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An Immunochemical Analysis of Human Apolipoprotein B-100 Structure-Function Relationships

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Submitted to the School of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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May 1998

Abstract

Low density lipoproteins (LDL), the major cholesterol carriers in human plasma contain a single copy of apolipoprotein B-100 (apoB-100), a 4536 residue, water-insoluble polypeptide as their exclusive protein component. ApoB-100-containing lipoproteins (LpB) are secreted from the liver as large triglyceride-rich lipoproteins and are converted in the circulation to smaller cholesteryl ester-rich LDL. As a ligand for the cell surface LDL receptor, apoB-100 has an important role in cholesterol metabolism and elevated apoB-100 levels increase the risk of atherosclerosis. Genetic polymorphism, oxidative modification, non-enzymatic glycation and the lipid environment of apoB-100 can affect its ability to mediate binding to the LDL receptor. In my Ph.D. program, I have produced and employed a panel of anti-apoB-100 monoclonal antibodies (mAbs) to study apoB structure and function. To this end, I developed and evaluated 3 novel immunization strategies to generate apoB-100 isoform-specific mAbs. Immunization of human apoB-100 transgenic mice proved to be an effective method for production of anti-apoB-100 mAbs having unusual specificities. This strategy could also be applied to other antigens. Several mAbs produced by this protocol are shown to be useful probes of apoB-100 conformation. I have characterized the binding specificities of a large panel of anti-apoB-100 mAbs and have analyzed the expression of their corresponding epitopes on LpB as a function of LpB intravascular metabolism. I show that the apoB-100 carboxy-terminus undergoes a major conformational change as large LDL are converted to smaller particles, a conformational change that could allow the particle to become

competent to mediate binding to the LDL receptor. Finally, I demonstrate that, when LDL undergoes non-enzymatic glycation, at least 6 sites in apoB-100 are modified, including 2 that flank the apoB LDL receptor-binding site. As the LDL receptor-binding site itself is not modified, the loss of binding activity may be secondary to a change in apoB conformation.

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Outline of the Thesis

In chapter I, I present our current concepts of the structure and biology of apolipoprotein (apo) B and the low density lipoprotein (LDL) receptor and their respective roles in lipoprotein metabolism. I discuss the pathological importance of modified LDL and the possible mechanisms by which modified LDL contribute to atherosclerosis. I then briefly describe how monoclonal antibodies (mAbs) have been used to study the structure and function of the apolipoproteins, cell surface receptors, lipid modifying enzymes and lipid transfer proteins that participate in the synthesis, assembly and metabolism of the plasma lipoproteins.

Chapters II, III and IV describe a part of the experimental results that I have obtained during my Ph.D. program. These chapters have been written in the form of manuscripts that have been, or will be, submitted for publication. In chapter II, I discuss in detail, the development and evaluation of three novel immunization protocols for the generation of anti-apoB isoform-specific mAbs. I also present the unusual binding properties of several of the mAbs that were produced using these strategies and elaborate on how these mAbs might be used as probes of apoB conformation.

In chapter III, I describe the characterization of the binding properties of a panel of anti-apoB mAbs including the mapping of their corresponding epitopes within apoB primary structure. I also present the results of experiments in which I have used these

anti-apoB mAbs to monitor the conformational changes of apoB that occur during its intravascular metabolism.

A series of experiments are described in Chapter IV in which I have used a large panel of anti-apoB mAbs to identify sites in apoB that are modified when LDL undergo non-enzymatic glycation. Glycation of LDL occurs in both diabetes and renal disease and potentially contributes to the increased risk of premature atherosclerosis that is associated with these conditions.

In chapter V, I elaborate upon the discussion of the results that are presented in Chapters II, III and IV. As I have chosen to condense my results into a three-manuscript format, I was not able to include all the relevant experimental findings that I have obtained as a part of my Ph.D. project. As a consequence, I have taken the opportunity to integrate some of these observations into the general discussion presented in Chapter V. Finally, I discuss at length the future directions that this project might take.

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Dedications

This thesis is dedicated to my parents and my family.

Acknowledgements

First and foremost, I would like to express my thanks to my supervisor, Dr. Ross Milne, for his insight, guidance and support during the years of research that led to the writing of this thesis. I have learned not only the techniques and methodology, but also the critical thinking and the profound love of science.

I am grateful for the advice and help from Dr. Zemin Yao and Dr. Roger McLeod, who are always available. I would also like to thank Drs. Marcel, McPherson, Sparks, and Schultz, mumbers of atherosclerosis group in Heart Institute, for providing a excellent scientific environment and essential training during my Ph.D. study. I thank Dr. Remy Aubin for his advice in supervisory committee. I acknowledge the great help from Tracey Neville and I always enjoy the discussion with Drs. Burgess, Vohl, and Ko. I will miss friends and follow graduate students Jay Wang, Robert Raffai, Vinita Chauhan and all others in our group. I am also in debt to Drs. Stephen Young, Thomas Innerarity, and Jan Borén of the Gladstone Cardiovascular Research Institute at the University of California, San Francisco for their transgenic mice. Dr. Jean Davignon from Institute de Research Clinique de Montreal has been very helpful in providing clinical material. Many thanks to all the members of Dr. Ross Milne's lab as well as the Atherosclerosis Group in Heart Institute.

Abbreviations

AG:	aminoguanidine
AGEs:	advanced glycation end products
AGE-LDL:	AGE-modified LDL
apo:	apolipoprotein
$apoB^{Gln3500}$:	point mutation at residue 3500 from arginine to glutamine
B/B_0:	B is radioactivity bound in the presence of competitor and B_0 is radioactivity bound in the absence of competitor
BSA:	bovine serum albumin
CE:	cholesteryl ester
CETP:	cholesteryl ester transfer protein
CPM:	count per minute
ED_{50}:	concentration of competitor required to reduce 50% of maximum binding
ER:	endoplasmic reticulum
FC:	free cholesterol
FDB:	familial defective $apoB^{3500arg \rightarrow gln}$
HDL:	high density lipoproteins
hLDL:	human low density lipoprotein
Ig:	immunoglobulin
IDL:	Intermediate density lipoproteins
LDL:	low density lipoproteins

LpB:	apoB containing lipoproteins
mAb:	monoclonal antibody
MTP:	microsomal triglyceride transfer protein
NaCl-Tween:	0.15 molar sodium chloride containing 0.025% (v/v) Tween-20
PBS:	phosphate buffered saline
PBS-BSA:	Phosphate-buffered saline containing 1% (w/v) bovine serum albumin
PL:	phospholipids
RIA:	radioimmunoassay
tg:	transgenic
TG:	triglyceride
Tween-Saline:	0.15 molar sodium chloride containing 0.025% (v/v) Tween-20
VLDL:	very low density lipoproteins

Chapter I

Introduction

Lipids are essential components of all eucaryotic cells where they play structural roles in cellular membranes, serve as a direct source of energy and regulate cellular functions. In humans, the major lipid species of plasma are phospholipids, cholesterol, triglycerides, and cholesteryl esters. As lipids are water insoluble, they are assembled into lipoproteins for transport from sites of absorption and synthesis to the sites of storage and utilization. The lipoprotein transport system is extremely complex and includes different classes of lipoprotein particles, lipases, lipid transfer proteins, and lipoprotein receptors. It is important to determine the mechanisms involved in the lipoprotein transport system and how levels of lipoproteins are controlled in circulation in order to understand the pathogenesis of diseases associated with malfunction of lipoprotein homeostasis and to elaborate effective pharmacological interventions.

Section I: Historical perspective of plasma lipoproteins

Soluble plasma lipoproteins were recognized as a unique class of conjugated proteins as early as 1897 by Schultz (Schultz, 1897), who observed that complete extraction of plasma lipids by diethyl ether could only be achieved after prior proteolytic degradation of plasma proteins. The notion that serum lipids are bound to proteins was

fully substantiated in the late 1920s by Macheboeuf (Macheboeuf, 1929), who first isolated a lipoprotein of constant composition from horse serum. During the 1930s and 1940s, evidence gradually accumulated that all serum lipids are combined with specific proteins into two major lipoprotein classes differing in physical and chemical properties. Blix and colleagues, (Blix *et al*, 1941) demonstrated that the major part of serum lipids migrated in an electric field with α1 and β-globulin mobility. This important finding confirmed both the existence of lipid-protein complexes and indicated the occurrence of at least two specific lipid-binding proteins. Owing to the characteristic electrophoretic mobility, these two lipoproteins were named α1 and β-lipoproteins.

The recognition of α1 and β-lipoproteins as two distinct lipoprotein classes provided the first classification of plasma lipoproteins on the basis of electrophoretic behavior and was a criterion that was applicable to both human and animal lipoproteins. However, the relatively wide boundaries and variations in electrophoretic mobility displayed in free electrophoresis by α1 and β-lipoproteins suggested further compositional complexity of the two lipoprotein classes. Application of zonal electrophoresis to the analysis of serum lipoproteins revealed lipid-stained bands not only in the α1 and β-globulin regions but also at the origin and in the α2-(pre-β), prealbumin and γ-positions substantiating the already suspected heterogeneity of lipoproteins (Kunkel *et al*, 1952, Dangerfield *et al*, 1955).

By ultracentrifugation, Gofman and co-workers, observed that plasma lipoproteins represent a wide spectrum of particle sizes and hydrated densities characterized by regions of maximal and minimal concentrations along a density gradient ranging from 0.92-1.21 g/ml (Gofman *et al*, 1949; DeLalla and Gofman, 1954). Lipoproteins can be separated, by fractional ultracentrifugal flotation in salt solutions of successively increasing densities, into four major classes designated as chylomicrons (d<0.94g/ml), very low density lipoproteins (VLDL, d=0.94-1.006 g/ml), low density lipoproteins (LDL, d=1.006-1.063 g/ml) and high density lipoproteins (HDL, d=1.063-1.021 g/ml) (Figure 1-1).

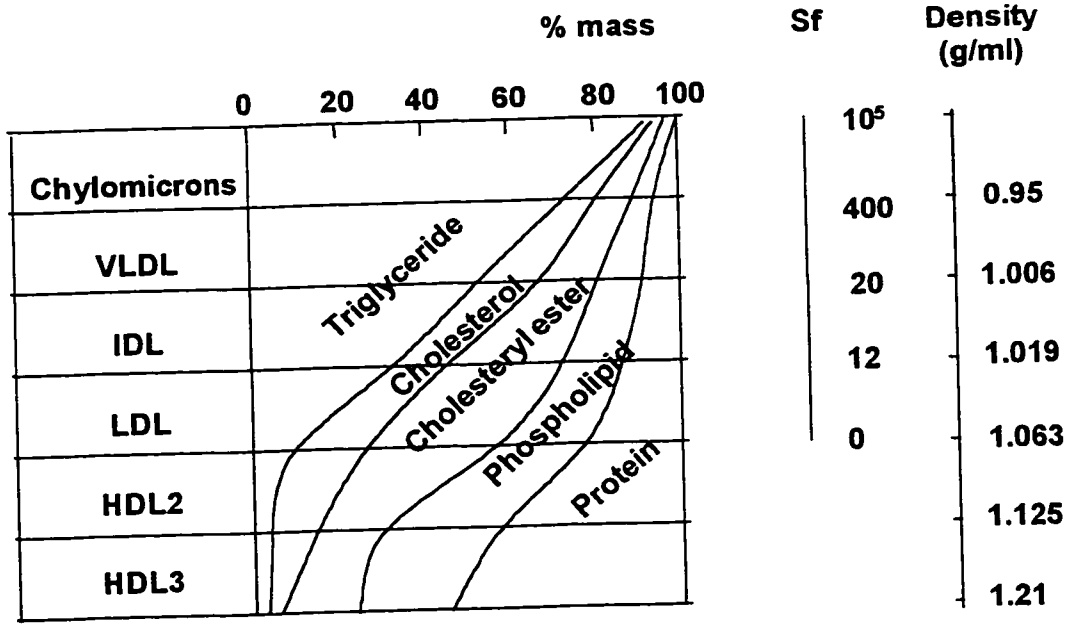


Figure 1-1. The classification, composition and hydrated density of normal human plasma lipoproteins.
With the increase of the protein content, the lipoprotein particles become denser mainly due to the loss of triglycerides. Sf, Svedberg flotation units. (Adapted from Mackner and Durrington, in *Lipoprotein Analysis.* ed. Converse, C.A., and Skinner, C.A.1992. p92)

However, there is considerable heterogeneity within each major lipoprotein density class with respect to the particle size and hydrated density (Lindgren *et al*, 1962 and Ewing *et al*, 1965). Application of analytical and preparative ultracentrifugation to studies of plasma lipoproteins has been of great practical and theoretical importance for further developments in the lipoprotein field. Separation of lipoproteins on the basis of their hydrated densities has become the most widely used methodology for the isolation of lipoprotein species and the main criterion for characterizing and classifying plasma lipoproteins.

Based on the immunological properties (Levine *et al*, 1955) and the characteristic N-terminal amino acids of the proteins (Avigan *et al*, 1956) of the major lipoprotein density classes, it was predicted that distinct apolipoproteins predominate in HDL and LDL, respectively. Furthermore, it was considered that these two apolipoproteins also constituted the protein moieties of chylomicrons and VLDL. However, other studies (Aladjem *et al*, 1957) suggested that chylomicrons and VLDL may contain additional protein constituents. In VLDL, three N-terminal amino acids were identified, glutamic acid, thought to be the amino terminal residue of β-protein, as well as serine and threonine (Shore and Shore, 1973). In human chylomicrons, Rodbell and Fredrickson described the fingerprints of α1- and β-proteins, referred to as A and B, and a third fingerprint of an unknown protein referred to as C (Rodbell and Fredrickson, 1959).

The detection of threonine and serine as the amino-terminal residues of apoC suggested a protein that consisted of either two non-identical polypeptides or two different proteins (McConathy *et al*, 1969). It was subsequently demonstrated that apoC was, in fact, comprised of three distinct polypeptides that were initially named apoLP-Ser, apoLP-Glu, and apoLP-Ala (Brown *et al*, 1970) based on their respective amino terminal residues. Shore and Shore established that apoA was also comprised of two non-identical polypeptides which they called apoLP-Thr and apoLP-Gln using the same criteria for the nomenclature (Shore and Shore, 1968 and 1969). This important discovery also provided an explanation for the initial failure to recognize the apolipoprotein heterogeneity of HDL. The apoLP-Gln was found to have a blocked amino terminus, which prevented its recognition on the basis on N-terminal amino acid analysis.

The complexity of the ApoA and apoC polypeptides prompted the introduction of a more systematic nomenclature that adequately and unambiguously denoted the already known apolipoproteins as well as those that might be discovered in the future. To this end, the ABC nomenclature was introduced in which apolipoproteins are designated by a capital letter, their constitutive polypeptides by Roman numerals, and the polymorphic forms of either apolipoproteins or polypeptides by Arabic numbers (Alaupovic, 1972 and 1991). For example, because of the frequently observed co-purified apoA or apoC polypeptides, they were considered to be the non-identical polypeptides of apoA and apoC, respectively. Therefore, the two apoA polypeptides were called apoA-I and apoA-

II, whereas those of apoC were called apoC-I, apoC-II, and apoC-III. The isomorphic forms of apoC-III were named apoC-III-0, apoC-III-1, and apoC-III-2.

The continuing search for additional apolipoproteins led to the discovery of a minor apolipoprotein first detected in very-high-density lipoproteins (VHDL, d>1.21 g/ml) (Alaupovic *et al*, 1966) and LDL, which was initially referred to as "thin-line" polypeptide and was later named apoD (McConathy and Alaupovic, 1973 and 1976). The next apolipoprotein, isolated from triglyceride-rich lipoproteins, was independently discovered by three groups of investigators (Shelburne *et al*, 1974 and Utermann, 1975, and Shore *et al*, 1973). Because of its relatively high content of arginine, this apolipoprotein was initially called the arginine-rich polypeptide. In accordance with the ABC nomenclature it is now called apoE. Olofsson *et al,* (Olofsson, *et al*, 1978) isolated a minor apolipoprotein from HDL that was characterized by a relatively low isoelectric point (pI=3.7). This acidic apolipoprotein, frequently found in association with apoA-I and apoA-II (Koren *et al*, 1982), was designated apoF. Another minor apolipoprotein differing in chemical, electrophoretic, and immunological properties from other apolipoproteins was identified in HDL and VHDL. This glucosamine-rich apolipoprotein was called apoG (Ayrault-Jarrier *et al*, 1978). Other apolipoproteins were discovered such as apoH (Schultze *et al*, 1961), apoI (Husby and Natvig, 1974), and apoJ (DeSilva *et al*, 1990). Most of these minor apolipoproteins also exist in a lipid-free form and their function in lipid metabolism is not understood.

Lipoprotein (a) (Lp (a)), first described as a genetic variant of LDL (Berg, 1963), represents a unique family of lipoprotein particles formed through the interaction of LDL and a highly glycosylated, hydrophilic protein called apolipoprotein (a) (apo (a)) (Dahlen, *et al*, 1986 and Utermann, 1989). The protein moiety of Lp(a) consists of apoB covalently bound through a disulfide linkage to apo(a) (Utermann, 1989). The structural, metabolic, and antigenic properties of Lp(a) are mainly due to apo(a), which was shown to possess a high degree of homology to plasminogen and, thus to belong structurally to a superfamily of proteins, including the proteases of the fibrinolytic and coagulation systems (Utermann, 1989 and Loscalzo, 1990). Although structurally an intriguing protein, apo (a) does not appear to form its own family of lipoprotein particles and has no known function in lipid transport processes. Elevated levels of Lp (a) have been shown to be a major risk factor for atherosclerosis (Somers and Chappell, 1996).

Section II: Lipoprotein Metabolism

Lipoproteins are macromolecular assemblies of specific lipids and proteins. They transport water insoluble nutrients through the bloodstream from their site of absorption or synthesis to peripheral tissue. Triglycerides are used for both energy expenditure and storage, where as phospholipids and cholesterol serve as building materials for cellular membranes and precursors for signaling molecules. Most of the lipids are derived from the diet; although the human body does have the ability to synthesize lipids from carbohydrate molecules. There are two defined pathways implicated in lipoprotein

metabolism according to the source of lipids and origin of the lipoproteins: the exogenous and endogenous pathways (Figure 1-2).

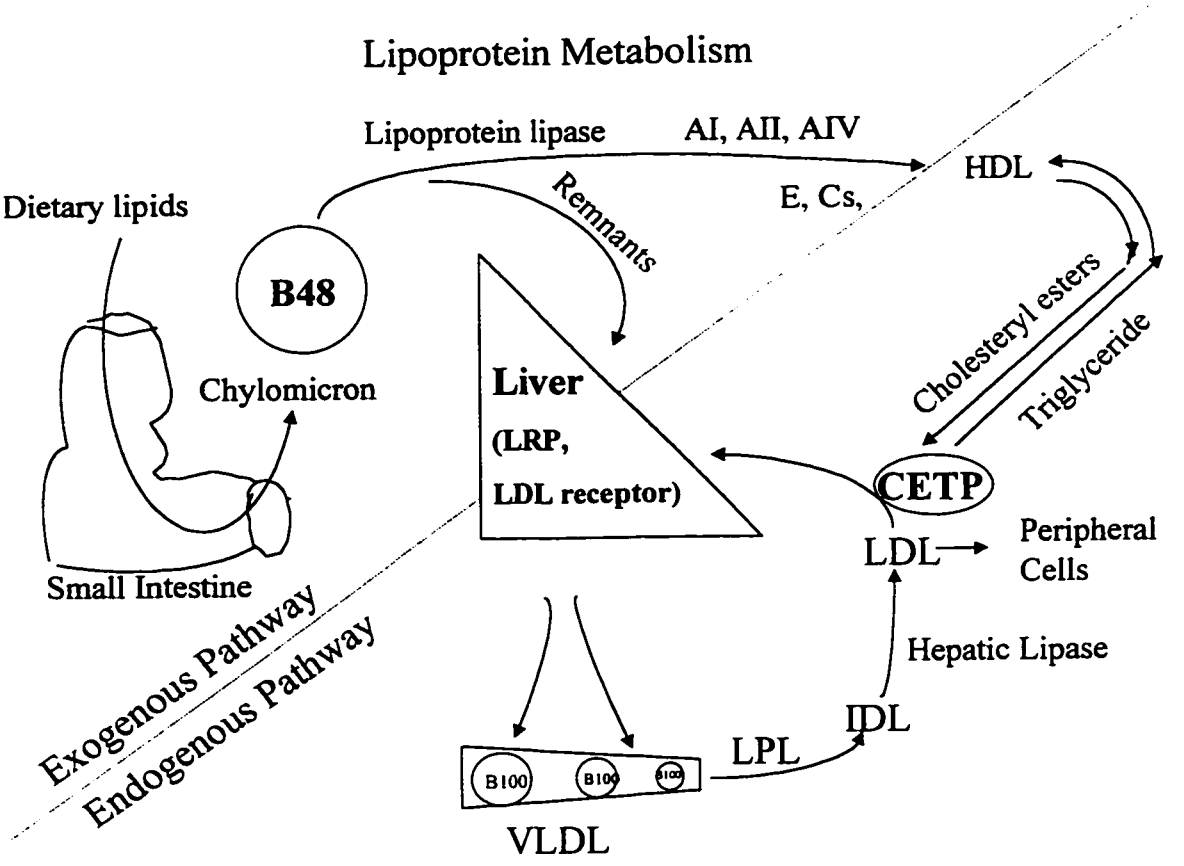


Figure 1-2. Triglyceride-rich lipoprotein metabolism: exogenous and endogenous pathways

As described in the text, dietary lipid is absorbed in the intestine. Free fatty acids are converted to triglyceride and assembled into chylomicrons within enterocytes that are secreted in the circulation. The chylomicrons undergo intravascular lipolysis and the remnants are rapidly taken up by the liver. This is the exogenous pathway. The liver secrets triglyceride-rich VLDL. Under the influence of lipases and the lipid transfer proteins, VLDL are converted into cholesteryl ester rich LDL that can be taken up by the LDL receptor. This is the endogenous pathway. (Modified from Schneider, W.J., in *Biochemistry of lipids lipoproteins and membranes* Vance, J., and Vance, D. (Edts), 1991, p462)

Exogenous pathway

Dietary lipids are absorbed by enterocytes of the small intestine and are secreted into circulation as chylomicrons whose lipid composition is a reflection of the composition of the diet. Chylomicrons are highly heterogeneous with particle diameters from 35 to over 250 nm. The ability of the enterocyte to change the size of chylomicrons in response to the availability of lipids ensures a mechanism to vary triglyceride secretion rapidly in response to dietary status (Davidson *et al*, 1986). Under the action of lipoprotein lipase, chylomicrons are lipolysed and converted into smaller chylomicron remnants that can be rapidly taken up by the liver through a receptor-mediated process (Herz *et al*, 1988). The apoE present in the chylomicron remnants is critical for this uptake as it is a ligand for both the LDL receptor, the LDL receptor-related protein (LRP) and cell surface proteoglycans (Koo *et al*, 1988; Hussain *et al*, 1996).

Endogenous Pathway

VLDL are synthesized in the liver by hepatocytes and are secreted into the bloodstream. VLDL are large particles with diameters of 40-60 nm and hydrated densities of less than 1.006. VLDL are composed of a triglyceride (TG)-rich core surrounded by phospholipid and cholesterol and are responsible for delivering *de novo* synthesized triglyceride from the liver to extra-hepatic cells. The principle structural protein of VLDL is apoB accounting for about 50% of VLDL protein. Within the plasma

compartment, VLDL acquire apoE and a complement of apoC molecules including apoC-II, which is the obligate co-factor of lipoprotein lipase (Breckenridge *et al*, 1978). Unlike the other apolipoproteins, Such as apoE and apoAI, apoB does not exchange between lipoprotein particles and, in this respect, behaves as an integral membrane protein.

During circulation within the bloodstream, VLDL are hydrolyzed by lipoprotein lipase that is anchored on the lumenal surface of the endothelial cells. Upon hydrolysis of triglycerides, VLDL become smaller, their hydrated density increases as they are converted from VLDL to IDL. Under the combined actions of lipoprotein lipase and hepatic lipase the IDL undergo further lipolysis. Concomitant with lipolysis, the apoCs and apoE are lost from the particle and there is transfer of cholesteryl ester (CE) from high density lipoproteins (HDL) to VLDL and IDL, a process which is mediated by cholesteryl ester transfer protein (CETP). In addition, some of the remnant particles are captured and endocytosed by cells, predominantly in the liver, through a process mediated by high affinity cell surface receptors. The ultimate product of the lipolytic cascade is LDL. LDL are spherical particles with a diameter of 20-25 nm and contain a single molecule of apoB-100 as their sole apolipoprotein. The surface of the particle consists of phospholipid and free cholesterol as well as apoB, whereas, the hydrophobic core is composed primarily of cholesterol ester and variable amounts of triglyceride (Beisegel *et al*, 1992) (Figure 1-3). The end product of the endogenous pathway, LDL, is taken up by LDL receptors situated on the surface of cells in the liver and peripheral tissues.

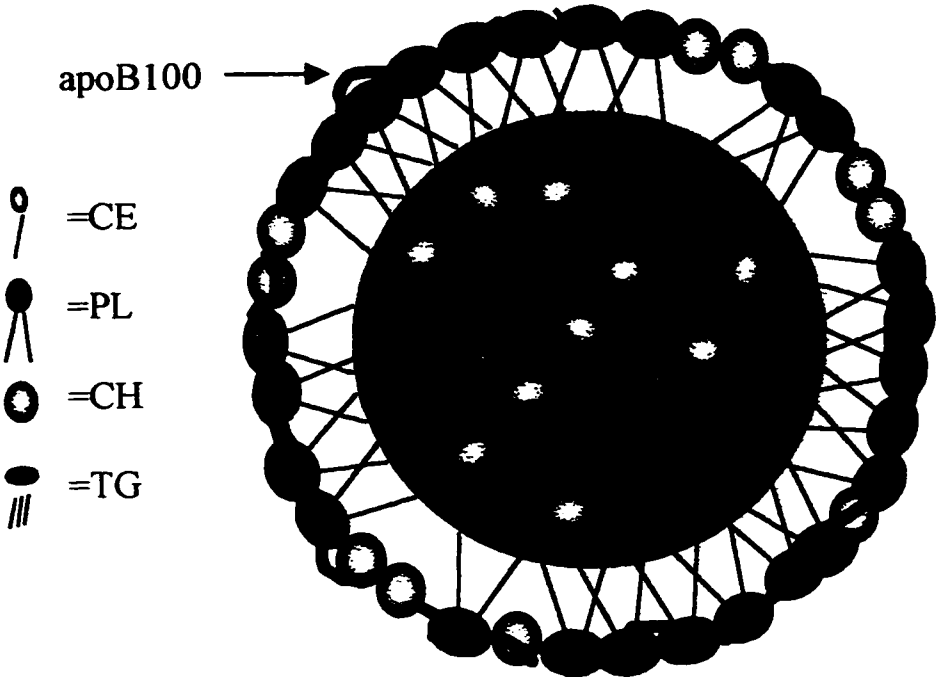


Figure 1-3. Low density lipoprotein particles
Low density lipoproteins are spherical particles with a core of triglycerides and cholesteryl esters that is surrounded by phospholipids, free cholesterol, and apolipoprotein B-100.

Heterogeneity of Lipoproteins

To a large extent the apolipoproteins dictate the metabolic fate of lipoproteins. A primary role for apolipoproteins is emulsification of lipid particles to assure that they remain water-soluble within circulation. For example, apolipoproteins B-100 (apoB-100) and apoB-48 are necessary for the assembly of VLDL and chylomicrons, respectively. In addition, cells have evolved other physiological functions for the apolipoproteins, which greatly enhance the metabolism of endogenous and exogenous lipids. ApoB-100 and apoE are ligands for cell surface receptors that are critical in appropriate delivery of lipid

to cells and in the maintenance of lipid homeostasis. Other apolipoproteins, such as apoCII and apoA-I, serve as cofactors for important lipid-modifying enzymes (lipoprotein lipase and lecithin:cholesterol acyltransferase, respectively) (Breckenridge *et al*, 1978).

While lipoproteins can be separated into classes and subclasses as a function of their hydrated densities, the particles within an isolated fraction are, nevertheless, heterogeneous in terms of their chemical compositions and physical properties. This heterogeneity both results from, and can modulate, metabolic events involved in plasma lipoprotein metabolism. Lipoproteins undergo continuous intravascular remodeling through the action of specific lipolytic enzymes, such as lipoprotein lipase and hepatic lipase, and lipid transfer proteins, including cholesteryl ester transfer protein and phospholipid transfer protein. In parallel, apolipoproteins, with the exception of apoB, can be lost from, or become associated with, individual lipoprotein particles. Lipoprotein particles with different chemical and physical characteristics within a given lipoprotein class can also have different biological properties. As an example, small dense LDL have a decreased affinity for the LDL receptor and an increased susceptibility to be oxidized (Campos *et al*, 1996; Tribble *et al*, 1992). Moreover, this heterogeneity has clinical relevance as it has been reported that individuals who have a predominance of small dense LDL ($d<1.040$ g/ml) have an increased risk for developing atherosclerosis (Austin *et al*, 1988; Lamarche *et al*, 1997).

Reverse Cholesterol Transport

Peripheral cells have the ability to both synthesize cholesterol and acquire exogenous lipoprotein-derived cholesterol but, with the exception of cells involved in the production of steroid hormones, lack the ability to catabolize it. As a consequence, the removal of excess cholesterol from peripheral cells and its transport to the liver is thought to be an important pathway in maintaining cholesterol homeostasis. It is thought that HDL participate in this reverse cholesterol transport. HDL are believed to be secreted by hepatocytes and to be generated within the circulation as products of the intravascular metabolism of triglyceride-rich lipoproteins. HDL are known to be implicated in delivery of cholesterol to steroid hormone-producing cells (Plump *et al*, 1996). A cell surface receptor that interacts with HDL has been cloned (Acton *et al*, 1994) and demonstrated to mediate the selective uptake of cholesterol by steroidogenic tissues (Rigotti *et al*, 1997). In addition to serving as a vehicle for delivery of cholesterol to these tissues, the inverse correlation between HDL levels and coronary heart disease in humans suggests that HDL has a protective effect against the development of atherosclerosis (Gordon *et al*, 1977). To support the protective role of HDL, transgenic mice that overexpress a transgene encoding the major protein component of HDL, apoA-I, are protected against the development of diet-induced atherosclerosis (Rubin *et al*, 1991). While it is not clear how HDL protect against coronary heart disease, it is thought that HDL may be responsible for an early step in reverse cholesterol transport (Glomset, 1968). A subpopulation of HDL particles has been shown to promote the removal of

cholesterol from cells by acting as an acceptor of free cholesterol from the cell membrane. The HDL particles could then transport cholesterol directly to the liver. Alternatively, through the action of lipid transfer proteins, cholesterol could be transferred to apoB-containing lipoproteins which could, in turn, deliver cholesterol to the liver for receptor-mediated uptake (Fielding and Fielding, 1995).

Section III: Apolipoprotein B

ApoB is synthesized in the liver and the intestine and is a major component of VLDL, IDL, LDL and chylomicrons. Elevated plasma concentrations of apoB and / or apoB-associated cholesterol increase the risk of developing premature coronary artery disease. The apoB gene has been mapped to human chromosome 2 (Knott *et al,* 1986 and Law *et al,* 1985). In human liver, apoB is secreted as a 4536 amino acid polypeptide with a calculated molecular weight of 513 kDa. The apparent molecular weight of apoB by SDS-polyacrylamide gel electrophoresis is 550 kDa due to glycosylation. In the intestine, the apoB transcript undergoes tissue-specific mRNA editing (Powell *et al,* 1987; Chen *et al,* 1987). As a result, the intestine-derived apoB (as found in chylomicrons) is truncated and is composed of the amino-terminal 2152 amino acids (48%) of the full-length apoB. Full-length apoB, as synthesized in the human liver, is called apoB-100 and the truncated form, synthesized in the intestine, is called apoB-48. In some rodents, there is also hepatic apoB mRNA editing and, as consequence, in rats

and mice, there is secretion of apoB-48 by both the liver and the intestine (Hodges and Scott, 1992).

Biosynthesis of Apolipoprotein B-100

Apo B-100 is synthesized primarily in the liver. Although the secretion of apoB requires its association with lipids, the apoB-100 polypeptide possesses several characteristics common to other secretory proteins. It is synthesized as a precursor with a 27 amino acid signal peptide that directs the nascent polypeptide to the endoplasmic reticulum (ER). The signal peptide contains a positively charged amino-terminal sequence, a central hydrophobic core region, and a more polar carboxy-terminal region that ends in the cleavage site. A length polymorphism involving the hydrophobic core region of the signal peptide of apoB has been described (Boerwinkle *et al*, 1989; Visvikis *et al*, 1990). While this polymorphism in the apoB signal peptide has been shown to affect the rate of translocation of a reporter protein across the membrane of rough ER in yeast (Sturley *et al*, 1994), it does not appear to influence apoB levels in humans (Boerwinkle *et al*, 1990).

For many genes, the primary control point in the regulation of expression is the frequency of transcription initiation, which depends on the interaction of transcription factors with promoter or enhancer elements. Such interactions control apolipoprotein gene expression during development and differentiation and govern tissue specific gene

expression and changes in expression in response to hormonal and metabolic stimuli. Sequences dictating the tissue-specific expression of apolipoprotein genes have been identified, (Sastry *et al*, 1988; Leff *et al*, 1989). In the case of hepatic apoB production, however, control is largely post-transcriptional at the level of translocation across the ER and intracellular degradation of nascent apoB polypeptides (Borchardt and Davis, 1987; Yao *et al*, 1997). Studies in primary rat hepatocyte cultures showed that VLDL-TG and apoB secretion is suppressed by insulin (Phung et al, 1997) and cellular apoB concentration are reduced due to increased intracellular degradation of newly synthesized apoB (Sparks and Sparks, 1990). In pulse-chase studies, insulin inhibited the transport of apoB from the ER to the Golgi (Patsch *et al*, 1983). In HepG2 cells, apoB mRNA levels were unaffected by oleate or insulin treatment in spite of alterations in apoB secretion (Dashti *et al*, 1989). Finally, apoB mRNA levels were unchanged in experimental animals whose circulating apoB levels were modulated by dietary manipulation (Sorci *et al*, 1989; Kroon *et al*, 1986; Kushwaha *et al*, 1991).

Assembly of apoB-containing lipoproteins appears to be, at least in part, a co-translational event (Borén *et al*, 1992). Early electron microscopic analyses of VLDL assembly in rat hepatocytes indicated that much of the lipidation of apoB occurred in the Golgi complex (Glaumann *et al*, 1975, Howell *et al*, 1982). More recently, however, it has been shown that the lipid to protein ratio of VLDL is the same in both ER and Golgi which would suggest that the assembly of rat hepatic VLDL is essentially completed within the ER (Rusiñol *et al*, 1993). Working with rat hepatoma cells, Boren et al.

demonstrated that B100-VLDL could be assembled in the microsomal lumen within 3 minutes, suggesting that B100-VLDL is assembled during or immediately after translation (Borén *et al*, 1994). Experiments in which truncated apoB variants were expressed in rat hepatoma cells demonstrated that the lipid content of these primordial particles was a function of the length of the apoB polypeptide (Yao *et al*, 1991; McLeod *et al*,, 1994). This is consistent with to a co-translational VLDL assembly mechanism. However, cumulative evidence suggests that the majority of lipid is incorporated into apoB to form VLDL after its translation. In McA-RH7777 cells, primordial B48-HDL could be converted to B48-VLDL (Borén *et al*, 1994). In addition, membrane-associated lipid-poor apoB100 can be a precursor for B100-VLDL. (Rustaeus *et al*, 1995). The post-translational lipidation step is independent of the length of the apoB chain but appears to be mediated by hydrophobic sequences within apoB48 (McLeod *et al*, 1996). This independence of lipidation on polypeptide length of the second step is likely responsible for ability of truncated apoB variants as short as apoB-37 to form VLDL (Linton *et al*, 1993). The co-translational step in the lipidation of apoB is clearly dependent on the activity of the ER resident protein, microsomal triglyceride transfer protein (MTP) (Leiper *et al*, 1994; Gordon *et al*, 1994; Wang *et al*, 1996). Whether MTP is necessary for post-translational lipidation is unclear (Gordon *et al*, 1996; Wang *et al*, 1997). Co-immunoprecipitation studies have demonstrated that MTP is associated with apoB in cell extracts (Wu *et al*, 1996).

In cell culture systems, most of the newly synthesized apoB-100 molecules are degraded within the cell and are never secreted. It is thought that the rate of apoB secretion is determined by the rate of apoB intracellular degradation which in turn is determined by lipid availability (Borchardt and Davis; 1987, Dixon *et al*; 1991, Sato *et al*, 1990; Yao *et al*, 1997). This allows the liver to respond to the changes in lipogenic state by varying the number of VLDL particles secreted and by changing their composition (Fungwe *et al*, 1992; Nakata *et al*, 1977). Davis and colleagues (Davis *et al*, 1990) demonstrated that translocation of apoB across the ER membrane could become arrested in rat primary hepatocytes. If apoB is misfolded within the ER or there is insufficient lipidation, apoB can undergo degradation. This can apparently occur at two distinct sites, within the ER lumen or in cytosol (Wu *et al*, 1997). The cysteine protease ER-60 has been implicated in apoB degradation within the ER lumen (Adeli *et al*, 1997). In addition, apoB has been shown to associate with the cytosolic heat shock 70 and to undergo degradation through the ubiquitin-proteosome pathway. Degradation through the latter pathway may be specific for partially translocated apoB nascent polypeptides and involve retrograde translocation (Fisher *et al*, 1997). This co-translational degradation of the nascent apoB can be decreased by the activity of MTP (Wang *et al*, 1996).

Lipid synthesis is another important regulatory step in the assembly of apoB-containing lipoproteins. It was shown that phospholipid synthesis is essential for secretion of apoB-containing lipoproteins (Yao *et al*, 1988). The initial interaction of

phospholipids with nascent apoB may create the lipoprotein surface that is necessary to further accommodate neutral lipids. The subsequent addition of triglyceride to the lipoprotein particle is needed for the maturation and secretion of the lipoprotein (Borén *et al*, 1993). Stimulation of apoB secretion by addition of oleic acid to the incubation medium has been demonstrated both in HepG2 cells (Dixon *et al*, 1991) and in rat hepatoma McArdle-7777 cells (White *et al*, 1992). In both cases, this effect was mediated by decreased intracellular degradation of apoB not by increased apoB synthesis. Wu *et al*, have suggested that oleic acid acts by promoting triglyceride synthesis and showed that oleic-acid stimulation of apoB secretion could be blocked by an inhibitor of fatty acyl coenzyme A synthase (Wu *et al*, 1994). However, others have presented evidence that it is cellular cholesteryl ester mass, not TG mass, that regulates the apoB secretion in HepG2 cells (Avramoglu *et al*, 1995). Impaired phospholipid biosynthesis causes a decrease in the number of VLDL particles in the Golgi, but no change in the number of particles in the ER (Verkade *et al*, 1993). Within the Golgi complex mature VLDL particles are packaged into secretory vesicles, exported to the plasma membrane and released into the circulation.

ApoB mRNA editing

The small intestine synthesizes a shorter form of apoB from the same gene that is responsible for apoB-100 production in the liver (Glickman *et al*, 1986). As indicated above, this shorter form, apoB-48, consists of the N-terminal 2152 amino acids of apoB-

100 and is necessary for the synthesis, assembly, and secretion of the triglyceride-rich chylomicrons (Kane, 1983). Some apoB-100 may also be synthesized in intestinal cells (Dullart *et al*, 1986). In rats and mice, both liver and intestine produce apoB-48 (Hodges and Scott, 1992). The tissue-specific post-transcriptional editing of apoB mRNA that is responsible for the generation of apoB-48 causes the conversion of codon 2153 from CAA, that encodes Gln, to UAA, a translational stop codon (Powell *et al*, 1987; Chen *et al*, 1987). The catalytic subunit of the apoB mRNA editing complex was cloned and characterized (Teng *et al*, 1993). The protein, apoB mRNA-editing enzyme catalytic polypeptide 1 (APOBEC-1), is a cytidine deaminase (Navaratnam *et al*, 1993) that is capable of deaminating nucleotide 6666 in apoB mRNA causing its conversion from C to U. The sequence specificity of editing appears to involve a mooring sequence, a spacer sequence and a regulator region that flank C-6666 within the apoB message (Hodges and Scott, 1992). A second constituent of the editing complex, the APOBEC-1-interacting protein (ABBP-1), that appears to be necessary for editing activity, has recently been cloned (Lau *et al*, 1997).

Primary structure of apoB

The complete amino acid sequence of apoB-100 was deduced from its cDNA sequence and, in part, from direct protein sequencing. (Knott *et al*, 1986; Yang *et al*, 1986, 1989; Law *et al*, 1986; and Cladaras *et al*, 1986). Human apoB-100 is encoded by a mRNA of 14,121 nucleotides that consists of a 5' untranslated region of 128

nucleotides, a coding region of 13,689 nucleotides, the TAA stop codon, and a 3' untranslated region of 301 nucleotides. Following translation, the 27-residue precursor peptide is cleaved before secretion of apoB-containing lipoprotein particles to produce a 4536 amino acid mature protein. The gene coding apoB exists as a signal copy at the end of the short arm of chromosome 2 (2p23) and spans 43 kb and contains 28 introns and 29 exons (Knott *et al*, 1985; Blackhart *et al*, 1986). Exon 26 is composed of 7275 base pairs and is amongst largest exons that have been characterized.

It is thought that basic amino acids of apoB participate in binding to the LDL receptor, possibly through ionic interactions with acidic residues within the ligand-binding domain of the receptor. This was originally based on the observation that selective chemical modification of Arg and Lys residues on apoB-100 in LDL eliminates its ability to mediate lipoprotein binding to the LDL receptor (Mahley *et al*, 1977; Weisgraber *et al*, 1978). This is also the case for the other ligand of the LDL receptor, apoE. When the apoB-100 primary sequence was analyzed for local regions that are enriched in basic residues and for similarity to the apoE LDL receptor-binding domain, two regions, 3147-3157 (A site) and 3359-3367 (B site) were identified as potential apoB LDL receptor-binding sites (Knott *et al*, 1985). In human apoB, a disulfide bridge is thought to exist between cysteines 3167 and 3297 that may bring site A and site B into spatial proximity in apoB tertiary structure (Knott *et al*, 1986; Milne *et al*, 1989). These two cysteines are not, however, conserved in apoBs of other species (Law and Scott, 1990). The strong net positive charge that characterizes site B in human apoB is also

found in apoB of other species, while this is not the case for site A (Law and Scott, 1990).

There is experimental evidence that site B may be directly implicated in binding to the LDL receptor. A synthetic peptide that represents human apoB residues 3345-3381 can mediate lipoprotein binding to the LDL receptor (Yang *et al*, 1986) and the replacement of basic amino acids by neutral amino acids within site B by mutagenesis eliminates apoB interaction with the LDL receptor (Borén *et al*, 1998). Milne *et al*, (Milne *et al*, 1989) have tested a panel of anti-apoB mAbs for their ability to block binding of LDL to LDL receptor. Antibodies that were specific for epitopes situated between apoB residues 3000-4000 block the binding of LDL to LDL receptor, while mAbs whose epitopes are located outside of this region had no effect. Furthermore, it was demonstrated that epitopes between residues 3000-4000 were inaccessible in LDL-LDL receptor complexes whereas mAbs that were specific for epitopes elsewhere in apoB could bind, in a stoichiometric manner, to receptor-bound LDL. The apoB LDL receptor-binding domain has also been localized by the respective abilities of truncated apoB variants to mediate binding to the LDL receptor. An apoB variant that terminates at residue 3387 can bind to the LDL receptor (Krul *et al*, 1992) whereas a second variant that terminates at residue 3040 can not (Welty *et al*, 1995).

Secondary structure of apoB

The large size and hydrophobicity of apoB have been the major obstacles in structural studies of apoB. Analysis of the apoB primary structure using an algorithm for prediction of secondary structure suggested that native apoB would contain 43% α-helix, 21% β-sheet structure, 20% random coil structure, and 16% β-turn (Chan, 1992). This predicted secondary structure is very different from that of the smaller exchangeable apolipoproteins, such as apoA-I and apoE, that are primarily composed of amphipathic α helices. Yang and colleagues (Yang *et al,* 1989) have proposed that apoB is composed of 5 domains that differ in their relative affinities for lipid. This was based on the identification of peptides that remained with, or dissociated from, the LDL particle after trypsin digestion. In this model, the amino terminal 1000 amino acids of apoB compose a domain that has relatively low affinity for lipids. A second domain (residues 1000-1700) contains both trypsin-releasable (TR) and trypsin-nonreleasable (TN) peptides. Domain 3 (residues 1700-3000) is composed primarily of TN peptides that would indicate that this region is tightly associated with lipids. Domain 4 (residues 3070 to 4100) that includes the putative apoB LDL receptor-binding site was identified as containing both TN and TR peptides. The carboxy-terminal domain appears to have a high affinity for lipid (Figure 1-4A). Segrest and colleagues (Segrest *et al*, 1994) have also proposed a pentapartite model for apoB secondary structure based on a sophisticated computer-assisted analysis of apoB primary structure (Figure 1-4B). In this model, three amphipathic α-helices are separated by two amphipathic β-sheets. The first α-helix is

predicted to fold as a globular domain with low affinity for lipids. Two β-sheet-enriched regions would bind to lipid irreversibly whereas the α-2 and α-3 domains would exhibit reversible lipid-binding. Recently, it has been shown that when short amphipathic β sheet peptides composed of apoB residues 1306-1642, 1643-1695, and 1696 to 1880, were expressed in rat hepatoma cell as apoA-I/apoB fusion proteins, they were able to form lipoprotein particles having the density of VLDL (McLeod *et al*, 1996). This would suggest that regions of apoB that have an amphipathic β structure could participate in the recruitment of core lipids during VLDL assembly. It has recently been proposed that the β1 and β2 domains of apoB directly bind to core lipids and provide a relatively rigid backbone whereas the α-2 and α-3 domains are flexible and permit the lipoprotein to accommodate varying amounts of lipid during its intravascular metabolism (Chauhan *et al*, 1998).

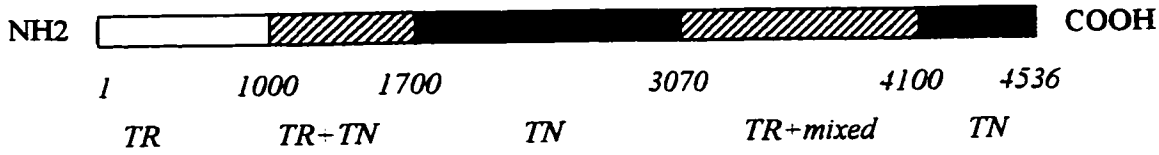


Figure 1-4A: Domain structure of apolipoprotein B in LDL predicted by the relative ability of peptide to remain associated with the particles after trypsin digestion (*Yang et al, 1989*).
See text for details.
TR: Trypsin releasable, TN: Trypsin nonreleasable

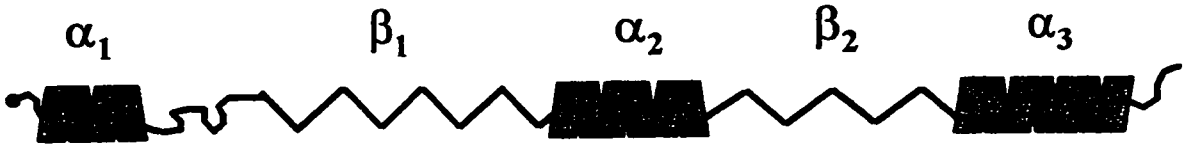


Figure 1-4B: Secondary structure of apolipoprotein B in LDL predicted by computer modeling (*Segrest et al, 1994*).
The pentapartite model of apoB secondary structure is composed of the: α_1 58-476, β_1 827-1961, α_2 2103-2560, β_2 2611-3867, and α_3 4061-4338 domains.

Chatterton and colleagues (Chatterton *et al*, 1991, 1995) have used immunoelectron microscopy to study the topography of apoB on the surface of LDL. Based on these analyses a "ribbon and bow" model for the conformation of apoB in native lipoproteins was proposed (Chatterton *et al*, 1995). According to this model, the amino-terminal 89% of apoB is in an extended conformation and wraps around the particle (the ribbon). The carboxy terminal 11% doubles back to cross the ribbon close to the putative apoB LDL receptor-binding site (the bow) (Figure 1-5). It was predicted that the "bow" may limit the accessibility of the LDL receptor-binding site (Chatterton *et al*, 1995) and thus could modulate the ability of the particle to be taken up by cells. Recent studies have provided strong evidence that the apoB carboxy terminus does, in fact, serve as a negative regulator of apoB-mediated binding to the LDL receptor (Borén *et al*, 1998).

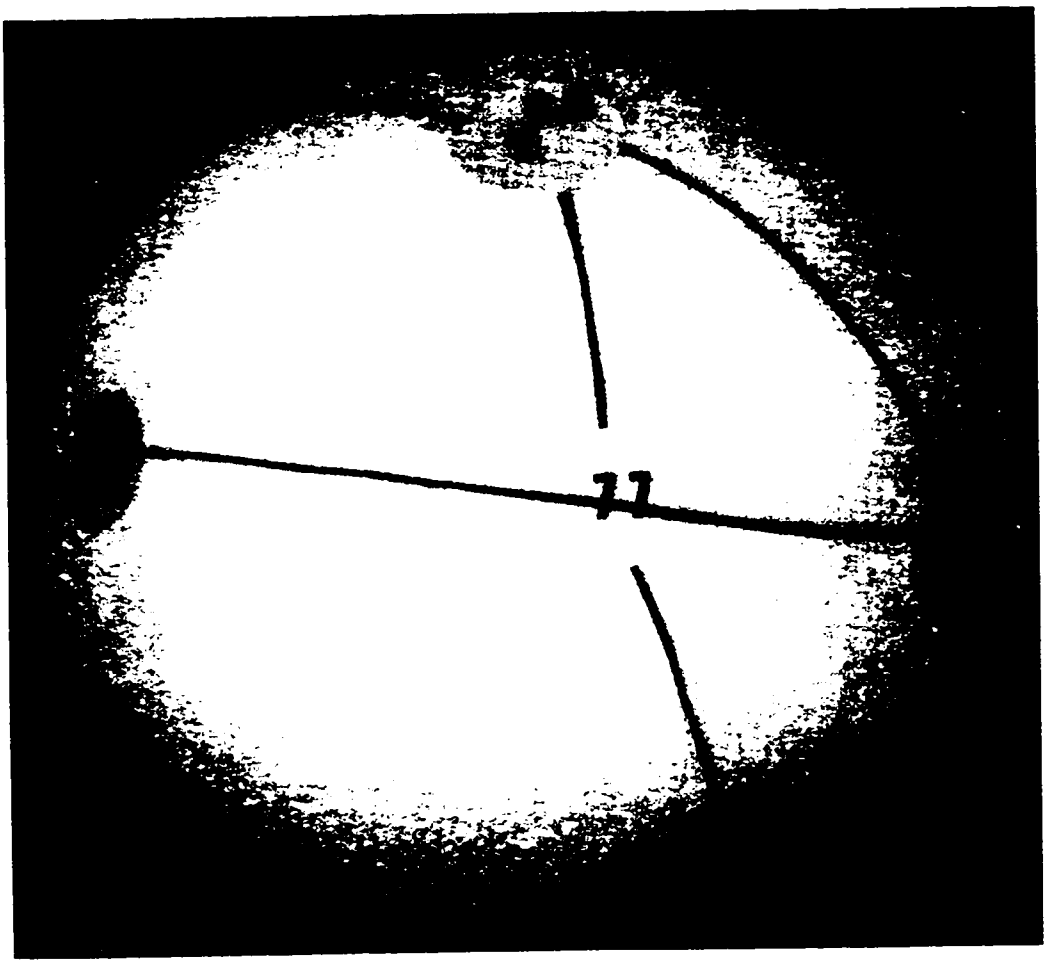


Figure 1-5: Ribbon and Bow model for apoB
ApoB wraps around the LDL particle and finishes a circle at a point close to B89 (89% of full-length apoB-100) (the ribbon). The orientation of the C-terminal 11% of apoB (the bow) moves backward from B89 to B92, and then crosses the ribbon near the apoB LDL receptor-binding site (apoB77) (Chatterton *et al*, 1995).

Heterogeneity of apoB-containing lipoproteins that alters apoB conformation and function

ApoB structure is also greatly affected by the physical and chemical properties of the lipoprotein particles with which apoB is associated. Conversion of VLDL to LDL in plasma by lipolysis and lipid transfer generates heterogeneity in apoB-containing

particles with respect to their size, density and lipid and apolipoprotein composition. This produces heterogeneity in apoB conformation that is, in turn, manifested in the immunoreactivity, protease sensitivity and receptor-binding properties of the lipoproteins. As VLDL particles are converted from large triglyceride-rich VLDL to the smaller cholesteryl ester-rich LDL, there are concomitant increases in apoB epitope expression (Tikkanen et al. 1984, Marcel et al. 1988) and alterations in the accessibility of protease-sensitive sites within apoB (Chen et al, 1989, 1991). The increase in apoB immunoreactivity of the particles during their intravascular metabolism coincides with a switch from apoE- to apoB-mediated binding to the LDL receptor (Krul et al. 1985, Marcel et al. 1988). Ishikawa et al, (Ishikawa et al, 1988) have, furthermore, proposed that a change in apoB conformation induced by the lipolysis of nascent VLDL is necessary for incorporation of apoE into the particles.

LDL, themselves, are heterogeneous in size, charge, hydrated density, and lipid composition and can be separated into distinct subclasses. This LDL heterogeneity has important clinical ramifications. Sniderman and colleagues first described a group of subjects with an increased risk of coronary artery disease who have normal plasma cholesterol levels and increased concentrations of apoB, a phenotype that was named hyperapobetalipoproteinemia (Sniderman et al, 1980). The LDL from these patients are characterized by an over-representation of small dense lipid-poor particles when compared to the LDL of normal subjects. It is now recognized that most individuals can be classified into two groups based on their LDL subclass pattern. The majority of

individuals have a pattern A LDL profile in which there is a predominance of larger more buoyant particles. The LDL of individuals with the pattern B are characterized by a predominance of small LDL particles, a phenotype which is associated with increased plasma triglyceride levels, decreased HDL cholesterol, and increased risk of coronary artery disease. The determination of LDL subclass pattern appears to have a genetic component with a gene for LDL particle size being linked to a locus on human chromosome 19 near the LDL receptor gene (Austin *et al*, 1988; Nishina *et al*, 1992). A factor that may contribute to the higher risk associated with the pattern B phenotype is the increased susceptibility of small dense LDL to oxidative modification (de Graaf *et al*, 1991). LDL heterogeneity also dictates apoB conformation and function. It has been observed that small dense as well as the most buoyant LDL particles have decreased reactivity with cell surface LDL receptors compared to LDL with intermediate density (Nigon *et al*, 1991; Campos *et al*, 1996). Moreover, the receptor-binding activities of LDL vary as a function of the LDL subclass pattern of the individual from whom the lipoproteins were isolated (Campos *et al*, 1996). The affinity of the buoyant LDL subfractions isolated from pattern B subjects is more dependent on apoE while buoyant LDL from pattern A individuals is mediated primarily by apoB. It has also been shown that the expression of epitopes close to the apoB LDL receptor-binding site differs among LDL subclasses (Teng et al, 1985).

There have been several attempts to determine the relative contributions of particle size and chemical composition to the modulation of apoB conformation and

function. It has been reported that modification of the triglyceride content of LDL by *in vitro* lipid exchange reactions (that produce little change in particle size) alter reactivity of the particles with cell surface receptors (Aviram *et al*, 1988; Kinoshita *et al*, 1990) and mAbs (Kunitake *et al*, 1990; Aviram *et al*, 1988 Kinoshita *et al* 1990) as well as apoB conformation as measured by ^{13}C-NMR (Aviram *et al* 1988). McKeone and colleagues (McKeone *et al*, 1993) compared triglyceride-rich LDL, either produced by *in vitro* lipid transfer or isolated from hypertriglyceridemic subjects, to normal LDL and found that no differences were detectable in their respective circular dichroic spectra. On the other hand, normal and triglyceride-rich LDL differed in reactivity with a mAb specific for an epitope near the amino terminus of apoB, in accessibility of protease cleavage sites, and in surface charge. Interestingly, triglyceride enrichment of the LDL decreased its uptake by fibroblasts whereas uptake of triglyceride-enriched LDL by HepG2 cells was increased. Based on the above studies, it was concluded that the conformation and function of apoB were modulated by the core composition of the LDL particle. Recent studies in which fluorescence depolarization of probes located either in the core or at the water / phospholipid interface of native and reconstituted LDL particles was monitored suggest that the motional states of lipids in the core and surface domains are relatively independent and that neither the conformation of apoB nor its ability to bind to the LDLr are likely to be affected by changes in the surface order caused by alterations in core lipids (Kroon, 1994). The author does, however suggest that LDL core composition could potentially influence apoB conformation by either affecting the surface / core distribution of unesterified cholesterol or through a direct interaction of apoB with neutral lipid.

Evidence that apoB does in fact penetrate the surface monolayer of LDL and establish hydrophobic interactions with core lipids has been obtained from experiments in which LDL was studied by limited proteolysis (Yang *et al*, 1989) and infrared spectroscopy (Banuelos *et al*, 1995). We have recently observed that about 70% of the LDL triglyceride remains bound to apoB following delipidation with sodium deoxycholate (Chauhan *et al*, 1998).

In contrast to the above studies, other investigators have concluded that LDL core composition is not a primary determinant of apoB conformation and function. Galeano and colleagues (Galeano et al, 1994) have demonstrated that the abilities of LDL to react with the LDL receptor of cultured human fibroblasts and with mAb 3F5, specific for an epitope near the LDL receptor binding site, appear to be independent of LDL triglyceride content but change as a function of LDL size. Moreover, changes in immunoreactivity and the affinity of LDL for the LDL receptor were correlated with changes in the apoB circular dichroic spectra. Chen and colleagues (Chen *et al*, 1994), who characterized normal and triglyceride-rich apoB by circular dichroic spectropolarimetry, by limited proteolytic digestion and by determination of the ability of the particles to bind to cell surface receptors also concluded that LDL particle size rather than core composition is the primary modulator of apoB conformation and function. Compositional analysis of LDL subspecies indicates that, as the core volume of the LDL particle decreases, the ratio of surface to core lipid decreases and it has been estimated that alterations in the conformation of apoB would be required to compensate for the surface changes

(McNamara et al, 1996). Again, this would be consistent with LDL particle diameter as being an important determinant of apoB conformation and function.

Post-translational modification of apoB

ApoB has 19 potential N-linked glycosylation sites, of which 16 are actually glycosylated in LDL, giving a molecular weight of 550,000. ApoB contains 8-10% carbohydrate in the form of galactose, manose, N-acetylglucosamine, and sialic acid residues (Swaminathan, *et al*, 1976: Chan, 1992). The importance of glycosylation in apoB secretion and metabolism has not been established. There are 25 cysteine residues in apoB100 that are asymmetrically distributed with a concentration in the amino terminal region. Sixteen of the cysteine residues are involved in intramolecular disulfide linkage (Yang *et al*, 1989) and, with the exception of the two N-terminal Cys pairs, Cys-1 / Cys-3 and Cys-2 / Cys-4, all are in disulfide linkage with immediately neighboring Cys residues. Cys-15, -16, -17, -18, -19 -22, -23, -24 and -25 exist in the reduced form (Sommer *et al*, 1991 and Coleman *et al*, 1989). Cys-25 (residue 4326) of apoB is responsible for the intermolecular disulfide bond with cysteine 4057 of apo(a) in Lp(a) particles (Callow *et al*, 1995; McCormick *et al*, 1995).

Several studies have shown that apoB is secreted as a phosphoprotein. Under normal physiological conditions, multiple serine groups are phosphorylated (Guevara *et al*, 1995). Livers from diabetic rats have been shown to secrete apoB that contains increased amounts of both phosphoserine and phosphotyrosine residues (Davis, R. A.,

1984). There are sequences of apoB that are similar to known phosphorylation signals but the specific sites involved in phosphorylation have not been defined. Serine phosphorylation of apoB occurs early in the secretion pathway in the ER. Once secreted, apoB is dephosphorylated in plasma. Several functions for apoB phosphorylation have been proposed, including regulation of secretion, targeting and protein stabilization (Davis *et al*, 1984). ApoB secreted by HepG2 cells has also been reported to contain covalently linked palmitate and stearate adducts. The multiple fatty acid acylation of apoB may play a role in the lipidation of apoB (Lee, 1991).

Genetic polymorphism of apolipoprotein B and molecules associated with apoB secretion

Much of our understanding of the structural and functional aspects of apoB has come from studies of inherited defects in apoB metabolism. Several relatively rare genetic disorders of apoB metabolism are directly linked to the apoB gene or to molecules that are implicated in the biosynthesis, assembly, and catabolism of apoB-containing lipoproteins. In addition, there are genetic polymorphisms of apoB that do not appear to have a major effect on its metabolism.

1. Ag polymorphisms of apoB-A number of genetic polymorphisms of apoB were discovered in the early 60s. The Ag (antigen group) genetic polymorphisms of LDL were initially identified serologically, based on the reactivity of anti-LDL isoantibodies present in the serum of multi-transfused individuals (Allison and Blumberg, 1961). Five

1961). Five pairs of epitopes were identified by these studies, with each pair representing alleles at closely linked loci. With the cloning and sequencing of the apoB gene, the molecular basis of all of these polymorphisms has been determined. Single base pair polymorphisms at five sites within the coding sequence of the apoB gene are responsible for the apoB isoforms that are detected serologically. To date, there have been no striking associations established between any of the Ag haplotypes and hyperlipidemias.

*2. **Familial Defective ApoB***-It is well known that the inheritance of alleles that encode defective forms of the LDL-receptor leads to elevated plasma cholesterol levels and premature atherosclerosis. This condition has been called familial hypercholesterolemia (FH). Hyperlipidemia and premature atherosclerosis can also result from the inheritance of genes encoding variants of apoB and apoE that are defective in mediating lipoprotein binding to the LDL receptor and to cell surface proteoglycans. Familial defective apoB (FDB) is a co-dominantly expressed genetic disorder that results from a mutant apoB allele whose gene product binds poorly to the LDL receptor (Soria *et al*, 1989). The clinical phenotype of FDB shows some resemblance to that of FH, although it is usually less severe. The genetic basis for the most common form of FDB has been shown to be a G→A substitution at nucleotide 10,708 of the apoB coding sequence that changes codon 3500 from CGG, coding for Arg, to CAG, coding for Gln (Innerarity *et al*, 1987). FDB occurs with an allelic frequency of 1/500 in Western populations. More recently, it has been reported that another apoB mutation that changes residue 3531 from Arg to Cys gives a biochemical and clinical phenotype that is indistinguishable from that of FDB

(Pullinger *et al*, 1995). The apoB^{Gln3500} mutation was originally observed in a patient who showed reduced clearance of autologous LDL, when compared to clearance of LDL from a normal donor (Vega and Grundy, 1986). Moreover, *in vitro*, the LDL of the patient had reduced affinity for the LDL receptor on cultured human fibroblasts. As a consequence of this reduced affinity for the LDL receptor, in FDB heterozygotes, there is a selective accumulation of LDL containing the apoB^{Gln3500} variant in plasma, and the defective particles can represent up to 87% of the circulating LDL (Arnold *et al*, 1994).

The poor reactivity of apoB^{Gln3500} LDL with the LDL receptor could indicate that, in normal LDL, Arg^{3500} participates directly in the interaction with the LDL receptor. Alternatively, Arg^{3500} may not be implicated in binding to the LDL receptor but its replacement by Gln may provoke a conformational change in apoB that alters the LDL receptor-binding site. There is now considerable evidence to suggest that the latter is, in fact, the case. Residue 3500 does not appear to contribute directly to apoB-mediated binding to the LDL receptor. A natural truncated apoB variant that terminates at residue 3386, and thus does not include residue 3500, binds to the LDL receptor with high affinity (Krul *et al*, 1992). Furthermore, a NMR spectral study revealed that, in normal human LDL, there are about 50 Lys with a pK of 8.9 ("active lysine") and 170 Lys with a pK of 10.5 ("normal lysine") (Lund-Katz *et al*, 1991). It was proposed that a maximum of ten of the active lysines are directly involved in binding to the LDL receptor. Analysis of LDL isolated from normal subjects and heterozygous FDB patients showed that LDL of the latter had fewer lysine residues with a pK of 8.9. When the analysis was repeated

on purified apoB^{Gln3500} LDL, at least seven lysine residues appear to have been redistributed from the active to the normal pool when compared to wildtype LDL. These results suggest that the Arg^{3500}→Gln substitution induces a major local conformational change in the receptor-binding region of apoB (Lund-Katz *et al*, 1991). The putative conformational change induced by the substitution at apoB residue 3500 would be responsible for the loss of over 95% of receptor-biding activity (Arnold *et al*, 1994). Marz *et al*, have recently identified the first homozygous FDB patient (Marz *et al*, 1993). Plasma LDL cholesterol levels were elevated but were lower than those commonly seen in FH patients. This would be anticipated as apoE-mediated lipoprotein binding to the LDL receptor would not be compromised in the FDB homozygote. Large buoyant LDL were cleared relatively normally in this patient by an apoE-mediated mechanism whereas there was accumulation of small dense LDL particles in the plasma (Marz *et al*, 1993).

3. ***Hypobetalipoproteinemia***-Hypobetalipoproteinemia is inherited as a co-dominant disorder that is characterized by decreased or absent plasma apoB-containing lipoproteins. Heterozygous individuals typically have total cholesterol and LDL cholesterol levels that are 25-50% of those of normal subjects. Co-segregation studies have shown that the mutations are within the apoB gene (Leppert *et al*, 1988). All the mutations to date resulted in truncated apoB variants and most have been initially identified at the protein level by SDS-PAGE gel electrophoresis. More than 20 different mutations have been described in many probands (Young and Linton, 1991). Affected individuals express apoB of different lengths. Apo B variants that represent 31% to 46%

of the amino-terminal of apoB are found associated with particles having the size and hydrated density of HDL. Longer truncated variants are assembled into larger lipoproteins of lower density. The frequency of apoB mutations causing hypobetalipoproteinemia is around 0.01% in the general population (Wagner *et al*, 1991), and this condition may decrease risk of coronary artery disease. When present in the homozygous form, hypobetalipoproteinmic patients have trace levels of apoB-containing lipoproteins and present with a clinical phenotype that is similar to that of abetalipoproteinemia. The clinical severity appears to be inversely related to the length of the truncated apoB protein and the ability of the variant apoB to be assembled into lipoproteins. A patient who was homozygous for an allele encoding apoB-25 (25% of full length apoB-100), presented with symptoms that are characteristic of abetalipoproteinemia (Huang *et al*, 1989). In contrast, several compound heterozygous patients were asymptomatic. In these latter cases, at least one of the truncated apoBs was capable of associating with lipid and of participating in the transport of fat-soluble vitamins.

4, ApoB deficiency disease: Abetalipoproteinemia-Abetalipoproteinemia is a recessive genetic disease in humans that is characterized by the virtual absence of apoB-containing lipoproteins in plasma. Plasma cholesterol (40 mg / dl) and triglyceride (10 mg / dl) are very low and all of the circulating cholesterol is found in the HDL fraction. Abetalipoproteinemia has been previously shown not to be associated with mutations in the apoB gene. In 1992, Wetterau and colleagues demonstrated that MTP was absent

from hepatocytes and intestinal enterocytes of abetalipoproteinemic subjects (Wetterau *et al*, 1992). MTP, which is found exclusively in the lumen of microsomes isolated from liver and intestine of normal subjects, is a soluble heterodimer composed of a small subunit of 55 kDa, protein disulfide isomerase, and a large subunit of 97 kDa (Sharp *et al*, 1993). As discussed above, MTP facilitates the assembly of TG-rich particles in cultured rat hepatocytes (Wetterau *et al*, 1992) and recruits triglyceride for association with apoB in a co-translational lipidation process (Wang *et al*, 1996). While patients with abetalipoproteinemia have normal apoB synthesis (Lackner *et al*, 1986; Bouma *et al*, 1990), the nascent apoB polypeptide chain can be neither lipidated nor secreted and, as a consequence, undergoes intracellular degradation.

Section IV: Modification of LDL and Its Implication in Atherogenesis

One of the characteristic features of the atherosclerotic plaque is the presence of lipid-laden cells. Based on their appearance they have been named foam cells and have been shown to be primarily derived from cells of the monocyte / macrophage lineage. It has been demonstrated that the regulatory homeostatic mechanisms that are inherent in the LDL receptor pathway prevent the conversion of monocytes / macrophages into foam cells when they are incubated in the presence of exogenous LDL (Brown and Goldstein, 1986). Hence, it was postulated that lipoproteins undergo post-secretory modifications which render them competent for endocytosis via a pathway that is distinct from the LDL receptor pathway. This alternate pathway would lack feed-back mechanisms and, when

exposed to modified LDL, would allow an unregulated accumulation of cholesterol esters within the cell. It was shown that exposure of monocytes to acetylated-LDL resulted in the cells acquiring a foam cell phenotype (Brown *et al*, 1980). While acetylated-LDL is not believed to be physiological, other modifications to LDL that have been shown to occur *in vivo* can also promote foam cell formation. Modification of LDL with malondialdehyde, a product of arachidonic acid metabolism, causes foam cell formation *in vitro* (Fogelman *et al*, 1980). Oxidation of LDL, a biological modification, also leads to a foam cell formation (Steinberg *et al*, 1989). It is now believed that oxidative modification of LDL plays a pivotal role in atherogenesis.

By the early 1980s, there was considerable evidence that cholesterol accumulation in the developing atherosclerotic lesion was probably due to the uptake of some modified form of LDL by a scavenger receptor that was present on the cell surface of macrophages (Henriksen *et al*, 1981). Incubation of LDL with endothelial cells, smooth muscle cells and macrophages in culture could induce the oxidation of polyunsaturated fatty acids on LDL. The lipid peroxides that are formed may fragment fatty acyl chains and may attach covalently to apoB, rendering the modified LDL a ligand for scavenger receptor. Immunological studies demonstrated oxidatively modified LDL present in atherosclerotic lesions (Yla-Herttuala *et al*, 1989) and animal experiments showed a protective effect of the antioxidant drug probucol (Kita *et al*, 1987).

The oxidative damage to LDL generates a series of modified forms of LDL that are in a number of ways more atherogenic than native LDL. It is widely accepted that LDL could increase the adherence of circulating monocytes to the arterial endothelial cells. At the same time, adhered monocytes increase the entry of LDL into the intima where LDL undergo oxidative modification catalyzed by any of the major cell types found in arterial lesions such as endothelial cells, smooth muscle cells, or macrophages. Even minimally-oxidized LDL could increase the adherence and penetration of monocytes into the intima. Severely oxidized LDL is directly chemotactic for monocytes and is a ligand for at least two cell surface receptors, the scavenger receptors A (SR-A) and B-I (SRB-I) (Kodama *et al*, 1990; Acton *et al*, 1994; Steinberg, 1997).

Another form of modification, non-enzymatic glycation (glycosylation), also occurs *in vivo*. Glucose reacts non-enzymatically with protein amino groups to initiate glycation, the early stage of the Maillard reaction. This process begins with the conversion of reversible Schiff base adducts to stable, covalently-bound Amadori rearrangement products (Figure 1-6). The levels of substitution of various plasma proteins with Amadori products are elevated in proportion to the degree of hyperglycemia in diabetes mellitus. In the intermediate stage of the Maillard reaction, the Amadori products can then undergo multiple dehydration and rearrangements to produce highly reactive carbonyl compounds called advanced glycation end products (AGEs) (Kennedy and Baynes, 1984). The structural features of AGEs are not well characterized due to the heterogeneity of the compounds derived from the Amadori rearrangement. Only a few

glycation products have been structurally identified, such as carboxy-methylysine (CmLys) (Fu *et al*, 1996) and pentosidine (Dyer *et al*, 1991).

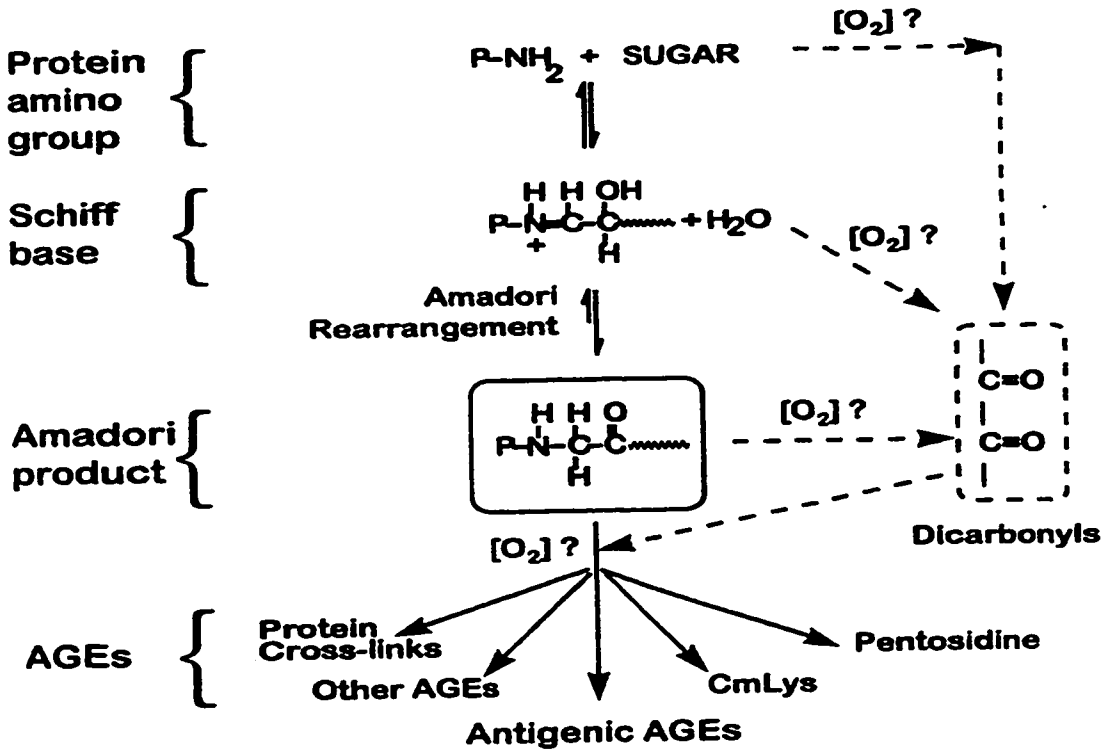


Figure 1-6. Pathway for non-enzymatic generation of advanced glycation end products (AGEs).
Free amino groups from protein react with reducing sugar to form unstable and reversible Schiff bases. The rearrangement of Schiff bases produce reactive Amadori intermediates that can then be converted to a variety of reactive substances, collectively called AGEs (Adapted from Booth *et al*, 1997).

Diabetes and end-stage renal disease are major risk factors for the development of atherosclerosis. Plasma lipoprotein profiles are abnormal in these conditions with an elevation in the level of apolipoprotein B containing lipoproteins. Another characteristic of diabetes and renal diseases is the elevated levels of protein-bound AGEs in the circulation (Makita *et al*, 1991). Glycated LDL has been recently identified *in vivo*

(Bucala *et al*, 1994). While AGE-modified LDL can not bind to the LDL receptor (Bucala *et al*, 1995), it can be taken up by other cell surface receptors that may be specific for the AGE adduct. A number of receptors for AGE-modified proteins have been identified, including the receptor for AGE (RAGE) (Skolnik *et al*, 1991), the macrophage scavenger A receptor (Horiuchi *et al*, 1996), p60, p90 (Yang et al, 1991), and galectin (Bierhaus *et al*, 1997). These receptors were identified by their ability to bind to AGE modified proteins, although the respective physiological roles of each receptor *in vivo* were not well understood. It has been demonstrated that upon binding to the receptors, AGEs stimulate a wide range of cell-mediated responses and can induce changes in cellular phenotype which, in turn can provoke vascular dysfunction, matrix expansion, glomerulosclerosis, and atherosclerosis (Brownlee *et al*, 1986). A recent study of the cellular responses that are stimulated by AGEs revealed a signaling pathway involving mitogen-activated kinases that leads to the activation of a series transcription factors (Lander *et al*, 1997).

Section V: The Low Density Lipoprotein Receptor

Regulation of cholesterol synthesis and metabolism was discovered in the early 1950s. When dogs were fed a high cholesterol diet, a negative feedback mechanism could be demonstrated in hepatic cholesterol biosynthesis (Gould *et al*, 1953). A similar conclusion was drawn from subsequent studies of various types of cells in culture (Bailey, 1973; Rothblat, 1969). When serum was present in the medium, cultured cells

produced little cholesterol from radioactive acetate. When serum was removed from the medium, cholesterol synthesis increased. The mechanisms involved in the cholesterol feedback system became evident with the discovery of the LDL receptor pathway (Goldstein and Brown, 1974 and 1986). Brown and Goldstein showed that the activity of HMG CoA reductase (3-hydroxy-3-methylglutaryl coenzyme A reductase), the rate limiting enzyme of cholesterol biosynthesis, is negatively correlated with the level of LDL in the serum. The initial point of control in this pathway is the cell surface LDL receptor that possesses high affinity for lipoproteins that contain apoB or apoE. Cells from patients with FH lack functional LDL receptors and are unable either to mediate the endocytosis of LDL or to suppress activity of HMG CoA reductase and endogenous cholesterol synthesis. The consequences of this inherited disorder are the extremely high plasma cholesterol levels and the serious clinical manifestations that characterize FH (Goldstein and Brown, 1974).

The LDL receptor pathway

The LDL receptor is the patriarch of a family of cell-surface receptors, including the LDL receptor, VLDL receptor, LDL receptor-related protein (LRP) and megalin/gp330. Members of the LDL receptor family of cell surface receptors are localized in clathrin-coated pits and function to internalize macromolecules into cells by receptor-mediated endocytosis (Goldstein *et al*, 1985b). The LDL receptor is the best-characterized receptor in this family in respect to structure and function.

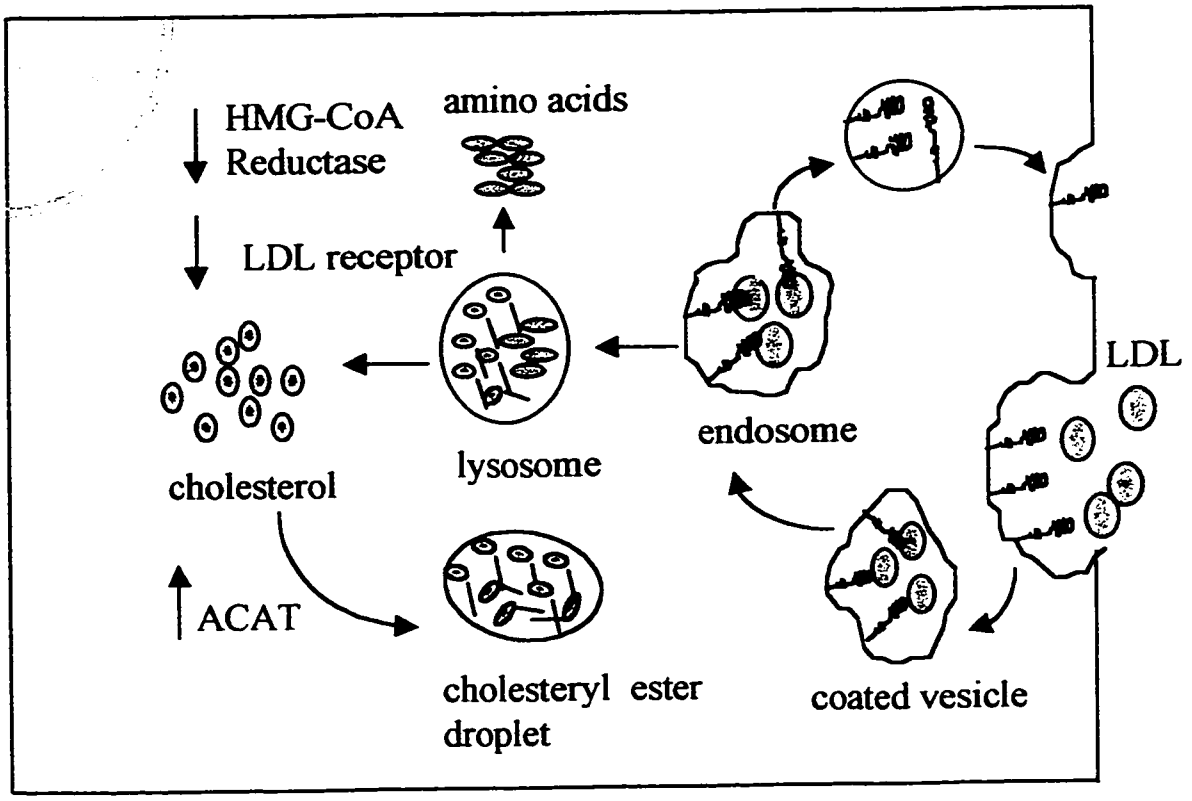


Figure 1-7: The LDL receptor pathway
LDL that bind to the LDL receptor are internalized into a clathrin coated vesicle that becomes uncoated and acidified, resulting in dissociation of the LDL-LDL receptor complex. The LDL are then delivered to lysosomes, whereas the receptor travels back to the cell surface in a recycling vesicle. Lysosomal degradation of LDL results in complete breakdown of apoB and liberation of cholesterol through hydrolysis of the cholesteryl esters. The LDL-derived cholesterol triggers several regulatory functions. There is: stimulation of acyl-CoA cholesterol acyltransferase, that leads to increased formation of cholesteryl esters for storage, suppression of 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMG-CoA reductase) that is a rate limiting enzyme for endogenous cholesterol synthesis, and decreased production of LDL receptor. (Modified from Brown and Goldstein, 1985)

The LDL receptor is a transmembrane glycoprotein consisting of 839 amino acids. It is expressed on the surface of all cell types, and has a high affinity for apoB-100

containing LDL as well as for apoE-enriched VLDL and chylomicron remnants (Brown and Goldstein, 1986). The LDL-receptor pathway (Figure 1-7) was first described by Brown and Goldstein, and it is the prototype of the pathways used for receptor-mediated endocytosis of other ligands that are taken up by receptor-mediated endocytosis (Goldstein *et al*, 1979; Pastan and Willingham, 1981; Bretscher and Pearse, 1984; Czekay *et al*, 1997). The LDL receptor is translated as a pro-protein in the endoplasmic reticulum, where its secretory sequence is cleaved. This 120 kDa protein undergoes extensive O-linked glycosylation in the Golgi apparatus to yield a 160 kDa mature protein (Tolleshaug *et al*, 1982). Following their emergence on the cell surface, some 45 minutes after synthesis, the receptors quickly cluster in clathrin-coated pits (Helenius *et al*, 1983; Anderson *et al*, 1976), whether bound to lipoprotein ligands or not. Within 3 to 5 minutes of their formation, the coated pits are internalized within the cell as coated endocytic vesicles, thereby removing lipoprotein-bound cholesterol, and other lipids from the circulation (Brown and Goldstein, 1986).

Once internalized within the cell, the vesicles rapidly lose their clathrin coat and fuse with many other newly formed vesicles resulting in the formation of large irregularly shaped organelles called endosomes or "receptosomes". These organelles become acidified through the action of ATP-driven proton pumps, which reside within the plasma membrane (Marsh *et al*, 1983; Helenius *et al*, 1983). When the intravesicular pH falls to 5.5, the LDL receptor dissociates from its lipoprotein ligand. The freed receptors then migrate, along with other receptors, to distal regions of the organelle, where they pinch

off and return to the cell surface for further use, whereas the lipoproteins are hydrolyzed within lysosomes. The recycling time for an LDL receptor is about ten minutes, and the LDL receptor can be reused up to 150 times, during its 10-30 hour life-span (Goldstein *et al*, 1979). The lipoprotein-derived cholesterol that is delivered to the cells is then esterified by free fatty acids, through the action of the intracellular enzyme acyl-coenzyme A cholesterol acyltransferase (ACAT), and stored as lipid droplets (Goldstein *et al*, 1974). Such accumulation of cholesterol within the cell is sensed, and causes the down-regulation of LDL receptor synthesis at the transcriptional level. As previously mentioned, the activity of the biosynthetic enzyme, HMG Co-A reductase is also down-regulated in the presence of excess cholesterol, through complex mechanisms that include modulation of transcription, mRNA stability and protein stability (Simonet and Ness, 1989). The importance of the LDL receptor in cholesterol uptake is exemplified in humans who lack functional LDL receptor and consequently suffer from the disorder FH.

Structure of the LDL Receptor Gene

The human gene for the LDL receptor is located on the distal short arm of chromosome 19 (p13.1-p13.3). It spans 45 Kb and is composed of 18 exons and 17 introns (Hobbs *et al*, 1990). The protein is composed of five discrete functional domains, encoded by the combination of various exons. Exon 1 encodes the signal sequence. Exons 2-6 encode the ligand-binding domain, which consists of seven tandem repeats of a 40-amino acid, cysteine rich sequence. Exons 7-14 encode a region that shares

sequence identity to the human epidermal growth factor precursor gene. This domain contains three 40-amino acid, cysteine-rich growth factor repeats. Exon 15 encodes a 58-amino acid sequence that is the attachment site for many O-linked carbohydrate chains. Exon 16 and the 5'-end of exon 18 encode the cytoplasmic domain that contains the signal that causes the receptor to cluster in clathrin-coated pits and to be internalized during receptor-mediated endocytosis. The structure of the LDL receptor gene shares an evolutionary history with exons from several other genes. It provides further evidence to support the hypothesis concerning the nature and function of introns (Gilbert *et al*, 1978). In this model, introns would permit functional domains that are encoded by discrete exons to shuffle between different proteins, thereby allowing proteins to evolve as mosaic combinations of pre-existing functional units. Members of the LDL receptor gene family are thought to have been assembled from a pool of ancestral exons that have also been used to assemble other genes that encode proteins that have diverse functions in the organism (Goldstein *et al*, 1985).

Functional domains of the LDL receptor

The structure of the LDL receptor is shown in Figure 1-8. The amino terminal domain, composed of 292 residues, is comprised of seven, 40 amino acid imperfect cysteine-rich repeats. Each of the seven repeats contains six cysteine residues that form three intra-repeat disulfide bonds. The three-dimensional structure of repeats 1 and 2 was solved using NMR spectroscopy (Daly *et al*, 1995), and the disulfide bond pattern of

repeat 1 has been established (Bieri *et al*, 1995). Within cysteine-rich repeat 1, the disulfide links were shown to consist of residues Cys 6 bound to Cys 18, Cys 13 to Cys

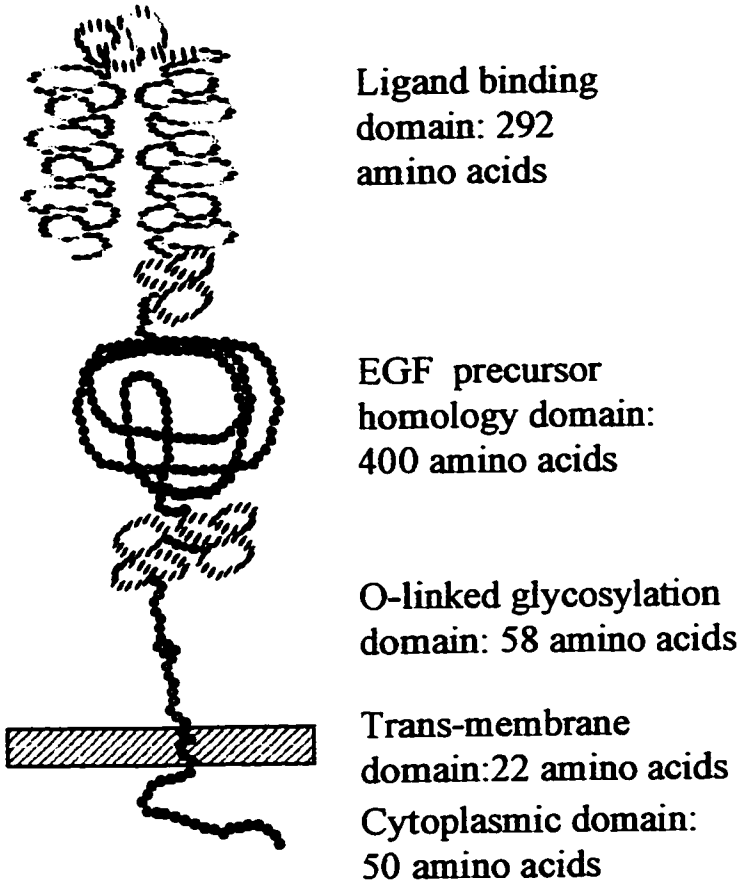


Figure 1-8: Structure of the LDL receptor
The LDL receptor is composed of structural domains, that are known to contribute to the various functions of the protein. The ligand-binding domain, that is made up of seven imperfect cysteine-rich sequences, is responsible for ligand binding. The EGF precursor homology domain is essential for receptor recycling and appears to indirectly modulate ligand binding. The O-linked sugar domain has no clear function, but may serve as a rigid region to project the receptor on the surface of the cell or increase the stability of LDL receptor. Finally, there is a membrane-spanning domain and a cytoplasmic domain that allows for clustering, internalization and recycling of the receptor. (Brown and Goldstein, 1985)

31, and Cys 25 to Cys 42. Other repeats are thought to share this pattern of disulfide bonding. A conserved motif that is characterized by a cluster of negatively-charged amino acids, Asp-X-Ser-Asp-Glu (DxSDE) is present near the carboxy-terminus of each of the seven repeats. It was originally thought that these clusters of negatively-charged residues interact with clusters of positively-charged residues on apoB-100 or apoE. (Figure 1-9) (Knott *et al,* 1985). More recently, however, it has been proposed that the DxSDE motif forms a calcium-binding site, that is essential for the proper folding of the repeat (Blacklow and Kim, 1996). Support for this latter concept has come from the

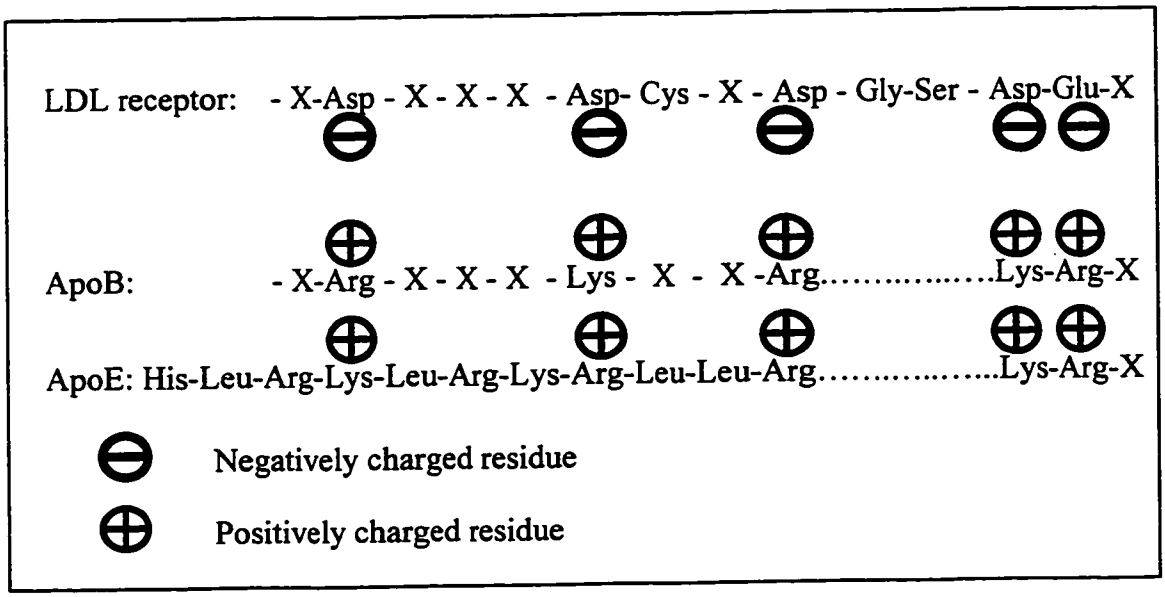


Figure 1-9: Consensus sequence for the binding sites of LDL receptor and its ligand, apoB and apoE
Sequence alignment of the LDL receptor, apoB, and apoE binding sites. The negatively-charged residues in the LDL receptor are thought to interact with positively-charged residues on apoB or apoE. The LDL receptor consensus sequence within the cysteine-rich repeats constitute the ligand-binding domain. (Modified from Beisiegel, U. in the *Structure and function of apolipoproteins* Rosseneu, M. (Edt) CRC p275)

recent determination of the crystal structure of cysteine-rich repeat 5 of the ligand-binding domain of the LDL receptor (Fass *et al,* 1997). In this structure, most of the negatively-charged residues within the DxSDE motif are coordinated with Ca^{++} and are, therefore, unavailable to bind to apoB or apoE. It is possible, nevertheless, that positively-charged residues within the respective LDL receptor-binding sites of apoB and apoE interact with negatively-charged residues that are situated elsewhere in the LDL receptor-binding domain. It could also be possible that, during binding of ligands to the LDL receptor, Ca^{++} is released which would free negatively-charged residues to interact with positively-charged residues in apoB or apoE.

The structural basis for the interaction of the LDL receptor with its ligands has been analyzed using natural LDL receptor variants from FH subjects or LDL receptor variants that were generated by *in vitro* mutagenesis. It has been shown that not all of the cysteine-rich repeats of the ligand-binding domain are necessary for lipoprotein binding (Russell *et al*, 1989). Moreover, certain of the variants demonstrate ligand-specific defects in binding. Whereas cysteine-rich repeat 1 was shown to contribute very little to either apoB- or apoE-mediated binding, deletion of any one of the remaining six repeats reduced apoB-mediated binding up to 95% (Russell *et al*, 1989). In contrast, β-VLDL binding, which is mediated almost exclusively through apoE, is reduced by 50% with the deletion of the fifth repeat, but is relatively insensitive to the deletion or mutation of any of the other repeats (Russell *et al,* 1989). A naturally occurring variant of the LDL receptor, which involves the loss of both the first and second repeats, results in normal

binding to β-VLDL, however, binding to LDL is reduced by 29% (Russell *et al*, 1989). The LDL receptor may use a combination of cysteine-rich repeats two to seven in order to bind LDL, whereas repeat five alone is sufficient to bind apoE-containing lipoproteins. The more stringent requirement for LDL versus β-VLDL binding by the LDL receptor, creates a situation in certain FH individuals in which the defect in apoB-mediated clearance of lipoproteins is partly compensated by normal apoE-mediated uptake by the LDL receptor. This selective defect in apoB-mediated lipoprotein binding that is exhibited by certain natural LDL receptor variants of FH patients has been shown to modulate the clinical phenotype (Hobbs *et al*, 1990).

The 400-amino acid epidermal growth factor (EGF) precursor homology domain was found to have 35 % homology to a portion of the extracellular domain of the EGF precursor (Yamanoto *et al*, 1984). This region includes three growth factor repeats, which consist of 40-amino acid, cysteine-rich sequences that differ from the cysteine-rich repeats found in the ligand-binding domain. The two first growth factor repeats are contiguous and are separated from the third repeat by a stretch of 280-amino acids that contains five copies of a conserved Tyr-Trp-Thr-Asp (YWTD) motif, that is repeated once every 40 to 60 amino acids. The EGF precursor homology domain is required for acid-dependent dissociation of lipoproteins from the receptor within endosomes during receptor recycling. Deletion of this domain results in a receptor that can localize in coated pits, bind and internalize apoE-containing lipoproteins but, within the endosome, the ligand fails to dissociate from the receptor and, as a consequence, the receptor is not

recycled to the cell surface (Davis *et al*, 1987). Thus, it is possible that the EGF precursor homology domain undergoes a pH-dependent conformational change that is, in turn, transmitted to the ligand-binding domain where it provokes ligand-receptor dissociation. This domain is also known to be calcium dependent for its proper folding and function (Downing *et al*, 1996). LDL receptor variants with mutations within this domain frequently fail to fold properly and undergo degradation within the endoplasmic reticulum or show retarded transport to the cell surface and are associated with clinical FH (Hobbs *et al*, 1990).

The third structural domain of the LDL receptor is composed of 58 amino acids, 18 of which are serines and threonines that are sites of attachment for O-linked carbohydrate chains (Yamamoto *et al*, 1984). While this domain of the LDL receptor is not essential for internalization of LDL by cultured fibroblasts, deletion of the exon encoding this domain in two families did lead to a heterozygous FH phenotype, indicating a possible role for this domain in the function of the receptor within the liver (Kuwano, 1991). The O-linked sugar chain were thought to play a role in maintaining the stability of the LDL receptor by protecting it from proteolytic cleavage (Kozarsky *et al*, 1988). It has also been postulated that the extensive glycosylation at this site may permit interactions with the cell surface heparin proteoglycans to keep the LDL receptor extended from the surface of the cell, and accessible to circulating plasma lipoproteins (Brown and Goldstein, 1985).

The fourth domain is a typical membrane-spanning domain composed of 22 hydrophobic amino acids, as demonstrated by proteolysis (Yamamoto *et al*, 1984). It is poorly conserved amongst species. The remaining 50 amino acids form the cytoplasmic domain. The cytoplasmic domain plays an important role in the clustering of LDL receptors in the clatherin-coated pits (Goldstein *et al*, 1979). The naturally occurring LDL receptor variants with mutations within this domain are synthesized and transported to the cell surface and are capable of binding to LDL with normal affinity. They do not localize to coated pits and can not internalize bound lipoproteins (Goldstein *et al*, 1985). A motif composed of Asn-Pro-x-Tyr in the cytoplasmic domain that is conserved amongst species and amongst other members of the LDL receptor gene family is thought to be a signal for directing the receptors to clatherin-coated pits (Chen *et al*, 1990).

Genetic defects in the LDL receptor causing familial hypercholesterolemia

There are more than 250 LDL receptor mutations that have been described, of which about 50 are point mutations that substitute individual amino acids within the seven cysteine-rich repeats of the ligand-binding domain (Goldstein *et al*, 1985). Approximately 41 % of the FH homozygotes are true homozygotes as determined by haplotype mapping as well as by mutational analysis. The remaining FH homozygotes are, in fact, compound heterozygotes, having inherited two different non-functional alleles. In certain ethnic populations, a specific mutant allele of the LDL receptor has achieved a high frequency through a founder effect. Examples of this occurrence include

the French Canadians, Afrikaners, Lithuanian Jews, Finnish, and Christian Lebanese (Hobbs *et al,* 1990).

Molecular analysis LDL receptor genes from affected individuals have been very informative with respect to the function of the various domains of the protein in relation to its structure (Brown and Goldstein, 1985b). All of the known mutant alleles can be subdivided into five classes that were determined by the functional behavior of the mutant receptors. Class 1 alleles fail to produce an immuno-precipitable protein (null alleles). Class 2 alleles are produced but are not transported to the cell surface. Presumably there is a problem with the intracellular folding of these alleles and they are simply retained within the cell and ultimately degraded. Class 3 mutants are expressed on the cell surface but are incapable of interacting with their apolipoprotein ligands. As stated previously, the binding properties of the receptor are conveyed by the collective co-operation of the cysteine-rich sequences that comprise the ligand-binding domain. Class 4 mutants fail to cluster within clathrin-coated pits, and hence are unable to mediate the endocytosis of bound lipoproteins. Finally, class 5 mutant receptors function normally in their internalization of bound LDL, but fail to undergo pH-dependent dissociation from the ligand and, as a consequence, are not recycled.

Section VI: Use of Monoclonal Antibodies to Study Structure and Function of Lipoproteins

Antibodies are host proteins produced in response to exposure to foreign molecules. They are synthesized primarily by plasma cells, a terminally differentiated cell of the B-lymphocyte lineage. Antibodies circulate in the blood and lymph where they can bind to foreign antigens. The antibody-antigen complexes are then removed from circulation primarily through phagocytosis by macrophages (Harlow and Lane, 1988).

Polyclonal antisera directed against lipoproteins and apolipoproteins have been used successfully as reagents in immunoassays for the quantification of apolipoproteins in plasma and other biological fluids. Even with the advent of the methodologies for the preparation of monoclonal antibodies (mAbs), polyclonal antisera remain the reagents of choice for some apolipoprotein immunoassays due, in large part, to their ability to recognize multiple epitopes on the antigen and to form insoluble complexes with specific antigen. Monoclonal antibodies are also used to quantify apolipoproteins and other molecules involved in lipoprotein metabolism. In addition, because of their specificity for a single epitope on the antigen, they have proved particularly useful for identifying functional domains of proteins and as probes of protein conformation. I will briefly describe several examples of the use of monoclonal antibodies for the study of lipoprotein structure and metabolism.

In 1975, Kohler and Milstein first reported the successful generation of hybridomas that secrete monoclonal antibodies using a protocol of cell fusion (Kohler and Milstein, 1975). Spleen cells of an immunized mouse are fused with a murine myeloma cell line using polyethylene glycol to mediate membrane fusion. A subpopulation of the resulting hybrid cells (hybridomas) have acquired the growth characteristics of the tumour cell parent and the ability to synthesize and secrete specific antibody from the splenic cell parent. Protocols have been established to identify and clone hybridomas that secrete antibodies with desired specificites. As a single hybridoma produces a single species of antibody and the hybridomas can be cloned, one has the ability to establish hybridoma cell lines that produce large amounts of antibody of a unique affinity and epitope specificity. The cells can be passaged indefinitely in culture or as tumours in mice or they can be frozen for long-term storage. One therefore has an unlimited source of antibodies that possess uniform binding properties. Each mAb is specific for a portion of an antigen, called an epitope or antigenic determinant. Depending on the monoclonal antibody the epitope could be either a conformational or a sequential epitope. Conformational epitopes are assembled from non-contiguous regions of the primary structure of the antigen and, as a consequence, are only expressed when the antigen is in its native conformation. Sequential or linear epitopes are composed of a contiguous sequence within the antigen and are largely independent of tertiary structure.

Monoclonal antibodies have been extremely useful in defining the effects on apolipoprotein conformation of the lipid environment of the lipoprotein. Early studies

using polyclonal antisera that had been raised against purified, delipidated apoA-I showed that only 5-10% of epitopes that could be detected in purified apoAI were expressed on native HDL. It was concluded that most of the molecule was not accessible because of either protein-lipid or protein-protein interactions (Schonfeld *et al*, 1976, 1977). Subsequent studies using a panel of anti-apoA-I monoclonal antibodies demonstrated that the lipid composition as well as the physical properties of the HDL particle have profound effects on apoA-I conformation and the expression of individual apoA-I epitopes (Marcel *et al*, 1991; Collet *et al*, 1991). Based on these immunochemical studies, Marcel *et al*, formulated a model of apoAI super-secondary structure (Marcel *et al*, 1991). Anti-apoB mAbs have also been used effectively to determine the role of lipoprotein particle size and lipid composition in modulating apoB conformation and function (Curtiss *et al*, 1982; Teng *et al*, 1984; Aviram *et al*, 1989; McKeon *et al*, 1994; Galeano *et al*, 1994)

There are now many examples in which mAbs have been used to study the structure of lipoproteins and to identify functional domains of proteins that are implicated in lipoprotein metabolism. The first reported anti-apolipoprotein mAbs were a panel of mAbs that were specific for human apoE (Milne *et al*, 1981). These mAbs were subsequently used for the identification of the heparin- and LDL receptor- binding sites on apoE (Weisgraber *et al*, 1983 and 1986) and to discriminate between apoE- and apoB-mediated binding to the LDL receptor (Hui *et al*, 1984; Marcel *et al*, 1988; Campos *et al*, 1996). Anti-apoB mAbs were used to provide the first experimental evidence that a LDL

particle contains a single molecule of apoB (Milne and Marcel, 1982), to identify the apoB LDL receptor-binding domain (Milne *et al*, 1989) and to determine the topography of apoB on native LDL (Chatterton *et al*, 1991, 1995). The neutral lipid-binding site of CETP was defined by mapping the epitope of an anti-CETP mAb that can neutralize CETP-mediated lipid transfer activity (Swenson *et al*, 1989).

Chapter II

New Immunization Protocols for the Generation of Anti-Human Apolipoprotein B Isoform-Specific Monoclonal Antibodies

Summary

Human apolipoprotein (apo) B is polymorphic. A relatively rare apoB allele that encodes a protein with an Arg→Gln substitution at residue 3500 and a greatly reduced affinity for the low density lipoprotein (LDL) receptor is responsible for familial defective apoB (FDB). The goal of this study was to develop immunization protocols that would elicit an anti-human apoB isoform-specific immune response in mice that could be used to generate monoclonal antibodies (mAbs) capable of discriminating between wildtype apoB and apoB^{Gln3500}. Initially we attempted to elicit neonatal immunological tolerance in mice to wildtype human apoB before immunization with LDL from an FDB individual. This was not successful. A second protocol was based on the chemical suppression of the immune response to wildtype human LDL with cyclophosphamide followed by immunization with LDL from an FDB individual. While the serum of mice immunized using this subtractive protocol showed preferential reactivity with apoBGln^{3500} LDL, of the 56 anti-human apoB hybridomas that were generated from these mice, only 2 selectively recognized the LDL used for immunization. One mAb was specific for apo (a) and the other for an epitope situated between apoB residues 1880 and 2100 that is differentially expressed in LDL isolated from different

individuals. The third strategy consisted of immunizing human apoB transgenic mice with LDL from an FDB individual. Five specific hybridomas were identified, of which one was specific for human apoE. The remaining mAbs were apoB-specific but could not distinguish between wildtype LDL and the apoB^{Gln3500} that had been used for immunization. To determine if immunological tolerance to the product of the transgene had been broken in the immunized mice, the mAbs were tested for their reactivity with human LDL and with lipoproteins isolated from human apoB transgenic mice. Two mAbs bound human apoB and transgenic human apoB, but only after the lipoproteins were adsorbed to plastic or subjected to SDS electrophoresis. Thus, the mAbs appeared to recognize epitopes not expressed on native LDL. One mAb reacted with human LDL but did not recognize human apoB in lipoproteins of transgenic mice and may be specific for a polymorphic epitope of human apoB. The final mAb was specific for an epitope that was expressed in native human LDL but not in lipoproteins of apoB transgenic mice, although it could be detected on both human apoB and transgenic human apoB after SDS electrophoresis. Immunization of human apoB transgenic mice is therefore an efficient method for generating anti-human apoB mAbs with novel specificities.

Introduction

In 1961, Allison and Blumberg described genetic polymorphism of human plasma based on the reactivities of iso-antibodies obtained from individuals who had received multiple blood transfusions (Allison and Blumberg, 1961). These Antigen group (Ag) polymorphisms were subsequently shown to result from multiple, co-dominantly-expressed alleles at the apolipoprotein (apo) B locus that differed by single-nucleotide interchanges at 5 sites within the apoB structural gene. Although the inheritance of certain Ag haplotypes has been reported to influence plasma lipid and lipoprotein levels in some studies, in general, Ag polymorphism does not appear to have a major impact on the clinical and biochemical phenotype. Clinically important polymorphisms of apoB have, however, been identified (Vega and Grundy, 1986; Innerarity *et al*, 1987). An apoB allele that encodes a protein with an Arg to Gln substitution at residue 3500 occurs with an allelic frequency of 1/500 in Western populations and leads to a condition known as familial defective apoB (FDB) (Soria *et al*, 1989). $ApoB^{Gln3500}$ has low affinity for the LDL receptor and, as a consequence, FDB individuals have a lipoprotein profile reminiscent of subjects with defective LDL receptors (Vega and Grundy, 1986). A natural truncated variant, apoB75, that terminates at residue 3386, and thus does not include residue 3500, binds to the LDL receptor with high affinity (Krul *et al*, 1992). Therefore, as residue 3500 itself does not appear to form part of the apoB LDL receptor-binding site, the poor reactivity of apoB ^{Gln3500} LDL with the LDL receptor appears to result from an indirect effect of the substitution. A ^{13}C NMR study has revealed that the

apoB$^{Arg\rightarrow Gln}$ substitution results in a significant loss of active lysine residues (Lund-Katz *et al*, 1991) and so it is probable that replacement of Arg^{3500} by Gln induces a major local conformational change in the receptor-binding region of apoB. More recently, Pullinger and colleagues (Pullinger *et al*, 1995) have identified an individual with a clinical and biochemical phenotype indistinguishable from that of FDB who has inherited an allele encoding an apoB$^{Arg3531\rightarrow Cys}$ variant. Other polymorphisms at the apoB locus that have an important impact on lipoprotein metabolism are alleles that encode truncated apoB variants that are responsible for hypobetalipoproteinemia (Young, 1990).

Monoclonal antibodies have been very useful probes for identifying functional domains of apoB and for determining apoB conformational changes that occur during its intravascular metabolism. To further define the structural basis of apoB-mediated binding to the LDL receptor we proposed to generate mAbs that would be capable of discriminating between a receptor-competent form of apoB, apoB^{Arg3500}, and its receptor-defective counterpart, apoB^{Gln3500}. Such mAbs could potentially be specific for an epitope(s) that includes residue 3500 or for an epitope(s) whose conformation is indirectly modulated by a substitution at position 3500 as has been proposed for the apoB LDL receptor-binding site. Here we will describe our efforts to develop immunization strategies that would promote apoB-isoform-specific immune responses in mice. Inter-species immunization generally results in the production of antibodies against species-specific epitopes that rarely discriminate between polymorphic forms of the antigen whereas intra-species immunization preferentially elicits an immune response against

allogeneic or polymorphic epitopes. An example of the latter is the Ag system of polymorphisms that were originally defined by antibodies in the sera of multi-transfused individuals and multiparous women. Similarly, the complex apoB polymorphisms of swine were identified using antibodies that were generated by allogeneic immunization (Rapacz *et al*, 1986). Exceptions to this general observation are the murine mAbs MB19 and BIP45 that differentiate between the products of human apoB alleles Ag(c) and Ag(g) (Young and Hubl, 1989) and MB47 that shows a slightly higher affinity for $apoB^{Gln3500}$ than for wildtype apoB (Young *et al*, 1994).

While intra-species immunization may be the protocol of choice for production of isoform-specific antibodies, there are serious ethical and technical considerations in the case of humans. As a consequence, our approach has been to try to mimic as closely as possible the conditions of intra-species immunization that favor the generation of an isoform-specific immune response while maintaining the advantages of using animals. We have developed and evaluated 3 immunization protocols for production of murine anti-apoB isoform-specific mAbs. We have attempted to elicit neonatal immunological tolerance in mice to wildtype human apoB before immunization with LDL from an FDB individual. A second protocol was based on chemical suppression of the immune response to wildtype human LDL followed by immunization with LDL from an FDB individual. Finally, we have immunized mice that carried a wildtype human apoB transgene with $apoB^{Gln3500}$ LDL.

Material and Methods

Preparation of Lipoproteins-ApoB^{Arg3500}

Normal LDL were prepared from blood collected by the Canadian Red Cross from healthy donors. ApoB^{Gln3500} LDL was isolated from plasma of FDB subjects that had been identified in the Lipid Clinic of the Institut de Recherches Cliniques de Montréal. The plasma was supplemented immediately with EDTA (1.0 mM), sodium azide (0.02%), phenylmethanesulfonal fluoride (0.5 mM), aprotinin (1 ug/ml), and butylated hydroxytoluene (BHT, 20 nM). LDL (1.019-1.063) were isolated by sequential ultracentrifugation at 40.000 rpm for 18 hr using Beckman L5-65 ultracentrifuge with a 50.2 Ti rotor (Beckman Instruments, Spinco Div., Palo Alto CA) (Havel *et al*, 1955). The isolated LDL were then filtered with 0.22 μm pore size and stored at 4 ^{0}C in the dark. The lipoprotein fraction was isolated from mouse plasma by ultracentrifugation at 100,000 rpm for 3 hr at a density of 1.063 g/ml. After ultracentrifugation, the d < 1.063 g / ml fraction was removed and dialyzed against phosphate-buffered saline (PBS) (pH 7.4) containing 1mM EDTA and 0.02% sodium azide.

Immunization protocols

a) Induction of neonatal tolerance to wildtype LDL and immunization with apoB^{Gln3500} LDL- Within the first 24 hr after birth, mice were injected intraperitoneally with 100 μg of LDL that had been isolated from normal subjects. After 4 weeks, they were immunized with 100 μg of FDB LDL in 200 μl PBS containing 50 μg of the

immunological adjuvant, N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl peptide) (Calbiochem, La Jolla. CA). The mice were given 2 additional identical boosts at 3-week intervals. Serum anti-apoB^{Gln3500} and apoB^{Arg3500} titres were monitored one week after each boost by a solid phase radioimmunometric assay (RIA).

b) Subtractive Immunization protocol to produce isoform specific mAbs for apoB$^{Gln\ 3500}$ - Female Balb/c mice, 6-8 weeks old, were injected intraperitoneally with 50 μg of apoB^{Arg3500} LDL in 200 μl PBS containing 50 μg of muramyl peptide. Cyclophosphamide (100 mg / kg body weight) in 100 μl water was injected intraperitoneally at 15 minutes, 1 hr, and 24 hr after injection of the LDL (Williams *et al*, 1992). Following immunosuppression, mice received tetracycline in their drinking water. After one month, mice were immunized with apoB^{Gln3500} LDL and muramyl peptide following the same schedule as above. Plasma titres of anti-apoB antibodies were monitored by solid phase RIA.

c) Immunization of transgenic mice carrying normal human apoB gene to produce mAbs specific for apoB^{Gln3500}-The transgenic mice carrying a human apoB^{Arg3500} transgene (Young *et al*, 1993) were immunized with apoB^{Gln3500}LDL / muramyl peptide as described above. Compared to other protocols, a more prolonged series of boosts was required before an anti-human apoB immune response could be detected in the serum using a solid phase RIA.

Production of anti-apoB hybridomas

Four days before the fusion and at least 3 weeks after the last intraperitoneal boost, a final bolus of 50 μg of apoB^{Gln3500} LDL in 100 μl PBS was administered by tail vein injection. The method for cell fusion of splenocytes from immunized mice with SP2-0 plasmacytoma cells has been described in detail (Milne *et al*, 1992). In brief, splenocytes from immunized mice were fused with SP2-0 cells mediated by polyethylene glycol. Fused cells were maintained selectively by supplementing with hypoxanthine, aminopterine and thymidine. Seven to ten days after the fusion, when the clones occupied >10% of the surface of the well, supernatants were tested for the presence of specific anti-apoB^{Gln3500} and anti-apoB^{Arg3500} antibodies by a solid phase RIA. In some cases the supernatants were also screened using a sandwich RIA. Positive hybridomas were recloned by limiting dilution, as described previously (Milne *et al*, 1992).

Solid phase radioimmunometric assay

Human LDL or the lipoprotein fraction of human apoB transgenic mice were diluted to 30 μg / ml in 5mM glycine pH 9.2 and distributed in 50 μl aliquots into Immulon II Removawells (Dynatech), and left overnight at room temperature. The excess antigen was then removed and the wells were washed 3 times with 0.15 M NaCl containing 0.025% Tween 20 (NaCl-Tween) and then saturated with 1% bovine serum albumin in PBS (PBS-BSA) for 30 minutes. After removal of BSA, 50 μl aliquots of hybridoma culture supernatant, purified mAbs or mouse serum, appropriately diluted in PBS-BSA, were transferred to the antigen-coated Removawells and incubated for a

minimum of 2 hr at room temperature. The wells were washed and 50 µl of ^{125}I-anti-mouse IgG in PBS-BSA were added. After a 2 hr incubation, the wells were again washed and bound radioactivity was determined. Positive wells were defined as having bound radioactivity of at least 5 times that of background or being in the range of the positive control. The assay has been previously described in detail (Milne *et al*, 1992)

Sandwich radioimmunometric assay

To avoid changes in apoB conformation that can occur when LDL is adsorbed to plastic, for certain fusions, we have also screened hybridoma supernatants using a solid phase sandwich RIA. The Fab fragment of mAb 1D1 (50 µl at 10 µg / ml in 5 mM glycine, pH 9.2) that is specific for an epitope of human apoB situated between residues 474-539 (Pease *et al*, 1990), was adsorbed to Removawells by an overnight incubation at room temperature. Following washing, 50 µl of hybridoma supernatant were added and, after a 2 hr incubation, the wells were again washed. The wells were incubated with 50 µl of affinity purified ^{125}I-anti-mouse Fc diluted in PBS-BSA. After washing, bound radioactivity was determined.

Competitive Radioimmunometric assay

The competitive apoB radioimmunometric assay has been described previously (Marcel *et al*, 1984). Immulon II Removawells (Dynatech Laboratories, Chantilly, VA) were coated by an overnight incubation with 200 µl of reference LDL (30 µg / ml in 5 mM glycine, pH 9.2). Plates were washed 3 times with NaCl-Tween and saturated by

incubation for 1 h with 250 µl of PBS-BSA. Serial dilutions (150 µl in PBS-BSA) of test and control LDL were prepared in separate microtitre plates, to which was added, 150 µl of the anti-apoB mAb, appropriately diluted in PBS-BSA. After a 2 hr incubation, 200 µl of the LDL-mAb mixture was transferred to the LDL coated wells. The plates were incubated overnight and again washed with Tween-NaCl. Two hundred µl of ^{125}I-goat anti-mouse IgG diluted to 70 ng/ml in 1% PBS-BSA was added to each well and incubated overnight. The wells were then washed with the Tween-NaCl as above and counted for bound radioactivity. Results are plotted as the CPM bound in the presence of competitor (B), divided by the CPM bound in the absence of competitor (Bo), as a function of the concentration of the competitor.

Production and purification of mAbs

Anti-human apoB-specific mAbs produced by hybridomas that originated from transgenic mice were purified from culture supernatants by affinity chromatography on Staphylococcal protein A sepharose (Pharmacia) (Ey *et al*, 1982). For hybridomas that originated from Balb/c mice, antibodies were isolated by affinity chromatography on Staphylococcal Protein G chromatography from ascitic fluid of hybridoma-bearing mice (Milne *et al*, 1992). The preparation of Fab fragments from purified monoclonal antibodies has been described previously (Milne *et al*, 1983). In brief, IgG was digested by papain and the mixture of Fab and Fc fragments was passed through protein A column. Fc fragment was retained by protein A column and Fab fragment was collected and concentrated from non retained fractions.

Iodination of immunoglobulin, Fc fragments, and LDL

The goat anti-mouse IgG and goat anti-mouse IgG Fc specific antibodies (Jackson Laboratories) were labeled with ^{125}I by the chloramine-T method (Milne *et al*, 1992). The specific activity of labeled IgG was between 10,000-30,000 cpm / ng of IgG. For the LDL receptor-binding assay, LDL was iodinated by the method of Bilheimer (Bilheimer *et al*, 1972). One mCi of sodium ^{125}I was used to iodinate 1 mg of LDL in the presence of 40 nmoles of iodine monochloride and 0.5 M glycine-NaOH, pH 10. Free ^{125}I was removed by gel filtration on Sephadex G-25 (pharmacia) followed by an overnight dialysis against PBS, pH 7.4. The specific activity ranged from 200-800 cpm / ng of apoB.

LDL receptor binding assay

For LDL receptor binding experiments, human skin fibroblast cells were plated in 35-mm dishes with 2 ml of DMEM supplemented with 10% (v/v) fetal bovine serum. When cells reached about 70% confluence, they were rinsed with 2 ml of sterile PBS and exposed to DMEM supplemented with 10 % lipoprotein depleted serum for 48 hr. Prior to the experiments, cells were placed at 4°C for 30 min, the medium was then removed and replaced by 1 ml of DMEM containing 25 mM HEPES, pH 7.4, supplemented with 10% lipoprotein depleted serum. Cells were then incubated with either ^{125}I-LDL alone or pre-formed ^{125}I-LDL-anti-apoB IgG complexes. The plates were then incubated for 3 hr at 4 ^{0}C. After washing, the bound ^{125}I-LDL were eluted with 1M NaOH and the effect of

the mAbs was determined by comparison with binding of ^{125}I-LDL alone. The mAbs 1D1 (no inhibition) and 5E11 (close to100% inhibition) (Milne *et al*, 1989) were used as negative and positive controls in the experiments. Assays were performed in triplicate.

Agarose gel electrophoresis

LDL from human plasma and LpB ($d < 1.063$) from plasma of transgenic and control mice were subjected to electrophoresis on pre-poured 0.5% agarose gel (Lipogel, Beckman Instruments) according to the manufacturer's recommendations. After completion of electrophoresis, the lipoproteins were transferred to nitrocellulose membrane, and the presence of human apoB, transgenic-apoB, and mouse endogenous apoB were probed by immunoblotting using mAbs specific for human or mouse apoB.

Western Blotting Analysis

Lipoproteins were subjected to SDS polyacrylamide gel electrophoresis (3-10% gradient) and transferred to nitrocellulose membranes (Milne *et al*, 1992). After saturation of the membranes with 5% milk powder in PBS, the transferred proteins were sequentially incubated with anti-apoB mAbs and horse radish peroxidase-conjugated anti-mouse IgG. Bound conjugate was visualized using an ECL kit (Amersham, UK) (Borén *et al*, 1992)

Measurement of Protein Concentration

Protein concentration was determined by a modified Lowry method (Markwell *et al*, 1978). Bovine serum albumin was used as the protein standard to determine the apoB concentration.

Results

Subtractive immunization:

Five adult BALB/c mice were injected with LDL isolated from a normal individual and, immediately thereafter, with a sublethal dose of cyclophosphamide. Several weeks later the mice were subjected to a normal immunization protocol using LDL isolated from a FDB subject. The reciprocal suppression / immunization protocol was also used with the goal of obtaining mAbs reactive with wildtype LDL and non-reactive with FDB LDL. For the initial experiment, control groups (5 mice each) included animals that received cyclophosphamide without antigen at the suppression step followed, several weeks later, by immunization with wildtype LDL and animals that received the same LDL for suppression and for immunization. Considerable mortality in suppressed mice was observed in the initial experiment (data not shown). This apparently resulted from opportunistic infections as it was avoided in subsequent experiments by the inclusion of tetracycline in the drinking water. Titration curves of circulating anti-apoB antibodies from the surviving mice are shown in Figure 2-1. Mice that were immunosuppressed with LDL from an FDB subject and later immunized with wildtype LDL had lower levels of circulating antibodies than did mice that had received cyclophosphamide without specific antigen at the suppression step. No immune response following immunization with apoB^{Arg3500}LDL was observed in the serum of mice that had received apoB^{Arg3500}LDL immediately preceding cyclophosphamide administration. Therefore, based on the specificity of circulating antibodies, it appears feasible to use

Figure 2-1; Anti-human LDL immune response of mice subjected to a subtractive immunization protocol.
Immunization group-Mice were injected with 50 μg of LDL isolated from a subject heterozygous for the apoB^{Gln3500} allele followed by 3 injections of 100 mg / kg body weight of cyclophosphamide at 0.25, 24 and 48 hrs (chemical immunosuppression step). At 3 and 5 weeks after immunosuppression, the mice were immunized with wildtype LDL (immunization step). One week later, the mice were analyzed for circulating anti-LDL antibodies by a solid phase RIA (3 / 5 mice survived). *Positive control group*-Mice received PBS followed by 3 injections of cyclophosphamide at the chemical immunosuppression step and were subsequently immunized with wildtype LDL. *Negative control group*-Mice were both immunosuppressed and subsequently immunized with wildtype LDL.

Figure 2-1

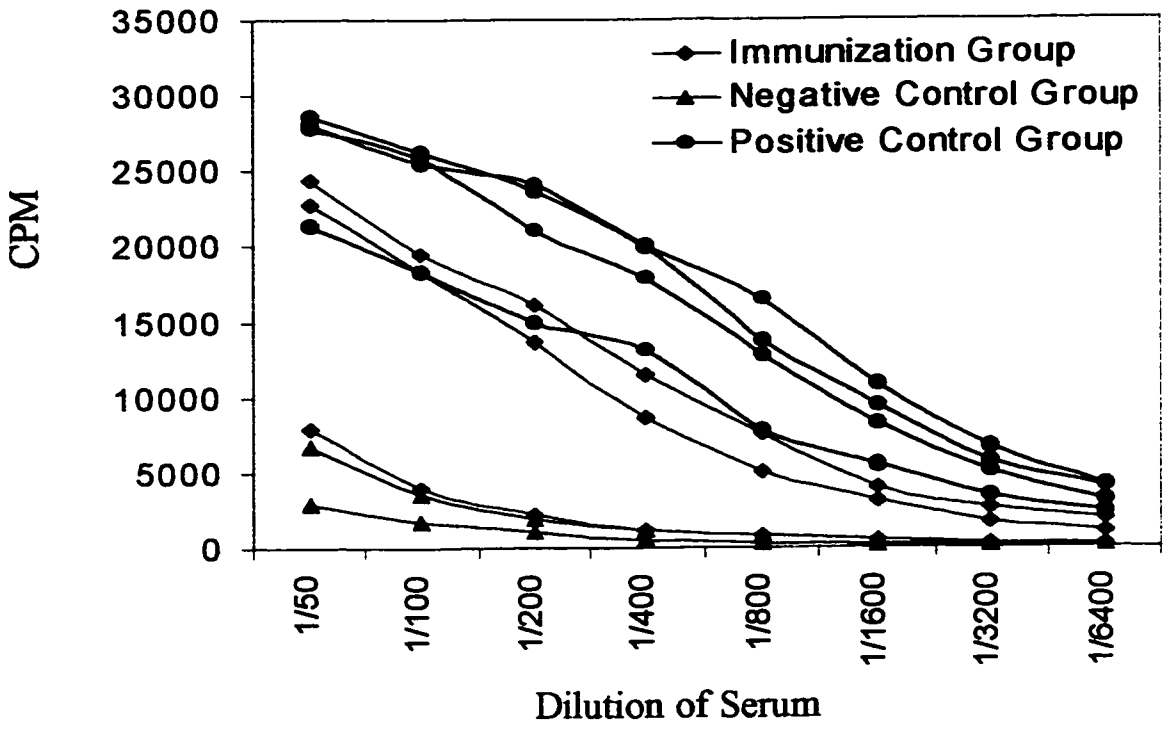
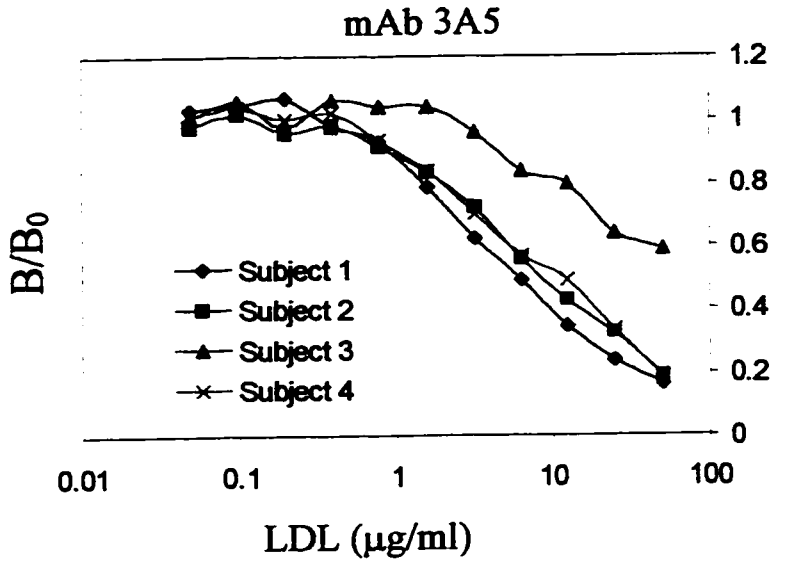
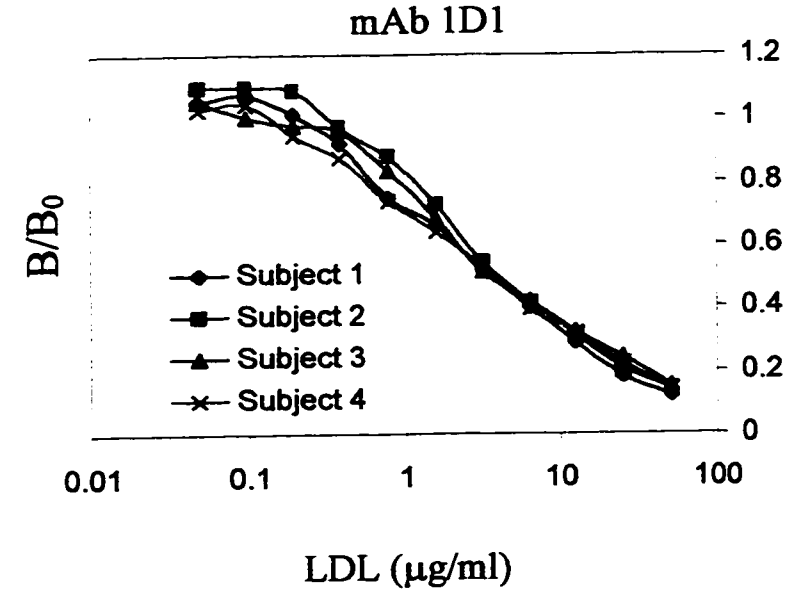

35000
30000
25000
20000
15000
10000
5000
0
CPM
Immunization Group
Negative Control Group
Positive Control Group
1/50
1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
Dilution of Serum

Figure 2-2; Reactivity of anti-apoB mAbs 3A5 and 1D1 with LDL isolated from the plasma of 4 normal subjects.
LDL from 4 subjects were tested for their relative reactivities with mAbs 3A5 and 1D1 in a competitive RIA. Results are expressed as B/B_o where B is radioactivity bound in the presence of competitor and B_o is radioactivity bound in the absence of competitor. LDL isolated from subject 3 showed an approximately 10-fold lower affinity for mAb 3A5. The mAb 1D1 reacts equally with the LDL from the 4 subjects.

Figure 2-2


mAb 3A5
B/B0
1.2
1
0.8
0.6
0.4
0.2
0
Subject 1
Subject 2
Subject 3
Subject 4
0.01
0.1
1
10
100
LDL (µg/ml)


mAb 1D1
B/B0
1.2
1
0.8
0.6
0.4
0.2
0
Subject 1
Subject 2
Subject 3
Subject 4
0.01
0.1
1
10
100
LDL (µg/ml)

subtractive immunization to generate an immune response that can differentiate between LDL of different individuals. Similar results were observed when the apoB^{Arg3500} LDL immune response was suppressed before immunization with LDL from an FDB subject (not shown). Nevertheless, of the 56 specific hybridomas that were generated from spleen cells of mice that had been subjected to the subtractive immunization protocol, only two secreted antibodies that could differentiate between the LDL used for immune suppression and that used for immunization. One of these mAbs, was specific for apo(a) and the other, 3A5, reacted with an epitope that is differentially expressed on LDL isolated from different individuals (Figure 2-2).

Production of mAbs using transgenic mice carrying a human wildtype apoB gene

Based on observations of the immunological status of other transgenic mice, one would predict that mice who carry a human apoB transgene gene would treat the product of the transgene as a self-antigen. When immunized with human LDL, they would presumably mount an anti-apoB immune response that is specific for epitopes that are present on the apoB used for immunization and absent from the human apoB expressed in the transgenic mice. We have, therefore, immunized human apoB^{Arg3500} transgenic mice with LDL from an FDB subject. In spite of multiple boosts with LDL, only a weak immune response was detected in the serum of the mice and, from 3 separate fusions, only 5 stable specific hybridomas were obtained. One hybridoma secreted a mAb that was specific for an epitope in the amino terminal 22kDa domain of human apoE (not shown). While the other 4 hybridomas secreted anti-apoB-specific mAbs, none could

distinguish between LDL isolated from normal and FDB subjects, respectively. To determine if tolerance in the transgenic mice to the product of the apoB transgene was broken by the immunization, we have compared reactivities of the four mAbs with normal human LDL and the d < 1.063 fraction of plasma from non-transgenic mice, human apoB^{Arg3500} transgenic mice and human apoB^{Gln3500} mice. When tested by a solid phase RIA, two of the mAbs, 2E1 and 3D11, reacted well with human LDL but showed no reactivity with lipoproteins isolated from either normal or transgenic mice (Figure 2-3). In contrast, mAb 9D3 and 2E12 and the control anti-apoB100 specific mAb, 4G3 reacted with both the human LDL and the transgenic lipoproteins. The same patterns of reactivity were seen with immunoblots of lipoproteins that had been separated by agarose gel electrophoresis (Figure 2-4). When the mAbs were tested for their respective reactivities with lipoproteins that had been separated by SDS PAGE and transferred to nitrocellulose membranes (Figure 2-5), 2E1 continued to show specificity for human LDL. In contrast, the specificity of mAb 3D11 for human LDL that was seen in the solid phase RIA and after agarose electrophoresis, was not apparent after SDS electrophoresis. Mab 3D11 showed reactivity with both human LDL apoB and with transgenic human apoB that had been subjected to SDS electrophoresis. The mAbs 9D3 and 2E12 as well as the control mAbs 1D1 and 4G3 recognized both human LDL and human transgenic apoB. It is interesting to note that 1D1 but neither 3D11, 9D3 nor 2E12 recognized a protein having the molecular weight of apoB48 in the plasma of the transgenic mice. To determine if the mAbs could recognize native apoB we have performed a competitive solid phase RIA (Figure 2-6). In this format, human LDL in the

Figure 2-3; Solid phase RIA of mAbs 2E1, 3D11, 9D3, 2E12 and 4G3 with LDL isolated from human plasma (h-HDL) and with lipoproteins isolated from the plasma of normal mice (mouse d<1.063) and from plasma of mice that express human wildtype apoB (tg-LpB d<1.063) or apoB^{Gln3500} (tg-LpB3500 d<1.063) transgenes.

Lipoproteins were adsorbed to plastic wells and then sequentially exposed to the anti-apoB mAbs and ^{125}I-anti-mouse IgG.

Figure 2-3

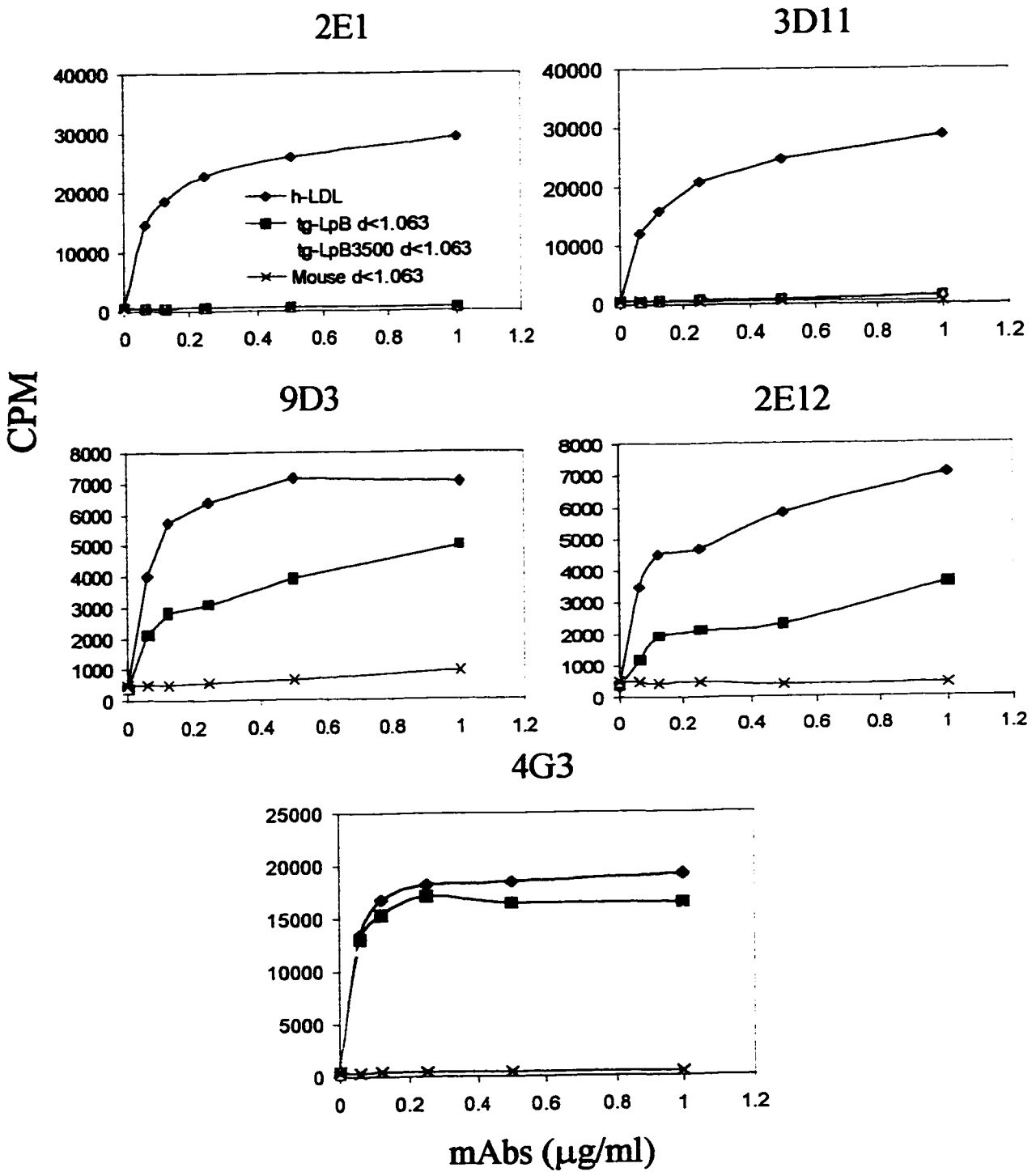

2E1
3D11
9D3
2E12
4G3
CPM
mAbs (µg/ml)
h-LDL
tg-LpB d<1.063
tg-LpB3500 d<1.063
Mouse d<1.063

Figure 2-4; Reactivity of mAbs 2E1, 3D11, 9D3, 2E12, 4G3 and LF5 with LDL isolated from human plasma (h-HDL) and with lipoproteins isolated from the plasma of normal mice (m-apoB) and from plasma of mice that express human wildtype apoB (tg-apoB) or apoB^{Gln3500} (tg-apoB3500) transgenes that had been subjected to agarose electrophoresis.

One μl of the lipoproteins was loaded onto a thin film agarose gel. Samples were run at 150 V for 30 min and transferred to nitrocellulose membranes. The membranes were sequentially exposed to the anti-apoB mAbs and ^{125}I-anti-mouse IgG. The mAb LF5 (a gift from Dr. Stephen Young) is specific for mouse apoB-100.

Figure 2-4

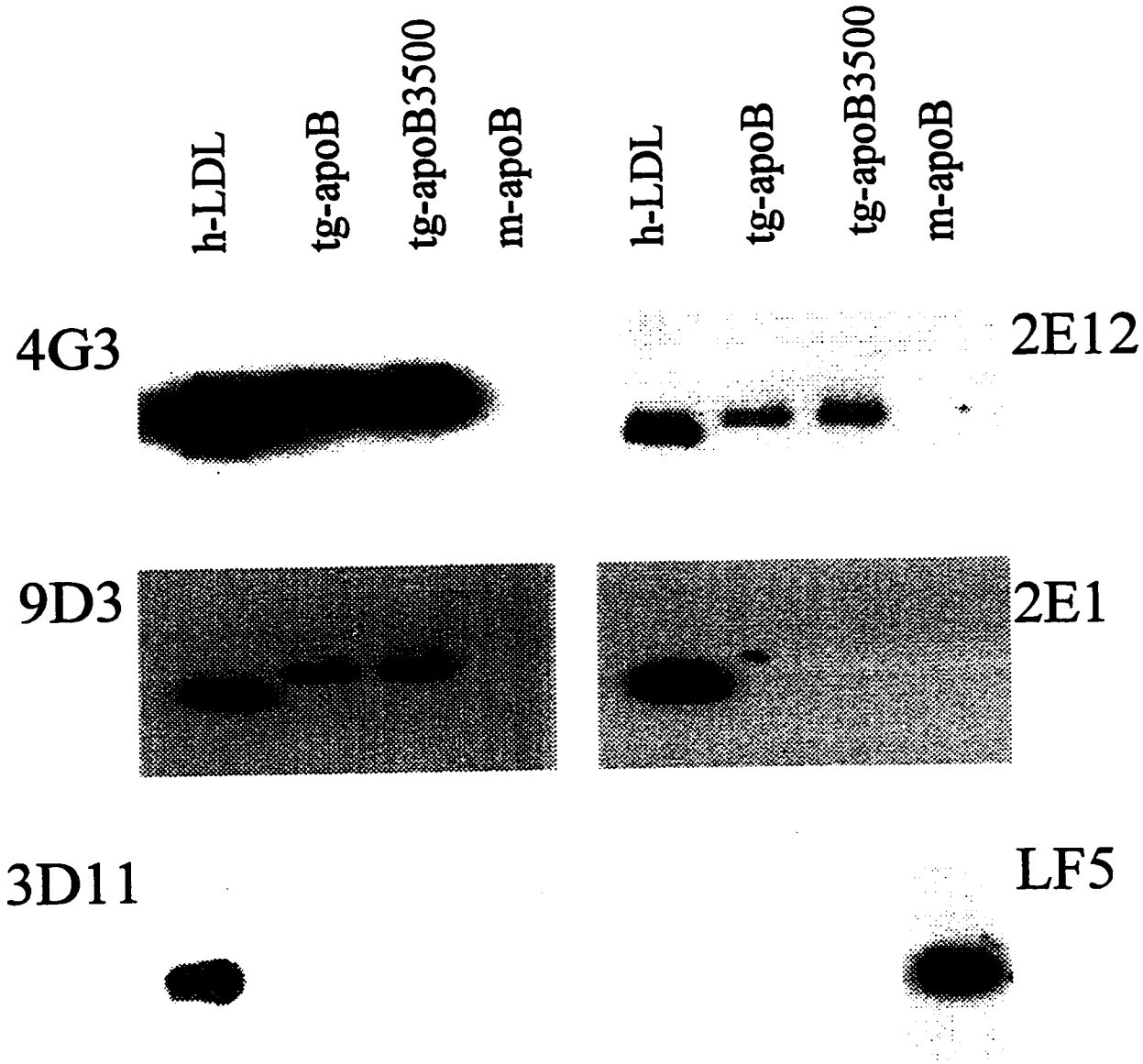
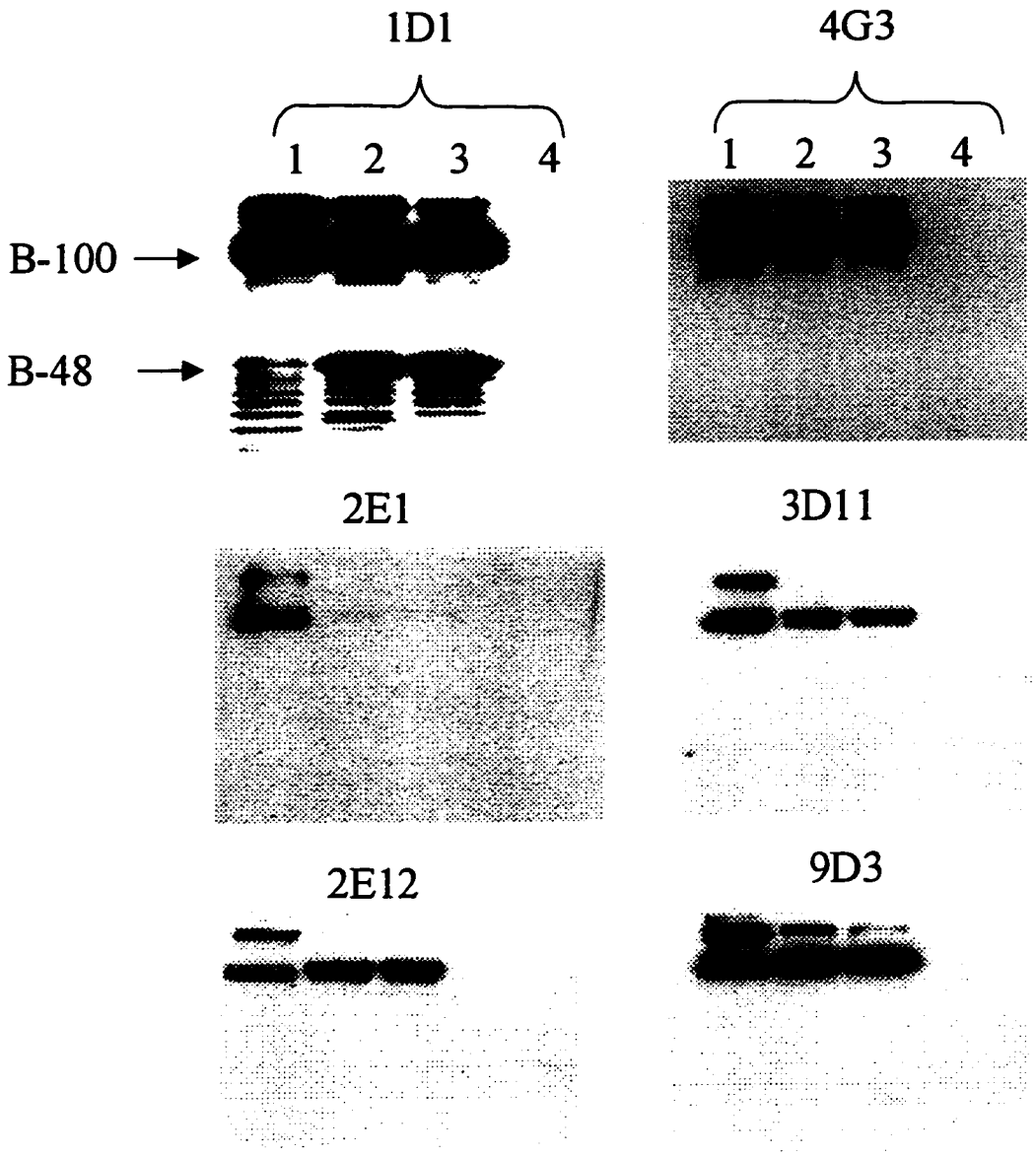

h-LDL
tg-apoB
tg-apoB3500
m-apoB
h-LDL
tg-apoB
tg-apoB3500
m-apoB
4G3
2E12
9D3
2E1
3D11
LF5

Figure 2-5; Reactivity of mAbs 2E1, 3D11, 9D3, 2E12, 4G3 and 1D1 with LDL isolated from human plasma (lane 1) and with lipoproteins isolated from the plasma of normal mice (lane 4) and from plasma of mice that express human wildtype apoB (lane 2) or apoB^{Gln3500} (lane 3) transgenes that had been subjected to SDS polyacrylamide electrophoresis.

Lipoproteins were subjected to SDS electrophoresis and were then transferred to nitrocellulose membranes. The membranes were sequentially exposed to the anti-apoB mAbs and ^{125}I-anti-mouse IgG. Note that only mAb 1D1 whose epitope is situated between apoB residues 474-539 reacts with human apoB-48 of the transgenic mice.

Figure 2-5



liquid phase could only compete with the immobilized LDL for binding mAbs 2E1 and 3D11. Lipoproteins isolated from non-transgenic mice were ineffective competitors with all of the antibodies. The reactivities of the four antibodies with human LDL and the lipoproteins isolated from normal and human apoB transgenic mice as determined by the different assay formats are summarized in Table 2-1.

Figure 2-6; Competitive solid phase RIA of mAbs 2E1, 3D11, 9D3, 2E12 and 4G3 with LDL isolated from human plasma (h-HDL) and with lipoproteins isolated from the plasma of normal mice (mouse d<1.063) and from plasma of mice that express human wildtype apoB (tg-LpB d<1.063) or apoB^{Gln3500} (tg-LpB3500 d<1.063) transgenes.

Dilutions of lipoproteins were mixed with an appropriate dilution of the mAbs and were added to polystyrene microtitre wells to which normal human LDL had been adsorbed. After incubation, the bound mAb was detected with ^{125}I-anti-mouse IgG. Results are presented as B/B_o where B is the radioactivity bound in the presence of competitor and B_o is the radioactivity bound in the absence of competitor.

Figure 2-6

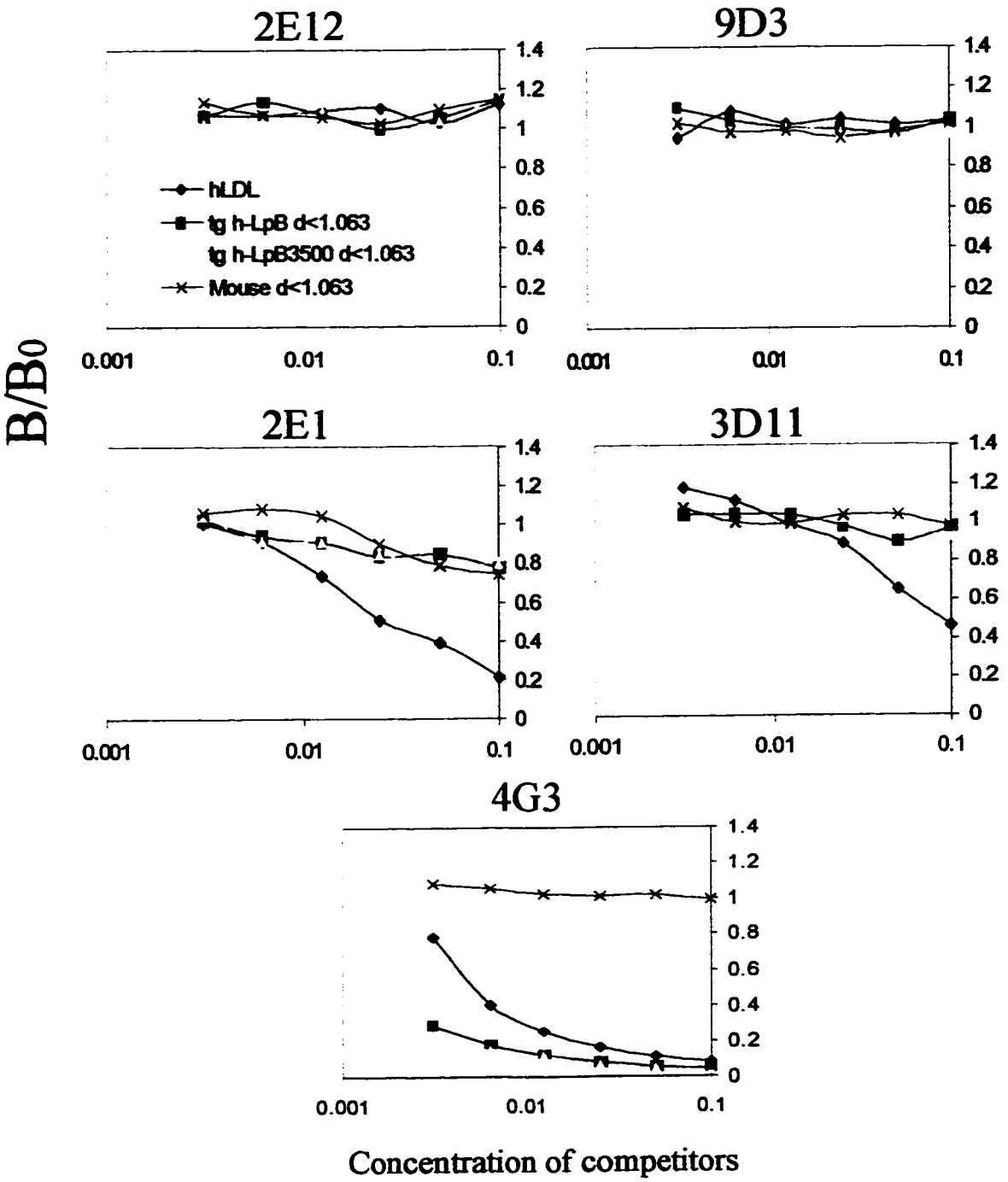

2E12
9D3
2E1
3D11
4G3
B/B0
hLDL
tg h-LpB d<1.063
tg h-LpB3500 d<1.063
Mouse d<1.063
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0.001
0.01
0.1
Concentration of competitors

Table 2-1

Reactivity of anti-apoB mAbs with LDL isolated from human plasma (hLDL) and with the lipoprotein fraction of normal mice (mouse d<1.063) and of mice that carry transgenes encoding wildtype human apoB (transgenic LpB) or human apoB^{Gln3500} (transgenic LpB^{Gln3500}).

MAb 2E1	Solid Phase RIA	Competitive RIA	Western Blot	Agarose Gel
HLDL	++++	++	+++	++++
transgenic LpB	-	+	-	-
transgenic LpB^{Gln3500}	-	+	-	-
mouse d<1.063	-	+	-	-

MAb 2E12	Solid Phase RIA	Competitive RIA	Western Blot	Agarose Gel
HLDL	++++	-	++	+++
transgenic LpB	++	-	+++	++
transgenic LpB^{Gln3500}	++	-	+++	++
mouse d<1.063	-	-	-	-

MAb 9D3	Solid Phase RIA	Competitive RIA	Western Blot	Agarose Gel
HLDL	++++	-	++++	++++
transgenic LpB	+++	-	++++	+++
transgenic LpB^{Gln3500}	+++	-	++++	+++
mouse d<1.063	-	-	-	-

3D11	Solid Phase RIA	Competitive RIA	Western Blot	Agarose Gel
HLDL	++++	++++	+++	++++
transgenic LpB	-	-	+++	-
transgenic LpB^{Gln3500}	-	-	+++	-
mouse d<1.063	-	-	-	-

4G3	Solid Phase RIA	Competitive RIA	Western Blot	Agarose Gel
HLDL	++++	++++	++++	++++
transgenic LpB	++++	++++	++++	++++
transgenic LpB^{Gln3500}	++++	++++	++++	++++
mouse d<1.063	-	-	-	-

Discussion

We have evaluated three methods to manipulate the immune response of mice in order to generate antibodies that are capable of discriminating between apoB isoforms. The three methods are based on the principle of immunizing mice that have natural or experimentally-induced immunological tolerance to one apo B isoform, with LDL having apoB of a different isoform. Assuming that a complete and antigen-specific non-responsive state could be established, we predicted that the immune response of the mice would be restricted to apoB epitopes that distinguish the apoB used for immunization from that to which the animal is tolerant. We have attempted to raise mAbs that could discriminate between LDL isolated from normal and FDB individuals, respectively. In addition to antibodies specific for epitopes that depend directly or indirectly on the nature of the residue at position 3500, we anticipated that we would also obtain antibodies specific for other apoB polymorphisms, including those that define the apoB Ag system. However, in addition to apoB epitopes that are differentially expressed on LDL due to polymorphism at the apoB locus, we show here that subtractive immunization and immunization of apoB transgenic mice can preferentially give rise to antibodies that are specific for epitopes that are only expressed on denatured or partially-denatured apoB, antibodies to minor proteins in the LDL fraction (apoE, apo(a)) and antibodies specific for apoB epitopes whose conformation is highly dependent upon the physical and chemical properties of the lipoprotein environment.

We were unsuccessful in our attempts to establish neonatal tolerance in mice to human LDL. It has long been recognized that, when animals are exposed to a foreign antigen during the neonatal period, specific long-lasting immunological tolerance to the antigen can be established. This phenomenon likely reflects immunological self / non-self recognition mechanisms that have been shown to include clonal anergy, active suppression (immune deviation) and clonal deletion (apoptosis of antigen-specific B and T lymphocytes) (Strobel, 1996; Liblau *et al*, 1994). Induction of neonatal tolerance has been used to manipulate the immune response for the production of specific antibodies (Golumbeski and Dimond, 1986; Williams *et al*, 1992). We have attempted to induce neonatal tolerance to $apoB^{Arg3500}$ LDL by injecting LDL from a normal individual into newborn mice. It was hoped that, when these mice had achieved immunological maturity and were immunized with LDL from an FDB subject, the immune response would be limited to epitopes that were present on the LDL used for immunization and absent from the LDL used to provoke neonatal tolerance. Such epitopes could include those that, directly or indirectly, result from the $apoB^{Arg3500 \rightarrow Gln}$ substitution. The immune response in these mice, however, showed no isoform specificity based on either the specificity of the anti-apoB response in the serum or that of the anti-apoB hybridomas that were generated (results not shown). Mice that had been injected with human LDL within the first 24 hr after birth contained circulating anti-human LDL antibodies when tested at 6 to 8 weeks of age. Moreover, the neonatal exposure to normal human LDL appeared to prime the mice for the subsequent immunization with FDB LDL. This has also been described for other antigens (Williams *et al*, 1992). Not surprisingly, therefore, in the

present study, neither the circulating antibodies in the mice nor the anti-apoB hybridomas that were generated from these mice showed any ability to discriminate between normal and FDB LDL. Others have reported that the induction of neonatal tolerance in mice can be used to modulate a subsequent immune response and the intra-molecular specificities of hybridomas that are generated (Golumbeski and Dimond, 1986). The conditions used in the present study to establish the non-responsive state may not have been optimal as it has been demonstrated that the dose of neonatally-administered antigen that is required to induce tolerance can vary greatly depending on the nature of the antigen (Golumbeski and Dimond, 1986).

A second approach that we have evaluated consists of chemical suppression of the immune response to one apoB isoform before immunization with a second isoform (Williams *et al*, 1992). Cyclophosophamide selectively kills rapidly dividing cells. If it is administered immediately following immunization, the rapidly dividing population that is targeted will include antigen-specific B and T lymphocytes that have been stimulated to proliferate on exposure to the antigen. When an appropriate dose of cyclophosphamide is administered at the time of immunization, antigen-specific immune suppression can result. (Bach and Strom, 1986; Turk *et al*, 1972; Matthew and Sandrock, 1987). In contrast to our inability to induce specific neonatal tolerance to human LDL in mice, we observed that the administration of a sublethal dose of cyclophosphamide to mice immediately after immunization with LDL totally suppressed a subsequent immune response to the same LDL. This suppression appeared to be epitope-specific as an

immune response, albeit reduced, was detected in mice in which different LDL preparations were used for suppression and immunization, respectively (Figure 2). Unfortunately however, the repertoire of anti-apoB hybridomas that were generated from mice subjected to this subtractive immunization protocol did not reflect the apparent specificity that had been observed in the serum. Only 2 of the 56 anti-apoB mAbs that were obtained preferentially reacted with the LDL used for immunization compared to that used for suppression and, in neither case, was the specificity related to the Gln→Arg substitution at apoB residue 3500. Although this immunization protocol was relatively inefficient for the generation of apoB isoform-specific monoclonal antibodies, subtractive immunization may, nevertheless, have influenced the repertoire of anti-apoB hybridomas that were obtained. Relatively few of the mAbs that were derived from mice that had been subjected to subtractive immunization would compete for binding to LDL with members of our large panel of anti-apoB mAbs that had been prepared using a conventional immunization protocol (Pease *et al*, 1990, Wang and Milne, unpublished observation). Administration of antigen and cyclophosphamide may have selectively suppressed the immune response to immunodominant epitopes of apoB. As a consequence, subsequent immunization may have elicited a humoral response directed primarily to apoB epitopes that are normally poorly immunogenic. The different repertoire of anti-apoB mAbs obtained from mice in these experiments may also reflect the use of the water-soluble muramyl peptide adjuvant which allows the LDL to be maintained in an aqueous solution. This may be less disruptive to apoB conformation than oil based Freund's adjuvant. Of the two mAbs that did show selective reactivity,

mAb 10D2 was specific for apo (a) that was present as a contaminant in the LDL that had been isolated from one of the FDB donors. The other mAb 3A5 recognized an epitope that was differentially expressed in apoB-containing lipoproteins prepared from different individuals. The epitope has been mapped to a region between apoB residues 1880-2100 (Wang *et al*, manuscript submitted). No differences in the genomic sequence encoding this region of apoB were detected between two individuals whose VLDL, IDL and LDL showed high or low expression, respectively, of the 3A5 epitope (Wang and Milne, unpublished results). It is possible that the substitution of amino acids outside of this region influence the expression of the 3A5 epitope.

Immunization of apoB transgenic mice with human LDL elicited an immune response that was weak but very specific for epitopes that differentiated the LDL used for immunization from the product of the transgene. This was also reflected in the number of hybridomas that were obtained from the mice and the specificity of the mAbs that they secreted. The mAb 1E9 was specific for apoE, a minor constituent of LDL that was isolated from human plasma. Two of the mAbs were specific for epitopes of human apoB that are only expressed when apoB conformation is modified by adsorption of lipoproteins to a solid phase. The mAb 2E1 is specific for an epitope that is present on the human apoB used for immunization but barely detectable on the product of the human apoB transgene. As this is the case for both denatured apoB and native LpB, this differential reactivity presumably reflects human apoB genetic polymorphism or

differences between humans and mice in their post-translational modification of human apoB.

Antibody 3D11 clearly has the most interesting specificity. It reacts with LpB isolated from human plasma but not with human LpB prepared from transgenic mice (Figures 2-3, 2-4, 2-6, Table 2-1). Unlike mAb 2E1, however, the ability of mAb 3D11 to discriminate between human LpB and transgenic human LpB, is lost when the apoB is subjected to the denaturing and delipidating conditions of SDS electrophoresis (Figure 2-5, Table 3-1). One possibility is that the expression of the 3D11 epitope on native apoB is highly dependent upon the physical properties and chemical composition of the LpB particle in which it is found. It has been shown that greater than 90% of the human apoB in the plasma of transgenic mice is found in lipoproteins that have a size, hydrated density and electrophoretic mobility that is characteristic of LDL. However, compared to LDL in normolipidemic humans, the human LDL in transgenic mice are triglyceride-enriched (Linton *et al*, 1993). This triglyceride enrichment does not appear to be the consequence of the absence of CETP activity in the transgenic mice (Grass *et al*, 1995) nor due to a particularity of human apoB (McCormick *et al*, 1996). It has been proposed that low hepatic triglyceride lipase activity or direct hepatic secretion of triglyceride-rich LDL could contribute to the unusual LDL composition in the transgenic mice (McCormick *et al* 1996). Thus, in transgenic human LpB, the 3D11 epitope could have an altered conformation due to the triglyceride enrichment or, alternatively, the epitope could be masked by the presence of other apolipoproteins.

Based on the specificity of the four anti-apoB mAbs, none would appear to be capable of high affinity binding to human apoB-containing lipoproteins in the circulation of the transgenic mice. Antibody 2E1 is specific for an epitope of human apoB that is absent or very poorly expressed on native or denatured transgenic human apoB. The 3D11 epitope is not expressed on intact LpB but can be detected on transgenic apoB that had been subjected to SDS-PAGE electrophoresis. Antibodies 2E12 and 9D3 react with intact transgenic human LpB in either a solid phase RIA or after agarose electrophoresis of the LpB. However, results with the competitive RIA show that expression of the 2E12 and 9D3 epitopes on transgenic human LpB only occurs when the lipoproteins are immobilized on a solid surface. We have previously shown that apoB undergoes a conformational change when LDL is immobilized and epitopes, that are not expressed on LpB in the liquid phase, can become accessible to mAbs (Milne *et al*, 1987). Finally, none of the mAbs showed reactivity with native or denatured mouse apoB. Thus, immunological tolerance to the product of the human apoB transgene and to endogenous mouse apoB in the transgenic mice was not broken by the immunization protocol.

Our lack of success in obtaining mAbs that can distinguish between $apoB^{Arg3500}$LDL and $apoB^{Gln3500}$LDL likely reflects the weak immune response that is elicited in the human apoB transgenic mice following immunization with human LDL. From three separate fusions, only four hybridomas were identified that secrete apoB-specific mAbs. More frequent boosts and / or alternative adjuvants may elicit a more vigourous immune response in the mice that would include antibodies whose epitopes are directly or indirectly modulated by residue 3500. Furthermore, as both human

apoB^{Arg3500}- and apoB^{Gln3500} transgenic-mice are now available (Borén *et al*, 1998), whose respective transgenes differ only at residue 3500, it may be possible induce an even more specific response in the human apoB transgenic mice by immunization with transgenic human LpB. Another possibility is that the immune response of the human apoB transgenic mice to human LDL was limited not only by tolerance to the product of the human transgenic but also by the tolerance to the endogenous mouse apoB. This could be especially critical for epitopes in the highly conserved region that includes the apoB LDL receptor-binding site. Human apoB transgenic mice whose endogenous apoB alleles have been inactivated by gene targeting may obviate this limitation.

The restricted repertoire of antibody specificities that we obtained using transgenic mice as a source of spleen cells for the generation of hybridomas demonstrates the potential of this immunization protocol. Of the four anti-apoB mAbs that were identified, one could apparently discriminate between apoB isoforms. Such a high percentage of isoform-specific antibodies would never be obtained using a standard immunization protocol. This approach is obviously not limited to apoB and could be used for the production of isoform-specific mAbs for any antigen for which the appropriate transgenic mice exist. An obvious example in the field of lipoproteins would be anti-apoE isoform-specific mAbs. In addition to the anti-apoB mAbs that we describe, it is also notable that we have isolated an anti-apoE-specific hybridoma from mice that had been immunized with human LDL. As apoE is present in very low concentration in LDL compared to apoB, it suggests that the immunization of transgenic mice could be an effective method of making mAbs against minor components in antigen preparations.

Chapter III

The Carboxy-Terminus of Apolipoprotein B-100 Undergoes A Major Conformational Change during Its Intravascular Metabolism

Summary

ApoB100-containing lipoproteins (LpB) are secreted from the liver as large triglyceride-rich, very low density lipoproteins (VLDL) into the circulation where they are transformed, through the action of lipoprotein lipase, hepatic lipase, and plasma lipid transfer proteins, into smaller, less buoyant, cholesteryl ester-rich low density lipoproteins (LDL). As a consequence of this intravascular metabolism, apoB-containing lipoproteins (LpB) are heterogeneous in size, hydrated density, surface charge, lipid, and apolipoprotein composition. This physical and chemical heterogeneity of LpB is, in turn, manifested in conformational and functional heterogeneity of apoB itself. To identify specific regions of apoB that undergo conformational changes during the intravascular transformation of VLDL into LDL, we have employed a panel of 29 well characterized anti-apoB monoclonal antibodies (mAbs) to determine if individual apoB epitopes are differentially expressed on VLDL, intermediate density lipoproteins (IDL) and LDL

isolated from six normal subjects. When analyzed in a solid phase radioimmunometric assay, the expression of most epitopes was not significantly different in VLDL, IDL and LDL. The carboxy terminal region between residues 4342-4536 and LDL receptor binding region between residue 2658-3268 of apoB were, however, significantly more immunoreactive in LDL than in VLDL and IDL. To see if apoB conformational heterogeneity within the LDL subfraction can also be detected with this panel of mAbs, a similar analysis has been performed on LDL subfractions from the same subjects that had been isolated by discontinuous gradient ultracentrifugation. Only the carboxy terminal epitopes of apoB were differentially expressed in the LDL subfractions, with the most buoyant subfraction (1.019-1.028 g / ml) being less reactive than the subfractions of medium (1.028-1.041 g / ml) and high (1.041-1.050 g / ml) density. The other epitopes were equally expressed among the subfractions of LDL, including the epitopes that are involved in LDL receptor recognition. It would appear, therefore, that the carboxy terminus of apoB undergoes a major conformational change as large LDL are converted into smaller, denser particles. Such a conformational change could have important functional implications as the apoB carboxy terminus has been proposed to modulate the accessibility and / or conformation of the apoB LDL receptor-binding site.

Introduction

Apolipoprotein B-100 (apoB-100), a 550 kDa glycoprotein composed of 4536 amino acid residues, is a predominant protein component of very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and low density lipoproteins (LDL) (Knott *et al,* 1986; Yang *et al,* 1986). It is a major transporter of both triglyceride and cholesterol in the plasma and, as a ligand for the LDL receptor, it plays an important role in the maintenance of cholesterol homeostasis. ApoB-100 is synthesized in the liver and secreted in the form of large, triglyceride-rich VLDL. Within the circulation, VLDL are transformed into smaller, denser, cholesteryl ester-rich LDL through the combined actions of plasma lipid transfer proteins, lipoprotein lipase, and hepatic lipase, a process that is accompanied by the loss of all apolipoproteins with the exception of apoB-100. As a consequence of the intravascular remodeling, apoB-containing lipoproteins (LpB) are constituted of subpopulations of particles that differ in terms of particle diameter, hydrated density, surface charge and apolipoprotein and lipid composition. This heterogeneity in the physical and chemical properties of LpB leads to apoB conformational heterogeneity that is manifested in the differential accessibility of protease-sensitive sites (Chen *et al,* 1989 and 1991) and expression of apoB epitopes (Curtiss and Edgington 1982; Tikkanen *et al,* 1984; Marcel *et al,* 1988). This has functional consequences, particularly in terms of the ability of apoB to mediate binding to the LDL receptor. While VLDL can bind to the LDL receptor, this binding is mediated almost exclusively by apoE. However, as VLDL

are converted into LDL within the circulation, there is a concomitant increase in the ability of apoB to mediate binding of the particle to the LDL receptor (Marcel *et al,* 1988; Krul *et al,* 1985). The LDL fraction, itself, is composed of discrete subfractions of particles that differ in their physical and chemical properties. LDL are heterogeneous in terms of apoB conformation (Aviram *et al,* 1988; McKeon *et al,* 1993; Galeano *et al,* 1994; McNamara *et al,* 1996), epitope expression (Aviram *et al,* 1988; McKeon *et al,* 1993; Galeano *et al,* 1994; Teng *et al,* 1985; Kinoshita *et al,* 1990), accessibility of protease sensitive sites (Chen *et al,* 1994) and affinity for the LDL receptor (Aviram *et al,* 1988; McKeon *et al,* 1993; Galeano *et al,* 1994; Kleinman *et al,* 1985; Campos *et al,* 1996). Whether it is lipid composition, particle diameter or another variable that is the major modulator of apoB conformation in LDL remains controversial. LDL heterogeneity is thought to have important clinical implications as a predominance of small, dense LDL particles has been reported to be a risk factor for atherosclerosis (Sniderman *et al,* 1980).

The large size of apoB-100, its hydrophobicity and its propensity to aggregate in the absence of lipid has hampered attempts at determining its native conformation. Immunoelectron microscopic analyses suggest that apoB-100 adopts an extended structure on the surface of LDL and wraps around the particle (Chatterton *et al,* 1991, 1995). Unlike most of smaller exchangeable apolipoproteins that are thought to be constituted primarily of tandem amphipathic α-helices, apoB-100 is predicted to contain both amphipathic α-helices and amphipathic β-sheets (Knott *et al,* 1986; Yang *et al,* 1986). By a number of experimental and theoretical criteria, apoB-100 appears to be

composed of distinct structural domains (Chen *et al,* 1989; Yang *et al,* 1989; Segrest *et al,* 1994). Results from limited proteolysis of LpB have suggested that apoB-100 is organized into 3 protease-resistant domains separated by two protease-sensitive regions located between residues 1280-1320 and 3180-3280, respectively (Chen *et al,* 1989). On the other hand, based on the identification of apoB fragments that were either released from, or remained with LDL particles following trypsin digestion, Yang *et al,* (Yang *et al,* 1989) have proposed that apoB100 is composed of five domains that differ in their relative affinities for lipid and / or trypsin accessibility. A five-domain model of apoB secondary structure has also been proposed (Segrest *et al,* 1994) based on a computer-assisted analysis of apoB-100 primary structure. In this pentapartite model, individual domains are characterized by their respective affinities for lipid and their predicted relative contents of amphipathic α-helices and amphipathic β-sheets.

Here I describe an immunochemical analysis of apoB in LpB fractionated as a function of their hydrated density. While there have been a number of reports in which mAbs have been employed as probes of apoB conformation, this study is unique in that I have used a panel of 29 well characterized anti-apoB mAbs whose corresponding epitopes are distributed throughout apoB primary structure. Moreover, I have compared epitope expression in lipoprotein fractions isolated from the same individual. I demonstrate that the carboxy terminus of apoB undergoes a major conformational change during the intravascular metabolism of LpB that may be implicated in regulating the ability of apoB

to mediate binding of the particle to the LDL receptor. Furthermore, the characterization of several new anti-apoB mAbs has helped to define the apoB LDL receptor-binding site more precisely.

Material and Methods

Preparation of lipoproteins

Fresh plasma from normolipidemic subjects was prepared from blood obtained from the Canadian Red Cross. The plasma was supplemented with 0.5 mM EDTA, 0.02% NaN_3, 0.5 mM phenylmethanesulfonal fluoride and 0.5 ug/ml leupeptin. Chylomicrons were removed by centrifugation of the plasma at 20,000 rpm for 30 min. at 4^0C. VLDL (d<1.006), IDL (1.006-1.019) and LDL (1.019-1.063) were isolated by sequential ultracentrifugation at 40,000 rpm for 18 hr using a Beckman L5-65 ultracentrifuge with a 50.2 Ti rotor (Havel *et al,* 1955). Solvent densities, including those for the preparation of LDL subfractions, described below, were adjusted by adding anhydrous KBr.

Preparation of LDL subfractions

The method of discontinuous density gradient ultracentrifugation for the isolation of LDL subfractions was based on that described by Teng *et al,* (Teng *et al,* 1985). To a 40 ml cellulose nitrate tube, 8 ml of the following solutions were added in succession: 1.130 g/ml, LDL (1.063 g/ml), 1.050 g/ml, 1.041 g/ml and 1.0286 g/ml. The gradient was

centrifuged in a Beckman SW 28 rotor at 28.000 rpm for 48 h at 8°C. Three fractions (1.019-1.028 g/ml) (large LDL), 1.028-1.040 g/ml (medium LDL), and 1.040-1.063 g/ml (small LDL) were then recovered based on visual inspection and reference density tubes. The subfractions were characterized in terms of particle diameter by electrophoresis on a 4-15% polyacrylamide gradient under non-denaturing conditions (Williams *et al,* 1990).

Production of anti-human LDL mAbs

Certain of the hybridomas used in the present study were obtained from mice that had been subjected to a subtractive immunization protocol that has been described on page 64. Briefly, BALB/c female mice of 4-6 weeks old were immunized by intraperitoneal injection of 50 μg of human LDL mixed with 50 μg of the adjuvant N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl peptide) (Calbiochem, La Jolla. Ca) in a total volume of 100 μl PBS. Cyclophosphamide (100 mg / kg body weight) was injected intraperitoneally, 10 minutes, 1 hour, and 24 hours after antigen injection (Williams *et al,* 1992). After one month, 50 μg of LDL that had been isolated from a subject with familial defective apoB (FDB-LDL) were immunized intraperitoneally with 50 μg of FDB-LDL in 200 μl PBS containing 50 μg of muramyl peptide. The anti-human LDL immune response was monitored by detection of anti-human LDL antibodies in plasma with a solid phase immunometric assay using both normal LDL and FDB-LDL as antigen. Four days before the fusion, the mice were given an intravenous boost with 10 μg of normal LDL in PBS. Hybridomas that secrete anti-human LDL mAbs were the product of a cell fusion between

cells of the plasmacytoma line SP2-0 and isolated spleen cells from the immunized mice. Details of the cell fusion, screening for hybridomas, solid phase immunometric assay and subcloning have been described in detail (Milne *et al,* 1992).

Mapping the epitopes for monoclonal antibodies

Bacterial expression of apoB fragments and preparation of the expressed protein from bacterial inclusion bodies have been described previously, as has the production of apoB fragments T1, T2, T3 and T4 by thrombin digestion of LDL (Pease *et al,* 1990; Knott *et al,* 1985). Immunoblotting was carried out on thrombin-digested LDL and fusion proteins after separation by SDS-polyacrylamide gel electrophoresis under reducing conditions. Proteins were transferred to nitrocellulose paper and probed by subsequent incubations of the membrane with anti-human LDL mAbs and ^{125}I-rabbit anti-mouse IgG (Kirkegaard and Perry Laboratories Inc, Gaithersburg, MD) (Milne *et al,* 1992). Transfected McArdle-RH7777 cell lines that secrete carboxy-terminally truncated human apoB variants (kindly provided by Drs. Zemin Yao and Roger McLeod) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% serum (Yao *et al,* 1991). Secreted lipoproteins were isolated in the d < 1.21 g/ml fraction after ultracentrifugation of the culture medium. Reactivity of the secreted human LpB variants with mAbs was determined by a sandwich radioimmunometric assay using the anti-apoB mAb 1D1 as the solid phase capture antibody (Raffai *et al,* 1995). Antibody competition studies were carried out as previously described (Pease *et al,* 1990).

Lipoprotein Composition

Total and free cholesterol and phospholipid and triglyceride content were determined enzymatically using Boehringer Mannheim (Indianapolis, IN) kits according to the manufacturer's recommendations. For LDL, protein concentration was determined by the modified Lowry method (Markwell *et al,* 1978) using bovine serum albumin as the standard. To determine apoB protein content of VLDL and IDL, apoB was precipitated with 50% isopropanol and the protein content of the precipitate was measured after solubilization with 2% sodium deoxycholate (Vega and Grundy 1996).

LDL receptor binding assay

To assay the reactivity of LDL subclasses with the LDL receptor, samples were tested for their ability to compete with ^{125}I-LDL for binding to the LDL receptor on the surface of cultured human fibroblasts (Arnold *et al,* 1992). In short, 3 μg of ^{125}I-LDL, alone or in the presence of unlabeled competitor, were added to human fibroblasts that had been cultured in the presence of 10% lipoprotein-deficient serum for 24 hours. After a 3 hr. incubation at 4°C, surface bound ^{125}I-LDL was determined. Results are expressed as a ratio of radioactivity bound in the presence of competitor to that bound in the absence of competitor expressed as a function of the concentration of the competitor. To evaluate the ability of anti-human apoB mAbs to block the binding of -LDL to the LDL receptor, ^{125}I-LDL was incubated with a molar excess of purified mAbs at 4^{0}C for 15 minutes prior to addition to cultured human cultured fibroblasts (Milne *et al,* 1989). Binding assays were carried out as above. Methods to determine the accessibility of apoB epitopes in

LDL-LDL receptor complexes have been described previously (Milne *et al*, 1989). In brief, cells were incubated with LDL at a concentration of 3 μg/ml in DMEM medium supplemented with 10% LPDS for 2 h at 4 ^{0}C. The cells were washed and exposed to ^{125}I-anti-apoB mAbs at 4 ^{0}C for 1 h. Cells were then washed and the ^{125}I-radioactivity was determined. Negative controls were performed with medium replacing LDL in the initial incubation step.

Competitive Radioimmunoassay

The competitive apoB radioimmunometric assay has been described previously (Milne *et al*, 1989). Immulon II Removawells (Dynatech Laboratories, Chantilly, VA) were coated by an overnight incubation with 200 μl of reference LDL (30 μg / ml in 5 mM glycine, pH 9.2). Plates were washed with a solution of 0.15 M NaCl containing 0.025% Tween 20, and then subsequently saturated by incubation for 1 hr with 250 μl of 1% bovine serum albumin in phosphate-buffered saline, pH 7.4 (PBS-BSA). Serial dilutions (150 μl) of test and control LDL were prepared in separate microtitre plates to which was added 150 μl of anti-apoB mAb appropriately diluted in PBS-BSA. After a 2 hr incubation, 200 μl of the LDL-mAb mixture was transferred to the LDL-coated Removawells. The plates were incubated overnight and again washed with the Tween-saline solution. Two hundred μl of ^{125}I-goat anti-mouse IgG labeled by the chloramine-T method (Milne *et al*, 1992) was diluted to 70 ng / ml in 1% PBS-BSA and added to each

well and incubated overnight. The wells then washed with the Tween-saline solution as above and counted for bound radioactivity.

Results

Production and mapping the epitopes of new anti-human apoB mAbs

In addition to a panel of previously described anti-human apoB mAbs, we have generated and characterized a new panel of anti-apoB mAbs from mice immunized with human LDL. While many anti-human apoB hybridomas were obtained from a total of 8 fusions, only the characterization of the mAbs that showed specificity different from those of our previous panel are described. The epitopes of the mAbs were mapped within apoB primary structure by their reactivity with i) T4 (residues 1-1297), T3 (residues 1298-3249) and T2 (residues 3250-4536) fragments of apoB generated by thrombin digestion of LDL, with ii) apoB fragments produced as β-galactosidase fusion proteins in E.coli and iii) with carboxy-terminally truncated apoB and apoA-I / apoB fusion proteins produced by appropriately transfected McA-7777 cell lines. In the latter case, mAbs were tested for reactivity with denatured apoB by Western blotting after SDS polyacrylamide electrophoresis and with native LpB using a sandwich radioimmunometric assay. The characterization of several of the mAbs that are of particular interest for the present study are presented in detail. Based on the reactivity of mAbs 4H11 and 605 with both the T2 thrombolytic fragment of apoB (Table 3-1) and the bacterial β-galactosidase fusion protein T2/8 (apoB residues 4081-4536) and their failure to recognize the T2/7 β-galactosidase fusion protein (residues 4081-4342) (Figure 3-1), the respective epitope(s) were assigned to a region encompassing apoB residues 4243-4536. A summary of the

Table 3-1.

Immunoreactivity of mAbs with full length and thrombolytic fragments of apoB.

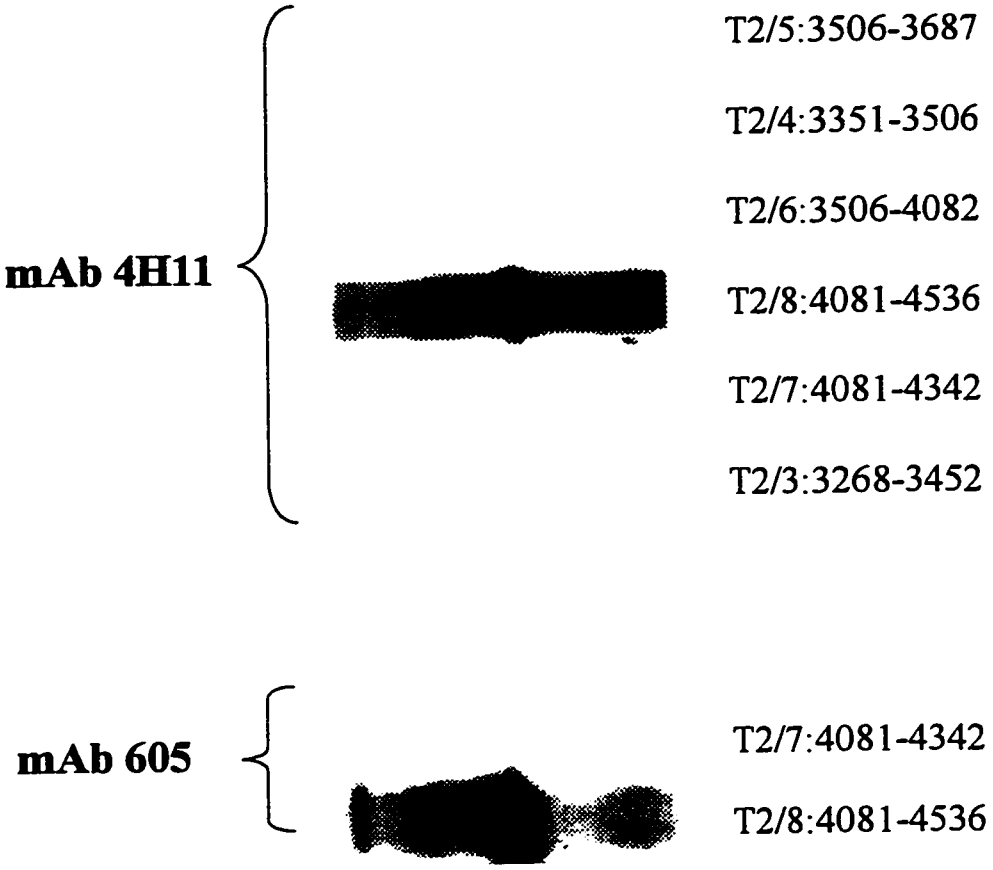
mAbs	376	746	3E11	1M1	1C4	374	2G4	3G9	278	390	588	4H11	3E8	234	605
ApoB	+++	+++	++	+	+	++	+++	+	++	++	+	+++	+	+++	+++
T4	+++	++	+	+	-	-	-	-	-	-	-	-	-	-	-
T3	-	-	-	-	+	+	++	+	++	+	-	-	-	-	-
T2	-	-	-	-	-	-	-	-	-	-	++	+++	+	+++	+++

Immunoblotting of mAbs with full length and thrombolytic fragments of apoB on SDS-PAGE (5%). Full length ApoB and its thromlytic fragments were transferred electronically to nitrocellulose paper and the mAbs against apoB were incubated and subsequently detected with appropriate ^{125}I-labeled secondary antibodies.

Antibodies 1D1 (474-539), Bsol 5 (2488-2543) and Bsol 7 (4521-4536) were used as positive controls for thrombin digested fragments and not listed in the table.

Figure 3-1: Representative immunoblots of mAbs with apoB fusion protein.
ApoB-β-galactosidase fusion proteins expressed in E.coli. were subjected to SDS-PAGE (5%), transferred to nitrocellulose membrane and probed with anti-apoB mAbs of interest and ^{125}I-anti mouse IgG secondary mAbs. Sequence of apoB fusion protein are T2/3: 3286-3452, T2/4: 3351-3506, T2/5: 3506-3687, T2/6: 3506-4082, T2/7: 4082-4352, T2/8: 4082-4536 (Pease *et al*, 1990).

Figure 3-1


mAb 4H11
T2/5:3506-3687
T2/4:3351-3506
T2/6:3506-4082
T2/8:4081-4536
T2/7:4081-4342
T2/3:3268-3452
mAb 605
T2/7:4081-4342
T2/8:4081-4536

reactivities of all the antibodies with apoB fragments generated by thrombin digestion is presented in Table 3-1 and with apoB fusion proteins and truncated apoB variants produced in E.coli or McA-7777 cells is presented in Table 3-2. A map of the epitopes of the new mAbs, along with those previously described anti-human apoB mAbs that are used in the present study is presented in Figure 3-2.

It is noted that the epitopes of several of the mAbs are located within the region of apoB that includes the LDL receptor-binding site. The epitopes for mAbs 278, 3G9 and 390 have been assigned to a region that includes residues 2658-3268, and all three mAbs mutually compete with 4G3 (residues 2980 to 3084) but not with 3F5 (residues 2835-2922) for binding to immobilized LDL (not shown). The 278 epitope could be distinguished from the 4G3, 390 and 3G9 epitopes by their respective sensitivities to reductive methylation (not shown). The mAb 588 was mapped to a region between apoB residues 3687-4081. Partial (25%), mutual competition between 5E11 (residues 3441-3506) (Pease *et al,* 1990) and 588 for binding to immobilized LDL was observed whereas mAb MB47 (residues 3429-3453, 3507-3523) (Young *et al,* 1994) did not compete with 588. All mAbs were tested for their ability to block binding of ^{125}I-LDL to the LDL receptor on cultured human fibroblasts. As would be predicted, mAbs specific for epitopes that are outside the previously defined LDL receptor-binding region of apoB (Milne *et al,* 1989) did not block binding of LDL to the LDL receptor. Antibodies 278, 3G9, 390 and 588 inhibit LDL binding to the LDL receptor, although in the case of mAbs 390 and 588, inhibition was only partial (Figure 3-3). The accessibility of all epitopes on

Table 3-2.

Immunoreactivity of mAbs with fusion protein produced by E.coli.

Epitopes in T4 (1-1297)

	97-474	97-689	582-851	851-1084	995-1328	851-1082	995-1328	Epitope position
376	-	+	-	-	-	-	-	474-582
746	-	++	-	-	-	-	-	474-582
3E11	-	-	-	-	-	-	+++	1082-1328
1D1	-	++	-	-	-	-	-	474-539

1D1 whose epitopes has been mapped previously is used as a control mAb.

Epitopes in T3 (1298-3286)

	1281-1328	1281-1480	1281-1880	1480-1880	1480-2240	1693-2148	2148-2375	2240-2658	2658-3268	2488-3215	3132-3494	Epitope position
374	-	-	++	-	-	-	-	-	-	-	-	1306-1542[a]
1C4	-	-	+	-	-	-	-	-	-	-	-	1696-1880[b]
3A5	-	-	-	-	-	-	-	-	-	-	-	1880-2100[c]
2G4	-	-	-	-	-	-	++	-	-	-	-	2148-2375
3G9	-	-	-	-	-	-	-	-	+++	-	-	2658-3268
278	-	-	-	-	-	-	-	-	+++	-		2658-3268
390	-	-	-	-	-	-	-	-	+	-		2658-3268
4G3	-	-	-	-	-	-	-	-	++	+		2980-3084

The previously characterized mAb, 4G3, is used as a control mAb which epitope has been mapped previously. [a,b]The epitopes of 374 and 1C4 were further narrowed to 1306-1542 and 1696-1880 based on their reactivity with apoB fusion proteins produced by McArdle-7777 cells. [c]The mAb 3A5 was mapped between 1880-2100 based on its immunoreactivity with carboxyl terminally truncated apoB expressed in McArdle-7777 cell line. 3A5 reacts with apoB 46 (terminates at residue 2100) and fails to react with B42 (terminates at residue 1880) (McLeod *et al*, 1996).

Epitopes in T2 (3287-4536)

	3132-3494	3214-3635	3214-3727	3286-3452	3351-3506	3506-3687	3506-4081	4081-4342	4081-4536	Epitope position
588	-	-	-	-	-	-	++	-	-	3687-4081
4H11	-	-	-	-	-	-	-	-	+++	4342-4536
3E8	-	-	-	-	-	-	-	-	+	4342-4536
234	-	-	-	-	-	-	-	-	+++	4342-4536
605	-	-	-	-	-	-	-	-	+++	4342-4536
Bsol 7	-	-	-	-	-	-	-	-	+++	4521-4536

Bsol 7 is used as a control mAb whose epitope has been mapped previously

Figure 3-2: A map of the apoB epitopes recognized by mAbs used in the present study.
The primary sequence of apoB is indicated as a solid line. Numbers under the apoB primary sequence are the amino acid residues, above the line are the mAbs. The mAbs colored red have not been previously reported. The mAbs colored green were the mAbs mapped previously (Pease *et al*, 1990)

Figure 3-2

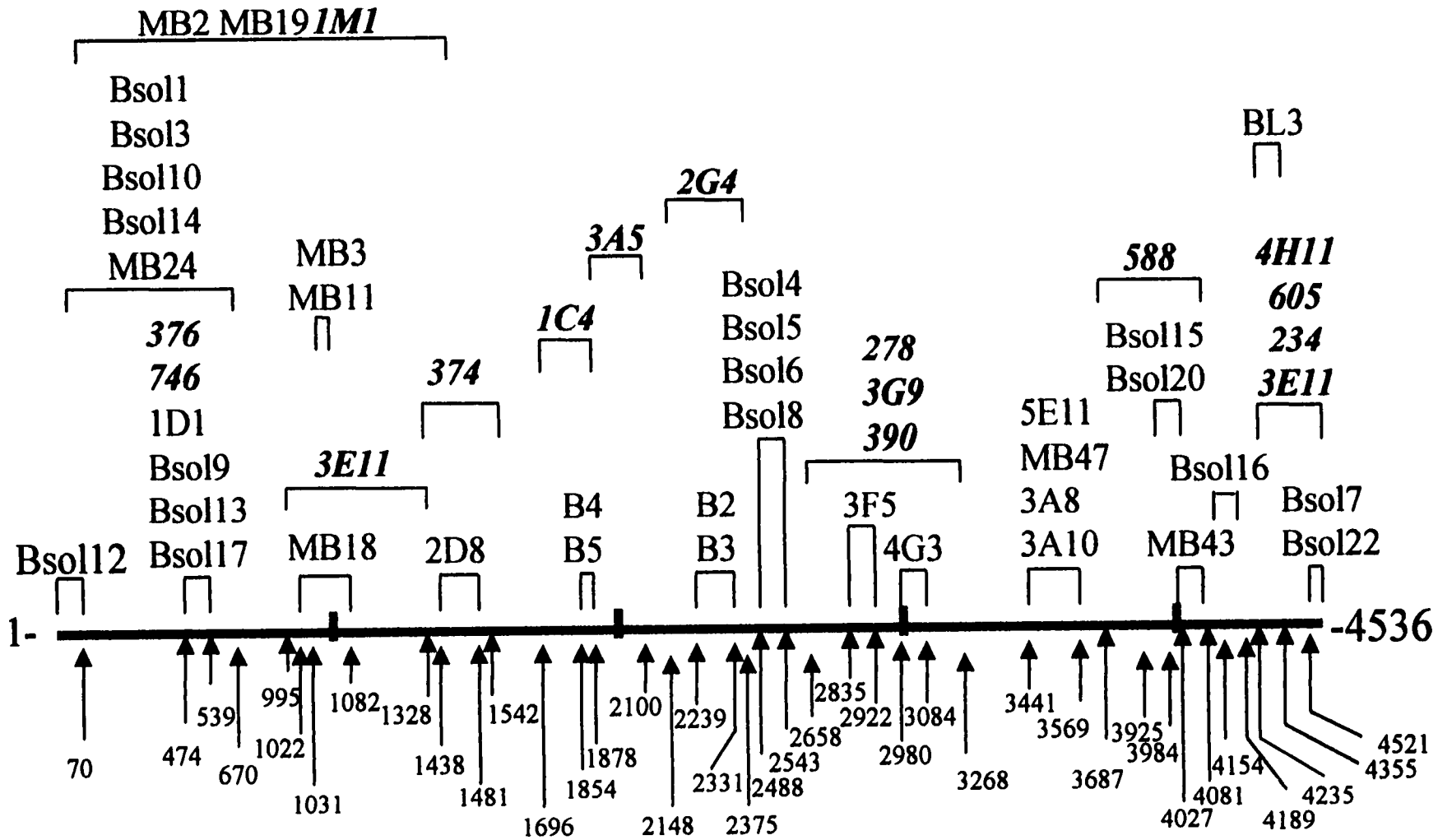
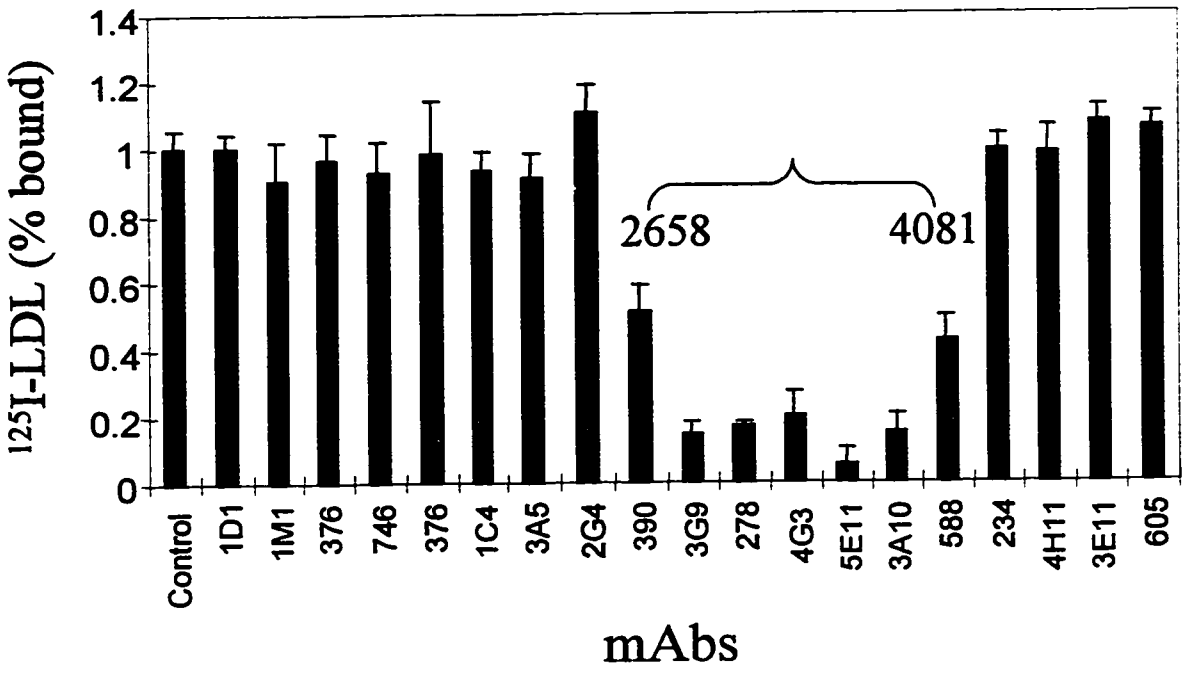

MB2 MB19 1M1
Bsol1
Bsol3
Bsol10
Bsol14
MB24
376
746
1D1
Bsol9
Bsol13
Bsol17
Bsol12
MB3
MB11
3E11
MB18
374
2D8
1C4
3A5
B4
B5
2G4
Bsol4
Bsol5
Bsol6
Bsol8
B2
B3
278
3G9
390
3F5
4G3
5E11
MB47
3A8
3A10
588
Bsol15
Bsol20
Bsol16
MB43
BL3
4H11
605
234
3E11
Bsol7
Bsol22
1-
-4536
70
474
539
670
995
1022
1031
1082
1328
1438
1481
1542
1696
1878
1854
2100
2148
2239
2331
2375
2488
2543
2658
2835
2922
2980
3084
3268
3441
3569
3687
3925
3984
4027
4081
4154
4189
4235
4355
4521

Figure 3-3: mAb-mediated of binding of LDL to the LDL receptor.
Newly generated mAbs were tested for their ability to inhibit the binding of ^{125}I-LDL to the LDL receptor on cultured human skin fibroblast (Milne, *et al.* 1989). The mAbs that are located within the brackets inhibit the binding of LDL to LDL receptor.

Figure 3-3


^{125}I-LDL (% bound)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2658
4081
Control
1D1
1M1
376
746
376
1C4
3A5
2G4
390
3G9
278
4G3
5E11
3A10
588
234
4H11
3E11
605
mAbs

the LDL-LDLr complex was also tested. The 278 and 3G9 epitopes are inaccessible on LDL-LDL receptor complexes whereas the epitopes for mAbs 390 and 588 are partially accessible (Figure 3-4).

Immunoreactivity of mAbs with VLDL, IDL, LDL and LDL subfractions.

To analyze how apoB conformation changes during the intravascular metabolism of LpB we have isolated VLDL, IDL, and LDL from the plasma of six normolipidemic individuals. LDL was further separated into 3 subfractions as described in Methods and Materials. The mean compositional analysis of the isolated lipoprotein fractions and LDL subfractions is presented in Table 3-3. The LDL subfractions were further analyzed by non-denaturing polyacrylamide gradient gel electrophoresis. As can be seen in Figure 3-5, we have observed the expected inverse relationship between LDL buoyant density and LDL particle diameter. We refer to the 1.019-1.028 g / ml, 1.028-1.040 g / ml, and 1.040-1.063 g / ml density subfractions as large, medium and small LDL, respectively. The relative distribution of total LDL apoB protein in the 3 subfractions was 6 ± 3%, 68 ±12% and 26 ± 8% in the large, medium and small LDL subfractions, respectively. Compared to large and medium LDL, the small LDL are depleted in cholesteryl ester and relatively enriched in triglyceride. Although there is slightly more apoE associated with large LDL by SDS-PAGE, no significant differences were seen between the three LDL subfractions in their ability to bind to the LDL receptor on cultured human fibroblasts (results not shown). Binding experiments were performed in the presence of purified 1D7

Figure 3-4: Binding of mAbs to receptor-bound LDL.
Results are expressed as the molar ratio of bound mAb to receptor-bound LDL. Molecular weight of 150,000 for IgG and 512, 000 for apoB were used to calculate the ratio. All results are normalized to 2D8 that was assigned a ratio of one. Results indicated with an asterisk were taken from Milne *et al*, (1989).

Figure 3-4

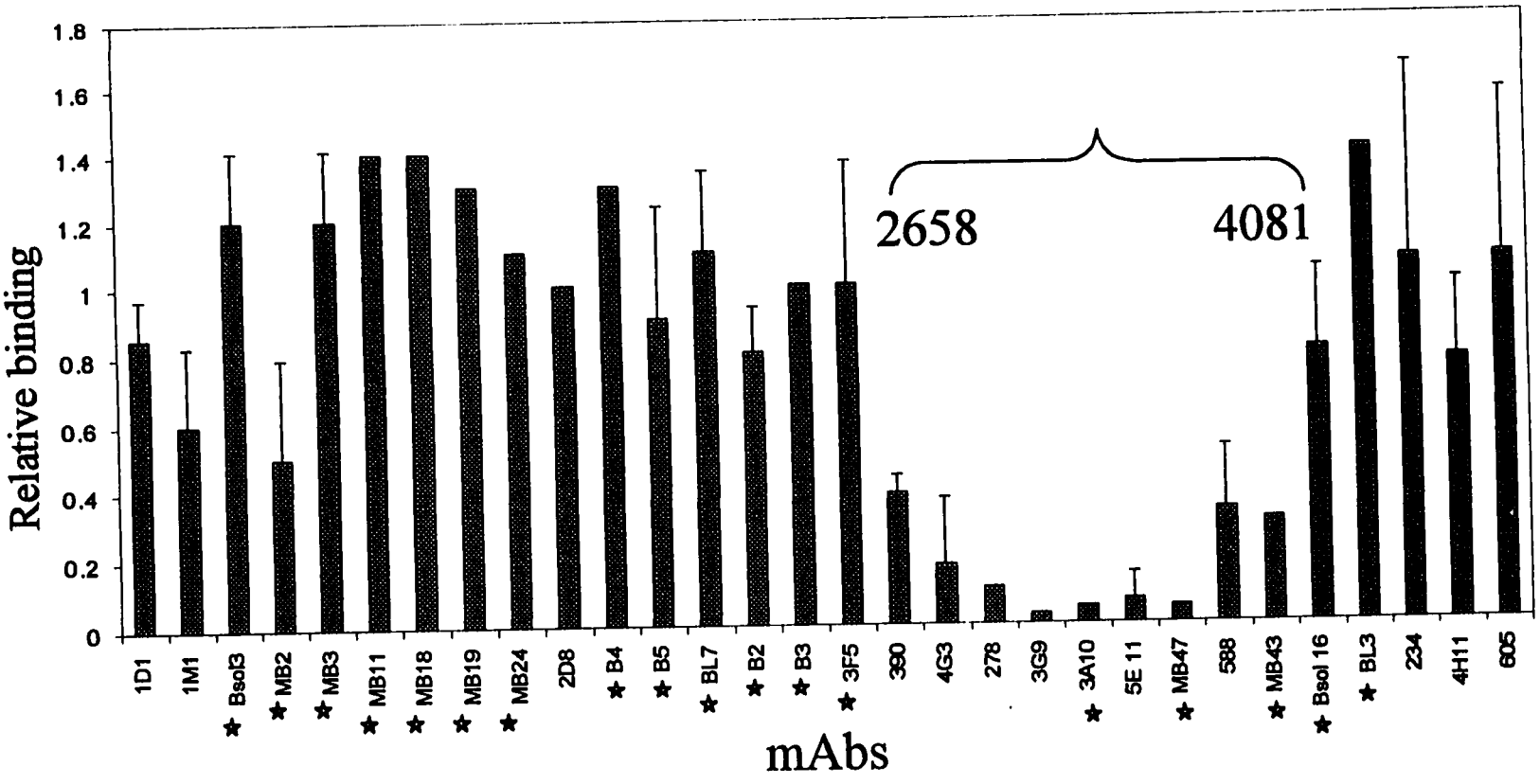

Relative binding
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2658
4081
1D1
1M1
★ Bsol3
★ MB2
★ MB3
★ MB11
★ MB18
★ MB19
★ MB24
2D8
★ B4
★ B5
★ BL7
★ B2
★ B3
★ 3F5
390
4G3
278
3G9
★ 3A10
5E 11
★ MB47
588
★ MB43
★ Bsol 16
★ BL3
234
4H11
605
mAbs

Figure 3-5: Non denaturing gel electrophoresis to determine the size of LDL subclasses.
One microlitre of LDL subfractions were subjected to non-denaturing 4-15% polyacrylamide gel electrophoresis under non-denaturing conditions according to manufacturer's specification. The gel was stained for protein.

Figure 3-5

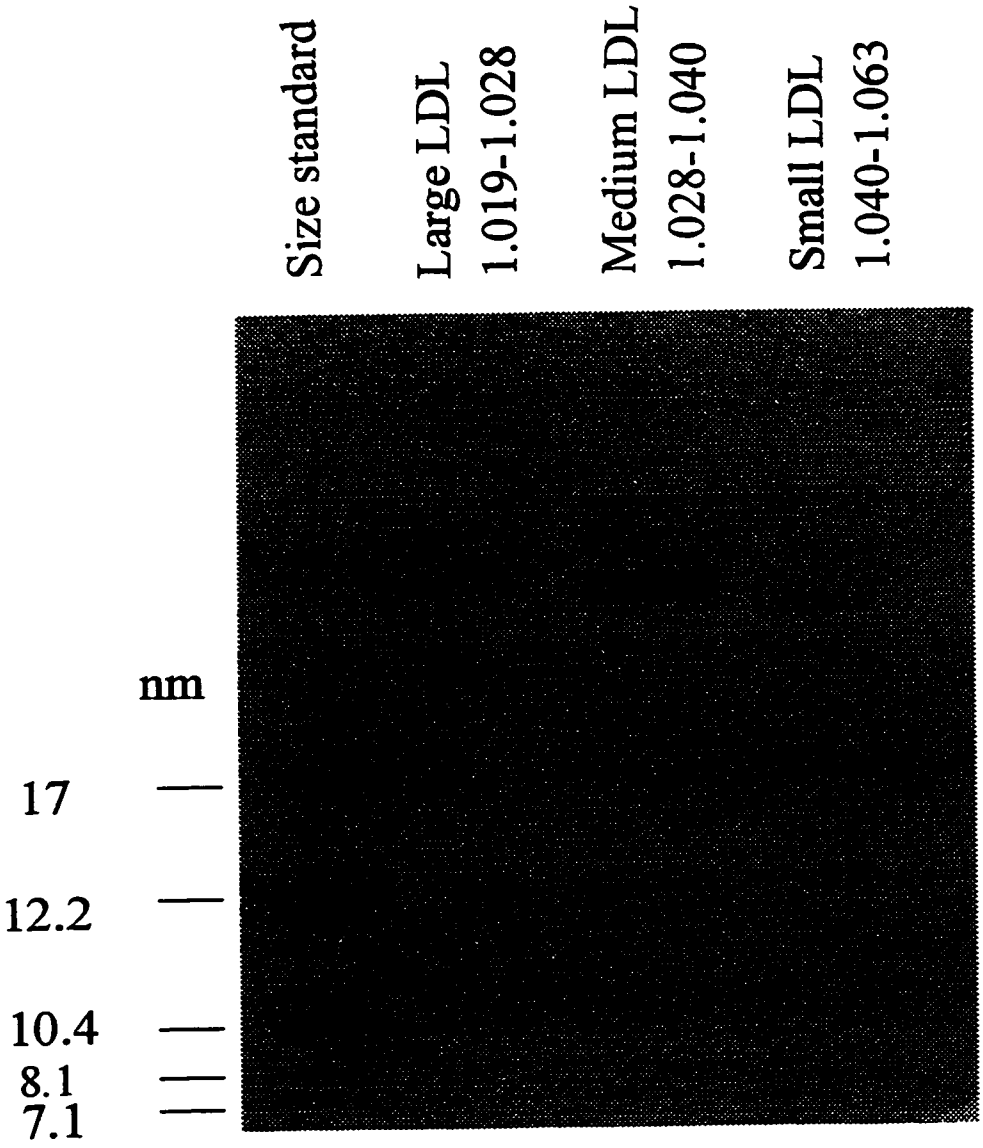

Size standard
Large LDL
1.019-1.028
Medium LDL
1.028-1.040
Small LDL
1.040-1.063
nm
17
12.2
10.4
8.1
7.1

Table 3-3.

Composition of VLDL, IDL and LDL and LDL subfractions

Mass percentage composition of VLDL, IDL and LDL

	n	TG	FC	CE	PL	ApoB	Protein
VLDL	4	52.0±12.1	8.9±1.1	10.9±1.5	20.1±3.4	2.8	8.0±0.8
IDL	4	23.3±1.5	9.4±1.5	24.6±1.6	21.9±0.9	11.6	20.9.±1.5
LDL	4	7.8±0.7	10.6±2.2	38.0±4.5	21.3±1.1	22.3	22.3

Mass percentage composition of LDL subfractions

	n	TG	FC	CE	PL	ApoB
Large LDL	4	4.3±1.9	9.8±2.7	42.9±17.3	23.4±3.3	19.6±3.4
Medium LDL	4	6.2±0.6	11.0±2.0	39.9±8.0	20.7±1.1	22.2±2.1
Small LDL	4	9.2±0.8	9.2±3.1	39.1±3.1	16.9±8.7	25.6±1.4

TG, triglyceride; FC, free cholesterol; PL, phospholipid; CE, cholesteryl ester

IgG, an anti-apoE mAb that blocks apoE-mediated binding to the LDL receptor (Weisgraber *et al,* 1983).

Immunoreactivities of VLDL, IDL, LDL and LDL subfractions in each subject were examined by solid phase competitive radioimmunometric assay using 29 mAbs specific for epitopes that range from the amino terminus to the carboxy terminus of apoB-100 (Figure 3-2). For VLDL and IDL, the results are expressed as a function of apoB protein (i.e. isopropanol-insoluble protein). In the case of LDL and LDL subfractions, virtually all of the protein was isopropanol-insoluble and so results are expressed as a function of total protein. The immunoreactivity of VLDL, IDL and LDL from the 6 subjects was determined for each of the mAbs. Representative competition curves are shown for two of the mAbs, 1D1, whose epitope is near the amino terminus of apoB and 4H11 whose epitope is near the carboxy terminus (Figures 3-6 and 3-7). Whereas, in each of the six subjects, the 1D1 epitope was expressed equally in the VLDL, IDL and LDL fractions, the 4H11 epitope was more reactive in LDL compared to VLDL and IDL. The relative ED_{50} values for all the mAbs are shown in Figure 3-8. As the lipoprotein fractions isolated from each subject were analyzed in separate assays, we have normalized the ED_{50} value to that of LDL of each subject which we have given a value of 1. Most of the epitopes that we have analyzed resembled that of 1D1 in not being differentially expressed in the different lipoprotein fractions. In contrast, two epitopes recognized by 4H11 and 6O5, situated between residues 4342 and 4536 were about three times more reactive in LDL than in VLDL and IDL. The two epitopes recognized by 4G3 and 278, situated

Figure 3-6: Competitive radioimmunoassay of mAb 1D1 with VLDL, IDL and LDL. The apoB concentration in VLDL and IDL was determined as isopropanol-insoluble protein whereas that of apoB was total protein. Competition curves are presented as the ratio of radioactivity bound in the presence of competitor to that bound in the absence of competitor as a function of the concentration of the competitor.

Figure 3-6

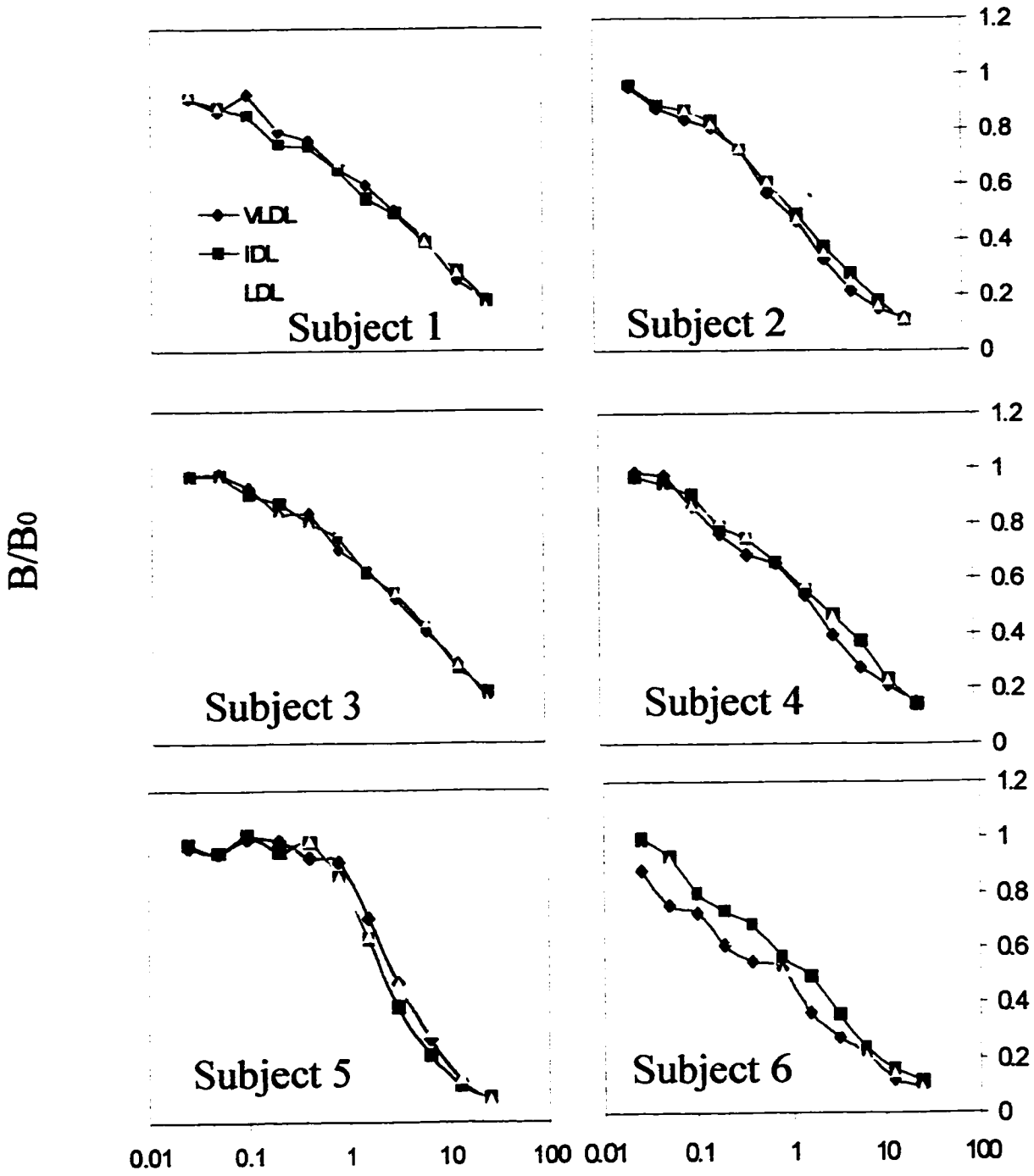

VLDL
IDL
LDL
Subject 1
Subject 2
Subject 3
Subject 4
Subject 5
Subject 6
B/B0
1.2
1
0.8
0.6
0.4
0.2
0
0.01
0.1
1
10
100
ApoB (µg/ml)

Figure 3-7: Competitive radioimmunoassay of mAb 4H11 with VLDL, IDL and LDL.
The apoB concentration in VLDL and IDL was determined as isopropanol-insoluble protein whereas that of apoB was total protein. Competition curves are presented as the ratio of radioactivity bound in the presence of competitor to that bound in the absence of competitor as a function of the concentration of the competitor.

Figure 3-7

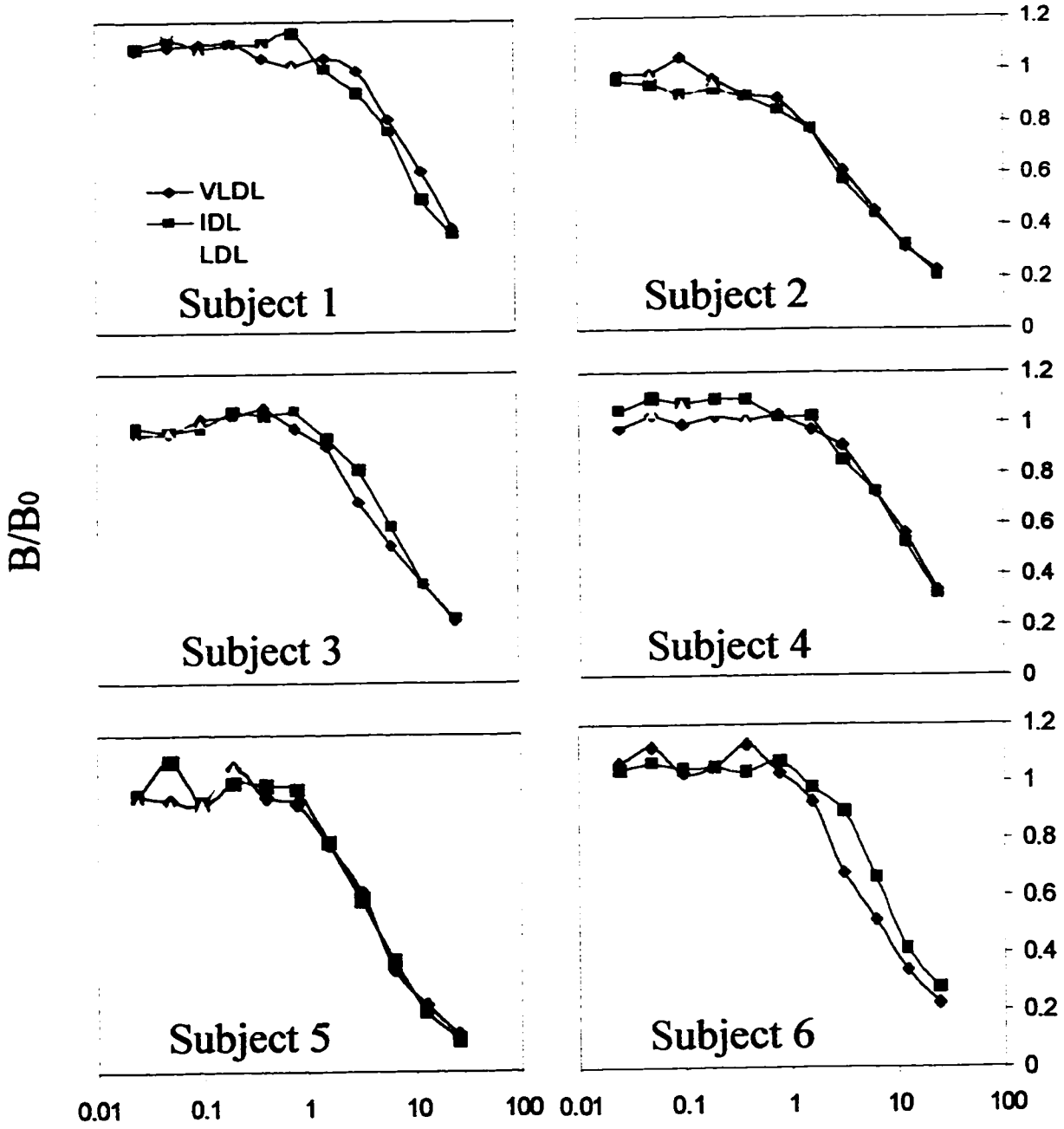

VLDL
IDL
LDL
Subject 1
Subject 2
Subject 3
Subject 4
Subject 5
Subject 6
B/B0
1.2
1
0.8
0.6
0.4
0.2
0
0.01
0.1
1
10
100
ApoB (µg/ml)

Figure 3-8: ED_{50} for VLDL IDL and LDL.
The immunoreactivity of VLDL, IDL, and LDL from 6 normolipidemic subjects was determined in a solid phase immunoassay with a panel of 29 anti-apoB mAbs. Results are expressed as the relative ED_{50}; the concentration of competitor required to reduce binding to 50% of maximum. The ED_{50} value obtained with LDL was normalized to unity. The paired t test was used to determine the significance of difference observed in the immunoreactivity between lipoprotein classes. For mAb 605 and 4H11, there are significant differences in its immunoreactivity between VLDL or IDL and LDL (605: pVLDL-LDL<0.001, pIDL-LDL<0.0005 pVLDL-IDL: NS; 4H11 pVLDL-LDL<0.01, pIDL-LDL<0.005; pVLDL-IDL, NS). Significant differences were seen for mAbs 4G3 (pVLDL-LDL<0.001, pIDL-LDL<0.01 pVLDL-IDL, NS) and 278 (pVLDL-LDL<0.05, pIDL-LDL<0.05, pVLDL-IDL, NS).

Figure 3-8

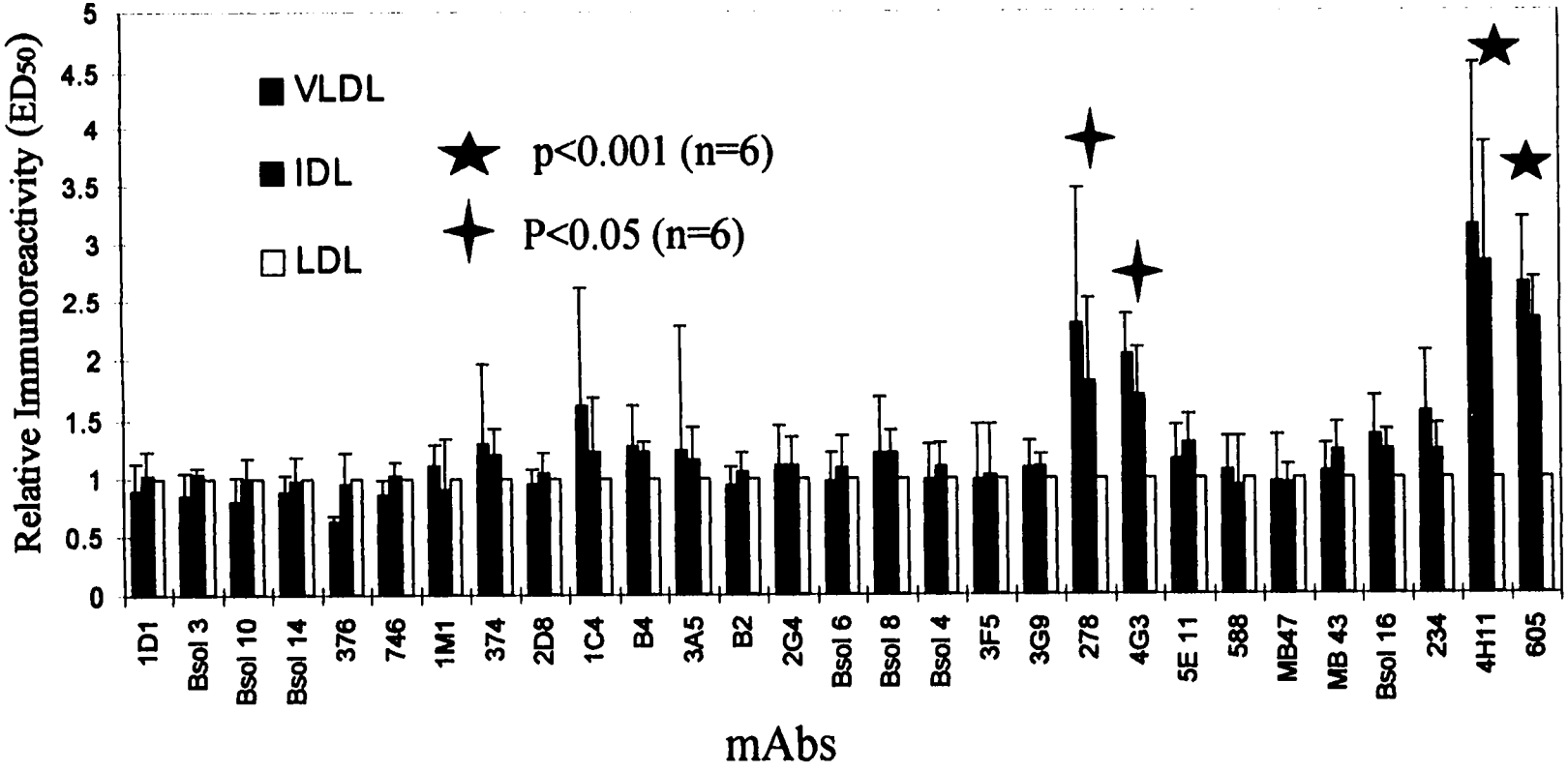
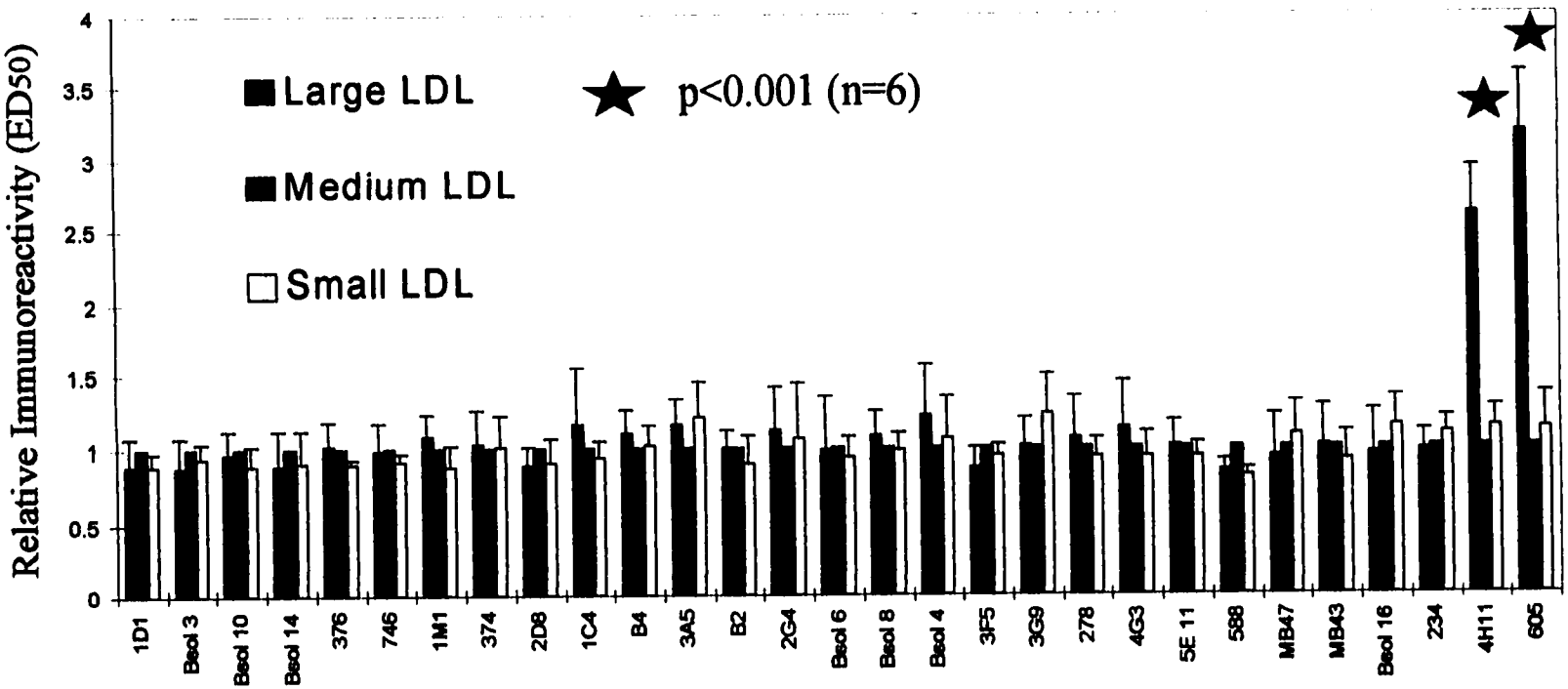

Relative Immunoreactivity (ED50)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
VLDL
IDL
LDL
p<0.001 (n=6)
P<0.05 (n=6)
1D1
Bsol 3
Bsol 10
Bsol 14
376
746
1M1
374
2D8
1C4
B4
3A5
B2
2G4
Bsol 6
Bsol 8
Bsol 4
3F5
3G9
278
4G3
5E 11
588
MB47
MB 43
Bsol 16
234
4H11
605
mAbs

Figure 3-9: ED_{50} for each LDL subfraction.
The immunoreactivity of large, medium, and small LDL from 6 normolipidemic subjects was determined. The ED_{50} value obtained with medium LDL was normalized to unity. The paired t test was used to determine the significance of difference observed in the immunoreactivity between LDL subfractions. The mAbs 605 and 4H11 showed statistically significant between Large LDL and medium or small LDL ($p<0.001$). No significant differences were found for 4G3 and 278 and any other mAbs.

Figure 3-9


Relative Immunoreactivity (ED50)
4
3.5
3
2.5
2
1.5
1
0.5
0
Large LDL
Medium LDL
Small LDL
p<0.001 (n=6)
1D1
Bsol 3
Bsol 10
Bsol 14
376
746
1M1
374
2D8
1C4
B4
3A5
B2
2G4
Bsol 6
Bsol 8
Bsol 4
3F5
3G9
278
4G3
5E 11
588
MB47
MB43
Bsol 16
234
4H11
605
mAbs

between 2658-3268 showed a reactivity, LDL > IDL > VLDL, that was statistically significant. The other two epitopes recognized by mAbs 374 (1328-1542), 1C4 (1764-1880) showed differences, but due to inter-individual variation, these were not significant.

The large, medium and small LDL subfractions were similarly analyzed for differential expression of apoB epitopes. The mean relative ED_{50} values for all of the mAbs is presented in Figure 3-9. Again, to reduce the effects of inter-assay variation, the ED_{50} values for the subfractions from each subject were calculated relative to that of the medium LDL, which was assigned a value of 1. Only the epitopes 4H11 and 6O5 were differentially expressed, with the large LDL being less immunoreactive than the medium and small LDL.

Discussion

Our results show that the carboxy terminus of apoB-100 undergoes a major conformational change as LpB are metabolized in the plasma of normolipidemic individuals. This change in conformation does not appear to be gradual but occurs abruptly as large buoyant LDL (density 1.019-1.028 g / ml) are converted to smaller denser particles (density > 1.028 g / ml). This conclusion is based on the observation that two epitopes, situated between residues 4342 and 4536, show an approximately 3-fold higher expression in total LDL compared to VLDL and IDL (Figure 3-8) and in medium and small LDL subfractions compared to larger more buoyant LDL (Figure 3-9). Chen *et al* (Chen *et al,* 1994), have also recently proposed that apoB undergoes a conformational change at this point in the intravascular metabolism of LpB. They have shown that there are major differences in the accessibility of protease-sensitive sites in apoB-100 between LpB < 1.033 and LpB > 1.033. These include *Staphylococcus aureus* V8 protease-sensitive sites at residues 1288 and 3199 and cathepsin D-sensitive sites at residues 2702 and 2666/2669. The concomitant change in the 6O5 and 4H11 epitope expression and in the accessibility of different protease-sensitive sites may reflect a global, rather than a local, conformational change of apoB as large buoyant LDL are converted to smaller denser particles. If this were the case, it is nevertheless surprising that such an alteration in the overall apoB conformation was not detected by any of the other mAbs used in the present study. It has been recently proposed, on theoretical grounds alone, that apoB

must adopt different conformations in different LDL subspecies but that it may be the tertiary structure of apoB that is predominantly modulated as a function of LDL size while secondary structure may be largely unchanged (McNamara *et al,* 1996). Such changes in tertiary structure could potentially include domain shifts that may not be manifested in changes in the immunoreactivity of epitopes located within domains but may be detected by markers specific for inter-domain linker regions that could include protease-sensitive sites. It should be noted that none of the mAbs in the present panel are specific for epitopes that coincide with the protease-sensitive sites reported by Chen *et al* (Chen *et al,* 1994). The carboxy terminus of apoB100, that includes the 6O5 and 4H11 epitopes, has been shown to have high affinity for lipids (Yang *et al,* 1989) and a predicted random coil structure (Segrest *et al,* 1994).

It may be important that epitopes that showed the greatest and most reproducible changes in expression between the LpB density fractions were located near the carboxy terminus of apoB. Regions downstream of the putative apoB LDL receptor-binding region (residues 3000 to 3500) have been proposed to play a role in regulating apoB-mediated binding to the LDL receptor. It has been shown that LDL, containing a natural apoB variant that lacks the carboxy terminal 11% of apoB primary structure (apoB89), are rapidly cleared from plasma and show an increased affinity for the LDL receptor (Parhofer *et al,* 1989, 1990). Recently, Borén *et al,* (Borén *et al,* 1998) have shown that VLDL from apoE-deficient mice that carry a human apoB80 transgene can bind to the LDL receptor with relatively high affinity whereas VLDL from apoE-deficient, human apoB100

transgenic mice bind poorly. Furthermore, truncation of the carboxy terminal 916 amino acid residues of human apoB can override the LDL receptor-binding defective phenotype that results from an $apoB^{3500Arg \rightarrow Gln}$ substitution. Together, these results suggest that the carboxy terminus of apoB100 is a negative regulator of apoB-mediated binding to the LDL receptor by either modulating the conformation or limiting the accessibility of the apoB LDL receptor-binding site. A model has been recently proposed for the topography of apoB on the LDL surface based on an electron microscopic study of LDL-anti-apoB mAb immune complexes which reveals a possible mechanisms for how the carboxy terminus might regulate apoB-mediated binding to the LDL receptor. Chatterton *et al,* (Chatterton *et al,* 1995) have proposed that, on the surface of LDL, apoB resembles a ribbon and bow, in which the amino terminal apoB-89 wraps around the LDL particle forming the ribbon. The remaining carboxy terminal 11% of apoB, forming the bow, loops back and crosses the ribbon at a point of intersection that brings the region apoB94-apoB96 (residues 4264 - 4354) close to the region of apoB that is responsible for binding to the LDL receptor. Lateral movement of the extreme carboxy terminus of apoB on the surface of the LpB that could occur during conversion of VLDL to LDL may expose the apoB LDL receptor-binding site. The increase in the immunoreactivity of mAbs 6O5 and 4H11 as large LDL are converted to medium LDL may also result from the same apoB conformational change.

In addition to the mAbs that are specific for epitopes near the carboxy-terminus of apoB, mAbs 278 and 4G3 also showed differential reactivity with VLDL, IDL and LDL.

The increased expression of the 278 and 4G3 epitopes as VLDL is converted to IDL and LDL may reflect the change of neutral lipid content. From the predicted apoB secondary structure, the 278 and 4G3 epitopes are located in regions that adopt a predominantly amphipathic β structure (Segrest *et al,* 1994). These amphipathic β-domains of apoB have been proposed to bind irreversibly to lipid (Segrest *et al,* 1994). It has been demonstrated that, when expressed in rat hepatoma cells as apoB / apoA-I fusion proteins, the amphipathic β-sequences of apoB are sufficient for recruitment of triglyceride into VLDL-like particles (McLeod *et al,* 1996). Furthermore, as neutral lipid is necessary for the expression of epitopes located in these regions (Marcel *et al,* 1984, Chauhan *et al,* 1998), it would appear that the amphipathic β-domains are intimately associated with the neutral lipid core of LpB. Two other epitopes, 374 and 1C4, that have been mapped to a region (residues 1328-1878) that is also predicted to have an amphipathic β-secondary structure, tended to be more reactive in LDL than in VLDL, although this did not reach statistical significance. As the amphipathic β-domains appear to be tightly associated with the neutral lipid core of the lipoprotein, the change in the immunoreactivity of epitopes in these domains could reflect the alterations in the core that occur during the intravascular metabolism of VLDL.

Apart from the change in immunoreactivity of the 6O5 and 4H11 epitopes and, to a lesser extent the 4G3 and 278 epitopes, the majority of apoB epitopes were remarkably independent of their lipoprotein environment. This may have been anticipated for epitopes

located in the amino terminus of apoB. The amino terminal 1000 residues of apoB are predicted to fold independently into a globular domain with relatively low affinity for lipids (Yang *et al,* 1989, Segrest *et al,* 1994). Furthermore, it has been demonstrated that apoB-18 (residues 1-781) can be secreted into the culture medium as a lipid-free protein with a density greater than 1.21 g / ml (Yao *et al,* 1991, McLeod *et al,* 1996). Amongst the other mAbs that recognized apoB equally in all lipoprotein fractions are 5E11 and MB47 whose epitopes are close to the apoB LDL receptor-binding site.

Several of the mAbs reported in the present study have been previously used to examine changes in epitope expression in LpB subfractions of moderately hypertriglyceridemic patients. In an earlier study, expression of the 5E11 epitope was found to increase progressively in LpB subfractions from $VLDL_1$ (Sf 100-400) through LDL, due to an increase in the apparent affinity of the mAb (Marcel *et al,* 1988). Changes in 5E11 immunoreactivity were not observed in the present study, nor was there differential expression of the overlapping / adjacent epitopes, MB47, and 588. The apparently contradictory results for the 5E11 epitope may reflect differences between LpB isolated from normal and hypertriglyceridemic individuals. It should also be noted that several of the mAbs, 2D8, 3F5, and 4G3 have been previously demonstrated to have reduced reactivity with the small dense LDL that characterize subjects with hyperapobetalipoproteinemia (Teng *et al,* 1985).

We have previously proposed that the portion of apoB primary structure that could be directly implicated in binding of apoB to the LDL receptor is limited to a region bounded by residues 3000 to 4000 (Milne *et al,* 1989). This was based on the observation that the epitope of mAb 3F5 (residues 2835-2922) is totally accessible in LDL-LDL receptor complexes whereas that of mAb 4G3 (residues 2980-3084) is not. Similarly, while the epitope for MB43 (residues 4027-4081) is almost inaccessible, that of mAb $B_{sol}16$ (residues 4154-4189) is fully accessible. Seventeen other epitopes situated elsewhere within apoB primary structure were also fully accessible. Therefore, an interesting observation to emerge from our characterization of the new panel of mAbs is that mAb 588, specific for an epitope situated between apoB residues 3687-4081, could partially block binding of LDL to the LDL receptor and its epitope is partially accessible in LDL-LDL receptor complexes. This observation would further define the region of apoB primary structure that is in close contact with the LDLr and would be consistent with the report that LDL containing apoB75 (terminating at residue 3386) can bind with high affinity to the LDL receptor (Krul *et al,* 1990). The inaccessibility of the MB43 epitope (4027-4081), that is located downstream of the 588 epitope, in LDL-LDL receptor complexes and the ability of the MB43 mAb to partially block binding of LDL to the LDL receptor may reflect a complex apoB tertiary structure in this region of the molecule.

In summary, our immunochemical analysis of LpB density subfractions has shown that apoB conformational changes that can be detected by altered epitope expression are localized to rather limited regions within apoB primary structure. Assuming that the

fractions which were analyzed represent discrete intermediates in the intravascular metabolism of LpB, it would appear that a major conformational change of apoB occurs as large buoyant LDL are converted to smaller less buoyant particles. The fact that this alteration in apoB conformation was detected by a change in expression of an epitope situated in a region that has been implicated in modulation of the apoB-mediated binding to the LDL receptor could indicate that it may have important functional consequences.

Chapter IV

Epitopes Close to the Apolipoprotein B Low Density Lipoprotein Receptor-Binding Site Are Modified in Glycated Low Density Lipoproteins

Summary

Advanced glycation end products (AGEs) are thought to contribute to the abnormal lipoprotein profiles and increased risk of cardiovascular disease that are associated with diabetes and renal failure, in part, by preventing apolipoprotein B (apoB)-mediated cellular uptake of low density lipoproteins (LDL) by the LDL receptors. It has been proposed that AGE modification at a single site in apoB, almost 1800 residues from the putative apoB LDL receptor binding domain, induces a change in the conformation of apoB that prevents its binding to the LDL receptor (J. Biol. Chem. 270, 10828-10832). To test this hypothesis, we have used a panel of 29 anti-human apoB monoclonal antibodies to identify other potential sites on apoB that are modified by *in vitro* LDL glycation. Glycation of LDL caused a time-dependent decrease in both their ability to bind to the LDL receptor and in the immunoreactivity of 6 distinct apoB epitopes, including two that flank the apoB LDL receptor-binding domain. ApoB appears to be modified at multiple sites as the loss of glycation-sensitive epitopes was detected on both native glycated LDL and denatured, delipidated, glycated apoB. In contrast, residues within the putative apoB LDL receptor-binding domain are not apparently modified in

glycated LDL. We propose that the inability of glycated LDL to bind to the LDL receptor is due to modification of residues adjacent to the putative LDL receptor-binding site that either eliminates their direct participation in binding or indirectly alters the conformation of the apoB LDL receptor-binding site.

Introduction

Nonenzymatic protein glycation by glucose is a physiological process that proceeds through a complex cascade of reactions that generates a heterogeneous mixture of products termed advanced glycation end products (AGEs) (Means and Chang, 1982; Baynes, 1991). AGEs are believed to contribute to the pathogenesis of diabetes (Vlassara and Bucala, 1996; Vlassara *et al,* 1994) and neurodegenerative amyloidal diseases such as Alzheimer's disease (Vitek *et al,* 1994; Harrington and Colaco, 1994). As nonenzymatic glycation is thought to also occur in normoglycemic individuals, albeit at a slower rate than in diabetic subjects, AGEs have been proposed to contribute to the pathogenesis of aging (Nonnier and Cerami, 1981; Dyer *et al,* 1983). The generation of AGE-modified proteins in the circulation is thought to result only, in part, from a direct interaction of glucose with serum proteins. Serum proteins can also be modified by low molecular weight, highly reactive AGE peptides that are present in the circulation, particularly under conditions of impaired renal function (Makita *et al,* 1991, 1994). These are degradation products of AGE-modified proteins that are released into the blood stream and are normally cleared by the kidneys. AGE-modified serum proteins prepared

in vitro have been shown to be toxic, immunogenic and capable of triggering cellular injury responses after uptake by specific cellular receptors. (Vlassara 1992; Yan *et al,* 1994). *In vivo*, AGEs can cause alterations to the extracellular matrix including cross-linking of collagen, thickening of basement membranes and the covalent binding of plasma proteins including low density lipoproteins (LDL) and immunoglobulin (Vlassara and Bucala, 1996).

It is well recognized that individuals with diabetes and renal insufficiency are at an increased risk for the development of atherosclerosis (Kannel and McGee, 1978). Plasma lipoprotein profiles are frequently abnormal in these conditions with an elevation in the level of the apolipoprotein B- (apoB) containing lipoproteins, including LDL. Defective lipoprotein uptake and metabolism in diabetic patients have been demonstrated (Hiramatsu *et al,* 1985) and the uptake and degradation of LDL isolated from diabetic subjects by normal fibroblasts has been shown to be impaired (Lopes-Virella *et al,* 1982). LDL modified by *in vitro* incubation with glucose shows retarded intravascular clearance in man (Kesaniemi *et al,* 1983) and animals (Steinbrecher and Witztum, 1984) and reduced LDL receptor-mediated binding and uptake by cultured human fibroblasts (Bucala *et al,* 1994). Similarly, in transgenic mice that express the human LDL receptor, there is impaired clearance of LDL that had been pre-exposed to AGE-peptides (Bucala *et al,* 1994). While there is reduced uptake of AGE-modified proteins via the LDL receptor, cell surface receptors for the AGE moiety are present on a number of cell types including monocytes, macrophages and endothelial cells (Makita, et al 1991). Two

receptors have recently been identified that can mediate the uptake of AGE-modified proteins, the class A scavenger receptor (El-Khoury *et al,* 1994) and the receptor for advanced glycation end products (RAGE) (Neeper *et al,* 1994). Binding of AGE-modified proteins to the AGE receptors triggers a number of cellular responses that could contribute to AGE-associated pathogenesis (Vlassara and Bucala, 1996).

A major site for AGE modification within apoB primary structure has recently been identified (Bucala *et al,* 1995). Although this site is far from the putative apoB LDL receptor-binding domain, it has been proposed that AGE modification at this site provokes a change in the conformation of apoB that prevents its binding to the LDL receptor. In the present study we have used a panel of 29 anti-apoB monoclonal antibodies (mAbs) to demonstrate that *in vitro* glycation of LDL results in modification at multiple sites in apoB, including two in the vicinity of the apoB LDL receptor-binding domain.

Materials and Methods:

Preparation of AGE-LDL and reductively methylated LDL

Plasma from healthy donors was collected and supplemented immediately with 1mM EDTA, 20 μM butylated hydroxytoluene (BHT). 0.5 mM phenylmethanesulfonal fluoride, and 0.02% sodium azide. LDL (density 1.019-1.063 g / ml) were isolated by sequential ultracentrifugation at 40.000 rpm for 18 hours (Havel *et al,* 1955). AGE-LDL were prepared by incubating LDL (2 mg / ml) with 200 mM glucose at 37 ^{0}C for up to 2 weeks in phosphate-buffer saline (PBS) containing 1 mM EDTA and 20 μM BHT with or without 300 mM aminoguanidine (Bucala *et al,* 1995). Control LDL were incubated under the same conditions without glucose or aminoguanidine. After incubation, the LDL were dialyzed against PBS containing 1 mM EDTA and 0.02% NaN_3. For the time course of glycation, aliquots of LDL were incubated at 37 ^{0}C, and glucose was added to individual samples at t_o or after 4, 8, 12 days to a final concentration of 200 mM. After 16 days of incubation all LDL were dialyzed as above. Control LDL were incubated for 16 days at 37 ^{0}C without glucose. LDL were reductively methylated by the method of Weisgraber *et al,* (Weisgraber *et al,* 1978).

LDL receptor binding assay

Glycated or control LDL were tested for their ability to compete with ^{125}I-native LDL (Bilheimer *et al,* 1972) for binding to the LDL receptor on the surface of cultured human fibroblasts as described previously (Milne et al, 1983). In short, ^{125}I- LDL (3 μg / ml) and the appropriately diluted competitor LDL, in a total volume of 1 ml, were

incubated for 3 h at 4 ^{0}C with cultured human fibroblasts. Bound ^{125}I-LDL was determined and the percentage of binding was plotted as a function of the concentration of the competitor.

Monoclonal antibodies

The production and characterization of anti-human apoB (Milne *et al,* 1983; Pease *et al,* 1990; Wang and Milne in preparation) and anti-AGE (Bucala *et al,* 1995) mAbs have been described elsewhere. The rabbit anti - peptide antiserum specific for apo B residues 3352-3371 was kindly provided by Dr. Tom Innerarity (Gladstone Institute of Cardiovascular Disease) (Innerarity *et al,* 1987).

Competitive Radioimmunometric assay (RIA)

The competitive apoB radioimmunometric assay has been described previously (Marcel *et al,* 1984). Immulon II Removawells (Dynatech Laboratories, Chantilly, VA) were coated by an overnight incubation with 200 µl of reference LDL (30 µg / ml in 5 mM glycine, pH 9.2). Plates were washed with a solution of 0.15 M NaCl containing 0.025% Tween 20 (Tween-saline), and then subsequently saturated by incubation for 1 hr with 250 µl of 1% bovine serum albumin in phosphate-buffered saline, pH 7.4 (PBS-BSA). Serial dilutions (150 µl) of test and control LDL were prepared in separate microtitre plates to which was added 150 µl of anti-apoB mAb appropriately diluted in PBS-BSA. After a 2 hr incubation, 200 µl of the LDL-mAb mixture was transferred to the LDL-coated Removawells. The plates were incubated overnight and again washed

with the Tween-saline solution. Two hundred μl of ^{125}I-goat anti-mouse IgG labeled by the chloramine-T method (Milne *et al,* 1992) was diluted to 70 ng / ml in 1% PBS-BSA and added to each well and incubated overnight. The wells were then washed with the Tween-saline solution as above and counted for bound radioactivity.

Western Blotting Analysis

LDL were subjected to SDS-PAGE (3-10% gradient) and were electrophoretically transferred to nitrocellulose paper (Towbin *et al,* 1979). The nitrocellulose membranes were exposed to diluted anti-human apoB or anti-AGE monoclonal antibodies or polyclonal anti-apoB peptide antibodies and then to the appropriate affinity-purified, horse radish peroxidase-conjugated anti-IgG secondary antibody. Bound IgG was detected using an ECL kit (Amersham, UK)

Other analytical methods

Protein concentration of the LDL was measured by the method of Lowry using bovine serum albumin as a standard (Lowry *et al,* 1951). Free amino groups on LDL were estimated by the trinitrobenzenesulfonic acid (TNBS) method (Habeeb, 1966) using valine as a standard. The relative LDL surface charge was assessed by agarose gel electrophoresis at 150 Volts for 30 minutes (Beckman, Palo Alto, CA).

Results

In order to study the structural and functional alterations that result from LDL glycation, freshly isolated LDL were incubated for 14 days at 37 ^{0}C under sterile conditions in the presence of 200 mM glucose, anti-oxidants and protease inhibitors. LDL were also similarly incubated in the absence of glucose and in the presence of both 200 mM glucose and 300 mM aminoguanidine, an inhibitor of advanced glycation (Brownlee *et al,* 1986). Although a glucose concentration of 200 mM is non-physiological, it has been shown to generate an AGE-modification of LDL that is immunochemically related, and comparable in concentration, to that observed in patients with diabetes or renal insufficiency (Mikita *et al,* 1992; Bucala *et al,* 1994). In 3 experiments the trinitrobenzenesulphonic (TNBS) acid reactivities of LDL incubated with glucose or with both glucose and aminoguanidine were reduced by 42 ± 6% and 8 ± 4 %, respectively, in comparison with LDL incubated in the absence of glucose. Even at the highest concentration tested (160 μg of apoB / ml), LDL that had been incubated with glucose could not compete with ^{125}I-LDL for binding to the LDL receptor of cultured human fibroblasts (Figure 4-1). LDL that had been subjected to the glycation conditions in the presence of aminoguanidine retained about 50 % of the binding activity of LDL that had been incubated in the absence of glucose. These results are similar to those previously reported (Bucala *et al,* 1995).

It has been proposed that AGE modification of one or more Lys residues between apoB residues 1388 and 1454 induces a conformational change in the apoB LDL-receptor-binding site that impairs its interaction with the LDL receptor (Bucala *et al*, 1995). To define the immunochemical changes of apoB that accompany LDL glycation, we have tested LDL, that have been subjected to glycation in the presence or absence of aminoguanidine, using a solid phase RIA with a panel of 29 anti-apoB mAbs. Complete competition curves for 4 of the mAbs from one experiment are presented in Figure 4-2 and the ED_{50} values for all antibodies with LDL from two subjects are shown in Figure 4-3, together with an apoB epitope map. Whereas glycated LDL are as reactive as LDL that had been incubated in the absence of glucose with certain mAbs (e.g. 1D1), they are much less reactive than non-glycated LDL with other mAbs (e.g. 2D8, B4 and 4G3). Interestingly, one of the epitopes that is particularly sensitive to glycation, 2D8, has been mapped to a region (residues 1438-1481) that overlaps the major site of AGE modification of apoB. Other epitopes, 4G3 and 588, that are glycation-sensitive are close to the putative apoB LDL receptor-binding site. Aminoguanidine can completely or partially prevent the loss of immunoreactivity of most epitopes that are sensitive to glycation. In contrast to other glycation-sensitive epitopes, however, the 2G4 epitope could not be protected by aminoguanidine.

Figure 4-1: The abilities of glycated LDL to compete with ^{125}I-labeled normal LDL for receptor binding.
LDL from two subjects were exposed to 200 mM glucose at 37°C for 14 days in the presence (LDL + Glu + AG) or absence (LDL + Glu) of 300 mM aminoguanidine according to the method of Bucala (Bucala *et al*, 1995). LDL from the two subjects were also incubated under the same conditions without glucose or aminoguanidine (LDL). LDL, LDL + Glu, and LDL + Glu + AG were tested for their respective abilities to compete with ^{125}I-LDL for binding to the LDL receptor on cultured human fibroblast.

Figure 4-1

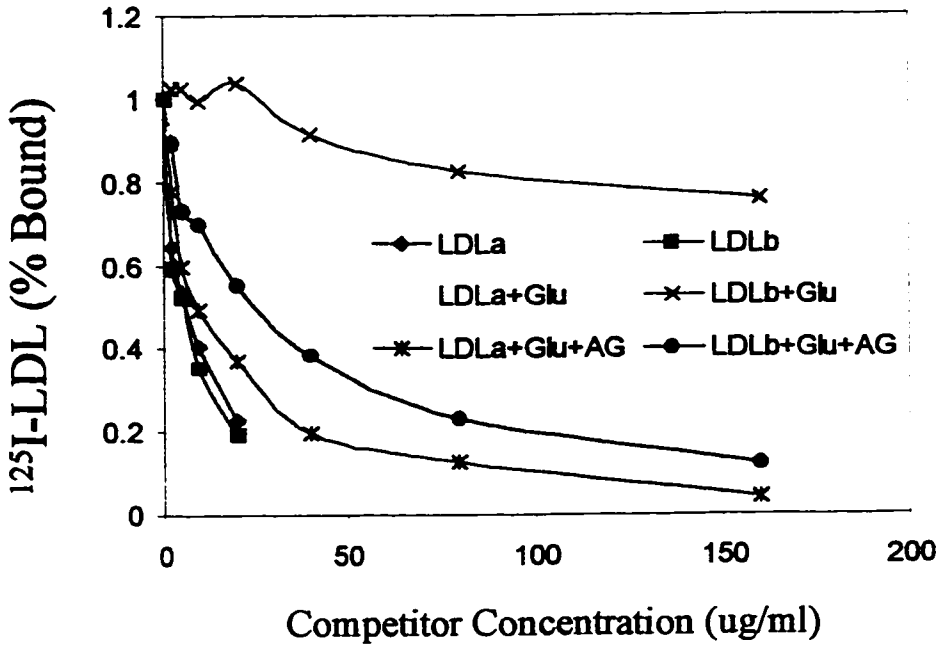

1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
125I-LDL (% Bound)
Competitor Concentration (ug/ml)
LDLa
LDLb
LDLa+Glu
LDLb+Glu
LDLa+Glu+AG
LDLb+Glu+AG

Figure 4-2: The immunoreactivity of glycated LDL.
The immunoreactivity of LDL that had been incubated for 14d at 37°C without glucose (LDL), with 200 mM glucose (LDL + Glu) or with 200 mM glucose and 300 mM aminoguanidine (LDL + Glu + AG) was determined by a solid phase RIA using a panel of 29 anti-apoB mAbs. Competition curves for mAbs 1D1, 2D8, 4G3 and B4 are presented.

Figure 4-2

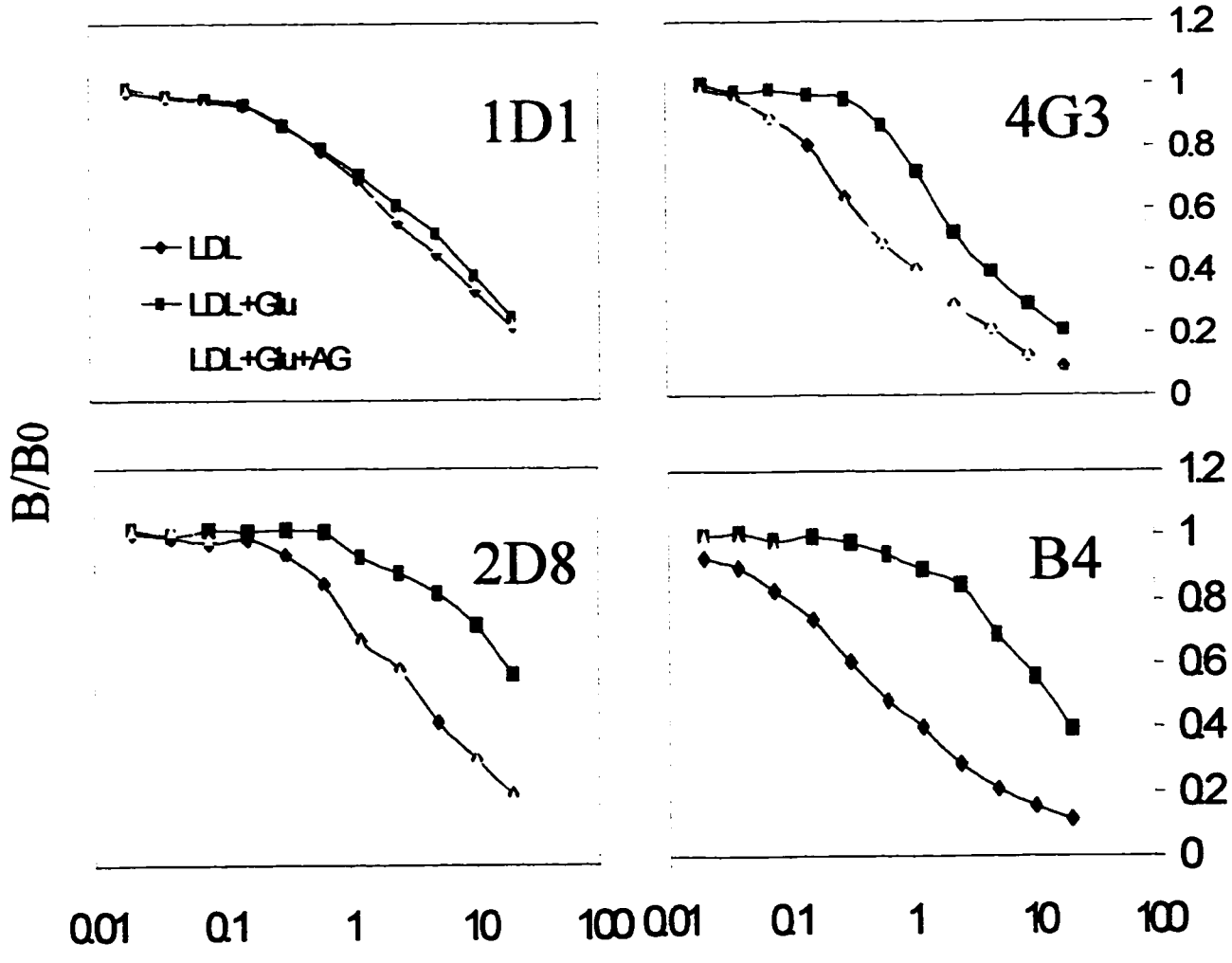
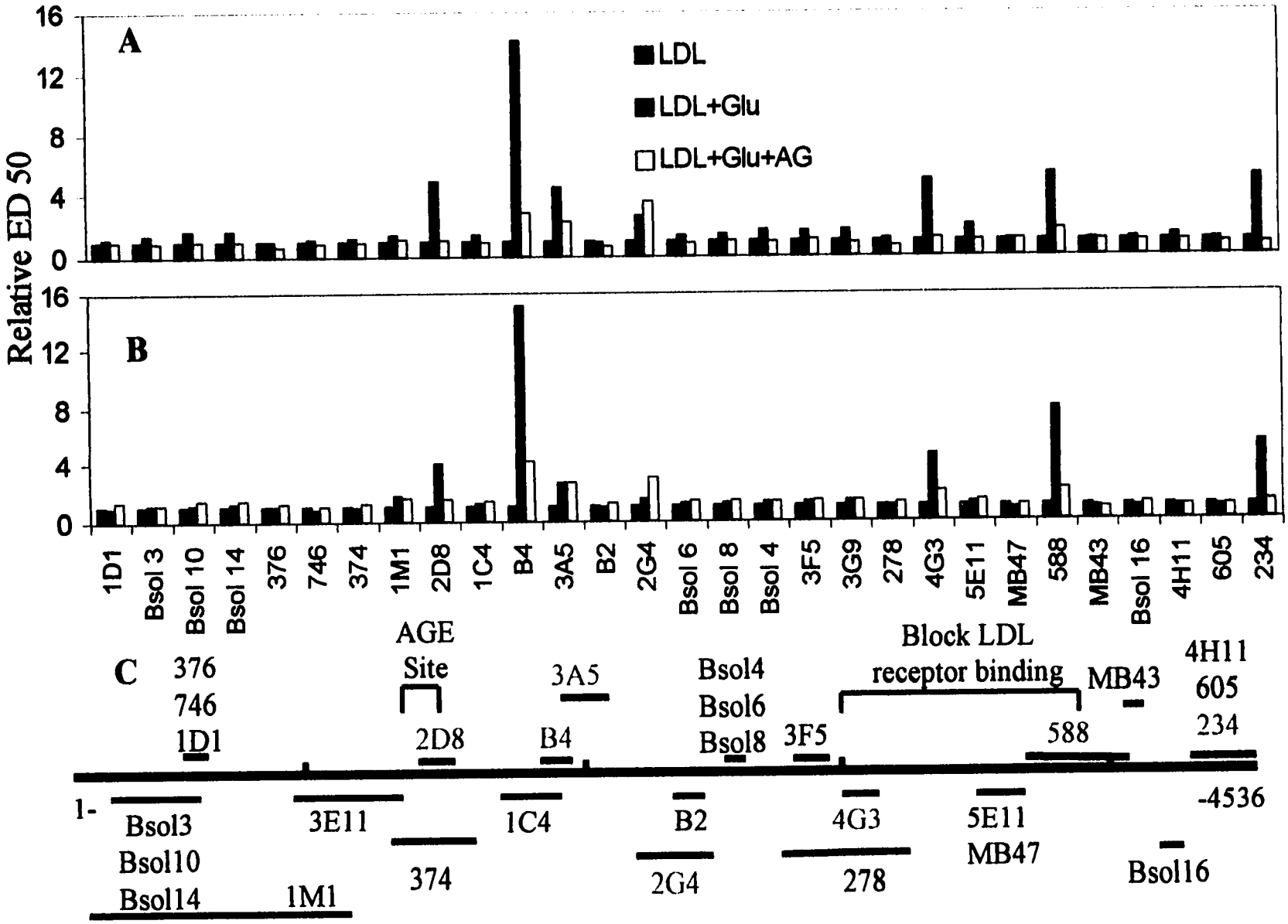

1D1
4G3
2D8
B4
LDL
LDL+Glu
LDL+Glu+AG
B/B0
1.2
1
0.8
0.6
0.4
0.2
0
0.01
0.1
1
10
100
LDL (µg/ml protein)

Figure 4-3: The immunoreactivity of glycated LDL.
The relative concentrations of LDL, LDL + Glu, and LDL + Glu + AG that were required to obtain 50% of maximum binding (ED_{50}) in the solid phase RIA. LDL from 2 subjects (**A** and **B**) were modified under the conditions described in the legend to figure 4-1. The ED_{50} for LDL that had been incubated in the absence of glucose was normalized to a value of 1 for each mAb. The positions of the epitopes within apoB primary structure recognized by the 29 mAbs are shown (**C**). The mAbs with red color indicate the reduced immunoreactivity for glycated LDL compared to control LDL.

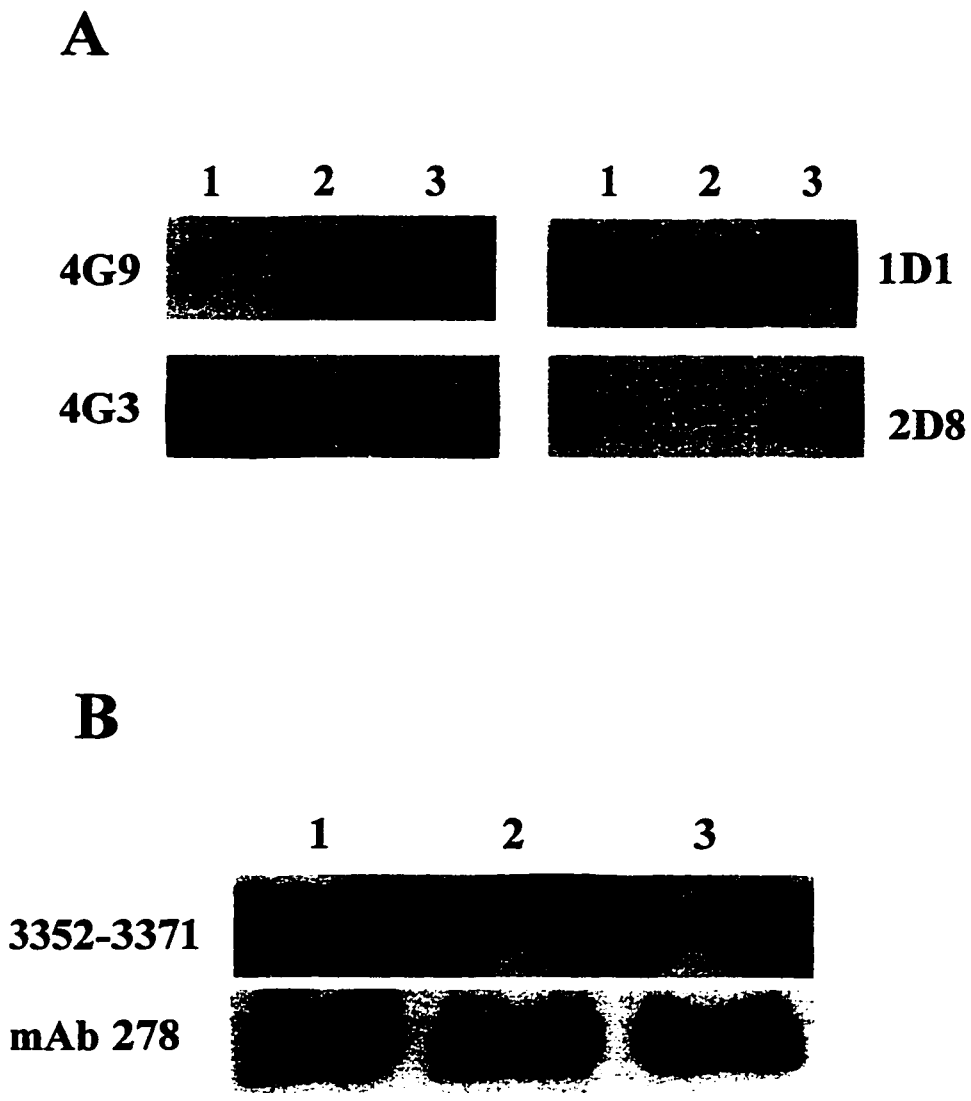
Figure 4-3


A
B
C
Relative ED 50
16
12
8
4
0
LDL
LDL+Glu
LDL+Glu+AG
1D1
Bsol 3
Bsol 10
Bsol 14
376
746
374
1M1
2D8
1C4
B4
3A5
B2
2G4
Bsol 6
Bsol 8
Bsol 4
3F5
3G9
278
4G3
5E11
MB47
588
MB43
Bsol 16
4H11
605
234
AGE
Site
Block LDL
receptor binding
Bsol4
Bsol6
Bsol8
1-
-4536
Bsol3
Bsol10
Bsol14
3E11
1M1
374
1C4
B2
2G4
4G3
278
5E11
MB47
Bsol16

The loss of immunoreactivity of epitopes close to the apoB LDL receptor-binding site could result from either glucose-mediated modification of residues that participate in the respective epitopes themselves, or from changes in conformation of these epitopes that are secondary to AGE modification of Lys residues between apoB residues 1388-1454. To distinguish between these two possibilities we have analyzed the samples for their reactivity with the mAbs on Western blots. As seen in Figure 4-4A, antibodies that showed a decreased reactivity with glycated LDL in the solid phase RIA also showed decreased reactivity with glycated apoB on Western blots. As it would be improbable that changes in epitope expression that are secondary to changes in apoB conformation could be detected by antibodies after SDS electrophoresis of LDL, the loss of reactivity of the mAbs likely reflects direct modification of the epitopes. A mAb specific for the AGE moiety, 4G9, (Bucala *et al,* 1995) reacted strongly with glycated apoB and very weakly with LDL that had been incubated without glucose or had been subjected to the glycation conditions in presence of aminoguanidine. As it is thought that apoB residues 3359-3367 may participate directly in binding to the LDL receptor, we have also tested glycated LDL and LDL that had been modified by reductive methylation for their reactivity with an antiserum prepared against a synthetic peptide that represents apoB residues 3352-3371 (Figure 4-4B). The antibody recognizes glycated but not reductively methylated LDL. Therefore, while lysine residues contribute to the epitope(s) recognized by this antiserum, they are not modified by glycation. In contrast, the 278 epitope is insensitive to either glycation or reductive methylation.

Figure 4-4: Immunoreactivity of glycated LDL after SDS-PAGE.
(**A**) Equal quantities of LDL (lane 1) LDL + Glu (lane 2) or LDL +Glu + AG (lane 3), modified under the conditions described in the legend to Figure 4-1, were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Immunoreactivity of the transferred proteins was tested with the panel of anti-apoB mAbs and mAb 4G9 that is specific for AGE adducts. Results with 3 anti-apoB mAbs and mAb 4G9 are presented. (**B**) Western blot analysis of the reactivities of LDL (lane 1), reductively methylated LDL (lane 2) and LDL + Glu (lane 3) with an antiserum specific for apoB residues 3352-3371 and with anti-apoB mAb 278

Figure 4-4



To study the time course of modification of epitopes of apoB and the loss of LDL receptor-binding capacity by glycation, LDL were incubated with 200 mM glucose for 4, 8, 12 and 16 days. LDL incubated in the presence of glucose showed a progressive loss in TNBS-reactive lysine residues (Figure 4-5) and a parallel increase in electrophoretic mobility (Figure 4-6). A decrease in the ability of glycated LDL to compete with ^{125}I-LDL for binding to the LDL receptor was detectable after only 4 days of glycation with a further loss of activity over the subsequent 12 days (Figure 4-7). There was also a time-dependent loss of immunoreactivity of glycation-sensitive epitopes as detected by either Western Blotting (Figure 4-8) or RIA (not shown).

Figure 4-5: Trinitrobenzenesulfonic acid (TNBS) - reactive lysine residues in LDL as a function of the time of glycation.
The TNBS-reactive lysine residues were determined in LDL that had been incubated with 200 mM glucose, with or without aminoguanidine, for 0, 4, 8, 12 or 16 days (see Materials and Methods). Fifty μg of LDL were mixed with 1 ml of 4% $NaHCO_3$ (pH 8.4) and 50 μl of 0.1% TNBS and incubated for 1 h at 37^0C. Absorbance was measured at 340 nm. The concentration of amino groups was estimated using valine as a standard.

Figure 4-5

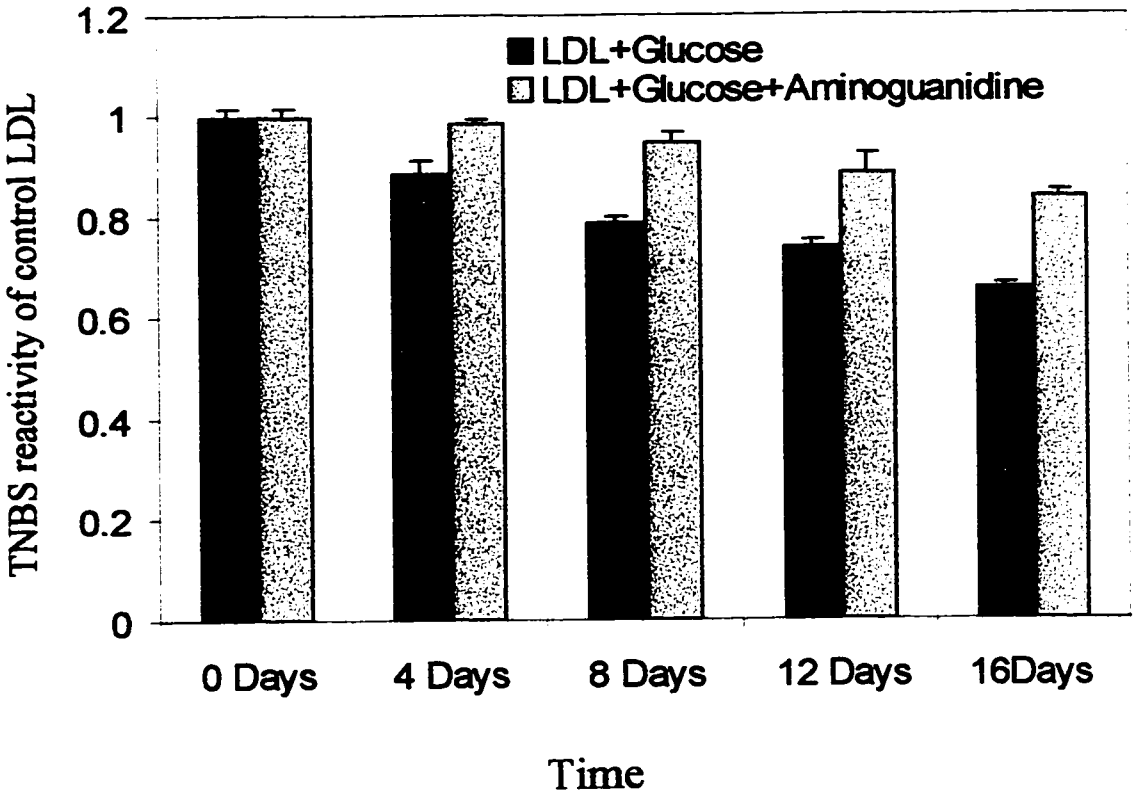

1.2
1
0.8
0.6
0.4
0.2
0
LDL+Glucose
LDL+Glucose+Aminoguanidine
TNBS reactivity of control LDL
0 Days
4 Days
8 Days
12 Days
16Days
Time

Figure 4-6: Electrophoretic mobility of LDL as a function of the time of glycation. LDL that had been incubated with 200 mM glucose, with or without aminoguanidine, for 0, 4, 8, 12 or 16 days (see Materials and Methods) were subjected to agarose gel electrophoresis. Samples were applied to a thin film agarose gel and were allowed to migrate for 30 min at 150V. The gel was stained for protein according to the manufacturer's recommendations.

Figure 4-6

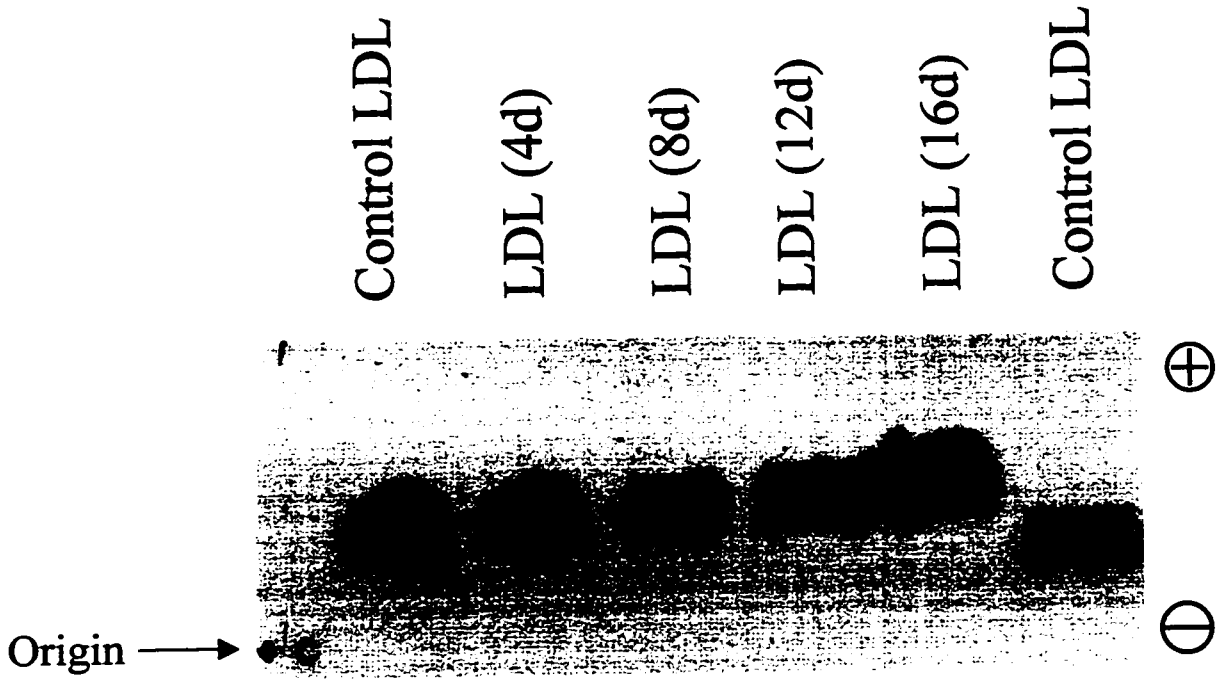


Figure 4-7: The ability of LDL to bind to the LDL receptor as a function of the time of glycation.
LDL incubated with 200 mM glucose, with or without aminoguanidine, for 0, 4, 8, 12 or 16 days (see Materials and Methods) were tested for their ability to compete with ^{125}I-LDL for binding to the LDL receptor on cultured human fibroblasts. Only the 16 day sample for LDL incubated with glucose and aminoguanidine is shown. Data points represent duplicate values.

Figure 4-7

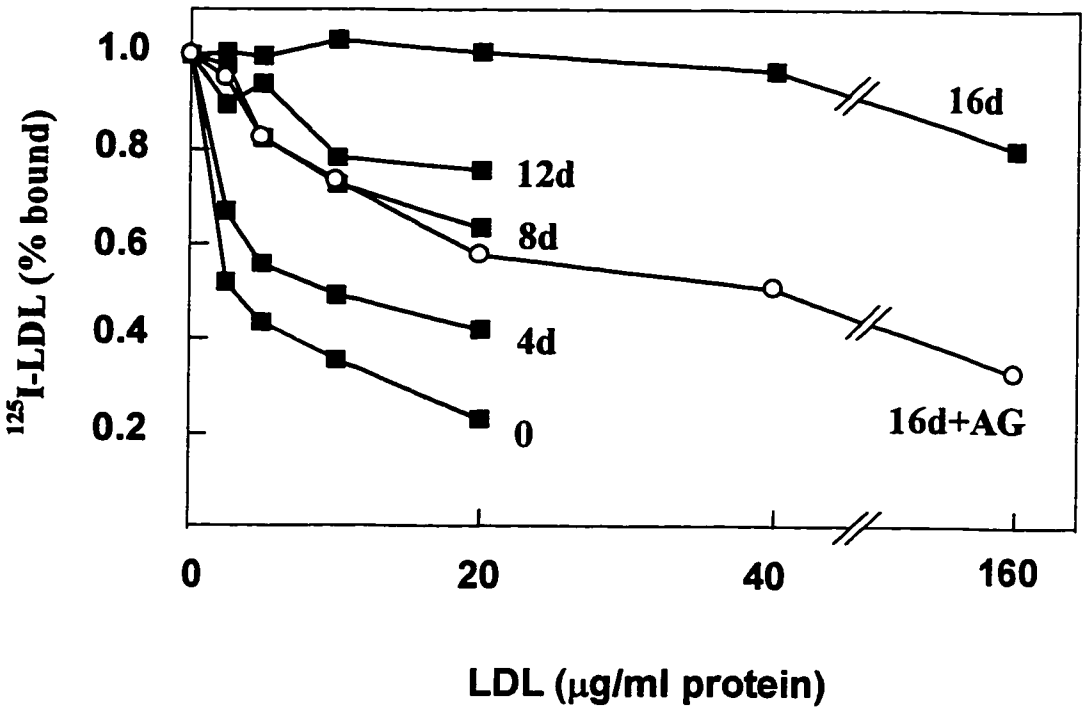
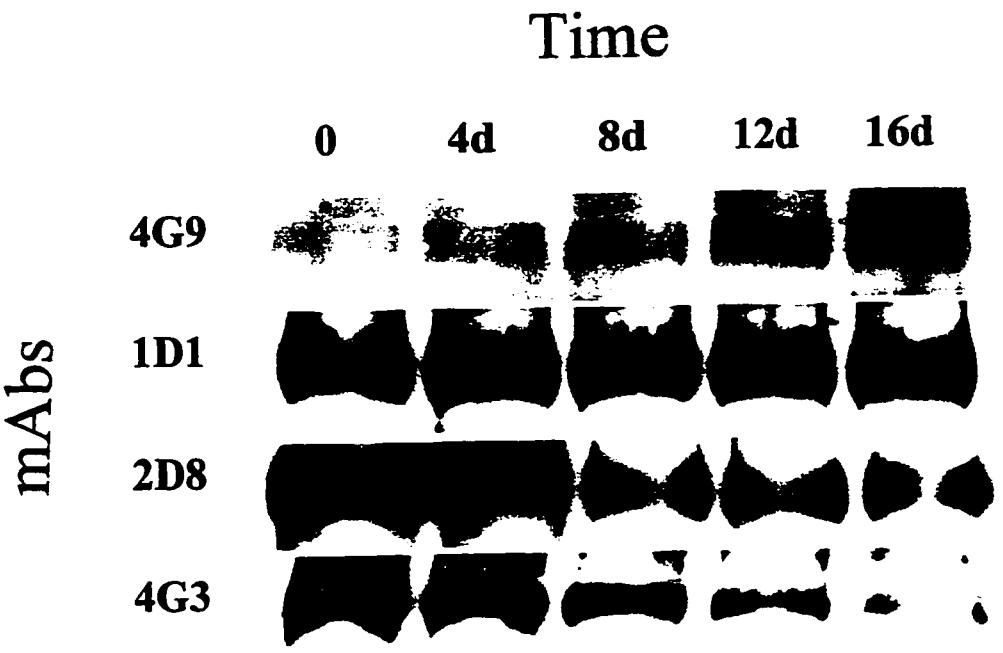

1.0
0.8
0.6
0.4
0.2
^{125}I-LDL (% bound)
16d
12d
8d
4d
0
16d+AG
0
20
40
160
LDL (μg/ml protein)

Figure 4-8: Immunoreactivity of LDL as a function of the time of glycation.
The immunoreactivity of LDL that had been modified under the conditions described in Figure 4-7 with anti-apoB and anti-AGE mAbs was determined by Western blotting. Results with the anti-AGE mAb, 4G9 and anti-apoB mAbs 1D1, 2D8 and 4G3 are presented.

Figure 4-8



Discussion

Basic residues of apoB are known to be critical for binding to the LDL receptor. This was originally deduced from the observation that chemical modification of either Arg or Lys residues in apoB prevents binding of LDL to the LDL receptor (Weisgraber *et al,* 1978). This could reflect modification of basic residues within the apoB LDL receptor-binding site or a change in apoB conformation due to modification of residues elsewhere in the molecule. As Lys residues are the primary targets of AGE modification, either mechanism could also be responsible for the inability of glycated LDL to bind to the LDL receptor. Consistent with an indirect effect of AGE modification on apoB-mediated binding to the LDL receptor, it has recently been demonstrated that the major site of apoB modification by AGE is located between residues 1388 and 1455, some 1791 residues upstream of the putative apoB LDL receptor-binding domain (Bucala *et al,* 1995). Based on this observation, it was proposed that AGE modification in the region of residues 1388 to 1455 induces a change in conformation of the apoB LDL receptor-binding site. In the case of apoB there are precedents for such a mechanism. A natural apoB variant that is characterized by an $Arg^{3500} \rightarrow Gln$ substitution is defective in its binding to the LDL receptor and is responsible for the condition known as familial defective apoB (Innerarity *et al,* 1990). While Arg^{3500} does not apparently form part of the apoB LDL receptor-binding site, the replacement of Arg^{3500} by Gln is thought to induce a conformational change that perturbs apoB-mediated binding to the LDL receptor (Lund-Katz *et al,* 1991).

The results presented here show that expression of a number of apoB epitopes is altered in glycated apoB. These include both epitopes that are, close to, and distant from, the major site of apoB AGE modification. The 2D8 epitope (residues 1438-1481), which has been mapped to a region that overlaps the major AGE modification site on apoB, is sensitive to glycation. In spite of this proximity, the 2D8 epitope and the major site of apoB AGE modification do appear to be distinct. We have observed that inclusion of the 2D8 mAb during the glycation of LDL could protect the 2D8 epitope from modification but could prevent neither the acquisition of AGE immunoreactivity by apoB nor the loss of receptor-binding activity of the LDL (Wang and Milne, unpublished observation). Two of the mAbs, 4G3 and 588, that show reduced affinity for glycated apoB are specific for epitopes (residues 2980-3084 and 3687-4081, respectively (Pease *et al,* 1990)) that flank the putative apoB LDL receptor-binding site and can block apoB-mediated binding to the LDL receptor. Other glycation-sensitive epitopes are located between residues 1854-1878 (B4), 1880-2100 (3A5), 2148-2375 (2G4) and 4342-4536 (234). In all cases the loss of expression of these epitopes in glycated LDL is apparent both in radioimmunoassay and after SDS electrophoresis. The loss of reactivity of the epitopes when tested under both native and denaturing conditions indicates that glycation directly modifies residues that compose the epitopes and that the loss of epitope expression may not be due to a change in apoB conformation. This would imply that the region of apoB comprised of residues 1388 through 1455 is not the only target of modification during *in vitro* glycation. This would be consistent with the respective magnitudes of the loss of

TNBS-active Lys residues and the increase in LDL electrophoretic mobility that were observed following glycation.

The non-enzymatic glycation of proteins is a complex cascade of reactions that gives rise to a mixture of AGEs that are heterogeneous and poorly characterized. As the identification of residues 1388-1455 as the major site of apoB AGE modification in the previous study (Bucala *et al,* 1995) was based on the reactivity of an AGE-specific mAb, modification of residues elsewhere in apoB may involve AGE adducts that are not recognized by this mAb and / or non-AGE modifications. It is notable that the epitope recognized by the anti-apoB mAb 2G4 could not be protected during glycation by aminoguanidine, an AGE inhibitor that is thought to block an initial step in glycation. It is possible that reactive AGE-forming intermediates can arise from oxidative reactions (glycoxidation) of free sugar or from initial Schiff base condensation products with protein amino groups, rather than from Amadori rearrangement products. In spite of the inclusion of antioxidants in the LDL preparations and our inability to detect an increase in lipid peroxides in the glycated LDL, we can not exclude the possibility that the loss of expression of certain of the epitopes resulted from oxidative modification of apoB. It has been shown that *in vitro* glycation of LDL can promote lipid oxidation (Bucala *et al,* 1993).

While our studies did identify glycation-sensitive epitopes in the proximity of the apoB LDL receptor-binding site, it is notable that glycation of LDL did not affect its

reactivity with an antiserum specific for apoB residues 3352-3371. ApoB residues 3359-3367 were initially identified as being potentially implicated in binding to the LDL receptor by their enrichment in basic amino acids and their homology to the LDL receptor-binding domain of apoE (Knott *et al,* 1985). The importance of this sequence in binding to the LDL receptor is now supported by strong experimental evidence (Boren *et al,* 1998). It is possible that glycation-sensitive lysine residues that are situated outside of the sequence 3357-3367 directly participate in binding to the LDL receptor. Alternatively, as was previously proposed (Bucala *et al,* 1995), the inability of glycated LDL to bind to the LDL receptor may result from an apoB conformational change that is secondary to the modification of residues outside of the apoB LDL receptor-binding site. However, as we have demonstrated that the expression of epitopes distributed throughout apoB primary structure is decreased in glycated LDL, it is no longer necessary to attribute this putative conformational change to the modification of residues far from the LDL receptor-binding site. Modification of lysine residues in the vicinity of the apoB LDL receptor-binding site may, in fact, be responsible.

Chapter V

General Discussion and Future Perspectives

In this Chapter, I will discuss in more detail the experimental results that were presented in Chapters II, III and IV, their implications with respect to our understanding of lipoprotein structure and metabolism and future directions for the work. In addition, I will briefly introduce a number of other observations that I have made during my Ph.D. program that were not included in the three manuscripts that constitute Chapters II, III and IV.

In Chapter II I describe the development and evaluation of novel methods to manipulate the immune response of mice in order to generate mAbs with unique specificities. The common principle of the three protocols that were developed was to exploit natural or experimentally-induced immunological tolerance to focus the immune response of mice on a very limited repertoire of epitopes of the immunogen. The initial goal was to use these immunization protocols to generate apoB isoform-specific mAbs and, in particular, mAbs that could discriminate between wildtype apoB and the apoB^{Gln3500} variant that is defective in its binding to the LDL receptor. While I have not yet been successful in obtaining mAbs that can distinguish between apoB isoforms that differ at residue 3500, I have clearly demonstrated the potential of using immunological tolerance as a tool to generate mAbs with specificities that one would not normally obtain using conventional immunization regimens.

The initial protocol that I evaluated was based on the induction of neonatal tolerance to one apoB isoform followed by immunization with a second isoform of apoB. Under the experimental conditions that I employed, I was unable to induce immunological tolerance to apoB. Moreover, not only did injection of LDL during the first 24 hours after birth fail to produce a specific immunological unresponsive state in the mice, it actually served to prime the immune system to mount a secondary immune response when the mice were later immunized. It is not surprising, therefore, that no apoB isoform-specificity was detected in the serum of the immunized mice nor in the mAbs secreted by hybridomas that were generated from their splenocytes. Given the success reported for other antigens (Golumbeski and Dimond, 1986), our inability to induce neonatal tolerance to apoB may reflect unique antigenic properties of apoB or a failure to establish the appropriate experimental conditions.

In contrast, using a subtractive immunization protocol based on chemical immunosuppression, I did observe that the humoral immune response to LDL could be selectively suppressed in an isoform-specific manner. Unfortunately, this isoform specificity was not reflected in the repertoire of mAbs that were generated from mice that had been immunized using the protocol. One complicating factor in these experiments was that I did not have LDL from a subject that was homozygous for the $apoB^{Gln3500}$ allele. As a consequence, the FDB LDL preparations that were used for immunosuppression, immunization, and screening were a mixture of wildtype LDL and $apoB^{Gln3500}$ LDL. It has been estimated that about 70% of LDL particles in plasma of

heterozygous FDB subjects contain the variant apoB^{Gln3500} due to their retarded clearance from the circulation (Arnold et al, 1994). This lack of homogeneous apoB^{Gln3500} LDL would be particularly problematic when I attempted to chemically suppress the immune response using FDB LDL, as apoB^{Arg3500} specific cells would presumably also be eliminated due to the presence of the wildtype LDL.

Antibody 4H11 was obtained from a fusion using splenocytes of a mouse that had been injected with FDB LDL followed immediately by cyclophosphamide and then later immunized with LDL from a normal individual. It was observed that mAb 4H11 reacted strongly with wildtype LDL and failed to recognize the FDB LDL that had been used for immunosuppression. It was therefore initially identified as a potential apoB isoform-specific mAb. Further analysis of the specificity of 4H11 revealed, however, that its reactivity was very sensitive to oxidative modification of apoB. When tested with freshly prepared apoB^{Gln3500} LDL, 4H11 reacted with high affinity. I had previously observed that FDB LDL were much more susceptible to oxidation than were LDL from individuals who had not inherited an apoB^{Gln3500} allele. If precautions were not taken, FDB LDL would rapidly become oxidized when stored at 4°C. FDB LDL were also much more susceptible than LDL from normal subjects to copper-mediated oxidation (results not shown). This observation was later confirmed by others (Marz *et al*, 1993). The increased susceptibility of apoB^{Gln3500} LDL to oxidation may represent an additional risk factor associated with FDB. With respect to the characteristic specificity of the 4H11 mAb, it is possible that, in chemically suppressing the immune response to FDB LDL, we

may have inadvertently eliminated B-lymphocytes that are specific for oxidatively-modified apoB.

Another mAb, 10D2, that was generated using the subtractive immunization protocol, was initially erroneously characterized as an $apoB^{Arg3500}$-specific mAb. When LDL was subjected to SDS-PAGE, 10D2 reacted with a species that was slightly larger that apoB-100, and the reactivity was lost when the sample was run under reducing conditions. When LDL or plasma was subjected to agarose gel electrophoresis, the 10D2-reactive particles migrated with pre-β mobility. We observed considerable inter-individual variability in terms of 10D2 reactivity and this correlated well with the concentration of Lp(a) in the samples. In collaboration with Dr. Marlys Koschinsky in the Department of Biochemistry at Queen's University, we demonstrated that 10D2 is specific for an epitope that was located in a region of apo (a) composed of kringle IV types 3, 4 and 5 domains. Measurement of the Lp (a) concentrations in the LDL preparations used for immunosuppression and immunization, respectively, confirmed that the FDB LDL used at the suppression step had less than 0.1 mg / dL of Lp (a) whereas the wildtype LDL used for immunization and for screening of hybridomas had 25 mg / dL Lp (a). It is probable, therefore, that by suppressing the immune response to apoB epitopes, we rendered the apo (a) more immunogenic. The subtractive immunization protocol appears, therefore, to be an effective method to raise mAbs to minor components of antigen preparations. Furthermore, I have noted that there is relatively little overlap in the intramolecular specificities of the anti-apoB mAbs generated using subtractive

immunization with those of the large panel of anti-apoB mAbs that was generated with conventional immunization protocols. Antigen-reactive B cells that are specific for highly immunogenic epitopes of apoB may be selectively targeted by chemical immunosuppression and, as a consequence, the subsequent immune response may be directed against epitopes that are normally, weakly immunogenic.

The third novel immunization protocol for generating apoB isoform-specific mAbs that I have evaluated is immunization of mice that carry a human apoB transgene. Five specific hybridomas were obtained from human apoB^{Arg3500} transgenic mice that had been immunized with LDL from FDB heterozygous individuals. Of these, two mAbs, 2E1 and 3D11 were of particular interest. The mAb 2E1 reacted with LDL isolated from normal and FDB individuals but showed no reactivity with the plasma lipoproteins of human apoB transgenic mice. It is possible that mAb 2E1 is specific for a human apoB isoform that is different from that expressed in the human apoB transgenic mice. To date, I have screened more than 20 human plasma samples by competitive RIA using mAb 2E1 and all of samples showed high reactivity. Therefore, if 2E1 does recognize a genetic polymorphism of human apoB, it would appear to specific for the product of an allele that is very highly represented in the population. An alternative possibility is that there are differences between mice and humans in their post-translational modification of apoB and that mAb 2E1 can detect such differences. This could include glycosylation, phosphorylation or fatty acid acylation of apoB. I am presently attempting to map the 2E1 epitope within apoB primary structure and to determine the structural basis for the

differential reactivity of 2E1 with LDL isolated from human plasma and LDL expressed in human apoB transgenic mice.

The mAb 3D11 is apparently specific for an epitope on apoB whose expression is highly dependent upon its lipoprotein environment. While it reacts with apoB in human LDL, it fails to recognize human apoB in the lipoproteins of human apoB transgenic mice. Under the denaturing conditions of SDS-PAGE, it is clear, however, that the residues that compose 3D11 are present in the product of the human apoB transgene. The differential reactivity of mAb 3D11 is only apparent with native lipoproteins. It is known that the "human" LDL in the plasma of human apoB transgenic mice are small and triglyceride-rich (Linton *et al*, 1993), a property that can not be attributed solely to the absence of CETP activity in mouse plasma (Grass et al, 1995). The unusual chemical and physical properties of the transgenic LDL could potentially alter apoB conformation and the expression of certain apoB epitopes. The reduced particle size and altered lipid composition of the LDL isolated from human hypertriglyceridemic subjects have been shown to affect the reactivity of the particles with anti-apoB mAbs (Teng et al. 1985, Galeano et al. 1994). I have not yet evaluated the 3D11 immunoreactivity of LDL from hypertriglyeridemic patients. The relatively low levels of hepatic lipase in mice and its altered tissue distribution compared to humans, could potentially be responsible for the characteristic properties of the LDL of human apoB transgenic mice. In collaboration with Dr. Joshua Schultz, I will test the 3D11 reactivity of lipoproteins of mice that overexpress both human apoB and human hepatic lipase. It has also been proposed that

there may be direct secretion of triglyceride-rich LDL from the livers of human apoB transgenic mice (Grass et al. 1995). In addition to their altered lipid composition, these particles could contain other apolipoproteins that could potentially mask the 3D11 epitope on apoB. While direct hepatic secretion of LDL in human apoB transgenic mice has not been demonstrated experimentally, it is known that triglyceride-rich LDL are the primary LpB secreted by HepG2 cells. Again, in collaboration with Dr. Joshua Schultz, I will determine the 3D11 immunoreactivity of LDL that are secreted by normal HepG2 cells and HepG2 cells that overexpress human hepatic lipase. Finally, I will replace cholesteryl esters in the core of normal human LDL with triglyceride by *in vitro* incubations with triglyceride-rich microemulsions and CETP and determine the effects on the expression of the 3D11 epitope. As the 3D11 epitope appears to be highly dependent on its lipoprotein environment, its expression could potentially be a marker for atherogenic lipoproteins.

The use of subtractive immunization protocols, and particularly immunization of transgenic mice, for the generation of mAbs having unique specificities has applications beyond apoB. There are now many murine lines that carry a wide variety of human transgenes that could potentially be used for the production of mAbs. In the case of human plasma lipoproteins, the obvious example would be human apoE. As a second ligand for the LDL receptor, the LRP, the VLDL receptor and heparin sulfate proteoglycans, apoE is an important modulator of lipoprotein metabolism (Weisgraber, 1994). The essential role of apoE in lipoprotein metabolism is underscored by the

dramatic hyperlipidemia and atherosclerosis that are seen in apoE-deficient mice. ApoE is polymorphic in terms of primary structure. The most common apoE isoforms, apoE2, apoE3 and apoE4 are encoded by 3 co-dominantly-expressed alleles. ApoE2 differs from the most common isoform, apoE3, by the substitution of a cysteine for an arginine at residue 158 whereas apoE4 differs from apoE3 by the substitution of an arginine for a cysteine at residue 112. ApoE2 is defective in its binding to the LDL receptor and to glycosaminoglycans and is responsible, in part, for the expression of type III dyslipoproteinemia, a condition that is characterized by an accumulation of triglyceride-rich lipoprotein remnants in the plasma. In addition to this well-documented association of apoE2 with type III dyslipoproteinemia, apoE genetic polymorphism influences plasma lipid levels (Davignon et al. 1988), postprandial lipemia (Weintraub et al. 1987), apoB metabolism (Demant et al. 1991), apoE distribution in lipoproteins (Gregg et al. 1986), and susceptibility to Alzheimer's disease (Stritmatter et al. 1993). As a consequence, determination of apoE genotype or phenotype has become an important criteria for the assessment of risk for atherosclerosis and neurodegenerative disease. The most common methods for the determination of apoE genotype / phenotype are based on the polymerase chain reaction and isoelectric focusing. Antibodies that are capable of discriminating between apoE isoforms could be incorporated into a rapid antibody-based assay that would offer advantages over the current techniques in terms of both time and cost. In addition, apoE isoform-specific mAbs could be useful immunohistochemical reagents to determine distribution of apoE isoforms in tissues and lesions and the mechanisms responsible for the observed association between the inheritance of the

apoe4 allele and Alzheimer's disease. Transgenic mice that express the 3 common human apoE isoforms are now available, and could be used for the generation of apoE isoform-specific mAbs. Now the transgenic mice lines carrying human $apoB^{Arg3500}$ and $apoB^{Gln3500}$ are available (Boren *et al*, 1998) that would allow us to mimic the scenario of blood transfusion which was demonstrated to be efficient to elicit antibodies against apoB isoforms in human (Allison and Blumberg, 1961).

Immunochemical techniques for the study of the structure and function of proteins have advantages not only in the field of lipoproteins, but also in the general field of biology. In particular, due to their monospecificity, mAbs have proven to be extremely useful as conformational probes of proteins and for the identification of functional domains. There are many examples in the literature in which mAbs have been used to study the structure-function relationships of biologically important processes. To cite just several examples, mAbs have used to study ligand-receptor recognition (Taub et al, 1989), T lymphocyte activation (van Seventer *et al*, 1991), viral infection (Smith, 1996), signaling pathways (Uhr *et al*, 1996) and enzymatic activity (Tawfik *et al*, 1994). In the introduction to the thesis I describe other examples in which mAbs have been used to examine the structure and function of molecules involved in the metabolism of plasma lipoproteins. Because of the large size of apoB and its insolubility in a delipidated form, indirect approaches have been taken to determine apoB conformation and to identify its functional domains. In this regard, anti-apoB mAbs have proved to be extremely important probes.

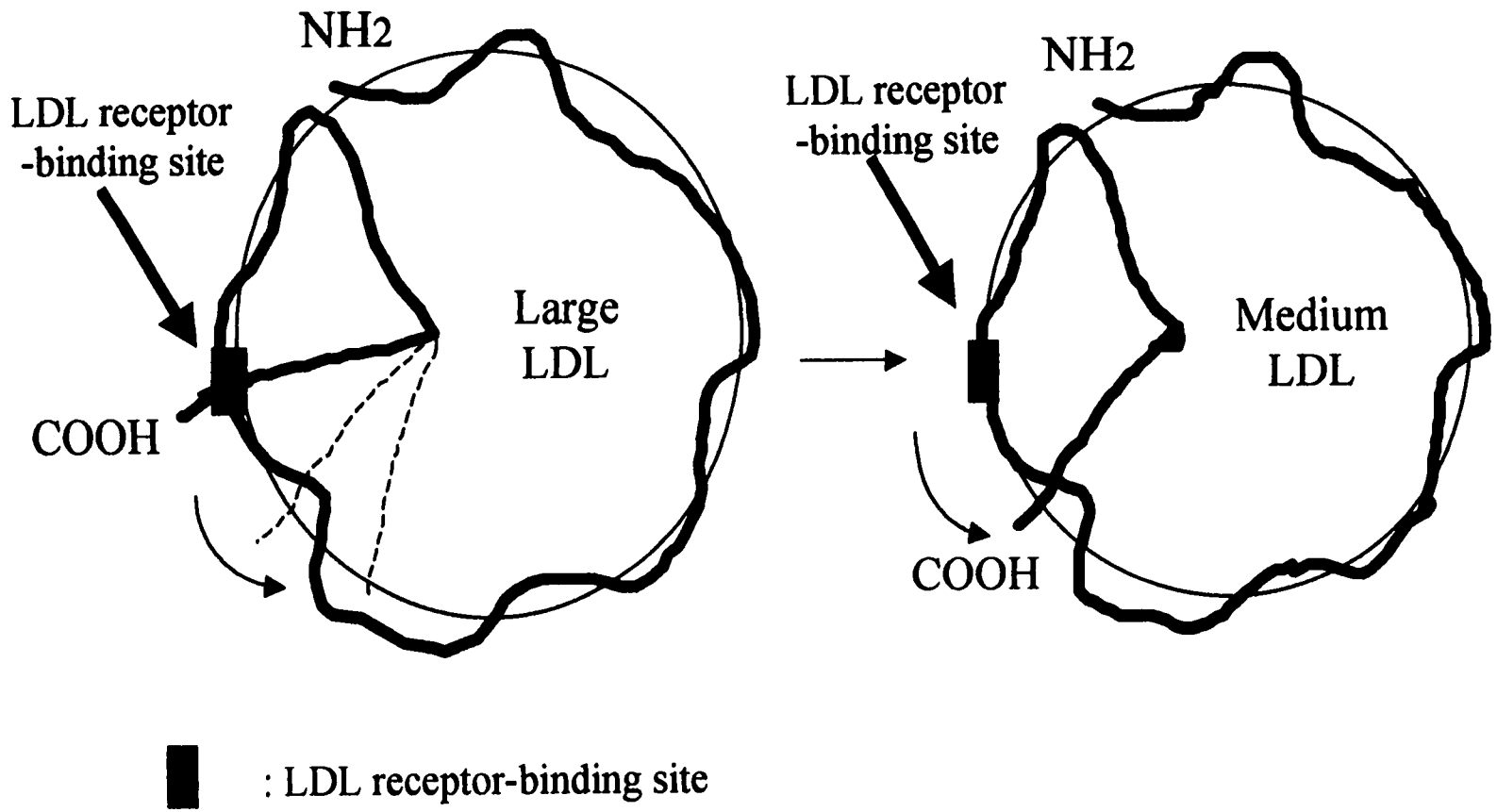
In Chapter III of the thesis, I describe the characterization of a new panel of anti-human apoB mAbs and the application of these mAbs to a study of the apoB conformational changes that occur during the intravascular metabolism of LpB. I have mapped the epitopes of the mAbs within apoB primary structure, identified several new mAbs that can block binding of LDL to the LDL receptor and I have determined if the epitopes of the latter mAbs are exposed on LDL receptor-bound LDL. In doing so, I have more accurately mapped the apoB LDL receptor-binding domain. Moreover, I have demonstrated that two of the new mAbs, 4H11 and 6O5, that are specific for epitopes near the apoB carboxy-terminus, detect an important change in apoB conformation that occurs as large LDL are converted to medium and smaller particles in the plasma. It is well established that larger triglyceride rich LpB can not be taken up by the LDL receptor through apoB-mediated binding. This property is acquired relatively late in the intravascular metabolism of LpB and is thought to result from an alteration in apoB conformation that is secondary to changes in the physical properties and lipid / apolipoprotein composition of the particles. The change in immunoreactivity of the 4H11 and 6O5 epitopes may be a manifestation of the same apoB conformational change.

As I have discussed in the preceding Chapters, there is strong evidence that the apoB LDL receptor-binding site is localized between apoB residues 3000-4000 and that apoB residues 3359-3367 appear to be directly implicated in the interaction with the LDL receptor. In addition to the LDL receptor-binding site, the carboxy-terminal region of

apoB would also include a negative regulatory domain for apoB-mediated binding to the LDL receptor. This was initially suggested by the observation that certain carboxy-terminally-truncated variants of apoB (Parhofer et al. 1990, 1992, Krul et al. 1992) have increased affinity for the LDL receptor. Clues to the mechanism of how this might occur came from electron microscopic analyses of LDL-anti-apoB mAb immune complexes that revealed the characteristic topology of apoB on the surface of LDL particles (Chatterton et al. 1991, 1995). Based on these observations, Dr. Verne Schumaker and his colleagues proposed the ribbon and bow model for apoB conformation in LpB (Figure 5-1). In this model, the carboxy-terminus of apoB could determine the accessibility of LDL receptor-binding site. One might predict that, as VLDL is converted to LDL, there would be lateral movement of the carboxy-terminal region of apoB that would allow the LDL receptor-binding site to become accessible for interaction with the LDL receptor. There is now strong experimental data to support a role of the carboxy-terminus of apoB as a negative regulator of LDL binding to the LDL receptor (Borén *et al*, 1998). It was shown that VLDL from apoE-deficient mice that carry a human apoB-80 transgene bind efficiently to the LDL receptor whereas VLDL from apoE-deficient that carry a full-length human apoB-100 transgene can not. It therefore appears that it is the extreme carboxy-terminus of apoB that prevents LpB from binding to the LDL receptor during its intravascular metabolism until it is converted to LDL. In the same study it was demonstrated that it is sequences downstream from residue 3500 that are responsible for the inability of apoB^{Gln3500} to mediate binding to the LDL receptor.

Figure 5-1

Model of the conformational change in the carboxy terminus of apoB and its modulation of LDL receptor-recognition



In collaboration with Drs. Tom Innerarity and Jan Borén, I am further investigating the mechanism by which the apoB carboxy-terminus acts as a regulator of apoB-mediated binding to the LDL receptor. A line of mice has been generated which carries a transgene that encodes a human apoB-100 variant in which residues 3359-3367 have been replaced with the residues that comprise the apoE LDL receptor-binding site (Borén et al. 1998). The transgenic LDL of these mice bind to the LDL receptor and are recognized by a mAb, 1D7, specific for the LDL receptor-binding site of apoE (Milne et al, 1981, Weisgraber et al, 1983). If the ribbon and bow model of apoB conformation on LDL is correct, and that the carboxy terminus of apoB serves as a regulator of binding to the LDL receptor-binding site, I would predict that LDL, but neither VLDL nor IDL, would react with 1D7. If, on the other hand, the 1D7 epitope is expressed in VLDL and IDL, I will also test our panel of anti-apoB mAbs for their ability to compete with mAb 1D7 for binding to VLDL, IDL and LDL. I would predict that mAbs that are specific for epitopes near the carboxy terminus of apoB (e.g. 6O5 and 4H11) may compete with 1D7 for binding to VLDL and IDL but there would be no competition for binding to LDL. A more sophisticated approach may be to use the technique of fluorescent resonance energy transfer to measure distances between pairs of anti-apoB mAbs in immune complexes with VLDL, IDL or LDL (Wang, J. *et al*, 1997).

It is well known that both diabetes and end stage renal disease are important risk factors for the development of atherosclerosis. One feature that is characteristic of the two conditions is the increased concentration of circulating proteins, including LDL, that

are modified with advanced glycation end products. As I discuss in Chapters I and IV, AGE-modified LDL could promote atherogenesis by a number of mechanisms. Because of the AGE adduct, modified LDL could become covalently cross-linked to proteins and proteoglycans in the arterial wall. AGE-modified LDL can be taken up by macrophages in the vascular wall via the SR-A and RAGE cell surface receptors. As these receptor pathways do not have the control mechanisms that are inherent in the LDL receptor pathway, the end result could be conversion of macrophages into foam cells. In addition, uptake of AGE-LDL or signaling via AGE- receptors has been shown to alter the cellular phenotype to one that is pro-atherogenic. Finally AGE-modified LDL is not a ligand for the LDL receptor and shows a prolonged residence time in plasma. This could have added importance as it has been demonstrated that AGE oxidation plays an important role in initiating lipid oxidation. All or some these properties associated with AGE modification of LDL could contribute to atherogenesis.

As I show in Chapter IV, when LDL is subjected to *in vitro* glycation, there are at least six sites of modification within apoB, including two that flank the apoB LDL receptor-binding site. It had been proposed that the loss of reactivity of glycated LDL with the LDL receptor was the result of a conformational change that was induced by the modification of a single site in apoB that is over 1700 residues from the putative LDL receptor-binding site. The demonstration of modified epitopes relatively close to the LDL receptor-binding site obviates the necessity of invoking such long-range effects on apoB conformation. The six sites of modification that I have identified are obviously a

minimum. The 29 apoB epitopes that were studied would clearly not cover all potential sites of modification in apoB. Moreover, we have observed a 42% reduction of free amino groups in glycated LDL, that could represent up to 147 of the 351 lysine residues of apoB. Interestingly, we observed that, while all epitopes that were sensitive to glycation were also sensitive to reductive-methylation, the contrary was not the case. There may be some sequence specificity in glycation.

An important observation in the present work was that *in vitro* glycation of LDL abolished its ability to bind to the LDL receptor but residues 3359-3367, the putative LDL receptor-binding site, did not appear to be modified. Although there is strong evidence that apoB residues 3359-3367 participate directly in binding to the LDL receptor (Yang et al. 1986, Boren *et al*, 1998), an additional contribution of residues outside of this region has not been excluded. Such residues could potentially be a target for modification by glucose or AGE-peptides. Another possibility is that modification of a residue outside of the apoB LDL receptor-binding site induces a change in the conformation of apoB such that it can no longer bind to the LDL receptor. This could be a local conformational change, as I have demonstrated that epitopes close to the apoB LDL receptor-binding site are modified in glycated LDL. A third possible mechanism by which glycation could modulate apoB-mediated binding to the LDL receptor is by modification of heparin-binding sites in apoB. It is thought that apoE-mediated binding of triglyceride rich lipoproteins to LDL receptor-related protein (LPR) is facilitated by interaction of apoE with heparin sulfate proteoglycans on the cell surface (Ji *et al*, 1994).

A co-operative effect between the heparin-binding and LDL receptor-binding sites of apoB could also be important in apoB-mediated binding to the LDL receptor. With the collaboration of Dr. Jim Burgess, I have shown in preliminary experiments that, compared to non-modified LDL, glycated LDL had decreased binding to heparin sulfate proteoglycans and dermatan sulfate proteoglycans whereas binding to chondroitin sulfate proteoglycans was normal.

It is not clear if the loss of LDL receptor-binding activity of glycated LDL is the result of the modification of a single critical lysine residue of apoB or it is the cumulative effect of modification of multiple residues. In an attempt to answer this question I will co-incubate anti-apoB mAbs and LDL during *in vitro* glycation in order to protect specific epitopes from modification. I have shown that the 2D8 mAb, that is specific for an epitope that is adjacent to the major site of apoB AGE-modification, could protect the 2D8 epitope from modification but did not prevent AGE modification of apoB or the loss of LDL receptor-binding activity. It will be interesting to repeat this experiment with other mAbs specific for glycation-sensitive epitopes. Of particular interest would be mAbs 4G3 and 588 that are specific for glycation-sensitive epitopes close to the apoB LDL receptor-binding domain. The glycation-sensitive 234 epitope would be another obvious candidate in view of the important regulatory role of the carboxy terminus of apoB in LDL receptor-binding. Another approach to evaluate the hypothesis that modification of lysine residues near the carboxy-terminus of apoB causes a conformational change and masking of the apoB LDL receptor-binding site would be to

compare the LDL receptor-binding properties of apoB-100 LDL and apoB-80 LDL following *in vitro* glycation. Appropriate transgenic mouse lines are available.

Another project that is currently underway in our laboratory involves the expression of a functional soluble form of the LDL receptor in the methylotrophic yeast, *Pichia pastoris*. It has been shown that the soluble ligand-binding domain of the LDL receptor is capable of binding apoB and apoE in a fashion that is similar to full length LDL receptor (Simmons *et al*, 1997; Dirlam *et al*, 1996). The availability of this soluble form of the receptor could be used to evaluate the contribution of cell surface proteoglycans to binding of LDL to the LDL receptor. As a corollary, one could test the hypothesis that the inability of glycated LDL to bind to the LDL receptor is secondary to the loss of binding to cell surface proteoglycans. Finally the soluble LDL receptor could potentially be used to determine the mechanism by which apoB mAbs, whose epitopes are separated by up to 1000 residues in apoB primary structure, can block apoB-mediated binding to the LDL receptor.

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RESEARCH EXPERIENCE:

1987,8-1988,9, research assistant, Division of Cellular Immunology, I worked on Human T cell surface antigens using immunochemical and biochemical techniques to identify, purify and quantify the various T cell surface antigens.

1988,9-1991,6, research assistant, Division of Molecular Immunology. I worked on Component Pertussis Vaccine. I have used several animal models to evaluate the antigenicity and toxicity from different components of the B Pertussis. Those works were supervised by Dr. Liya Wang, Director, Division of Cellular-Immunology, National Vaccine and Serum Institute, Beijing, China

1991,6-1992,12, visiting scholar, Laboratory of Metabolism of Lipoproteins, Clinical Research Institute of Montreal. I worked on the protocol of producing isoform-specific monoclonal antibodies. The project was supervised by Dr. Ross Milne, director, Lab of Immunochemistry.

1993,01-Present, Ph.D. candidate, Department of Biochemistry, University of Ottawa Heart Institute, Atherosclerosis Group. I moved from Montreal to Ottawa with my supervisor and continue my project. I am working on a project to generate isoform specific monoclonal antibodies against human apolipoprotein B and to develop a quick assay for familial defective apoB patients and to employ mAbs as conformational probes to study the structure and function of apolipoprotein B

PUBLICATIONS:

1, Yuwei Wang, **Xingyu Wang**, Vivian Franklin and Zemin YaoThe Second Step Assembly of Hepatic Apolipoprotein B48-Containing Very Low Density Lipoprotein Does Not Utilize Newly Synthesized Triglycerides (Abstract) *Circulation* 1995 Vol 92, Page 1080

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5, **Xingyu Wang**, and Ross W. Milne. Conformational Change of Apolipoprotein B during Metabolic Cascade Detected by Monoclonal

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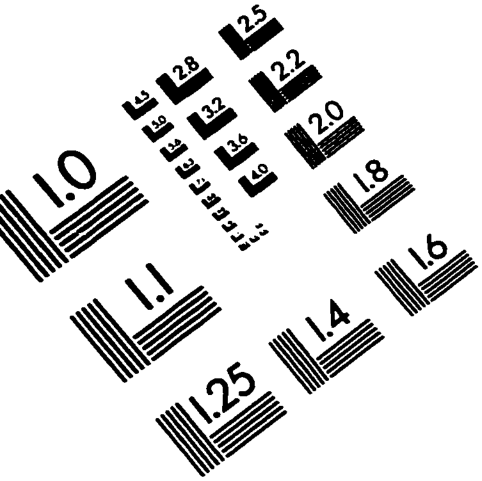
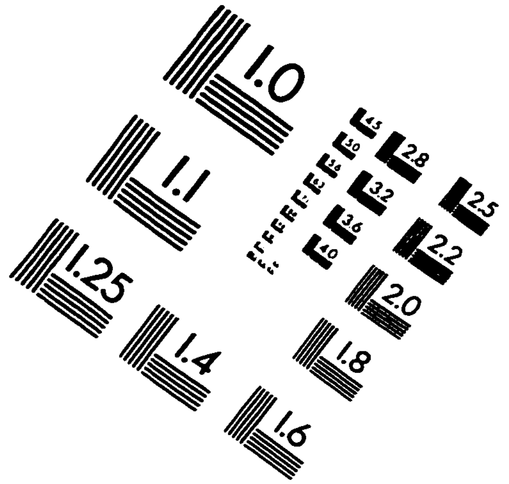
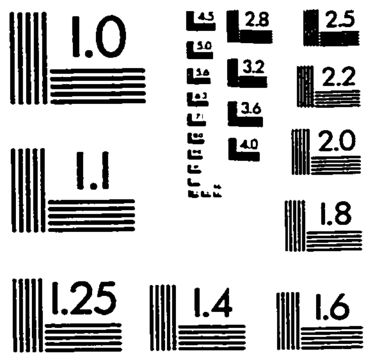
6, **Xingyu Wang**, Jean Davignon, Stephen Young, Jan Boren, Thomas Innerarity and Ross Milne. New Immunization Protocols for the Generation of Anti-Human Apolipoprotein B Isoform-Specific Monoclonal Antibodies (Submitted to *J. Lipid Res.*)

7, and 8, Two research articles published in 1991 in *Journal of Chinese Biological Products* in the topic of production of component pertussis vaccine (In Chinese).

IMAGE EVALUATION TEST TARGET (QA-3)







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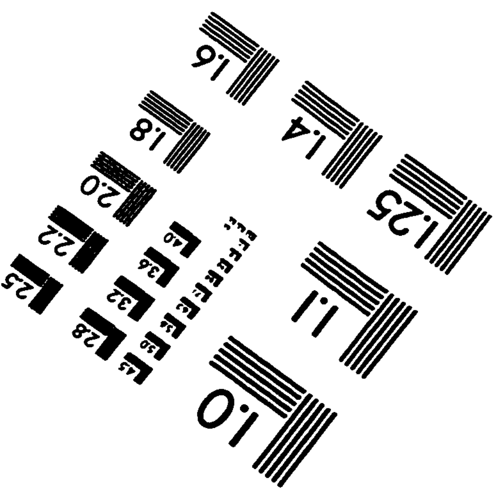

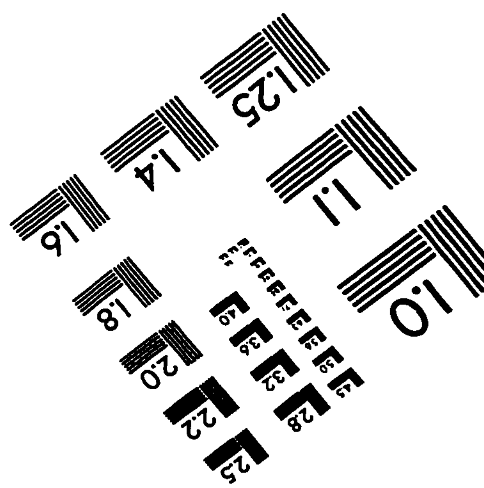

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