in anti-T in serum upon removal of IC produced by desialylation. After removal of IC following neuraminidase treatment (N+ and NH) supernatant was dialysed against PBS-T and agglutination titre of this sample against desialylated human RBC of the same blood group was measured. Titre was scored as the serial two-fold dilution upto which agglutination was positive. Results of six consecutive trials given.

Desialylation-dependent entry of IgA1 into IC was even more explicitly demonstrated in serum from which natural IC had been removed by PEG precipitation. After dialysis to remove PEG and incubation with active (N+) or inactive (NH) neuraminidase, secondary IC formed were collected by precipitation with 2% PEG. Secondary IC from N+ serum contained substantially higher IgA1 than did secondary IC from NH serum (P value < 0.01; $n = 6$) (Figure 15). Desialylation per se did not enhance binding of IgA to jacalin.

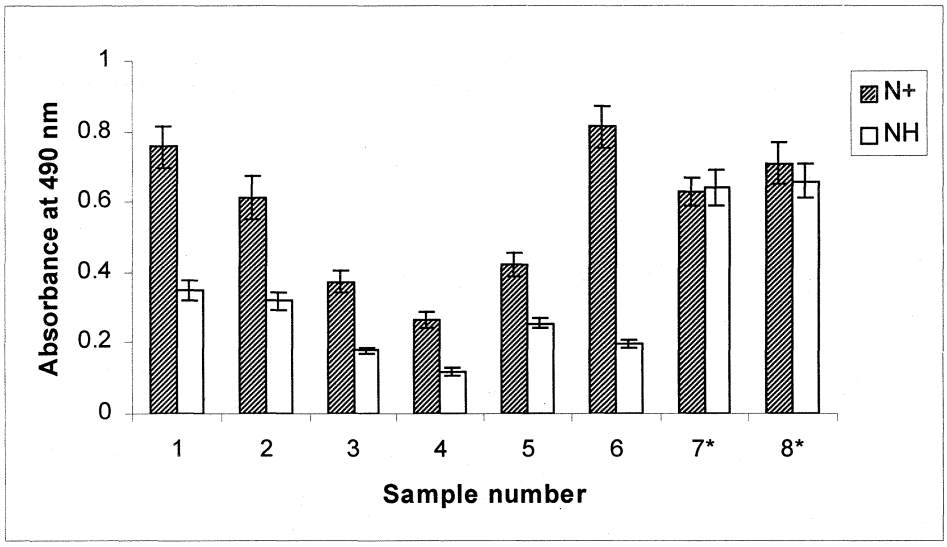

Figure 15. Increased entry of IgA1 to secondary IC upon desialylation of IC-free serum. Details are under 'Methods'. Results of six consecutive trials (samples 1 to 6) given. * : samples 7 and 8 are standard IgA and IgA1P respectively. Mean ± S.D. of triplicate trials shown. P value < 0.01.

Discussion

Exclusive specifity for O-linked oligosaccharides of glycoproteins regardless of sialylation and sugar-binding activity unaffected by immobilization on Sepharose or coating on polystyrene make jacalin an excellent tool for isolation and analysis of O-glycosylated proteins [Hortin and Trimpe, 1990]. In this study O-glycosylated proteins recognized by jacalin in solution (JPSP) as well as those that bind to Sepharose-immobilized jacalin (JSSP) from human serum were separately used to demonstrate the dominance of IgA1 in both populations.

Recognition of serum O-glycosylated proteins including IgA1 on dot blots and Western blots by anti-T akin to known T antigen-specific lectins as well as inhibition of anti-T-mediated hemagglutination by desialylated IgA1 suggested that *in vivo* this antibody may bind to serum O-glycosylated proteins, chiefly IgA1,

consequent to their desialylation. This was confirmed by demonstration of increased formation of immune complexes containing both IgM and O-glycosylated serum proteins including IgA1 following desialylation of serum. Desialylation-dependent formation of IC between IgA1 and IgM was even more decidedly demonstrated in serum from which natural IC had been removed prior to desialylation. Since hardly any serum immunoglobulin other than anti-T is known that recognizes desialylated O-linked oligosaccharides of serum in contrast to their native forms and since anti-T is largely IgM the amount of IgM that is in combination with O-glycosylated proteins or IgA1 was taken as a measure of IC formed between anti-T and the glycoproteins.

Formation of fresh IgM-desialylated IgA1 complexes upon addition of anti-T to desialylated serum from which IC had been removed, decrease in IgA1-containing IC following removal of anti-T prior to desialylation and the marked decrease in free anti-T titre in serum following desialylation offered further proof for formation of immune complexes between anti-T and O-glycosylated proteins including IgA1.

The potential of IC to cause vascular lesion by recruitment of PMN, leukocytes and macrophages through complement-dependent chemotaxis is well established and underlined by the prevention of vascular injury following complement depletion [Henson, 1977]. Circulating IC had been shown to be strong and independent risk factor for myocardial infarction in many studies [Lefvert et al., 1995; Mustafa et al., 2000]. Molecular pathology of several diseases has been suggested to involve IgA-containing IC. In IgA nephropathy and its systemic analogue Henoch-Schonlein purpura, both suggested to be mediated by IgA-

containing IC, the IgA deposited in glomerular and other lesion sites was found to be exclusively of IgA1 class [Mestecky et al., 1986; Scheinfeld et al., 2003]. When injected into experimental rats desialylated IgA1 in contrast to native IgA1 accumulated in glomeruli [Sano et al., 2002]. Reduced sialylation of IgA1 in polyethylene glycol-precipitated macromolecules of serum was observed in focal proliferative sclerosing IgA nephropathy patients, compared to controls [Xu et al., 2005]. Particularly, glomerular IgA in IgA nephropathy was enriched in T antigen-rich (ie, desialylated) IgA1 [Iwase et al., 2002]. Increased serum IgA against several periodontic pathogens and *Chlamydia pneumoniae* are risk factors for cardiovascular diseases [Pussinen et al., 2005; Saiku et al., 1992]. Influenza virus noted for secretion of neuraminidase as well as for induction of serum IgA [Janeway et al., 2001] is strongly associated with atherosclerosis [Madjid et al., 2003]. At the same time infectious etiology is now being increasingly attributed to cardiovascular diseases [Libby et al., 1997; Madjid et al., 2003]. Also, IgA nephropathy has been reported to be associated with infections of the upper respiratory tract [Donald and Grande, 2002].

Pathogen-derived antigens are often rich in polysaccharide structures that elicit predominantly IgA antibodies (most of which is IgA1) [Johnson et al., 1996]. Also many bacteria and viruses including those that are pro-atherogenic and pro-nephropathic are known to secrete the enzyme neuraminidase that can desialylate IgA1. Further investigations to assess the level of IgA1-containing IC in patients infected with such microbes are therefore relevant. Incidentally, diabetes which

causes pervasive vascular damage is often accompanied by high serum levels of IgA as well as neuraminidase [Rodriguez-Segade et al., 1996; Merat et al., 2003].

Immune complex between anti-T and desialylated IgA1 *in vivo,* if formed may be aided by its unique composition in bringing about vascular injury. Firstly, as is evident from present results anti-T-bound desialylated IgA1 still has binding sites left for galactose-binding lectins. For the most abundant human galactose-binding lectin, galectin-1 which is expressed on endothelial cell surface as well [Baum et al., 1995], IgA1 is the most prominent serum ligand and more so after desialylation [Sangeetha and Appukuttan, 2005]. Present results suggest the possibility of galectin-1-dependent anchoring of anti-T-desialylated IgA1 IC on vessel walls and tissues during infections and diabetes, both known to induce vascular damage. Secondly, IgM as well as IgG of anti-T-IgA1 immune complexes have their binding sites engaged so as to enable the respective Fc portions to attract complement and initiate an inflammatory cascade.

PART II

Desialylation-dependent recognition of serum O-glycosylated protein [IgA1] by anti-T leading to formation of immune complexes in diabetic patients.

Desialylation-dependent recognition of serum O-glycosylated protein [IgA1] by anti-T leading to formation of immune complexes in diabetic patients.

Circulating immune complexes (CIC) have been postulated to contribute to the development of the secondary complications of diabetes mellitus [Thiele and McDonald, 1987]. An increase in circulating IgA concentrations is a generalized phenomenon among diabetic patients [Rodriguez-Segade et al., 1996]. Since diabetic patients may have a number of secondary diseases, attempts were made to correlate the most common of these (acute and/or chronic bacterial infections) with elevated IgA levels. IgA levels of diabetic patients with infections versus diabetic patients without infections were significantly different (P less than 0.05) [Gill et al., 1981].

Desialylation-dependent entry of IgA1 into IC was even more explicitly demonstrated in diabetic serum than normal serum [Figure 16 and 17]

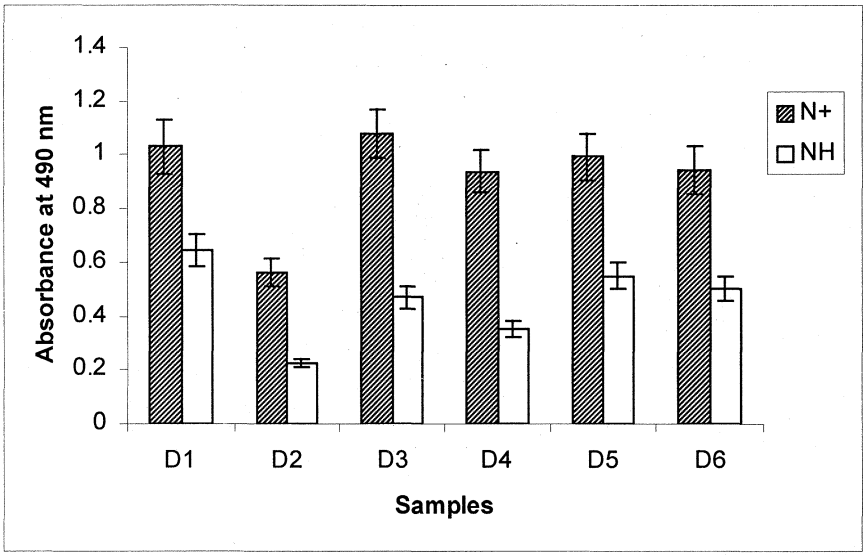

Figure 16. Increase in IgA content of IC following desialylation from sera of diabetic patients. IgA assay details are under 'Methods'. Results of six consecutive trials (samples 1 to 6) given. Mean ± S.D. of triplicate trials shown. P value = 0.

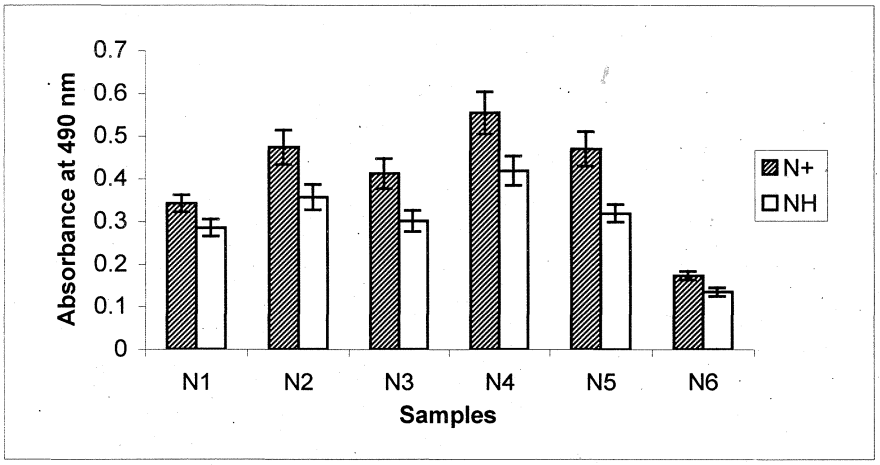

Figure 17. Increase in IgA content of IC following desialylation from sera of normal controls collected and analyzed on the same days as for Fig.16. IgA assay details are under 'Methods'. Results of six consecutive trials (samples 1 to 6) given. Mean ± S.D. of triplicate trials shown. P value = 0.003.

The percentage increase in entry of IgA1 to IC formed from neuraminidase treated serum (N+) over non- desialylated control (NH) is enormously higher (112% ± 42.6) in the case of diabetic sera, than in the case of normal sera (33% ± 9). The t-test shows highly significant results (P value = 0; n = 6) [Figure 18].

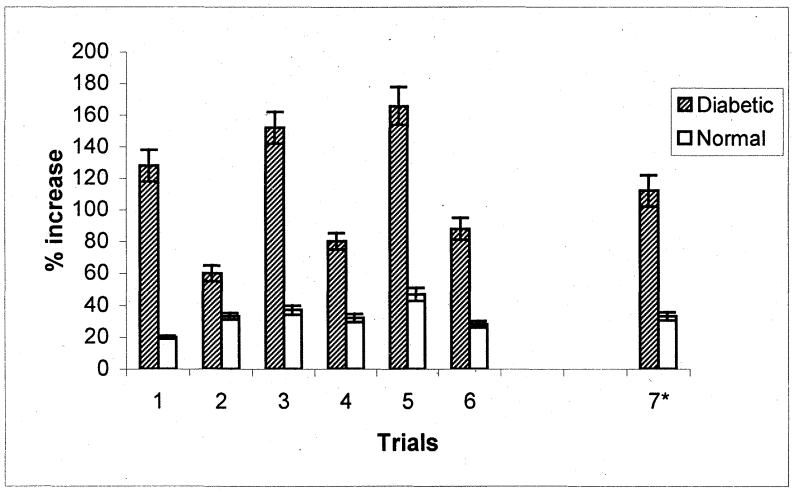

Figure 18. Percentage increase in IgA content of IC from normal and diabetic person's sera in N+ compared to NH cases. Results of six consecutive trials of one diabetic and normal case at a time (samples 1 to 6) given. * : sample 7 shows mean percentage increase. P value = 0.

Desialylation of fresh diabetic serum by *Clostridium perfringens* neuraminidase led to increased formation of immune complexes containing IgM, the major immunoglobulin type in anti-T on one hand and IgA1 on the other, than desialylated normal serum [Figure 19 and 20]. Hence, the percentage increase in IgA-IgM content in IC formed from neuraminidase treated serum (N+) over non-desialylated control (NH) is much higher (116% ± 29.3) in the case of diabetic sera, than in the case of normal sera (28% ± 9.5). The t-test shows highly significant results (P value = 0; n = 6) [Figure 21].

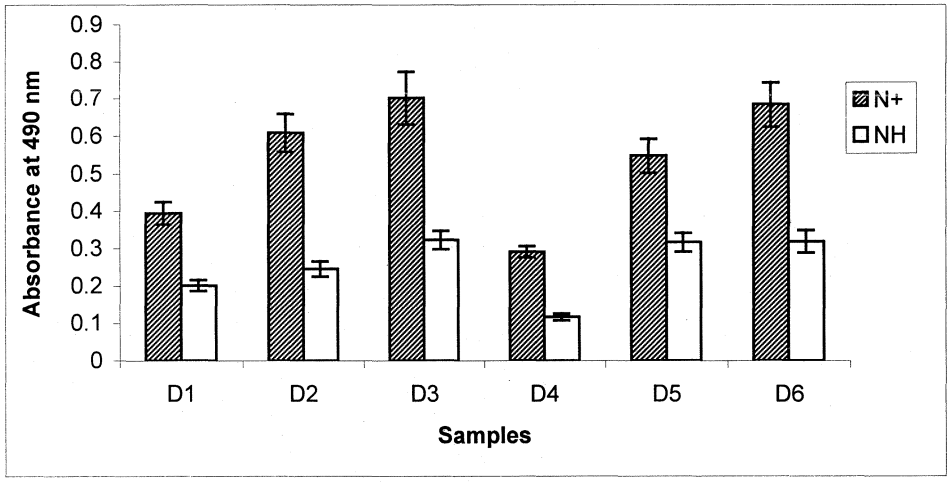

Figure 19. Increase in IgA-IgM content of immune complex following desialylation from sera of diabetic patients. Details are under 'Methods'. Results of six consecutive trials given. Mean ± S.D. of triplicate trials shown. P value = 0.001.

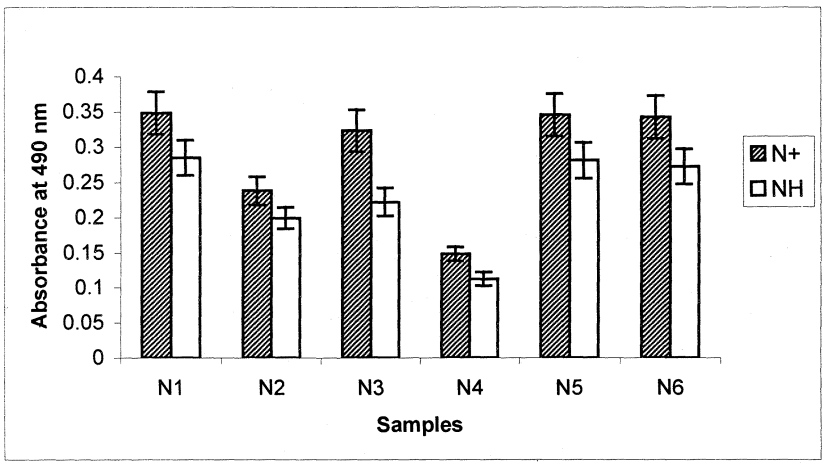

Figure 20. Increase in IgA-IgM content of immune complex following desialylation from sera of normal controls collected and analyzed on the same days as for Fig.19. Details are under 'Methods'. Results of six consecutive trials given. Mean ± S.D. of triplicate trials shown. P value = 0.001.

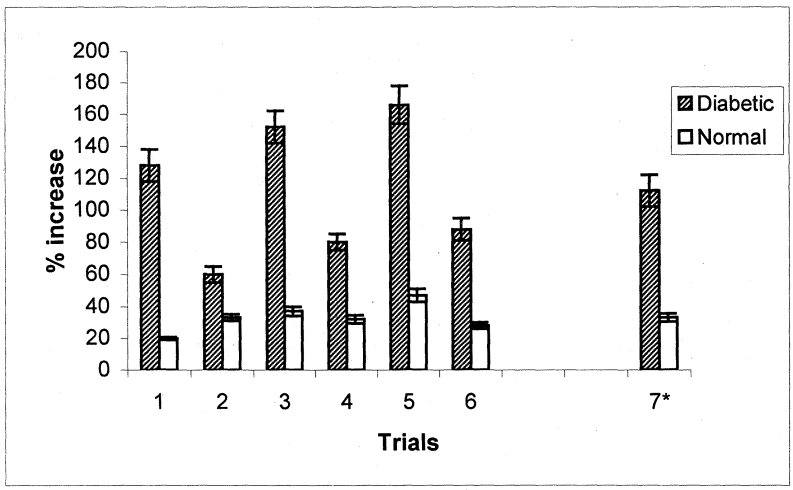

Figure 21. Percentage increase in IgA-IgM content of IC from normal and diabetic person's sera in N+ compared to NH cases. Results of six consecutive trials (samples 1 to 6) given. Each pair from 1-6 was collected on the same day.* : sample 7 shows mean percentage increase. P value = 0.

Discussion

The present results suggest that immune complexes involving IgA1 and anti-T is a hallmark of diabetes. High serum IgA concentration [S.Rodriguez-Segade et al., 1996] and increased serum neuraminidase titre [Merat et al., 2003] that have been shown to accompany diabetes may together contribute to this phenomenon. On the other hand prospective studies have reported associations among various markers of inflammation and incidence of diabetes [Pradhan et al., 2001], and it has been proposed that inflammation has a causal role in the development of diabetes [Pickup and Crook, 1998]. Whether inflammation causes or follows diabetes or both, diabetes is a risk factor for a host of vascular disorders including myocardial infarction and stroke [Vilbergsson et al., 1998; Kannel et al., 1990]. Elevated levels of immune complexes (CIC) have been detected in the circulation of all types of diabetic humans [Irvine et al., 1977; Iavicoli et al., 1982; Abrass et al., 1983] and have been postulated to play a role in the development of diabetic complications [Minick & Murphy, 1973; Irvine et al., 1978]. The precise relationship of immune-mediated injury to the pathogenesis of diabetic microangiopathy remains to be determined. The decreased rate of plasma clearance of model immune complexes in diabetic rats is consistent with previous reports of elevated levels of circulating immune complexes in both diabetic humans and animals [Abrass et al., 1983; Abrass, 1984]. An increase in circulating IgA concentrations is a generalized phenomenon among diabetic patients; IgA concentrations above the reference range are more common among male than female diabetics; and diabetic complications are associated with a significant increase

in serum IgA concentration [Rodriguez-Segade et al., 1996; Singh and Kulig, 1992]. Also, 80-87% of IgA in diabetes are polymeric [Triola et al., 1984]. All the type 1 and type 2 diabetic subgroups defined on the basis of their micro- or macrovascular diabetic complications (nephropathy, retinopathy etc.) had higher serum IgA concentrations than the corresponding groups of patients without complications. This suggests that monitoring IgA may provide early warning of the possible presence of complications [Rodriguez-Segade et al., 1996; Cheta et al., 1982].A role of IgA system abnormality has been suggested in the pathogenesis of diabetic vascular complications [Casiglia et al., 1990]. Abnormal immunoreactive IgA-containing renal glomerular deposits are found in diabetic nephropathy patients [Miller et al., 1988]. Accumulating evidences also suggest that immune complexes containing IgA of mucosal origin may be involved in microangiopathy production in Type 2 diabetes [Triola et al., 1984]. CIC are known to persist in the blood for long periods of time. Such CIC following deposition in the small blood vessels have the potential to lead to microangiopathy with debilitating clinical consequences [Nicoloff et al., 2004]. It was postulated that the elevations of serum IgA and IgA-CIC were due to subclinical infection of the mucosa and/or deterioration of IgA clearance in patients with NIDDM [Eguchi et al., 1995]. Sialic acid is a terminal component of the non-reducing end of carbohydrate chains of glycoproteins and glycolipids [Ng and Dain, 1976]. In support of increased desialylation of glycoconjugates in diabetes is the fact that serum sialic acid concentration increases in type 2 diabetes mellitus associated with microvascular complications [Crook et al., 1993; Crook et al., 1994]. Crook M

et.al found that serum sialic acid was significantly higher in men with diabetic complications than in those without any of the complications [Crook et al., 2001].

The present observation that IgA1 desialylated by neuraminidase forms IC with anti-T in serum and that IgA1-anti-T IC are significantly increased in diabetes along with reports that serum IgA as well as neuraminidase are elevated in diabetes, together offer IgA1-anti-T immune complex as a possible cause for vascular injury attending diabetes.

PART III

A faster and inexpensive method for Lp(a) assay.

A faster and inexpensive method for Lp(a) assay

Lp(a) was shown to be an independent risk factor for coronary atherosclerosis, conferring a relative risk ranging from 1.6 to 3.6 [Dahlen et al., 1986]. Increased Lp(a) is also a risk factor, with similar magnitudes of effect, for Ischemic peripheral vascular disease, Ischemic stroke and abdominal aortic aneurysm [Jones et al., 2007]. So the determination of lipoprotein (a) in human serum with high accuracy and reproducibility may help establish the diagnostic value of this lipoprotein as a risk factor of atherosclerosis.

A faster and inexpensive method was developed for Lp(a) assay, which involves microplate-coating of jacalin, incubation with Lp(a) dilutions and probing with anti-apo (a)-HRP. The new assay method is less expensive than commercial ELISA [ELITEST] kit method as the former requires only anti-apo (a) antibody, while the latter requires two highly specific antibodies against Lp(a) (microplate-coating of anti apo (a), incubation with Lp(a) dilutions and probing with anti-apo B-HRP) which makes the assay very expensive.

The new jacalin-based assay method is more sensitive than commercial ELISA Lp(a) kit method (the limit of detection of Lp(a) being as low as 5 ng/ ml). Figure 22 demonstrates the dose response with jacalin-based assay vs standard Lp(a) assay. At higher concentrations of Lp(a), the response for jacalin-based assay is better than the commercial assay [Figure 22 and Table 8].

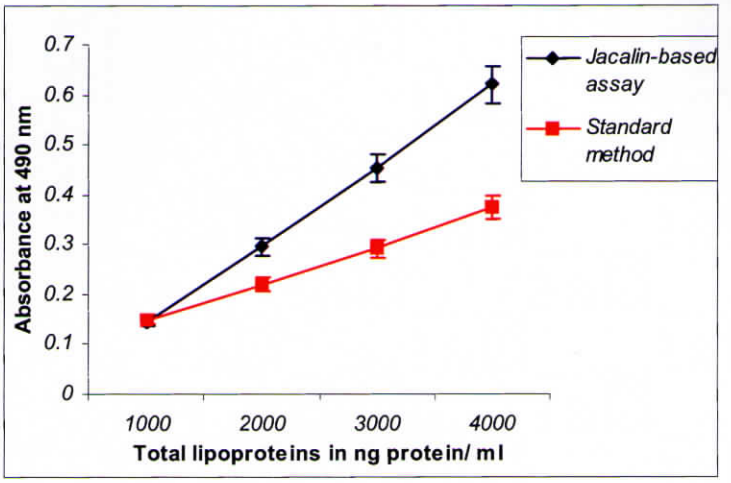

Figure 22. Dose response with jacalin-based assay versus standard Lp(a) assay. The floating protein-free lipid layer (total lipoproteins) obtained from density-gradient ultracentrifugation of Lp(a) rich serum was used as the source of Lp(a).

Table 8. Enhancement of sensitivity in Jacalin-based assay compared to Standard assay.

Concentration of lipoprotein in (ng/ ml)	Percentage increase in Jacalin-based assay
1000	0
2000	35
3000	55
4000	65

Figure 23 shows the linearity of response of jacalin method using varying serum dilutions (3 serum samples used). The method is linear over a wide range of Lp(a) concentrations of serum.

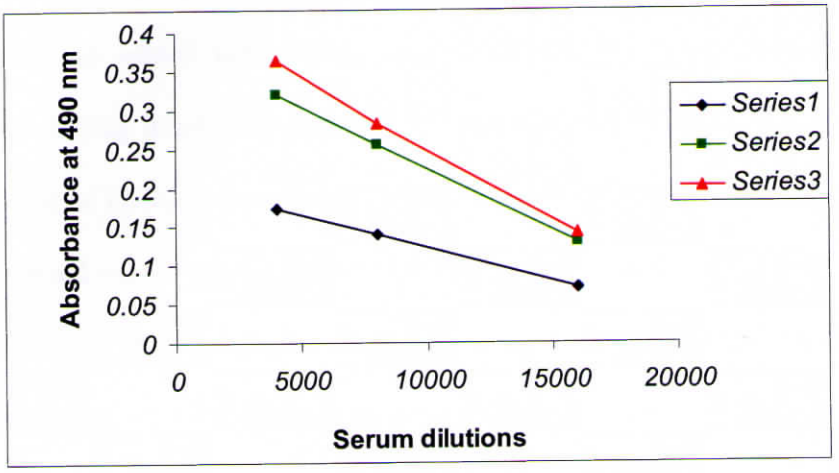

Figure 23. Linearity of response of jacalin method using varying serum dilutions.

As shown in Figure 24, response curve for the new assay is also linear over a wide concentration range of standard Lp(a) (from commercial Lp(a) assay kit, Wako Chemical Industries, Japan).The detection limit of Lp(a) determination was around 5 ng / ml.

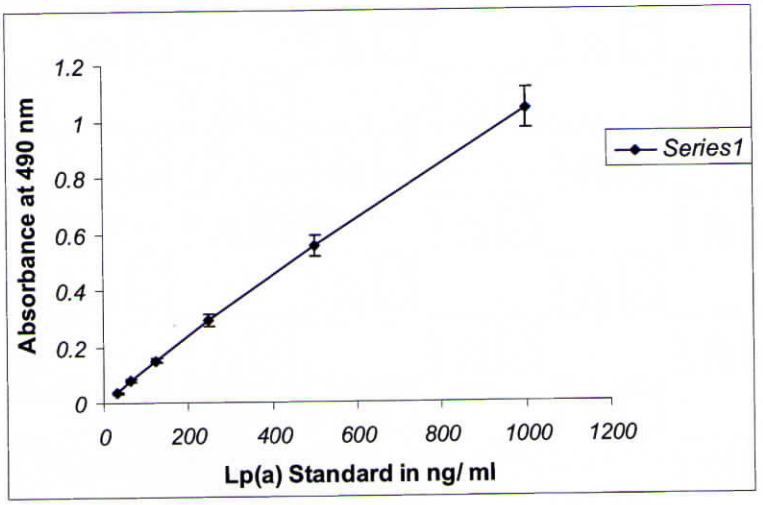

Figure 24. Response curve of jacalin method using standard Lp(a) (from commercial Lp(a) assay kit, Wako Chemical Industries, Japan). Error bars for triplicate trials shown

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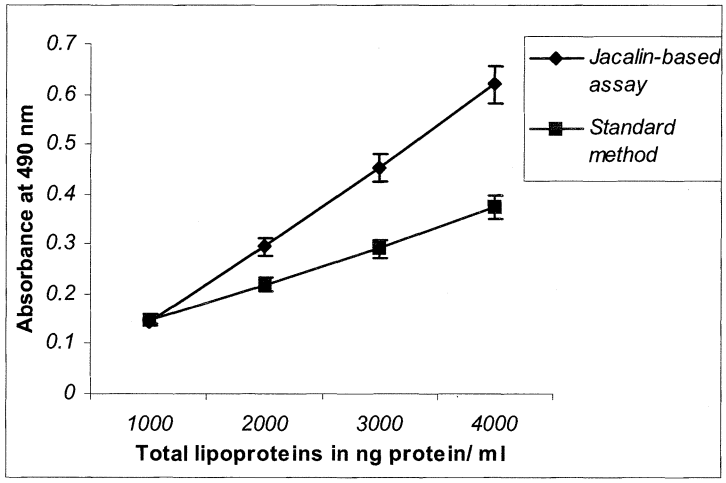

Figure 22. Dose response with jacalin-based assay versus standard Lp(a) assay. The floating protein-free lipid layer (total lipoproteins) obtained from density-gradient ultracentrifugation of Lp(a) rich serum was used as the source of Lp(a).

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Figure 25 demonstrates the percentage cumulation of response on addition of increasing lipoproteins to serum. This was done to check whether the assay response to added lipoproteins is cumulative. Percentage cumulation was calculated by adding increasing amounts of lipoproteins to a fixed sample of serum with base value of Lp(a) concentration and the response of the mixture was compared with the expected values (sum of serum Lp(a) and value due to added Lp(a) obtained from Fig. 24 data). This comparison was used to calculate percentage cumulation. As shown in the figure, 90-95% cumulation was observed.

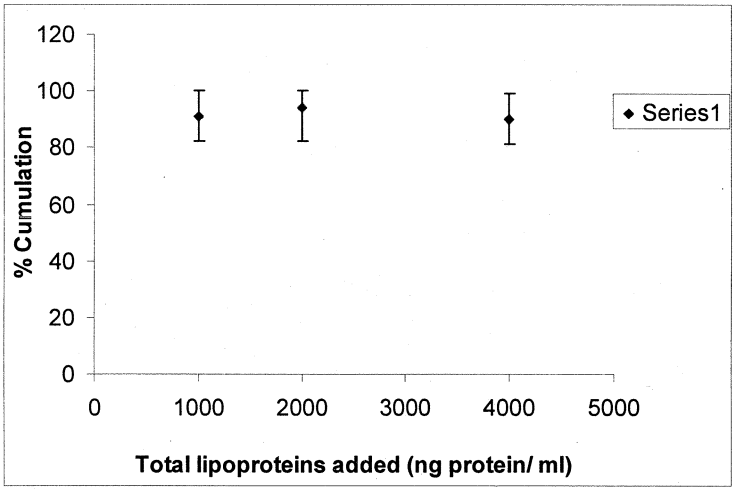

Figure 25. Percentage cumulation of response on addition of increasing lipoproteins to serum. The floating protein-free lipid layer (total lipoproteins) obtained from density-gradient ultracentrifugation of Lp(a) rich serum was used as the source of Lp(a).

Figure 26 shows comparison of jacalin-based assay and standard Lp(a) assay (commercial Lp(a) assay kit, Wako Chemical Industries, Japan using Dade dimension AR autoanalyzer) in detecting Lp(a) in human sera. Lp(a) concentrations was calculated in 6 human serum samples by both methods and the value obtained expressed in mg/dl. As shown in the figure, there is strong correlation between the

content of Lp(a) determined by jacalin-based assay and the standard assay. The correlation coefficient is 0.99 (n = 6, P = 0).

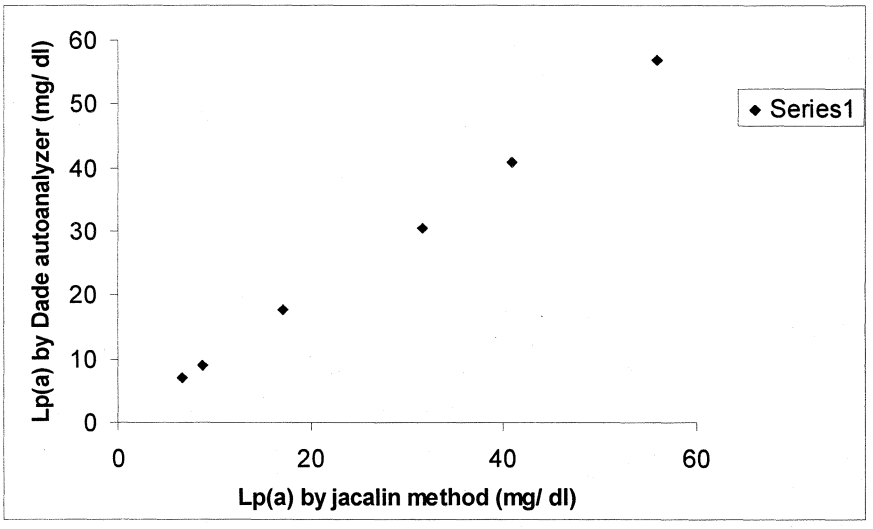

Figure 26. Comparison of jacalin-based assay and standard Lp(a) assay (commercial Lp(a) assay kit, Wako Chemical Industries, Japan using Dade dimension AR autoanalyzer) in detecting Lp(a) in human sera. Each point represents the mean of duplicate measurements by each method.

Figure 27 shows comparison of jacalin-based assay and standard Lp(a) assay (commercial [ELITEST] ELISA assay kit, Hyphen Biomed, France) in detecting Lp(a) in human sera. Lp(a) concentrations was calculated in 6 human serum samples by both methods and the value obtained expressed in mg/dl. As shown in the figure, there is strong correlation between the content of Lp(a) determined by jacalin-based assay and the standard assay. The correlation coefficient is 0.99 (n = 6, P = 0).

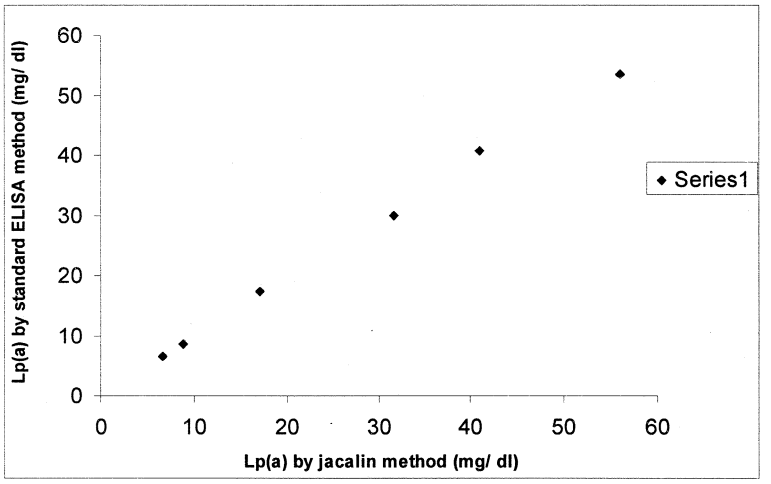

Figure 27. Comparison of jacalin-based assay and standard Lp(a) assay (commercial [ELITEST] ELISA assay kit, Hyphen Biomed, France) in detecting Lp(a) in human sera. Each point represents the mean of duplicate measurements by each method.

As shown in Table 9, in almost all the cases the percentage difference in the Lp(a) concentrations between the new assay and standard assays is less than 5, which validates the jacalin-based Lp(a) assay method.

Table 9. Percentage difference between Jacalin-based Lp(a) assay and Standard assays.

Sample Number	% difference between Dade and Jacalin-based assays.	% difference between ELITEST and Jacalin-based assays.
1	3.7	1.5
2	1.1	2.1
3	4.0	1.2
4	3.8	5.7
5	0.3	1.0
6	1.4	4.7

Discussion

The Jacalin-based lectin sorbent assay procedure described displays high sensitivity and reproducibility. The method is linear over a wide range of Lp(a) concentrations and showed high percentage cumulation in response. Comparison of the values obtained with this assay and commercial standard Lp(a) assays showed good correlation. Thus, this method allows accurate and reliable determination of the concentration of Lp(a) without isolation of the lipoprotein fraction.

Serum levels of Lp(a) have been widely reported to be higher in CAD patients than in healthy controls [Genest et al., 1991, Sandholzer et al., 1992, Kario et al., 1994; Bostom et al., 1996], and thus Lp(a) has been suggested to be a CAD risk factor [Utermann, 1989, Loscalzo, 1990, Stein and Rosenson, 1997]. High levels of Lp(a) (> 30 mg/dl) appear to increase the risk of premature CAD. Lp(a) levels more than 30 mg/dl by itself lend a 3-fold risk of CAD [Enas, 1996] The risk is increased several-fold in the presence of high levels of other lipid and non-lipid risk factors.

The method for determining Lp(a) described in this study allows assessment not only of the pathogenetic role of Lp(a) in atherogenesis but also of the diagnostic and prognostic significance of this lipoprotein as a risk factor of atherosclerosis.

PART IV

Desialylation-dependent recognition of principal O-glycosylated lipoprotein [Lp(a)] by anti-T leading to formation of immune complexes.

Demonstration of carbohydrate structure and T-antigen prevalence of serum lipoproteins.

Carbohydrate side chains on glycoconjugates whether resident on cell membranes, secreted or intracellular play an important role as recognition moieties in various biological processes. The mechanism of Lp(a)-mediated pathology being hardly known, this thesis details an attempt to elucidate recognition of the disaccharide T antigen that gets exposed in desialylated human serum Lp(a), by the serum anti-T antibodies leading to formation of immune complexes. Possible pathophysiological consequences of the resulting immune complexes are discussed in view of the affinity for Lp(a) of galectin-1 that is expressed on several tissues including blood vessel walls. The role of carbohydrate structure on Lp(a), particularly their glycoprotein components apo(a), in the development of atherosclerotic plaque is lacking in literature.

Recognition of Western blotted desialylated and native serum O-glycosylated lipoproteins by T antigen -specific lectins and anti-T

O-glycosylated serum lipoproteins purified by jacalin-mediated precipitation followed by ultracentrifugation were resolved by alkaline pH electrophoresis (Figure 28). On Western blot of these lipoproteins, Lp(a) and LDL were identified by antibodies against apo (a) and apo B chains. Only the slow moving band of strip 2 is recognized by anti apo (a) while this along with a faster moving band are recognized by anti apo B in strip 1. This suggested that the slow and fast moving bands of strip 1 were Lp(a) and LDL respectively.

Binding of anti-T to Lp(a), though negligible to the native lipoprotein (strip 4), was significant following its desialylation (strip 3), while LDL was not

recognized even after desialylation. This is in line with the finding that Lp(a) is unique among lipoproteins as it is very rich in T antigen (Fless et al.,1986). The strips 5 & 6 are controls. They rule out any non-specific binding.

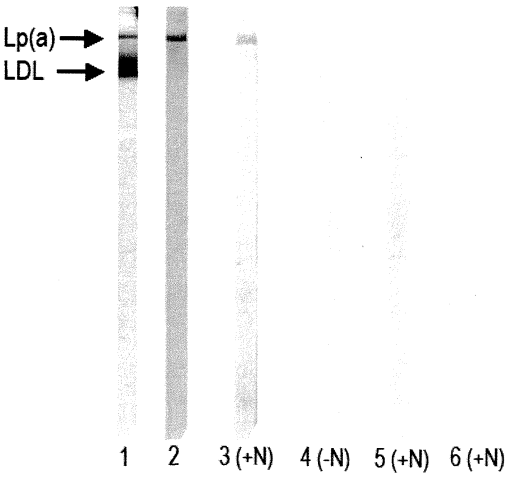

Figure 28. Recognition of Western blotted desialylated and native serum O-glycosylated lipoproteins by anti-T. Western blotted serum O-glycosylated lipoproteins were probed with anti apo B-HRP (1); with anti apo(a)-HRP(2). Strips 3&4 with (+N) or without (-N) neuraminidase treatment were probed with anti-T followed by second antibody-HRP mixture; strip 5 was probed with second antibody-HRP mixture alone and strip 6 with (+N) neuraminidase treatment was probed with ABG (anti-ß–glucoside antibody) followed by second antibody-HRP mixture.

Upon desialylation, human placental galectin-1(HPL)-HRP recognition of Lp(a) increased (strip 3 of Figure 29) remarkably. Similarly, (Peanut agglutinin) PNA-HRP recognition of Lp(a) increased upon its desialylation (strip 5). The requirement of desialylation for recognition by PNA-HRP indicates that the T antigen moieties of Lp(a) are invariably covered by sialic acid moieties.

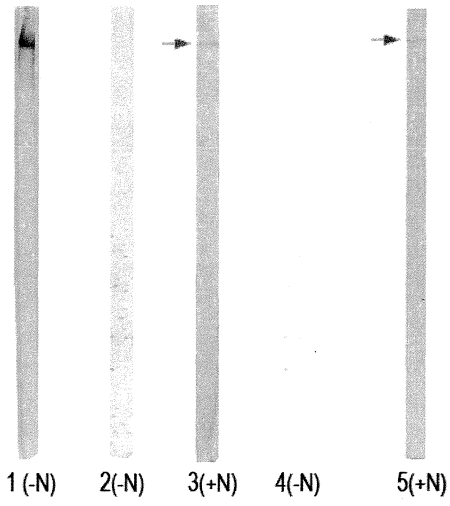

Figure 29. Recognition of Western blotted desialylated and native serum O-glycosylated lipoproteins by T antigen -specific lectins. Western blotted serum O-glycosylated lipoproteins were probed with jacalin-HRP without (-N) neuraminidase treatment (strip 1); strips 2 &3 were probed with HPL-HRP without (-N) or with (+N) neuraminidase treatment and strips 4 &5 were probed with PNA-HRP without (-N) or with (+N) neuraminidase treatment.

The types of glycosylations present on Lp(a) was analyzed by ELISA method using HRP-conjugated lectins. Polystyrene microplate-coated purified Lp(a) was recognized by serum anti-T, only after desialylation of the former. Among O-glycosylation specific lectins, bovine heart galectin -1 (BHL) and PNA recognition of Lp(a) was significantly higher upon its desialylation (Figure 30). The present result suggests that T-antigen containing lipoprotein Lp(a) upon desialylation becomes ligand for anti-T.

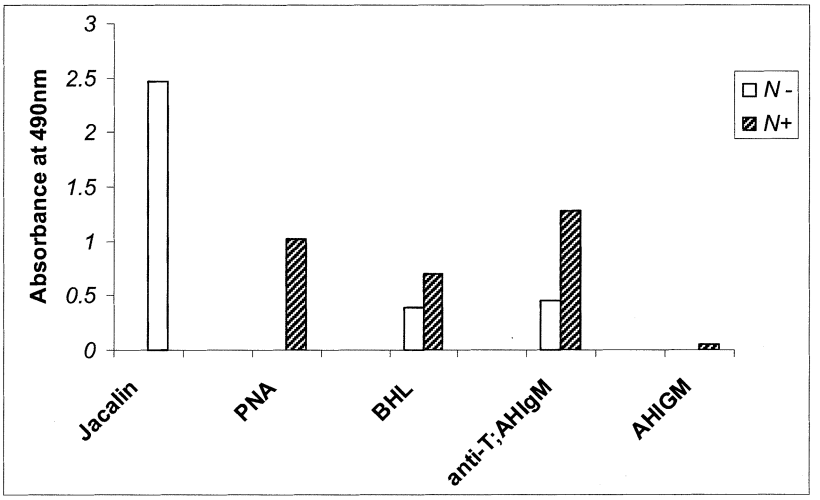

Figure 30. Lectin as well as anti-T binding to native and desialylated Lp(a), coated (1μg/well) on polystyrene microwells.

Dot-blotted Lp(a) was tested as a ligand for anti-T and HPL. Results show that unlike native Lp(a), desialylated Lp(a) was a very efficient ligand for anti-T and T-antigen specific lectin, HPL (Figure 31).

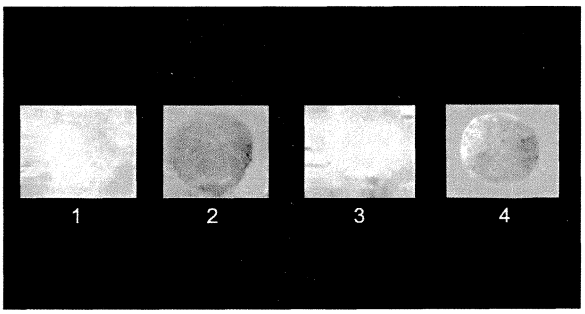

Figure 31. HPL and Anti-T recognition of native and desialylated human serum Lp(a). Dot blotted Lp(a) (2 μg) treated with (2 & 4) or without (1 & 3) neuraminidase were probed with anti-T-HRP (1 & 2) and HPL-HRP (3 &4) respectively.

Desialylation-dependent recognition of principal O-glycosylated lipoprotein [Lp(a)] by anti-T leading to formation of immune complexes.

Lp(a) immune complex formation is a necessary step in lipid accumulation in human intimal cells (Tertov and Orekhov,1994). Also, it is suggested that excessive uptake of lipoprotein-antibody complexes by macrophages leading to formation of foam cells may play an important role in atherogenesis [Klimov et al., 1985]. Results presented in earlier sections indicate the possibility of formation of such immune complexes through anti-T.

Desialylation of fresh serum by *Clostridium perfringens* neuraminidase gave rise to far more Lp(a)-containing immune complexes than did native serum Lp(a) or serum Lp(a) treated with heat-inactivated neuraminidase (P value=0; n = 6) (Figure 32).

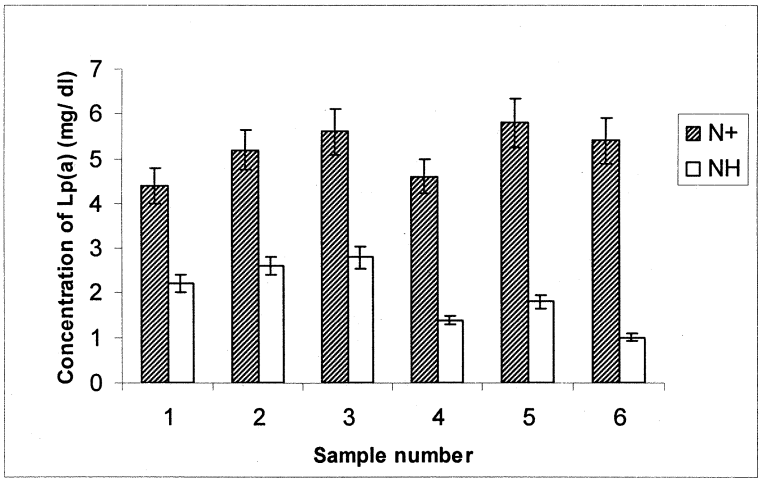

Figure 32. Increase in Lp(a)-containing IC in serum following desialylation. Jacalin (1 µg/well) coated on polystyrene wells of 96 well ELISA plate was incubated with 200 µl 80 times dilution in PBS of IC samples (N+ or NH) for 2 h at 4°C. After washing followed by 2 h incubation at 4°C with 200 µl HRP conjugate of anti-apo(a) (3 µg/ml antibody in PBS-T) and washing, bound HRP was measured as described under 'Methods'. Results of six consecutive trials given. Mean ± S.D. of triplicate trials shown. P value = 0.

Desialylation-dependent entry of Lp(a) into IC was even more explicitly demonstrated in serum from which natural IC had been removed by PEG precipitation. After dialysis to remove PEG and incubation with active (N+) or inactive (NH) neuraminidase, secondary IC formed were collected by precipitation with 2% PEG. Secondary IC from N+ serum contained substantially higher Lp(a) than did secondary IC from NH serum (P value=0; n = 12) (Table 10)

Table 10. Increased Lp(a) incorporation into immune complexes following desialylation of serum, demonstration in serum-free of naturally occurring immune complexes.

Sample Number	Concentration of Lp(a) in N+ IC (mg/ dl)	Concentration of Lp(a) in NH IC (mg/ dl)	Percentage increase
1	1.6 (± 0.12)	0.2 (± 0.01)	700
2	4.4 (± 0.40)	1.2 (± 0.09)	267
3	3.2 (± 0.30)	1.8 (± 0.15)	78
4	10.6 (± 0.90)	4.4 (± 0.40)	141
5	3.6 (± 0.35)	1.6 (± 0.12)	125
6	7.6 (± 0.70)	2.6 (± 0.25)	192
7	4.6 (± 0.40)	2.0 (± 0.18)	130
8	6.6 (± 0.64)	3.6 (± 0.30)	83
9	7.6 (± 0.72)	2.2 (± 0.20)	245
10	8.6 (± 0.80)	5.6 (± 0.52)	54
11	2.8 (± 0.25)	1.2 (± 0.10)	133
12	9.2 (± 0.85)	5.6 (± 0.54)	64

Lp(a) concentration in IC calculated using std.Lp(a) provided with ELITEST Kit. Results of twelve consecutive trials given. * Mean ± S.D. of triplicate trials shown. P value = 0.

Involvement of anti-T in shifting desialylated Lp(a) to the IC phase was examined alternatively by withdrawing anti-T from plasma. Plasma depleted of anti-T by treatment with desialylated RBC of the same group and control treated with unmodified RBC were both desialylated, IC separated and Lp(a) in IC assayed after capture on microwell-coated jacalin and probing with HRP conjugate of anti-apo (a). Results of six consecutive trials (Table 11) show very significant decrease in transfer of Lp(a) to IC in serum in which anti-T was absent, compared to control serum (P value = 0.001; n = 6).

Table 11. Involvement of anti-T in transferring Lp(a), after desialylation of plasma, to IC.

Trial No.	Concentration of Lp(a) in IC from*(mg/dl)		Decrease of Lp(a) in IC following depletion of anti-T (%)
	Normal serum	Anti-T-depleted serum	
1	3.0 (± 0.2)	1.6 (± 0.1)	88.0
2	5.0 (± 0.4)	2.6 (± 0.2)	92.3
3	7.4 (± 0.6)	4.2 (± 0.3)	76.2
4	4.2 (± 0.3)	2.2 (± 0.2)	91.0
5	8.6 (± 0.7)	4.6 (± 0.4)	87.0
6	8.8 (± 0.8)	6.0 (± 0.5)	47.0

Immune complex dissolved in PBS of original serum volume was diluted 80 times in PBS-T before addition to wells coated with jacalin (1μg/well). After 2 h incubation, bound Lp(a) was assayed using HRP conjugate of anti-apo (a) (1.5 μg antibody per ml) and OPD as substrate. Results of six consecutive trials given. * : Mean ± S.D. of triplicate trials shown. P value = 0.001.

Control experiments were carried out to rule out the possibility of desialylation of Lp(a) per se affecting its recognition by coated jacalin or its precipitation by PEG. Results indicated that coated jacalin recognized native and desialylated Lp(a) equally. Also, desialylation as such did not increase the precipitability of serum protein-free Lp(a) by PEG (data not shown).

Discussion

Lp(a) was reported to be an independent risk factor for the presence of coronary atherosclerosis, conferring a relative risk ranging from 1.6 to 3.6 [Dahlen et al., 1986]. Increased Lp(a) is also a risk factor, with similar magnitudes of effect, for ischemic peripheral vascular disease, ischemic stroke and abdominal aortic aneurysm [Jones et al., 2007]. Also, there is compelling evidence from numerous studies that individuals with high plasma Lp(a) levels (>30 mg/dl) are at a significantly increased risk of atherosclerosis, coronary heart disease and ischemic stroke [Craig et al., 1998; Kronenberg et al., 1999; Evans, 2002; Von Eckhardstein et al., 2002]. Lp(a) is considered to be ten times more atherogenic than LDL [Lawn, 1992; Wilken et al., 1993; Seed et al., 1990]. Relative risk of CAD is increased three-fold if the levels of Lp(a) are more than 30 mg/dl [Maher et al., 1995; Armstrong et al., 1986]. Serum Lp(a) levels have been shown to correlate well with the presence, extent, severity and score of atherosclerotic lesions on coronary angiography [Rosengren et al., 1990; Budder et al., 1994; Wang et al., 1994].

The mechanism of action of Lp(a) in atherosclerosis is still not clear, but Lp(a) has been shown to be able to interfere with various factors

involved in thrombogenesis and thrombolysis [Scanu 1992, Stein and Rosenson 1997]. It has also become apparent that Lp(a) can be modified by oxidative events and by the action of lipolytic and proteolytic enzymes with the generation of products that exhibit atherothrombogenic potential. The role of the O-glycans linked to the inter-kringle linkers of apolipoprotein(a) is also emerging. This information is raising the awareness of the pleiotropic functions of Lp(a) and is opening new vistas on pathogenetic mechanisms whose knowledge is essential for developing rational therapies against this complex cardiovascular pathogen [Scanu, 2003]. Present study shows that Lp(a), reported as unique among lipoproteins as it is very rich in T-antigen (by Fless et al. in 1986), becomes ligand for anti-T upon desialylation.

Lp(a), which consists of LDL and apo(a), contains six times as much sialic acid as LDL [Ehnholm et al 1972, Utermann 1989]. Lp(a) from CAD patients was reported to be sialic acid -poor and atherogenic in cell culture compared with Lp(a) from healthy controls and their formation was strongly correlated with the extent of intracellular cholesterol deposition [Tertov and Orekhov 1994].Also neuraminidase concentration in serum has been found to be significantly higher ($P< 0.001$) in CAD+ than CAD- subjects (Sonmez et al,1998). Present results suggest that increased desialylation of Lp(a) followed by its recognition by anti-T may be a major mechanism by which the lipoprotein gets anchored on vessel walls, especially as Lp(a) is the best lipoprotein ligand for tissue galectin-1[Chellan et al., 2007].

. Interestingly, hypercholesterolemic subjects had higher plasma Lp(a)-IC levels. The circulating levels of these immune complexes are likely to vary with

plasma concentrations of Lp(a) [Wang et al., 2004]. Higher and significant levels of immune complexes were found in the patients with atherosclerotic lesions [Romano et al., 1984]. Substantial evidence suggests that complement activation may be a link between lipoprotein deposition and subsequent lesion development in atherosclerosis [Torzewski, et al., 1997] It is suggested that excessive uptake of lipoprotein-antibody complexes by macrophages leading to formation of foam cells may play an important role in atherogenesis [Klimov et al., 1985]. Lp(a) immune complex formation is a necessary step in lipid accumulation in human intimal cells (Tertov and Orekhov,1994).Present results suggest a plausible mechanism for formation of such immune complexes through anti-T.

Chapter 5

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

SUMMARY AND CONCLUSIONS

Recent reports have identified infection-mediated immune inflammations as major contributor towards atherosclerosis [Lefvert et al., 1995; Mustafa et al., 2000] as much as towards nephritis [Mestecky et al., 1986] and autoimmune neuropathy. Pathogen burden remains an independent predictor for the presence of CAD even after adjustment for other risk factors. Together with conventional risk factors, pathogen burden imposed an additional independent risk for the presence and severity of CAD and endothelial dysfunction. The odds of having CAD were 4.1-fold higher in patients with 4 or 5 previous infections compared with those with 0 or 1 previous infection [Prasad et al., 2002]. Molecular mechanisms by which infections cause vascular dysfunction and predispose to atherogenesis is yet to be provided.

In tropical populations, high pathogen burden and serum immunoglobulin titre may aggravate immune inflammatory events. Sialylated or cryptic T-antigens (Gal $\beta 1 \rightarrow 3$ GalNAc) are present in normal cells and tissues [Springer, 1984]. Also humans have naturally occurring serum anti-T antibodies induced predominantly by the intestinal flora so that its titre goes up with pathogen burden [Boccardi et al., 1974; Springer and Tegtmeyer, 1981]. Tissue deposition of IgA1 has been observed in inflammations ranging from nephropathies [Mestecky et al., 1986], atherosclerosis [Pussinen et al., 2005; Saiku et al., 1992] to neuropathies of infectious etiology .On the other hand, atherosclerotic plaques have been repeatedly shown to be rich in Lp(a) [Smith and Cochran, 1990]. Lp(a), which is the most O-glycosylated lipoprotein has been recently reported to be an independent risk factor

for coronary artery disease and stroke [Koschinsky, 2005]. Incidentally IgA1, the only O-glycosylated immunoglobulin type is as well the sole immunoglobulin type which gets deposited in the glomeruli in IgA nephropathy. Also glomerular IgA in IgA nephropathy was enriched in T antigen-rich (ie, desialylated) IgA1 [Iwase et al., 2002]. Evidences are emerging for a physiological role for T antigen as a natural ligand for the most prevalent mammalian tissue lectin, galectin-1 whose expression increases in tumor tissues [Raz et al., 1986] and whose sugar inhibitors retard tumor colonization [Koshik et al., 1997]. Also, primary cultures of human aortic endothelial cells express galectin -1 [Baum et al., 1995]. Notably, the best serum glycoprotein inhibitor for human heart galectin-1 was IgA1, the lone O-glycosylated (T antigen-bearing) immunoglobulin while enzymatic removal of sialic acid moiety from IgA1 to expose free T antigen substantially increased its efficiency as a galectin-1 ligand [Sangeetha and Appukuttan, 2005].Among human serum lipoproteins, Lp(a) which is unique in being profusely O-glycosylated, was the best ligand for human galectin-1 [Chellan et al., 2007].

Localized or systemic release of neuraminidase is a characteristic of many bacterial and viral infections [Soong et al., 2006; Gimsa et al., 1996]. Interestingly enough, the T-antigens in both IgA1 and Lp (a) as they occur in serum are masked (from recognition by anti-T antibody) by a terminally attached sialic acid moiety. Removal of this sialic acid moiety is easily achieved at physiological pH and ionic strength by sialidase enzymes. These enzymes are secreted in serum and tissues by pathogenic bacteria such as *Vibrio cholerae*, *Streptococcus pneumonia* and viruses

such as *influenza* A and B, *parainfluenza*, measles etc. during their infection cycle [Yarnell, 2001].

Anti-T titre goes down substantially during malignancy due to cancer cells possessing exposed surface T antigen and is recovered consequent to surgical/otherwise removal of malignant tumors [Springer, 1976]. This shows the surveillance of exposed T antigens by serum anti-T. On the other hand, the most prominent T antigen bearing glyconjugates in serum, IgA1 and Lp(a) are involved in vascular and perivascular inflammations. It remains a lacuna that recognition of desialylated IgA1 or Lp(a) by anti-T leading to immune complex formation and tissue deposition of the latter as cause for inflammation has not been investigated. In this study we demonstrate the interaction of human serum anti-T with the premier T antigen-containing serum glycoproteins, IgA1 and Lp(a) following desialylation of the latter by bacterial neuraminidase. Possible pathophysiological consequences of the resulting immune complexes are discussed in view of the affinity for IgA1 and Lp(a) of galectin-1 that is expressed on several tissues including blood vessel walls. The mechanism of IgA- or Lp(a)-mediated pathology being hardly known, this work elucidates the possible primary event in the process, namely recognition by human serum antibody (anti-T) of the disaccharide T antigen that gets exposed when IgA1or Lp(a) is desialylated to form immune complexes.

In a mixture of serum O-glycosylated proteins (separated from serum using exclusive selectivity of jacalin for them) IgA1 was identified as the major constituent, since a) the major band moved identically in electrophoresis with IgA, b)

treatment with anti-IgA-Sepharose deleted the major band selectively and c) anti-IgA recognized the major band on Western blot of the mixture.

The preference of affinity-purified anti-T for desialylated, but not native IgA1 was demonstrated using dot blotted human IgA1. Anti-T-mediated hemagglutination of desialylated human RBC was inhibited by purified desialylated IgA1 in solution, but not by its native form or other immunoglobulin types. Desialylation of fresh serum by *Clostridium perfringens* neuraminidase led to the formation of immune complexes containing IgM, the major Ig type in anti-T on one hand and O-glycosylated proteins/ IgA1 on the other. Excess desialylated IgA1 in supernatant after immune complex precipitation could in turn form immune complex with freshly added anti-T. Decrease in anti-T content of serum after immune complex precipitation using desialylated IgA1 could be demonstrated in terms of agglutination of desialylated RBC. Depletion of anti-T from serum by treating with desialylated human RBC led to decreased formation of immune complexes with IgA1, following desialylation.

Western blot of the entire range of serum O-glycosylated proteins (prepared by jacalin-mediated precipitation from serum) when probed with anti-T confirmed the desialylation-dependent recognition by anti-T of almost all O-glycosylated proteins of serum, chiefly IgA1. Most of these bands were also recognized by the T antigen-specific animal lectin galectin-1 though the relative intensities of bands were different.

Polystyrene microplate-coated purified Lp(a) was recognized by serum anti-T, only after desialylation of the former. O-glycosylated serum lipoproteins

purified by jacalin-mediated precipitation followed by ultracentrifugation were separated by alkaline pH electrophoresis. On Western blot of these lipoproteins, Lp(a) and LDL were identified by antibodies against apo (a) and apo B chains. Binding of anti-T to Lp(a), though negligible to the native lipoprotein, was significant following its desialylation, while LDL was not recognized even after desialylation.

A faster and inexpensive method was developed for Lp(a) assay, which involves microplate-coating of jacalin, incubation with Lp(a) dilutions and probing with anti-apo (a)-HRP. The new jacalin-based assay method is more sensitive than commercial ELISA Lp(a) kit method (the limit of detection of Lp(a) being as low as 5 ng/ ml). The method is linear over a wide range of Lp(a) concentrations and showed high percentage cumulation in response and strong correlation with standard Lp(a) assays.

Desialylation of fresh serum by *Clostridium perfringens* neuraminidase gave rise to far more Lp(a)-containing immune complexes than did native serum Lp(a) or serum Lp(a) treated with heat-inactivated neuraminidase. Desialylation-dependent entry of Lp(a) into IC was even more explicitly demonstrated in serum from which natural IC had been removed by PEG precipitation. Secondary IC from N+ serum contained substantially higher Lp(a) than did secondary IC from NH serum. Depletion of anti-T from serum by treating with desialylated human RBC led to decreased formation of immune complexes with Lp(a), following desialylation .

Desialylation of fresh diabetic serum by *Clostridium perfringens* neuraminidase led to increased formation of immune complexes containing IgM, the major immunoglobulin type in anti-T on one hand and IgA1 on the other, than

desialylated normal serum. The percentage increase in entry of IgA1 to IC formed from neuraminidase treated serum (N+) over non- desialylated control (NH) is enormously higher (112% ± 42.6) in the case of diabetic sera, than in the case of normal sera (33% ± 9). Similarly, the percentage increase in IgA-IgM content in IC formed from neuraminidase treated serum (N+) over non-desialylated control (NH) is much higher (116% ± 29.3) in the case of diabetic sera, than in the case of normal sera (28% ± 9.5).

Immune complex between anti-T and desialylated IgA1 *in vivo,* if formed may be aided by its unique composition in bringing about vascular injury. Firstly, as is evident from present results anti-T-bound desialylated IgA1 still has binding sites left for galactose-binding lectins. For the most abundant human galactose-binding lectin, galectin-1 which is expressed on endothelial cell surface as well, IgA1 has been reported to be the most prominent serum ligand and more so after desialylation. In view of the biology of tissue galectin-1 reported earlier from this laboratory and elsewhere present results suggest the possibility of galectin-1-dependent anchoring of anti-T-desialylated IgA1 IC on vessel walls and tissues during infections and diabetes, both known to induce vascular damage. Secondly, IgM as well as IgG of anti-T-IgA1 immune complexes have their binding sites engaged so as to enable the respective Fc portions to attract complement and initiate an inflammatory cascade. This interaction may provide part of the mechanism of the short serum half life of IgA1, the mechanism of which is currently unclear as the ASPGR was recently found to be responsible almost exclusively for turnover of IgA2 and not IgA1 [Rifai et al., 2000].

Present results also suggest that immune complexes involving IgA1 and anti-T could be a key component in diabetic vascular immune inflammations, especially since high serum IgA concentration as well as increased serum neuraminidase titre has been shown to accompany diabetes.

Lp(a) from CAD patients was reported to be sialic acid-poor (2.5 fold) and atherogenic in cell culture compared with that of healthy subjects. Also neuraminidase concentration in serum has been found to be higher in CAD+ than CAD- subjects. Present results suggest that increased desialylation of Lp(a) followed by its recognition by anti-T leading to immune complexes may be a major mechanism by which the lipoprotein gets anchored on vessel walls, especially as Lp(a) is the best lipoprotein ligand for tissue galectin-1.

Figure 24 shows the schematic summary of the proposed hypothesis of this Ph.D study which envisages the possibility of galectin-1-dependent anchoring of anti-T-desialylated Lp(a) IC on vessel walls and tissues during early stages of atherosclerosis resulting in immune mediated vascular damage. IgM as well as IgG of anti-T-Lp(a) immune complexes have their binding sites engaged so as to enable the respective Fc portions to attract complement and initiate an inflammatory cascade. Complement factors are released following IC- complement interaction which can attract monocytes or macrophages. Activated monocyte/ macrophage can release leukotrienes and cytokines that induce endothelial cell surface to synthesize more of adhesion molecules such as selectins and galectin-1. Increased vascular permeability caused by these immune events on surface endothelial layer helps transfer of immune

complexes and macrophages into the sub-endothelial layers. This may be a seeding event towards development of foam cells in the smooth muscle cell (SMC) layer.

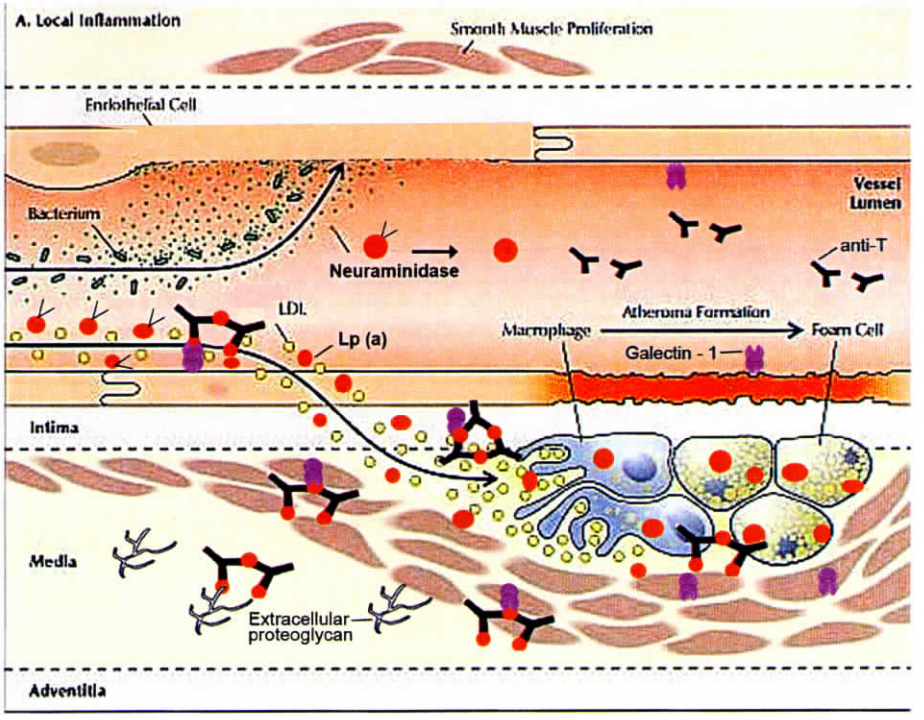

Figure 24. Schematic summary of the proposed hypothesis of this Ph.D study which envisages the possibility of galectin-1-dependent anchoring of anti-T-desialylated Lp(a) IC on vessel walls and tissues during early stages of atherosclerosis resulting in immune mediated vascular damage. The binding may also favor transendothelial transport of galectin-1- IC to the subendothelial space, where they may be engulfed by tissue macrophages. This may be a seeding event towards development of foam cells in the SMC layer.

FUTURE DIRECTIONS

Pathogen-derived antigens are often rich in polysaccharide structures that elicit predominantly IgA antibodies (most of which is IgA1). Also many bacteria and viruses including those that are pro-atherogenic and pro-nephropathic are known

to secrete the enzyme neuraminidase that can desialylate IgA1. The following lines of investigations are pertinent in this direction.

1) To check whether in infections involving neuraminidase release (eg. Influenza), the IC is richer in desialylated Lp(a) or IgA1.

2) To study whether diabetes (reported to entail higher serum neuraminidase) is accompanied by desialylated Lp(a)/IgA1-rich IC.

3) To check the desialylation status of foam cell Lp(a): For which an ELISA method involving attachment of cell extract to microtitre-coated anti apo(a) and probing with HRP-conjugate of a lectin-specific for desialylated glycoproteins (Peanut agglutinin) should be employed.

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