METABOLIC AND PHYSICOCHEMICAL BASES OF HYPERAPOBETALIPOPROTEINEMIA

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ABSTRACT

The goal of this thesis was to elucidate the physicochemical and metabolic bases of Hyperapobetalipoproteinemia (HyperapoB). This disorder, which is likely the commonest metabolic abnormality associated with premature coronary artery disease, was defined as a combination of a normal, or near-normal, LDL cholesterol in the face of an elevated LDL apoB.

LDL, even in normals, is heterogeneous. The experimental findings herein confirm this. They also extend this concept to indicate that familial hypercholesterolemia (FH) and HyperapoB each imprint LDL in different and characteristic ways, each an exaggeration of the typical relations between LDL composition and size in normals. At one extreme is HyperapoB, which is characterized by most of the LDL particles being smaller and denser than normal because they contain less cholesteryl ester but the same amount of apoB as normal. At the other is FH, which is characterized by larger, cholesteryl ester-enriched particles. There is, as well, a predictable relation between LDL particle size and the immunoreactivity of certain apoB epitopes.

Turnover studies of hepatic apoB using traditional analytic models showed that hepatic apoB is overproduced in HyperapoB, a finding which stands in marked contrast to the impaired catabolism of apoB in FH. A new multi-compartmental model of LDL metabolism has been developed which appears to elucidate several of the basic mechanisms involved in the pathogenesis of HyperapoB. All the data to date indicate that the characteristic abnormalities of LDL in HyperapoB are all consequences of the overproduction of hepatic apoB. Obviously, the goal for future research must be to understand the basis for this overproduction. A preliminary study with adipose tissue suggested that the overproduction of hepatic apoB might be secondary to a defect in peripheral tissue triglyceride biosynthesis.

RESUME

Le but de cette thèse est d'élucider le fondement chimique, physique, et métabolique de l'hyperapobetalipoprotéinemie (HyperapoB). Cette anormalité métabolique est probablement la plus frequente chez les malades prematurés des artères coronaires, et elle a été définie comme une combinaison d'un taux elevé de apoB de LDL avec un taux normal, ou presque normal, de cholestérol de LDL.

La LDL est heterogène, même chez les sujets normaux. Ceci est confirmé par les resultats presentés ici. De plus, on montre que le rapport normal entre composition et grandeur des particules LDL est modifié de façon différente et caractéristique dans les cas de l'hypercholestérolemie familiale et de l'HyperapoB. Dans le cas de l'HyperapoB la plupart des particules LDL sont plus petites et plus denses que les normales parce qu'elles contienent moins de cholestérol esterifié mais la même quantité d'apoB. Dans le cas de l'hypercholestérolemie familiale, les particules sont plus grandes parce qu'elles sont enrichies en cholestérol esterifié. Il y a aussi un rapport entre la grandeur des particules LDL et l'immunoreactivité de certain épitopes de l'apoB.

Nos études de turnover de l'apoB du foie, en utilisant des modèles analytiques traditionnels, ont demontré que l'apoB hepatique est surproduite dans l'HyperapoB. Cette observation est en contraste avec le catabolisme defectueux de l'apoB qui est caractéristique de l'hypercholestérolemie familiale. On a developé un nouveau modèle multicompartimental qui semble élucider plusieurs des mécanismes fondamentaux impliqués dans la pathogènese de l'HyperapoB. Toutes les donnés recueillies jusqu'à date montrent que les anormalités de LDL chez l'HyperapoB sont une consequence de la surproduction d'apoB. La recherche future devra se pencher sur les causes de cette surproduction. Une étude préliminaire sur le tissu adipeux a suggéré que la

surproduction hepatique d'apoB peut être due à un défaut dans la biosynthèse de triglycerides chez le tissu périphérique.

PROLOGUE

The reader is informed that I have chosen the option of including manuscripts of original papers published in journals as part of this thesis. Chapters 2, 3, 4, 5, and 6 have their own Abstract, Introduction, Materials and Methods, Results, Discussion, and References sections. Chapters 2, 3, and 4 have been published while Chapters 5 and 6 represent original work not yet published.

The list of published papers included in this thesis is as follows:

Chapter 2: Teng B, Sniderman AD, Thompson GR, Forte TM, Krauss RM, and Kwiterovich PO. (1983) Composition and distribution of low density lipoprotein fractions in hyperapobetalipoproteinemia, normolipidemia, and familial hypercholesterolemia. Proc. Natl. Acad. Sci. USA 80, 6662-6666.

Chapter 3: Teng B, Sniderman AD, Krauss RM, Kwiterovich PO, Milne RW, and Marcel YL. (1985) Modulation of Apolipoprotein B antigenic determinants in human low density lipoprotein subclasses. J. Biol. Chem. 260, 5067-5072.

Chapter 4: Teng B, Sniderman AD, Soutar A, and Thompson GR. (1986) Metabolic basis of hyperapobetalipoproteinemia: Turnover of apolipoproteins in low density lipoprotein and its precursors and subfractions compared with the normal and familial hypercholesterolemia. J. Clin. Invest. 77, 663-672.

Each of these articles was coauthored by my thesis supervisor, Dr. Allan D. Sniderman. In Chapter 2, Dr. Gilbert R. Thompson provided blood from patients suffering from familial hypercholesterolemia. Dr. Trudy M. Forte performed the electron microscopic study to measure LDL particle size. Dr. Ronald M. Krauss performed the analytical ultracentrifical studies of low density lipoprotein. Dr. Peter O. Kwiterovich has been a collaborator for several years.

In Chapter 3, Dr. Ronald H. Krauss provided much fruitful discussion. Dr. Ross W. Milne and Dr. Yves L. Marcel supplied us with the monoclonal antibodies used and Dr. Marcel also helped write and criticize the manuscript.

In Chapter 4, Dr. Gilbert R. Thompson supervised the preliminary study and the writing of the manuscript. Dr. Ann Soutar contributed many valuable insights in our discussions. Although all the turnover studies in the patients with familial hypercholesterolemia were done in London, England, I performed all of the analyses.

The work presented in Chapter 5 was done under the supervision of Dr. Lorne Zech of the National Institute of Health, Bethesda, Maryland.

Dr. Armor Force was responsible for measuring adipocyte size for the work cited in Chapter 6.

For all of the work in this thesis, I have received valuable technical assistance from Mrs. Hai Vu.

Except for the supervision and assistance mentioned above, I performed all the experimental work and analysis of the results included in this thesis.

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ABBREVIATIONS

The abbreviations used throughout this thesis are listed here. In most cases they are also defined at their first appearance in each chapter.

ACR	: absolute catabolic rate
ACT	: acyl CoA:cholesterol acyltransferase
apo AI	: apolipoprotein AI
apo AII	: apolipoprotein AII
apoB	: apolipoprotein B
apoC	: apolipoprotein C
apoE	: apolipoprotein E
B-LDL	: buoyant LDL
BSA	: bovine serum albumin
CAD	: coronary artery disease
cDNA	: complementary DNA
CE	: cholesteryl ester
CHD	: coronary heart disease
cm	: centimeter
cpm	: counts per minute
d	: day or density g/ml
D-LDL	: dense LDL
^{o}C	: centigree
dl	: deciliter
EDTA	: disodium ethylendiamine tetraacetate
FC	: free cholesterol
FCHL	: familial combined hyperlipidemia
FCR	: fractional catabolic rate

FH	: familial hypercholesterolemia
FHTg	: familial hypertriglyceridemia
g	: gram
h	: hour
HDL	: high density lipoprotein
HMG-CoA reductase	: 3-hydroxy 3-methylglutaryl CoA reductase
HSA	: human serum albumin
HTg	: hypertriglyceridemic
HyperapoB	: hyperapobetalipoproteinemia
IDL	: intermediate density lipoprotein
kD	: kilodaltons
kg	: kilogram
LCAT	: lecithin:cholesterol acyltransferase
LDL	: low density lipoprotein
uEq	: microequivalent
min	: minute(s)
ml	: milliliter
mM	: millimolar
nEq	: nanoequivalent
NTg	: normotriglyceridemic
PBS	: phosphate-buffered saline
PL	: phospholipids
P/S	: polyunsaturated fatty acids/saturated fatty acids
RBW	: relative body weight
rpm	: revolutions per minute
S.A.	: specific activity
S.D.	: standard deviation of the mean

SDS	: sodium dodecyl sulfate
sec	: second(s)
S.E.	: standard error of the mean
S_f	: flotation rate expressed in Svedberg units (10^{-13} sec) corrected for concentration dependence and standard conditions (26°C)
Tg	: triglyceride
uCi	: microcurie
ug	: microgram
ul	: microliter
VLDL	: very low density lipoprotein

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CHAPTER 1

INTRODUCTION

PREFACE

This introduction will examine the relation of serum lipoproteins to the risk of coronary artery disease with particular reference to Hyperapobetalipoproteinemia (HyperapoB), a disorder of hepatic apoB metabolism, which we first described in 1980. The experimental work presented in this thesis will establish the physicochemical and metabolic bases of this disorder.

First, though, the relations of serum lipids, lipoproteins, and apoproteins to coronary artery disease will be briefly reviewed; then the physiologic roles of the plasma lipoproteins will be briefly examined. Particular attention will be given to LDL heterogeneity and the synthesis and catabolism of LDL since these concepts are fundamental to the understanding and study of HyperapoB. This chapter concludes with a discussion of the regulation of hepatic apoB synthesis.

Even at the outset, we should note that if there is ever to be prevention of coronary artery disease, the factors responsible must be recognizable well in advance and their impact on the arterial wall interdicted. This thesis deals with HyperapoB, a disorder that appears to be present in a considerable number of patients with premature coronary artery disease. The purpose of this research is to move closer to the goal of preventing the clinical disease produced by this disorder.

SERUM LIPIDS, LIPOPROTEINS, AND APOPROTEINS WITH CORONARY ARTERY DISEASE

Atherosclerosis is a complex lesion within the arterial wall with different features at different times in its natural history. When mature, the lesion usually includes an acellular necrotic core, rich in cholesterol, overlaid by a thickened, raised intima. Within the lesion there are several cell types including smooth muscle cells, monocytes or macrophages, plus abundant collagen, elastin, glycosaminoglycans, fibrinogen, fibrin and lipoproteins (1). Although much remains to be learned about its development, there can be no doubt that atherosclerosis is almost always the underlying cause of myocardial infarction. Many lines of evidence, epidemiologic, pathologic, and clinical, lead to the view, particularly in the case of coronary artery disease, that the serum lipoproteins play a pivotal role in both the initiation and development of the atherosclerotic lesion. The principal objective of this section, therefore, will be to examine the evidence relating serum lipids, lipoproteins, and apoproteins to the development of coronary artery disease.

Serum cholesterol, low density lipoproteins, and premature coronary artery disease: Windaus (2) showed that cholesterol is a major component of atherosclerotic lesions in diseased arteries, and since then, in a variety of animal models (3,4) it has been possible to show that atherosclerotic lesions appear rapidly after the experimental induction of hypercholesterolemia. There is also a large, now classical, body of knowledge in humans relating levels of serum cholesterol and lipoprotein cholesterol to the risk of coronary artery disease (5-14). Amongst these, the

best known is the Framingham Study which demonstrated, at least in men under 50, a clear relationship between serum cholesterol and the risk of coronary artery disease (7). This initial formulation was however restated with the recognition that serum lipids were carried in different lipoproteins which themselves had different relation to the chance of disease. This advance became practical only after the advent of analytic and preparative ultracentrifugation. The individual most responsible for this change in thinking, Gofman (8), proposed an atherogenic index based on the measurement of different lipoprotein fractions and he and his colleagues concluded that the Sf 0-20 lipoproteins were a particularly powerful predictor of risk in subjects under the age of 50. This initial observation was subsequently confirmed by Kannell and his colleagues (9) who showed, within the Framingham Study, a clear relationship between risk and the level of low density lipoprotein cholesterol. Because low density lipoprotein (LDL) is the major carrier of cholesterol in plasma, the metabolism of this lipoprotein became a principal focus for the investigation of atherosclerosis.

The primary importance of low density lipoprotein as a cause of atherosclerosis is seen most clearly in familial hypercholesterolemia (FH). In its homozygotic form, the LDL cholesterol level in this disorder averages about five times higher than the normal population with those so affected dying of either coronary disease or aortic stenosis -- the latter due to lipid infiltration within the aortic valve leaflets. Unfortunately, in homozygotes, death almost always occurs before the second

decade (10). Most important for the argument that LDL cholesterol levels are linked to the risk of disease has been the recent report of the LRC-Coronary Primary Prevention Trial (14). This study of hypercholesterolemic males showed that reducing total and LDL cholesterol level with cholestyramine treatment significantly diminished the frequency of coronary artery disease morbidity and mortality. Thus not only has it been shown in the experimental animal that hypercholesterolemia can produce atherosclerosis, it has now been demonstrated in a study of one large human population at risk that reduction of cholesterol level diminishes cardiovascular risk. It should also be noted that in humans regression of coronary atherosclerosis documented angiographically has been shown in a small group of patients by medical therapy (15), by ileal bypass surgery (16), and by long-term plasma exchange (17,18). In animal models there is also mounting evidence that atherosclerosis can regress in the face of various interventions, such as diet (19,20), cholestyramine therapy (21), and ileal bypass surgery (22) -- all aimed at reducing LDL cholesterol level.

There can be no doubt then that LDL cholesterol level is correlated with the risk of coronary disease. However, despite all this evidence, one can not ignore the fact that most patients with premature coronary artery disease have normal, not elevated, LDL cholesterol levels. But LDL is a complex macromolecule made up of several lipids, free cholesterol, cholesteryl ester, triglyceride, phospholipids, and virtually a single protein -- apolipoprotein B (apoB). LDL can differ in composition in different situations and this heterogeneity in makeup appears to have an

important relation to atherosclerosis. Thus in our study of patients with premature coronary artery disease, a substantial proportion had normal levels of LDL cholesterol but increased levels of LDL apoB -- a combination we called HyperapoB. Further data confirm that study of LDL heterogeneity is essential to an understand of the role of LDL in atherosclerosis (48-50).

LDL heterogeneity related to coronary artery disease has been described by several investigators. Gofman (52) showed that a larger, more buoyant LDL with a flotation rate between Sf 12-20 was found in many patients with coronary artery disease. Rudel et al. (4) also showed a population of larger, cholesteryl ester enriched LDL particles in cholesterol-fed monkeys, while St. Clair et al. (54) demonstrated cholesteryl ester accumulation in cultured cells incubated with these LDL particles. Patsch et al. (53) showed LDL from patients with FH were larger and contained more cholesteryl esters and less triglyceride than LDL isolated from normal subjects from the same kindred. We have shown that patients with coronary artery disease frequently have a cholesteryl ester-poor and relatively protein-enriched subfraction of LDL (48). The same observation has been reported in familial combined hyperlipidemia (49) -- a monogenic disorder often associated with premature coronary artery disease (36,37).

HDL cholesterol and coronary artery disease: Only recently has considerable attention been paid to high density lipoproteins (HDL). Before this, they were considered relatively unimportant since only about a fifth of the plasma cholesterol was contained in this fraction. Certainly, the first report of

lowered HDL concentration and atherosclerosis -- a report which appeared in 1951 (23) -- stimulated little interest. By now, however, many epidemiological studies have demonstrated a strong association between lowered levels of HDL cholesterol and the risk of coronary artery disease (24-27). Indeed, when the Framingham population was reexamined, it was found that HDL cholesterol levels had greater predictive value than the measurement of either LDL or total serum cholesterol (25). It should be noted, however, that the survey was done 20 years after the beginning of the study, the results applying therefore to an older population. The interest in HDL cholesterol levels certainly intensified after the presentation of the HDL hypothesis (28,29). In brief, this hypothesis states that HDL clears cholesterol from extrahepatic tissue and is the most important factor in determining the efficiency of reverse cholesterol transport. That is to say, HDL can remove cholesterol from peripheral tissues and transport it to the liver, the only tissue in the body with the capacity to catabolize and excrete cholesterol. Indeed, there are experimental data showing that HDL may inhibit cholesterol accumulation in arterial wall and thus possibly prevent the formation of atnerosclerotic lesions (28-30). In vitro studies have also confirmed that HDL is capable of transporting cholesterol back to the liver (31,32). Therefore, it is widely believed that a low HDL concentration might well be associated with impaired clearance of cholesterol from the arterial intima and HDL particles play an antiatherogenic role.

Serum triglycerides and premature coronary artery disease:

It has long been known that patients with coronary artery disease, on average, have clearly higher levels of plasma triglyceride than do normals. By the same token, however, there remains considerable controversy as to whether plasma triglyceride levels are, in fact, an independent risk factor for coronary artery disease (33). The controversy exists, at least in humans, because there have been conflicting results reported, with most epidemiologic studies failing to show an independent association of coronary artery disease risk and plasma triglycerides. Since in the fasting state most of the plasma triglycerides are present in very low density lipoproteins (VLDL), the relationship of VLDL with coronary artery disease was then explored. Several studies have indeed found a direct correlation of VLDL level with risk, if univariate analysis is employed, but not if multivariate analysis is used (9,34,35). This suggests that the correlation of VLDL level with risk may derive from a secondary association and triglycerides or VLDL themselves may not be atherogenic. For example, a recent report from the Framingham study showed, in both men and women, that triglyceride level has little impact on risk in people who have average or high levels of HDL and an increase in coronary artery disease is seen only when the HDL cholesterol level is below 40 mg/dl (13). However, hypertriglyceridemic patients with familial combined hyperlipidemia have shown higher risk of coronary artery disease (36,37). We have also demonstrated that hypertriglyceridemia with hyperapobetalipoproteinemia is often associated with coronary disease (51). Beyond this, though, one should note that there are no animal

models of pure hypertriglyceridemia producing atherosclerosis.

Remnant particles and premature coronary artery disease: In addition to the role that VLDL, LDL, and HDL play in the pathogenesis of atherosclerosis, one must also consider whether remnant lipoprotein particles are independent and important determinants of risk. Of course, formation of a remnant particle is a normal event in the metabolism of triglyceride rich lipoproteins. For example, chylomicrons transport dietary triglyceride to various cells through the body. After lipolysis of the triglyceride in chylomicrons, remnant particles are formed which are enriched in cholesterol and rapidly cleared by the liver. Zilversmit has suggested if hepatic uptake of chylomicron remnants were saturated, they might subsequently be internalized by arterial smooth muscle cells and by this sequence chylomicron remnant particles might be atherogenic (39).

There is certainly at least one situation in humans in which remnant particles are known to be present in large quantities and to be associated with premature atherosclerosis. This clinical disorder has been called familial dysbetalipoproteinemia or type III hyperlipoproteinemia. The particles present in this syndrome have been named beta-VLDL and are generally thought to represent exaggerated forms of the remnant particles normally created during the catabolism of the triglyceride rich lipoprotein (40,41). In vitro studies make it evident that such particles can lead to the accumulation of cholesteryl ester in macrophages, confirming the atherogenic potential of these particles (42,43).

Apoprotein levels and premature coronary artery disease:

The relation of plasma apoprotein levels to coronary artery disease has only recently been noted, but promises to shed considerable light on the risk and pathogenesis of coronary artery disease. In 1963, Cramer (55) suggested that patients who suffer a myocardial infarction had higher LDL protein levels than normal controls even though there was little difference in their cholesterol levels. Later, Lees (56) pointed out that some patients with type IV hyperlipoproteinemia, whose LDL cholesterol concentration is by definition normal, have elevated LDL protein (apoB) levels. Avogaro et al. (57,58) measured total cholesterol, triglyceride, apoB and apolipoprotein AI (apo AI) levels in controls and post-infarction patients and, in normolipidemic post-infarction patients, found that the total apoB levels were higher and apo AI level lower in comparison with controls. Therefore, altered apoprotein levels, either elevated apoB, or decreased apo AI level, seem to be indicative of disease in normolipidemic patients. Since then, other studies which differ in methodology have examined these or similar parameters in various groups of patients. For example, in post-infarction patients, Vergani et al. (59) observed lower apo AI levels but no difference in apoB levels in those with coronary disease, compared to controls. Fager et al. (60) observed much the same except for a lower value of apolipoprotein AII (apo AII), rather than apo AI, in disease. On the other hand, DeBacker et al. (61) found higher apoB and lower apo AI and HDL cholesterol levels in post-infarction patients when compared to controls matched by age and body mass. Onitiri et al. (62) measured the levels of VLDL apoB and LDL apoB

and found both were higher in myocardial infarction survivors. Sniderman et al. (51) demonstrated that many post-infarction patients had elevated levels of plasma LDL apoB.

Patients with angiographically-documented coronary artery disease have also been studied. Sniderman et al. (47) found that a group of these patients had normal or near normal levels of LDL cholesterol, but elevated plasma LDL apoB levels -- a condition we named hyperapobetalipoproteinemia (HyperapoB). Similar findings have been demonstrated by some workers (63-71), although negative findings have also been reported (72-74). Wayne et al. (63) measured total apoB levels in patients with angiographically documented coronary artery disease and when hypercholesterolemia was excluded, apoB level was the best discriminator between coronary artery disease and controls. Kaldetzky et al. (64) also reported elevated levels of apoB and lower apo AI in coronary angiography patients. However, the apoB level was the best parameter to separate controls and coronary artery disease patients. Reisen et al. (65) reported that the levels of apo AI and apo AII were lower and apoB were elevated in coronary disease patients and Fruchart et al. (66) have also reported elevated apoB levels in coronary artery patients. Recently, Van der Heiden et al. (67) found a significant relationship between the extent of coronary artery occlusion and the level of apoB. Kukita et al. (68) in their study of patients with coronary artery disease and their relatives showed that both groups have elevated levels of apoB and serum triglycerides with lower levels of serum HDL cholesterol and apo AI. They (69) then demonstrated that both apo AI

and apoB had significant discriminative power between coronary artery disease patients and controls which was independent of the serum triglyceride level. Crouse et al. (70) have reported that the levels of LDL apoB correlated better with coronary disease than did LDL cholesterol. They (71) then found that LDL molecular weight was lower in patients with coronary disease than that of controls, that is to say, LDL in these patients had a higher hydrated density. This agrees with our results from HyperapoB patients, who have denser and smaller LDL particles (48). Vega et al. (72) used a colorimetric method to measure LDL apoB in normolipidemic patients with documented coronary artery disease. Their data suggest that the level of LDL apoB is not a better indicator of risk in normolipidemic coronary artery disease patients, but could be a predictor of risk in hypertriglyceridemic coronary artery disease patients. Schmidt et al. (73) studied angiographically-defined coronary atherosclerosis patients and concluded, after multivariate logistic regression analysis, that the ratio of HDL cholesterol to plasma cholesterol may be a superior predictor of coronary artery disease than other parameters such as LDL cholesterol and LDL apoB levels. Lehtonen et al. (74) studied 83 patients from Finland with three-vessel coronary artery disease and indicated that lower levels of HDL cholesterol and apo AI were the best discriminator between disease and control groups. Curiously, the apoB level of coronary artery disease patients was actually lower on average than controls.

These controversies in reports of apoprotein levels in patients with coronary artery disease may relate either to difference in patient populations or differences in methodology. The

latter probably is the main problem. Patton et al. (75) used four monoclonal antibodies to human plasma LDL to study apoB levels in patients with angiographically documented coronary artery disease. With a polyclonal antibody they confirmed that plasma apoB levels were significantly increased in patients with coronary artery disease. With the monoclone LP-22, this difference was even larger, with less overlap of apoB concentrations in patients with or without disease. They then concluded that perhaps monoclonal antibodies will be useful in identifying the various determinants of apoB, and offer greater predictive value.

Terminology of Hyperlipoproteinemias: The emphasis on lipoproteins in the pathogenesis of coronary artery disease led to a classification developed at the National Institutes of Health (44) and since widely applied. The hyperlipoproteinemias were divided into five types:

Type I hyperlipoproteinemia - increased chylomicrons

Type IIa hyperlipoproteinemia - increased LDL

Type IIb hyperlipoproteinemia - increased VLDL and LDL

Type III hyperlipoproteinemia - increased VLDL of abnormal composition and electrophoretic mobility

Type IV hyperlipoproteinemia - inc eased VLDL

Type V hyperlipoproteinemia - increased chylomicrons and VLDL

It must be noted however that these phenotypes do not correspond to genotypes. That is to say, a phenotype can be produced by more than one mechanism and not all of these mechanisms are genetic. A genotypic correspondence to the phenotypic classification

(45,46) is shown below:

Type I	Familial lipoprotein lipase deficiency
Type IIa	Familial hypercholesterolemia Familial combined hyperlipidemia
Type IIb	Familial combined hyperlipidemia
Type III	Familial dysbetalipoproteinemia
Type IV	Familial hypertriglyceridemia (mild form) Familial combined hyperlipidemia
Type V	Familial hypertriglyceridemia

LIPOPROTEINS AND LIPOPROTEIN COMPOSITION

In order to understand the physiologic role of lipoproteins, I will first review their composition and then discuss their functions as transporters of lipids within the body. Lipoproteins are made up of apoproteins -- apo AI, AII, AIV, B-48, B-100, CI, CII, CIII, E, and (a) -- and lipids -- free and esterified cholesterol, phospholipids, and triglycerides. These apoproteins, combined with lipids, form spherical particles which circulate within the plasma compartments. Carbohydrates found in glycosphingolipids and/or glycoproteins are present in small amounts but their physiological significance is not yet well-defined.

The hydrophilic surface of lipoproteins is composed of apoproteins, free cholesterol, and phospholipids whereas the hydrophobic core consists mainly of triglycerides and cholesteryl ester. Apoproteins obviously have vital functions as determinants of lipoprotein structure, cholesterol metabolism, and atherogene-

sis. The surface location of apoproteins is critical to their function, particularly with regard to modulating the catabolism of lipoprotein particles.

The density of lipoproteins in plasma is a function of their relative content of protein and lipid, while their electrophoretic mobility depends on the net charge of the apoproteins. The plasma lipoproteins can be divided into several general classes. Chylomicrons and VLDL are the major triglyceride-carrying lipoproteins. LDL, the major cholesterol-carrying lipoprotein in plasma, is a product of VLDL metabolism while HDL are relatively protein-rich and lipid-poor particles which may be important in preventing cholesterol deposition or in promoting the efflux of cholesterol from the extrahepatic tissues to the liver, so-called reverse cholesterol transport (28). In one sense, HDL may be viewed as modified products of redundant surface lipids and proteins generated during the process of triglyceride transport. They are first secreted as 'nascent' or discoidal structures from hepatic and intestinal cells (76,77) and afterwards modified. The origin of HDL is certainly considerably more complicated than that of the other lipoproteins. Finally, there is a minor lipoprotein class, Lp(a) lipoprotein with a hydrated density of 1.050-1.12 g/ml and slow pre-beta electrophorectic mobility on agarose gel electrophoresis and which contains about 30% protein by weight (78).

Presently, there are 14 known apoproteins; the characteristics of only the main apoprotein constitutent of plasma lipoproteins will be discussed. Apo AI and Apo AII are the major

proteins of HDL and have been reported to enhance the enzyme activity of lecithin: cholesterol acyltransferase (LCAT) and hepatic lipase (HL), respectively (79,80). Apo AI and Apo AII are also found in lymph chylomicrons, as is apo AIV which has been shown to be a potent activator of LCAT in vitro (81). Apo AIV also exists in lipoprotein-free fraction of plasma. ApoB is a structural protein of chylomicrons, VLDL, LDL, and Lp(a). Apo B-100 accounts for over 95% of LDL protein mass, and approximately 35% of VLDL protein mass (82). Apo B-100 plays a crucial role in the binding of LDL to cell-surface receptors (83). Apo B-48 is characteristic of and a major protein component of lymph chylomicrons and its remnant (84). Its apparent molecular weight on 3.5% polyacrylamide gel is approximately 48% of that of apo B-100 (82,84). The C apolipoproteins are protein constituents of lymph chylomicrons, VLDL, and HDL. Apo CI enhances the enzyme activity of LCAT (85); apo CII activates lipoprotein lipase (86), while apo CIII has been shown to inhibit lipoprotein lipase as well as decrease chylomicron remnant uptake (87). ApoE is a protein constituent of chylomicrons, VLDL, and HDL and is important in hepatic receptor-mediated uptake of chylomicron remnants and also interacts with apoB/E receptor (88). ApoE consists of several isoforms, as determined by isoelectric focusing at pH 4-6 (89). This genetic heterogeneity has significant metabolic consequences: Apo E3 has normal binding to liver receptors, while apo E2 binding is defective (90). Apo Lp(a) is the major protein constituent of Lp(a) (78).

LIPOPROTEINS AS LIPID TRANSPORTERS

The physiological role of lipoprotein is to transport lipids among tissues. In this section, two of these lipid transport systems, the triglyceride and fatty acid transport system and the cholesterol transport system, will be briefly reviewed.

Dietary lipids represent a major portion of the total daily calories of humans and animals and play an important role in the nutritional and physiological process of the body. The normal dietary intake of fat of the adult in the western hemisphere varies from 60 to 100 g/day. Most of this is ingested as triglycerides, with the remainder comprised of phospholipids and cholesteryl esters. In response to fat absorption in the intestine, the resynthesized triglycerides, phospholipids, and cholesteryl esters are combined with free cholesterol and small amounts of apoprotein, apo B-48, apo AI and apo AIV, to form chylomicrons. Chylomicrons are secreted from the absorptive cells into the lymphatics and subsequently enter the plasma via the thoracic duct. Therefore, both dietary triglyceride and cholesterol are transported by this system.

Triglyceride and fatty acid transport system: The triglyceride and fatty acid transport system delivers energy as required by the body. These appear in the blood in two forms: triglyceride as carried by lipoproteins particles, and free fatty acids (FFA) which under physiological conditions are carried by albumin.

Triglyceride: The two major carriers of triglyceride are chylomicrons and VLDL. The actual size of chylomicron is largely

determined by the transport rate of the triglyceride through the intestinal cells (91). There is conflicting evidence as to whether two classes of triglyceride-rich lipoprotein are present in mesenteric lymph. During fasting VLDL-size triglyceride particles are in the lymph. However, their apoproteins are quite different from plasma VLDL and resemble instead lymph chylomicrons; these particles are thus usually regarded as small chylomicrons (92). During higher rates of lipid absorption, particle size increases to accomodate the increased lipid flux across the intestinal mucosa, and large chylomicrons become the predominant particles in the lymph (92).

Active synthesis of intestinal apoprotein is necessary for normal chylomicron formation. Studies (93,94) suggest that apo AIV and apoB are the two principal apoproteins that are structurally required for the formation of chylomicrons. Upon entering plasma, chylomicrons acquire apoE (92,95,96) and apoC (97) from the HDL particles. Apo CII activates the lipoprotein lipase (LPL), the core triglyceride is then hydrolyzed, and this results in the formation of partial glycerides, di- or monoglycerides, fatty acids, and glycerol. These products can be taken up by the tissues (98) and in adipose tissue they can be reesterified to triglyceride for storage. From adipose tissue free fatty acids may be released back to the blood where they bind with albumin and subsequently are taken up by the liver or other tissues (99). After hydrolysis, the compositions of chylomicrons are markedly altered both as to lipids and apoproteins. Some of the surface phospholipids, together with most of the apoproteins C, AI, and AIV, are transferred to HDL. The remnant

particles are then removed by the liver through apoE receptors (95,100). The half-life of chylomicrons is five minutes in humans (101,102) and of chylomicron remnant, about 30 minutes (103). Thus the function of chylomicrons is to move triglycerides from the intestine either to adipose tissue where energy can be stored or to supply fatty acids to other tissues such as muscle, where fatty acids can be used immediately by the cells.

The second major source of plasma triglyceride is VLDL which is synthesized endogenously -- that is, not arising directly from dietary triglyceride. Many tissues are able to esterify fatty acids to form triglyceride, but only the liver secretes them in the form of VLDL into the blood in significant amounts. The fatty acids esterified in the liver come from two sources: first, fatty acids can be synthesized de novo from acetyl-CoA, which is derived mainly from lactate, alanine or glucose; second, fatty acids can be taken up into the hepatocytes extrcellularly, either from the hydrolysis of chylomicron triglyceride or as fatty acids from hydrolysis of adipose tissue triglyceride. VLDL are then defined as the particles which transport triglyceride from the liver to peripheral tissues. This pathway thus maintains lipid homeostasis in the liver. Compared to the flux in chylomicron formation and secretion associated with absorption of dietary fat, the hepatic VLDL formation rate is relatively constant, providing a reasonably continuous source of triglyceride for plasma. However, hepatic triglyceride synthesis and secretion of VLDL by the liver is affected by genetic, dietary, and hormonal factors. It is of considerable interest to

determine whether they affect the number of VLDL particles secreted, or the size and lipid content of these secreted particles. At present, it is believed that nascent VLDL particles, regardless of size, contain a constant mass of apoB-100 (149). Nascent VLDL contains less apoC and relatively more phospholipids than plasma VLDL, which acquires apoC and free cholesterol from HDL after secretion (105). It has been shown *in vitro* that the surface components of VLDL, apoC, free cholesterol, and phospholipids, are removed from the lipoprotein concommitantly with the hydrolysis of triglycerides (106). The acceptor of the surface components is HDL (106-108), although Eisenberg and Olivecrona (109) suggest that this process could be independent of the presence of an acceptor lipoprotein and may take the form of a surface fragment particle.

In humans, more than 50% of the VLDL will be converted to LDL (40,231), whereas in rats and guinea pigs only 5 and 15%, respectively, are so converted, the remainder being rapidly taken up and catabolized by hepatocytes (111). It has been suggested that the uptake of remnant particles into the liver is greatly facilitated by apoE, and inhibited by apoC (112). Although the remnant uptake concept has been applied to the metabolism of VLDL and chylomicrons, the receptor that mediates hepatic uptake is thought to be different. In Watanabe heritable hyperlipidemic (WHHL) rabbits, which lack hepatic LDL receptors, VLDL remnant-like particles accumulate in the blood, whereas chylomicron remnants do not (113). Therefore, these two triglyceride transport lipoprotein particles have the same function, yet their catabolic fates may be different.

Fatty acids: Most of the long-chain fatty acids present in plasma are contained in glycerides, phospholipids, and cholesteryl esters and are transported via lipoproteins. In addition, a small amount of plasma fatty acid exists in unesterified or free form. Under physiological conditions, they are bound by albumin. Two sources of plasma free fatty acids are known; one is from lipolysis of chylomicrons and VLDL, the other, by far the main source of plasma free fatty acids, is triglyceride stored in the adipose tissue. An enzyme, hormone-sensitive lipase (116) hydrolyzes triglyceride into free fatty acids, the mobilization of which from the adipocytes is regulated by dietary, hormonal, and nervous stimuli (114). Free fatty acids that are taken up by cells can be used for esterification or for tissue oxidation to generate energy.

Free fatty acids are of great importance in supplying energy to tissues, because of their rapid turnover rate. Indeed, the half-life of plasma free fatty acids in humans is only one to two minutes (115). Studies in normolipidemic subjects (99,117) showed that the output of triglyceride in VLDL was dependent upon the uptake of free fatty acids in the splanchnic region. Howard et al. (118) further demonstrated in an obese population with low plasma lipids that VLDL triglyceride synthesis was not significantly related to fasting free fatty acids levels, but was significantly correlated with postprandial free fatty acids. Havel et al. (99) pointed out that the uptake of free fatty acids in the splanchnic region in a hyperlipidemic group is greater than in a normolipidemic group. In all cases, studies have sug-

gested that increased transport rate of free fatty acids to the liver can increase VLDL production (99,117-121).

Therefore, recycling of fatty acids is present. That is, a series of fatty acid-carrying particles exists -- chylomicrons, albumin, and VLDL -- which can transport fatty acids from the intestine or liver to tissues such as cardiac or skeletal muscle, where they are used, or to adipose tissue where they are stored and from which they can be hydrolyzed to be used again. There is thus a continuous flow or shuttle of fatty acids within the circulation.

Cholesterol transport system: Cholesterol is present in the diet; if necessary, however, quantities sufficient for normal requirements can be synthesized in the liver, intestine, and other tissues. Cholesterol is an essential structural component of cell membranes, and also the precursor of steroid hormones and bile acids. Plasma lipoproteins play an important role in the transport of cholesterol between sites of absorption, synthesis, catabolism, and excretion.

As described above, dietary cholesterol is transported by chylomicrons. After chylomicron are hydrolyzed by lipoprotein lipase in the capillary bed, part of the surface components, primarily apoC, phospholipids, and free cholesterol, are transferred to HDL (93,95,122). The remnant particle retains its cholesteryl ester, apoB and apoE to be catabolized in the liver (87,88). It has been suggested that during the hydrolysis of chylomicrons in capillaries of adipose tissues, cholesterol can cross the capillary wall by lateral diffusion and contribute to

membrane proliferation of fat cells during storage of triglycerides (124). Cholesteryl ester, in contrast, is not taken up by extrahepatic tissues. This pathway of chylomicron metabolism is quite efficient. Thus, the plasma level of cholesterol rises very little, if at all, after a single high cholesterol meal. On the contrary, in patients with familial dysbetalipoproteinemia (41,123), the remnant particles remain in plasma and become further enriched in cholesteryl ester and these particles can be taken up by macrophages (38,43,272), perhaps causing accumulation of cholesterol in the arterial wall.

The liver, which rapidly takes up dietary cholesterol in the form of chylomicron remnants, disposes of some of the sterol in the bile, either as unesterified cholesterol or as bile acids. Much of the cholesterol and bile acid secreted by the liver is reabsorbed in the intestine and again delivered to the liver for secretion, thus forming an enterohepatic circulation (125). Therapy to lower the plasma cholesterol level by interruption of this enterohepatic circulation of bile acids has been achieved by drugs (126) and surgery (127), so that during each cycle a portion of the cholesterol and bile acid is lost in the feces. With the typical high-cholesterol western diet, about 1100 mg of sterol is lost from the body each day. In the steady state, about 850 mg of this sterol is derived from endogenously synthesized cholesterol and approximately 250 mg from dietary cholesterol (128,173).

When VLDL is secreted from the liver, cholesterol will be delivered to extrahepatic tissue. However, since the composition of nascent VLDL is unknown, the amount of cholesterol that leaves

the liver by this route is also unknown. The liver probably uses dietary cholesterol as the source for this lipoprotein synthesis when it is available; otherwise, the liver synthesizes its own cholesterol by increasing the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (129). In rats (130), the activity of liver microsomal enzyme acyl CoA:cholesterol acyltranferase (ACAT) activity is high and nascent VLDL contains cholesteryl ester, whereas in humans, ACAT activity in the liver is low (130). Therefore, in humans, it has been suggested that most of the cholesteryl ester in VLDL is synthesized within the plasma through the action of LCAT (131), or cholesteryl esters are tranferred from HDL by the plasma cholesteryl ester exchange protein (132,133) and/or, as we have suggested, via an exchange mechanism of triglyceride and cholesteryl ester between nascent VLDL and LDL in the splanchnic bed (134). In the vascular space, VLDL particles are hydrolyzed in capillaries via the action of lipoprotein lipase. As the size of the VLDL particles diminishes, the particles are converted to intermediate density lipoprotein (IDL) and then LDL. Kinetic apoB studies have shown that in normal subjects, 70 to 50% of VLDL is converted to LDL (40,231). Studies with fibroblasts have shown that VLDL from hypertriglyceridemic patients, but not normolipemic VLDL, are capable of interacting with the apoB/E receptor and regulating cholesterol synthesis (135,136). However, a study with the HepG2 cell line indicated that VLDL can be removed by a receptor which is mediated independently of the LDL apoB/E receptor and that there is no substantial regulation of cholesterol metabolism (137).

In normal subjects, the cholesterol in LDL constitutes about two-thirds of the total plasma cholesterol. The LDL particles are removed from the plasma with a fractional catabolic rate of about .45 of the plasma pool per day (138). LDL can then transport cholesterol to extrahepatic cells where, via apoB/E high affinity receptor-mediated endocytosis, the cholesterol can be used for cellular reactions (83). This uptake mechanism also regulates intracellular cholesterol metabolism by turning off the activity of HMG-CoA reductase and decreasing the synthetic rate of apoB/E receptors (83). LDL can also be degraded by macrophages of the reticuloendothelial system. The exact uptake mechanism is still unclear, except that the macrophage has a receptor for modified LDL (139). Macrophages can store and excrete cholesterol, and in FH homozygotes who lack the apoB/E receptor, much of the LDL might be cleared through this system. When macrophages are overloaded with cholesteryl esters, they are converted into "foam cells" which may be components of atherosclerotic plaques (140). Finally, most of the LDL is catabolized by the liver (141,142) and therefore cholesterol is transported back to the liver. The hepatic LDL recognition in humans is probably quite complex since there appears to be a recognition site in addition to the apo B/E receptor which is probably regulated differently from the classic apo B/E receptor (143-145).

In steady state, tissues excrete cholesterol into the plasma in amounts equal to that taken up from LDL. Such excretion results from cell death, as well as membrane turnover in living cells. Free cholesterol leaving the cell is believed to be ab-

LCAT, after which the cholesteryl esters are tranferred to VLDL and LDL; alternatively, HDL is taken up by the liver (148). This is the reverse cholesterol transport system. Therefore, the body cells acquire cholesterol from the catabolism of lipoproteins and then return the cholesterol to other lipoprotein particles. Studies with animals have demonstrated that the cholesteryl ester in HDL is preferentially taken up by the liver and adrenals, probably through a specific, saturable receptor (32,148). Therefore, an amount of cholesterol equal to that which leaves the liver in the form of lipoproteins must return to the liver each day. The cholesterol in plasma is continually turning over owing to entry of cholesterol into the circulation in association with plasma lipoproteins, the removal of cholesterol by intracellular degradation of lipoproteins, and possibly by a shuttle system in which lipoproteins release part of their cholesterol load to the liver and then return it to the circulation. In addition to the net flux of cholesterol through the plasma, there is a flux of free and esterified cholesterol between the different lipoproteins with a net transformation of free into esterified cholesterol. Turnover within the plasma involves simple molecule-for-molecule exchange, together with bulk transport involving carrier proteins which solubilize esterified cholesterol.

HETEROGENEITY OF VLDL AND LDL

VLDL: VLDL of human serum are highly polydisperse with respect to particle diameter, hydrated density, and flotation rate. Their size is dependent on triglyceride synthesis by the

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are removed rapidly from the blood, and only a small fraction are converted to IDL and eventually to LDL. By contrast, most particles containing VLDL B only are converted to LDL, rather than removed directly. They hypothesized that the presence of apoE is a major determinant of the metabolic fate of VLDL particles. This is a very intriguing hypothesis and may be supported by *in vitro* studies by Gianturco et al. (136) and Krul et al. (159), who pointed out that apoE is the preferred recognition site for cellular binding of large VLDL particles, characteristic of hypertriglyceridemia.

LDL: Despite evidence to the contrary (160-169), LDL has usually been considered as a homogeneous entity, both clinically and experimentally. Adams and Schumaker (160), using a buoyant density gradient, showed that the LDL is made up of two or three discrete components. Hammond and Fisher (162) showed four components in the LDL by density gradient ultracentrifugation. Their compositional studies indicated an increasing lipid content with increasing molecular size. Fisher et al. (162,169) then used analytical ultracentrifugation to show that, among individuals, LDL can be either monodisperse or polydisperse; subjects with hypertriglyceridemia generally had polydisperse LDL. Krauss and Burke (168) then examined LDL heterogeneity by gradient gel electrophoresis, a technique which separates particles by diameter, in a polyacrylamide matrix of decreasing pore size. They demonstrated heterogeneity of LDL in plasma and in isolated LDL fractions. Shen et al. (166) also used density-gradient ultracentrifugation to show marked diversity of LDL in normolipidemic

subjects, both in terms of size and composition, the larger particles being enriched in cholesteryl ester, compared to the smaller protein-enriched particles. They then suggested that these particles may have different metabolic pathways and that the most buoyant LDL fraction, as well as a smaller and denser LDL subfraction, may be more prevalent in some patients with coronary artery disease (49,166,168). We (48) have used equilibrium density gradient ultracentrifugation and found that patient with hyperapobetalipoproteinemia have a cholesteryl ester-poor and relatively protein-enriched LDL subfraction. This subfraction is smaller in size and has a higher average hydrated density. These findings tend to be even more marked in hypertriglyceridemia (48). We (48) and others (170-172) have also studied LDL composition and heterogeneity in patients with familial hypercholesterolemia. Patsch at al. (172) showed that LDL in patients with familial hypercholesterolemia were larger and contained more cholesteryl ester and less triglycerides than the LDL of normal individuals. We (48) also have shown that patients with familial hypercholesterolemia are characterized by a subfraction of LDL which is physically larger, containing relatively more cholesteryl ester and free cholesterol and less protein. All these findings of LDL heterogeneity in composition and density are compatible with the spherical model for LDL, in which particle size decreases if the contents of core and surface lipids decrease, while the particle becomes denser due to relative protein enrichment. On the other hand, when the content of the core and surface lipids increase, the particle enlarges and becomes more buoyant. In cholesterol-fed monkeys, large

cholesteryl ester-enriched LDL particles are found in plasma, the size of which correlates positively with the severity of coronary artery atherosclerosis (4,20).

Changes in LDL composition in humans was clearly demonstrated in 1970 by Lees (56). He showed variations in the LDL cholesterol to protein ratios among normal, type I, type II, type IV, and type V hyperlipidemia and indicated that in many type IV patients, plasma and LDL cholsterol were normal, whereas the LDL protein was higher, therefore producing a lower LDL cholesterol to protein ratio than in normal subjects. Metabolic perturbation caused by drugs, diet, and weight-reduction could also alter the composition of lipoprotein. Wilson and Lees (174) documented changes in LDL cholesterol to protein ratios among three groups of patients who underwent weight reduction, carbohydrate induction, or clofibrate treatment. The changes in LDL composition in terms of cholesterol to protein ratio were inversely related to VLDL cholesterol levels. Witzum et al. (175) and others (176-178) have also observed profound changes in VLDL metabolism in subjects undergoing colestipol therapy. Within a few days of the onset of therapy, there appeared to be an increasing amount of larger VLDL particles, which were triglyceride enriched, together with a fall in LDL cholesterol level. This rise in VLDL levels, however, was transient and was followed later by a decrease in both VLDL and LDL cholesterol to protein ratios (175), which suggests a change in density and/or in size distribution of the apoB-containing lipoprotein. The metabolic consequence of these pertubations and the physicochemical base

for these observation has been elucidated by Sniderman et al., *in vivo* (134) and others, *in vitro* (180-182).

Diet effects on lipoprotein composition in humans appear to vary. A diet rich in polyunsaturated fat decreases plasma cholesterol and triglyceride levels, but the effects on lipoprotein composition are controversial. Some groups have noted a decrease in the LDL cholesterol to protein ratio (183,184), while others have not (185-187). The reason for this disagreement is unclear, but may be a function of the patient populations chosen for the studies. In non-human primates, the effect of dietary polyunsaturated and saturated fat on the development of atherosclerosis was measured after five years; the severity of atherosclerosis in the coronary arteries was significantly less in the polyunsaturated fat-fed animals, in whom LDL concentration and size had decreased (50).

Mechanisms of LDL heterogeneity: In normal human plasma, net transport of cholesterol from cells, cholesterol esterification by LCAT reactions, and the ability to exchange cholesteryl ester with triglycerice among lipoproteins, influence the composition of plasma lipoproteins. The polar lipids in lipoproteins, free cholesterol, and phopholipids turnover at a very fast rate. It has been shown, both in humans and in rats, that lipolysis of triglyceride-rich lipoprotein is apparently the major source of HDL phospholipids (100,188). Once phospholipid molecules are in HDL, they are either metabolized or exchanged with the same molecules in other lipoproteins or cell membranes. The dynamics of free cholesterol are similar to those of phospholipids. Free

cholesterol is consumed by the LCAT reaction and may be used by cells (189). The mechanism of this exchange process is likely to be via transient contact between two lipoprotein particles, or between a lipoprotein particle and a cell surface, during which exchange of polar lipids occurs by lateral diffusion (190). The core lipids, cholesteryl ester, and triglyceride, can either be exchanged or transferred from one lipoprotein to another. All these processes are facilitated via lipid transfer proteins. The original idea that cholesteryl esters of plasma are not exchangeable was based on the observation that isolated rat lipoproteins do not exchange their cholesteryl esters *in vitro* (191). But we now know that rat plasma has no exchange protein (192) and that this is also true in the guinea pig (193) and pig (194). In humans and rabbits (193), however, cholesteryl ester and triglyceride exchange and transfer among lipoproteins does occur.

Nicols and Smith (195) were the first to show that during 37°C incubation of human plasma, cholesteryl ester is transfered from HDL to LDL, in exchange for triglyceride. These observations suggested the presence of a carrier complex in whole plasma, by which esterified cholesterol is transferred from one lipoprotein fraction to another. Zilversmit et al. (196) then described a protein in d > 1.25 g/ml fraction of the cholesterol-fed rabbit plasma that facilitates the exchange of cholesteryl ester between VLDL and LDL. We (182) and others (197) then reported cholesteryl ester exchange between HDL and LDL mediated by a protein factor in the d > 1.25 g/ml fractions of human plasma. Chajek and Fielding (198) then reported an equimolar exchange of cholesteryl ester and triglyceride from HDL to VLDL and LDL but the transfer

activity was confined to the denser than 1.063 g/ml fraction. Barter et al. (199) then reported mass transfer, in humans, of esterified cholesterol from LDL to VLDL and a net mass transfer of triglyceride in the reverse direction from VLDL to LDL. This report also provided good evidence in support of the hypothesis that, in vivo, the pools of esterified cholesterol in LDL and VLDL are not in equilibrium. Hopkins and Barter (133) suggested the cholesteryl ester and triglyceride mass transfer may be an independent process -- that is, the transfer is carried out by different proteins. Incubation experiments with either rabbit or human VLDL and HDL particles have shown that the thiol-blocking agent, p-chloromercurphyenyl sulfonate, markedly reduced the rate of triglyceride transfer, while having little or no effect on the rate of transfer of cholesteryl ester (133). However, Morton and Zilversmit (200) further purified the protein factor and concluded that it is the same plasma protein with molecular weight of 58,300 to 66,400 daltons. This protein is now designated as lipid transfer protein and is characterized as a hydrophobic glycoprotein with an isoelectric point of 5.2 (197). The existence of transfer proteins that mediate bidirectional transfers of esterified cholesterol and triglyceride between plasma lipoprotein fractions provide the potential for an equilibrium of each among all lipoproteins. In vivo, in humans, Nestel (132) demonstrated that labelled esterified cholesterol transfers rapidly from HDL to LDL, thus creating a potential pathway for the disposal of HDL cholesterol. We (134) showed that, in humans, the cholesteryl ester in LDL is decreased, triglyceride is in-

creased, and VLDL cholesterol is increased reciprocally across the splanchnic bed. Therefore, at least in humans, the cholesteryl ester exchange process and the cholesteryl ester and triglyceride transfer process among lipoproteins are active and the most probable site at which they are most active is the splanchnic bed.

Apart from the question about the physiological role of the cholesteryl ester and triglyceride exchange system, one may ask how a relatively large protein can interact with lipoproteins, so as to transfer lipids among them. According to current concepts, the nonpolar lipids are present in the hydrophobic cores of the lipoproteins. The exchange reaction raises the possibility that the exchange protein can penetrate into this core or that at least some of these nonpolar lipids are present close to the lipoprotein surface. A composite model of the arrangement of lipid and protein in porcine LDL seems to support the latter (201).

We have suggested that cholesteryl ester-triglyceride exchange is the basic mechanism which is responsible for the generation of smaller, cholesteryl ester-poor LDL particles. This mechanism has been demonstrated by Deckelbaum et al. (181), *in vitro*. Even though this working hypothesis for the mechanism of formation of small LDL seems reasonable, it is not certain that small LDL particles, per se, are atherogenic. However, the factors associated with the formation of small LDL, such as overproduction of VLDL apoB, may be responsible for the predisposition of some invididuals to premature atherosclerosis. In humans with elevated VLDL production, the cholesteryl ester for trigly-

ceride exchange process would be enhanced and smaller LDL would result. In addition, it is certainly possible that the increased transport rate of small LDL to the arterial wall might promote atherosclerosis.

In contrast with the small LDL in HyperapoB, patients with familial hypercholesterolemia have large cholesteryl ester-enriched LDL particles. These patients have normal production of VLDL particles but oversynthesize LDL and, most strikingly, have markedly decreased LDL catabolism (202,234). These patients must have abnormal exchange/transfer processes of cholesteryl ester and triglyceride; the prolonged residence time of LDL particles might result in the accumulation of cholesteryl ester in LDL particles. This oversynthesis of LDL might be caused by delayed clearance of IDL particles resulting in the accumulation of cholesteryl ester-enriched LDL particles (203,204,220). Cholesterol-fed monkeys have large cholesteryl ester-enriched LDL particles (4), yet the apoB-containing lipoproteins secreted by the liver in monkeys are actually triglyceride-rich lipoproteins within the LDL density range (205). This suggests that the lipolytic process might be slow and that abnormal LDL particles might accumulate more cholesteryl ester for exchange of triglyceride. In both cases, the increased concentration and residence time of LDL may result in enhanced deposition of these particles in the arterial wall.

Consequences of LDL heterogeneity: The changes in LDL composition cause the heterogeneity in LDL. This, in turn, might influence LDL apoB conformation and possibly influence the patho-

genesis of coronary atherosclerosis. Using a spherical model for the LDL particle, the spatial relationship of the phospholipids and free cholesterol to apoB antigenic determinants must change as the lipid to protein ratio changes. Studies with polyclonal and monoclonal antibodies (156,157) demonstrated that, after lipolysis, VLDL particles gradually decrease in size and VLDL immunuoreactivity increases. Delipidation of apoB results in loss of reactivity to epitopes exposed on intact LDL (206). However, binding reactivity of the monoclonal antibodies to the epitopes can be restored by association of apoB with lipid structure having a cholesteryl ester hydrophobic core, but not with cholesterol-phospholipid liposomes, suggesting that the cholesteryl ester core influences the conformation of apoB (206). Temperature changes should result in modification of lipoprotein conformation and fluidity; a study by Mao et al. (207) did show that LDL immunoreactivity varied as a function of temperature. Circular dichroism studies have also suggested a temperature-induced change in apoB conformation; however, it is not clear whether the change was related to core cholesteryl ester transitions, or surface lipid rearrangement (208-210). Furthermore, the heterogeneity of LDL particles might influence the interaction of LDL with cells or with the arterial matrix. Proteoglycans, the structural matrix of the arterial wall, have been extracted from arteries and tested for binding reactivity with LDL from different human subjects (211). The more-reactive LDL were enriched in cholesterol and were relatively depleted of triglycerides and protein. These data might support the observations of enhanced

binding of large, cholesteryl ester-enriched LDL particles, obtained from FH patients (211) or monkeys fed with a cholesterol-rich diet (54) with arterial smooth muscle cells. By contrast, in HyperapoB, small LDL particles, because of their size, might have an increased transport rate into the arterial wall and as a consequence of this, atherogenesis be accelerated.

APOB KINETICS

LDL formation is a dynamic process that occurs intravascularly and involves remodeling and delipidation of precursor VLDL and IDL. In this lipolytic cascade, apoB-100 is retained within the lipoprotein particles. Therefore, studying apoB kinetics gives us an understanding of VLDL, IDL, and LDL metabolism. In normal humans, an average of 50% of the VLDL is converted to IDL then to LDL, most of the rest is catabolized irreversibly except for a small amount which becomes a slow turnover pool, which is eventually removed from VLDL (40). Two-thirds of the LDL particles are metabolized through the LDL apoB/E receptors, the remaining one-third of the LDL is catabolized by the alternative receptor-independent pathway (212). However, the actual proportion differs among individuals and pathological conditions (40,212,213).

With the availability of radioactive tracers, we have been able to study complex biological systems. A number of isotope kinetic studies have been done previously to define the metabolism of apoB-containing lipoproteins in humans. Techniques for studying apoB metabolism include the use of labelled isolated

tracers or radioactive amino acids such as ^{75}Se-selenomethionine and ^{3}H-leucine as precursors of apoB.

The classic method of analyzing kinetic data by means of differential equations provides a limited interpretation of metabolic kinetics for a general, non-linear, time-dependent system (214,215). The advantage of this kind of computation is its simplicity. However, one cannot make any predictions beyond what the data provide. Some investigators have developed detailed models which overcome most of these limitations (40,253, 254,257,262,263). The greatest advantage of these lies in their potential to express all the information contained in the data, to propose novel hypotheses, and to design new experiments to validate the model further. In any case, as stated by Berman (257), the results obtained from both approaches should always agree. The common concern which does remain is the interpretation of kinetic data in terms of metabolic processes.

Turnover of VLDL apoB: Most studies of VLDL apoB turnover have used radioactive iodine to label VLDL apoprotein and, after injection, follow the decay of specific activity in the plasma to obtain an estimate of turnover rates. There are various methods to determine specific activity of VLDL apoB: PAGE-urea gels and sephadex gel filtration (40,216,217,253), sephadex G-150 gel filtration followed by protein determination (110,219,223,234), 10% TCA precipitation after lipid extraction (250), TMU precipitation of apoB and protein measurement (218,224,225,228,229, 232,236,246,248,262), RIA for apoB (244), and isopropanol precipitation of apoB and protein measurement (245). These

methodologies have generally paralleled the evolution of techniques and understanding of apoprotein chemistry, with all of the methods aiming to prepare a purified apoB fraction from VLDL particles.

Following injection of ^{125}I-labelled VLDL, the resulting decay curve for VLDL-apoB usually has two components. The first, which accounts for most of the decline in specific activity, is rapid, with a half-life in the range of 2-14 hours. The second and much smaller component conforms to a 'tail' on the curve, with a much longer half-life, similar to that of LDL apoB. The simplest approach to analyze VLDL-apoB kinetics is to determine the fractional catabolic rate (FCR) from the initial slope of the decay curve (single-exponential analysis). This method has been employed by several investigators and has continued to be used (110,218,231,233,244). In this method, the tail of the curve is not considered to contribute significantly to the FCR of VLDL-apoB. Another approach is to measure the FCR from the area under the decay curve by multiexponential curve-fitting techniques (Chapter 4). This method takes both components into consideration. Readon et al. (224,242) used a two-pool model which also takes into account the two components of the specific-activity decay curve. The fourth approach for estimating FCR of VLDL apoB has been to employ multicompartmental analysis. This method was introduced by Berman et al. (40). Their model contains two pathways: first, a pathway with stepwise hydrolysis of VLDL triglyceride is used. As triglycerides are removed, the particles get smaller and denser, finally ending in the IDL range. This process is a "cascade" and consists of a four-step delipidation chain

terminating in an IDL compartment. This pathway largely accounts for the shoulder of the VLDL apoB specific activity/time curve. The second processs arises from an incomplete expression of the first. In it, the particle remains in the VLDL range as a modified "lingering" particle. Such particles form a separate population and are eventually removed from VLDL, either by VLDL receptors, or by scavenger pathways, or else they are hydrolyzed very slowly until they drift into the IDL range. In Type III hyperlipidemia patients this slow turnover pool is very large (25-30% of total VLDL apoB mass) (40). Most patients with this disorder have absence of apoE-3 and apoE-4, possessing instead E2 -- a natural mutation of apoE-3 -- which results in delayed clearance. In vitro studies demonstrate that such lipoproteins can be directly taken up by macrophages (38,43,272). This, then, validates the prediction from kinetic modeling that beta-VLDL disappears directly from plasma. In normal subjects, this part only represents 5% of the total VLDL apoB mass. These are the normal remnant particles which will eventually enter into the IDL range or be catabolized by VLDL receptors, or the scavenger pathway. Eaton et al. (253) and Fisher et al. (252), using endogenous tracer, added a third pathway, a fast-turnover pool, in which VLDL particles are removed from plasma irreversibly, in a single step, with perhaps some reappearing in the LDL range. In rats, most of the VLDL is probably removed irreversibly from this pathway without re-entering the LDL range. A similar, but simplified, model has been employed by Le et al. (327). They simplified the model of a four-step delipidation chain with a rapidly-

catabolizing pool into one subcompartment, plus a slow-turnover pool. Recently, Beltz et al. (263) found that the number of compartments of the VLDL delipidation cascade can be varied among each subject studied. The FCR was dependent on the number of pools such that increasing the chain length decreased the FCR.

In several studies, the VLDL apoB was followed into other lipoprotein fractions to examine precursor-product relationship. Early reports suggested that, in most normal subjects, VLDL apoB was converted quantitatively to LDL (218,224). These results indicated that in normotriglyceridemic subjects, 90% of VLDL apoB mass is converted into LDL apoB (218,224) whereas in hypertriglyceridemic subjects, only one-third of the VLDL apoB was converted to LDL apoB (327). More recently, however, it has been reported that a significant fraction of VLDL apoB can be removed irreversibly. While some investigators have suggested that removal of VLDL apoB by this pathway occurs mainly in hypertriglyceridemic patients (110,222,224,229), other studies (231, 245,246) imply that this pathway can be significant, even in normal subjects. This phenomenon has been demonstrated with endogenous tracer studies (252,253), where preferential irreversible catabolism of VLDL, without prior conversion to IDL and LDL, was especially found in hypertriglyceridemia. This same pathway was also observed in animal studies (204,265). Removal of VLDL remnants appears to be mediated by LDL apoB/E receptors (273,274). However, VLDL from patients with hypertriglyceridemia has been reported to be taken up via apoE, rather than apoB (136,159).

Turnover of LDL apoB: The standard procedure for estimating turnover of LDL apoB is to isolate LDL by ultracentrifugation, iodinate it with radioactive iodine, reinject the LDL and then follow its decay in total plasma radioactivity over a period of two to three weeks. The pool size of LDL apoB in plasma is obtained by determination of total protein in LDL. LDL apoB usually has a biexponential decay, and the FCR of LDL apoB is estimated from this curve using the two compartmental model of Matthews (259). Another way to determine the FCR of LDL apoB is from the ratio of radioactivity in urine and plasma (221).

The actual procedures used for estimating turnover of LDL apoB vary from laboratory to laboratory. The density range used for labeling is usually 1.019-1.063 g/ml, but the range 1.020-1.050 g/ml has also been used. Some investigators have used reultracentrifuged LDL. We, however, have found this procedure will cause loss of LDL particles. All of these variations, of course, may influence the interpretation of the kinetic data.

Three models have been suggested to analyse LDL apoB kinetics and we have developed another. Generally, a two-compartmental model (259) has been used to analyze LDL apoB kinetics. This model hypothesizes a single intravascular pool in equilibrium with an extravascular pool. The plasma decay then represents the disappearance of a single homogeneous pool of LDL in plasma, and the shape of the curve is determined in large part by the exchange process with extravascular space. This model is difficult to apply since LDL has now been demonstrated to consist of heterogeneous particles. The second model was developed by Fisher et al. (252), who used this model to explain data from

patients with "polydisperse" LDL. They showed two species of LDL, one peaking at Sf 10, the other at Sf 4, both of which demonstrate different kinetic curves. They suggested that when polydisperse LDL is present, two intravascular compartments of LDL are needed. This, however, was not required for subjects with "monodisperse" LDL. Compartment Sf 10 decayed faster than compartment Sf 4 and they presumed only Sf 4 equilibrated with an extravascular pool. The third model was suggested by Goebel et al. (269). They also proposed two plasma compartments of LDL with different turnover rates from studies on two homozygotic and five heterozygotic FH patients.

Data from various subjects (231,232,234,245,246) indicate that total synthesis of LDL apoB can exceed estimated amounts of VLDL apoB converted from VLDL. This phenomenon suggests that LDL can be directly synthesized independent of the VLDL cascade. At present, four possible pathways for LDL formation have been suggested: VLDL conversion to LDL; direct secretion of IDL, with rapid conversion to LDL (270); secretion of LDL-size particles directly into plasma (234); or, the input of LDL could be from rapid and complete lipolysis of newly-secreted VLDL which would not be detected in the isotope kinetic data because they would be, in effect, unlabelled (263).

It has been suggested that VLDL metabolic heterogeneity might impact on LDL formation. Stalenhoef et al. (275) studied VLDL from a patient with lipoprotein lipase deficiency and postulated that this VLDL is, in composition, very close to nascent VLDL; this patient's VLDL, when injected into normal subjects,

was rapidly removed from the circulation and only a small portion converted to LDL. Packard et al. (276) studied a different population of VLDL particles in normal subjects. They demonstrated that only 10% of the larger triglyceride-rich VLDL were converted to LDL, whereas more than 40% of the small VLDL became LDL. They concluded that only small VLDL secreted into plasma would be converted into LDL, whereas the small VLDL derived from large VLDL would be degraded irreversibly, without conversion to LDL. This metabolic heterogeneity may depend on the quantity and/or the type of apoprotein present on VLDL particles. For example, Havel (277) reported that apoE is found in large amounts on large VLDL and the catabolic remnants produced from them, but only one or two apoE molecules may be present on the remnants from small VLDL. Gianturco et al. (136) and Krul et al. (159) also showed that large VLDL contained more apoE than apoB and apoE is the determinant by which this large VLDL is taken up by fibroblasts. The ratio of apoC to apoE may also be an important determinant of the fate of the particle; particles containing high amounts of apoC more likely to become LDL since they would more likely escape uptake and catabolism in the liver (279). Furthermore, the rate of triglyceride synthesis relative to the rate of apoB synthesis may determine the size of the VLDL particle produced by the liver (327) and consequently the proportion of VLDL particles converted to LDL. The last point suggests that larger VLDL can accept more cholesteryl ester from LDL than smaller particles and so have a higher content of cholesteryl ester and so may not be converted to LDL. These large VLDL particles, therefore, might be cleared directly from plasma due

to their enrichment in cholesteryl ester and apoE. Eisenberg et al. (283) have shown that bezafibrate treatment of patients with hypertriglyceridemia decreases their plasma triglyceride levels and increases their lipoprotein lipase and hepatic triglyceride lipase activity. This, in turn, decreases the cholesteryl ester content in VLDL, producing smaller VLDL particles, and increases conversion of VLDL to LDL.

Direct synthesis of LDL has been demonstrated in humans (216,234,235,254) and in animals (284-286). Soutar et al. (234) showed that, in FH homozygotes, the absolute synthetic rate of LDL is twice that of VLDL. Following a protocaval shunt in one patient, the rate of LDL apoB synthesis dropped and could then be accounted for by the VLDL synthesis, thereby implicating the liver as the source of newly synthesized LDL. This has been confirmed by Janus et al. (235) and Eaton et al. (254). By blocking VLDL catabolism in squirrel monkeys with Triton WR1339 and monitoring the incorporation of ^{14}C-leucine into LDL apoB, Illingworth (284) demonstrated that 10-19% of LDL apoB was secreted directly into the plasma. Goldberg et al. (285) also showed that, in monkeys, from 25-75% of LDL was secreted directly into plasma. Direct hepatic secretion of lipoprotein particles within the LDL density range (1.019-1.063 g/ml) has also been described in liver perfusion studies in pigs (286) and in non-human primates (205). These LDL-like particles contain apo B-100 plus excess surface constituents and a triglyceride-rich core (205). It has been suggested that these particles are also LDL precursors, but since they are present in the LDL density range,

they would be missed in kinetic studies using labelled VLDL.

The liver transports more than 3 grams of cholesterol per day and has the highest level of LDL receptor activity (288,289). In humans, it has been demonstrated recently that the liver has an LDL receptor which is different from the peripheral LDL receptor (143,290,291) in that it is present in FH homozygotes, only partically blocked by EDTA, and does not lead to the regulation of HMG-CoA reductase activity. The degree of LDL receptor regulation in liver appears to vary widely from species to species (292-294). In humans, the presence of LDL heterogeneity within and among individuals suggests that the turnover rate will be affected by the presence of different receptors. Results of studies in WHHL rabbits (204), which do not possess a functional LDL receptor in the liver, showed that IDL was accumulated in addition to LDL during the delayed clearance of VLDL and more of the IDL was converted into LDL. It has been suggested, therefore, that the LDL receptor may play a role in LDL formation from IDL and VLDL, in addition to its role in LDL clearance from plasma. However, whether this is the case in humans requires further study.

Kinetic data of apoB turnover: The kinetic data for both VLDL apoB and LDL apoB in normal or control subjects, FH, FCHL, familial hypertriglyceridemia (FHTg), and unclassified hypertriglyceridemia will be reviewed next.

"Normal" or Control Subjects: In studies of VLDL-apoB kinetics, the plasma concentration of VLDL apoB has varied over a considerable range, from 1.5 to 15 mg/dl. The estimated FCR for

VLDL-apoB has also varied; thus, in studies using radiolabelled VLDL, the fractional catabolic rate has ranged from 2.5 to 13.9 per day. In these studies, the synthetic rates of VLDL-apoB varied from 9.1 to 15.3 mg/kg/day (40,110,218,223-225,233,235, 244). Eaton et al. (253), using an endogenous tracer, found that their VLDL apoB FCR ranged from 10.6 to 15.1 per day while the synthetic rate ranged from 9 to 29 mg/kg/day.

With regard to LDL, plasma LDL apoB concentrations have ranged from 34 to 108 mg/dl. In some studies (218), the LDL has included LDL and IDL (density: 1.006-1.063 g/ml). Also, some investigators have used correction factors to compensate for differences in chromogenicity of bovine serum albumin (BSA), while others have not. The FCR for LDL apoB has ranged from 0.26 to 0.462 per day, while the synthetic rate has ranged from 7.7 to 14.4 mg/kg/day (40,215,218,220,221,224-229,231).

Familial Hypercholesterolemia (FH): VLDL apoB and LDL apoB kinetics have been studied in FH homozygotes and heterozygotes. For FH homozygotes, the concentration of VLDL apoB in plasma was in the normal range and the synthetic rate of VLDL apoB in four patients averaged 11 mg/kg/day, whereas one patient had a synthetic rate of 35 mg/kg/day. The fraction catabolic rate was within the normal range -- 7.20 per day (202,234). As expected, the LDL apoB synthetic rate was markedly elevated, varying from 20 to 40 mg/kg/day and the fractional catabolic rate was substantially decreased with an average rate of 0.10 per day (202,227,234,238,247). For FH heterozygotes, the synthetic rate of VLDL apoB was in the normal range, varying from 13 to 27 mg/kg/day in the 24 patients studied by different investigators

(110,202,231,234,235) and its fractional catabolic rate averaged 6.0 per day. LDL apoB synthetic rates in these patients varied from normal to elevated levels (9 to 18 mg/kg/day) whereas their fractional catabolic rates were always lower than normal subjects (202,215,227,231,234, 235,238,254). Soutar et al. (202) first noted there was no precursor/product relationship between VLDL apoB and LDL apoB in FH homozygotes. After quantitative analysis they noted that not all LDL apoB was derived from VLDL apoB; they then suggested there was independent synthesis of LDL apoB (202). These observations were later confirmed by Janus et al. (231) and Eaton et al. (254) in FH heterozygotes.

Familial Combined Hyperlipidemia (FCHL) and Familial Hypertriglyceridemia (FHTg): The kinetics of VLDL apoB and LDL apoB in these patients have been studied by several groups (235, 244,248). For FCHL, the VLDL apoB synthetic rate is increased and the fractional catabolic rate decreased compared to normal (22.2 vs. 12.9 mg/kg/day, 3.98 vs. 5.81 per day, respectively) (244). This was also demonstrated by Janus et al. (235) where in FCHL the VLDL apoB production rate ranged from normal to elevated (13.9 to 44.4 mg/kg/day) and the fractional catabolic rate was decreased compared to normal (2.5 vs. 7.2 per day). Kissebah et al. (243) showed that FCHL patients had increased synthetic rates and decreased fractional catabolic rates compared to normal (33.8 vs. 18.6 mg/kg/day, 2.4 vs. 4.8 per day, respectively). In summary, patients with FCHL consistently demonstrated increased VLDL apoB synthetic rates and decreased fractional catabolic rates compared to normal.

For FHTg, the VLDL apoB synthetic rate is also increased with a relatively low fractional catabolic rate compared to normal (244) (17.6 vs. 12.9 mg/kg/day, 2.74 vs. 5.81 per day, respectively). This is also shown by Kissebah et al. (243) with an increased synthetic rate and a decreased catabolic rate compared to normal (34.9 vs. 18.6 mg/kg/day, 3.8 vs. 4.8 per day, respectively). However, Janus et al. (235) found that the level of VLDL apoB synthetic rate in FHTg was not elevated, but rather these patients had reduced catabolic rates compared to normal (1.2 vs. 7.2 per day).

When the turnover rate of VLDL triglyceride and VLDL apoB was compared in FCHL and FHTg, it was found that in the former the turnover rate of VLDL triglyceride and apoB were both increased (235,244), whereas in the latter the turnover rate of VLDL triglyceride is disproportionately greater than for VLDL apoB. It was then suggested that the primary defect in FCHL is overproduction of VLDL apoB, while in FHTg it is oversecretion of triglyceride-enriched VLDL.

With respect to LDL apoB kinetics in FCHL, Kissebah et al. (248) showed that these patients had an increased synthetic rate and increased fractional catabolic rate compared to normal (29 vs. 13.2 mg/kg/day, 0.66 vs. 0.42 per day, respectively). However, Janus et al. (235) found the FCR of LDL apoB in FCHL was similar to normal (0.28 vs. 0.31 per day), whereas the LDL apoB synthetic rate was increased compared to normal (12.2 vs. .77 mg/kg/day). Thus, it seemed that an increased apoB synthetic rate in both VLDL and LDL is a characteristic feature of FCHL. In FHTg, there is a trend to overproduction of LDL apoB, but these

patients had a significantly higher catabolic rate than normal and so normalize their LDL apoB concentration in plasma (248). Sigurdsson et al. (222) also concluded that the low plasma levels of LDL frequently observed in patients with very high plasma triglyceride levels are due to a high removal rate of LDL in these patients, rather than to abnormal LDL synthesis.

Coronary Artery Disease: Finally, Kesaniemi et al. (239) studied patients with coronary heart disease with normal to mild hypercholesterolemia and showed that the plasma concentration of LDL apoB was positively correlated with the synthetic rate of LDL apoB. By contrast, the fractional catabolic rate of LDL apoB did not vary. They then studied 8 normolipidemic patients with coronary heart disease (271) and found no difference in LDL apoB concentration between these patients and controls. These patients also had an elevated LDL apoB transport rate but an increased fractional catabolic rate, but an increased fractional catabolic rate (271). They concluded that this was a subset of patients with increased synthetic rate and fractional catabolic rate. Vega et al. (240) also studied patients with coronary artery disease and separated those with hypertriglyceridemia (Tg $\geq$ 361 mg/dl) and with normolipidemia (Tg $\leq$ 246 mg/dl). They suggested (240) that hypertriglyceridemic patients with coronary heart disease and some of the normolipidemic patients with coronary heart disease had both increased transport rate and fractional catabolic rate of LDL apoB compared to normal (21.7 vs. 10.6 mg/kg/day, 0.56 vs. 0.31 per day, respectively); however, the majority of normolipidemic patients with coronary heart disease

did not have a defect in LDL metabolism. Kesaniemi et al. (246) recently studied VLDL apoB and LDL apoB kinetics in patients with coronary heart disease. These kinetic data were analyzed by multicompartmental analysis. These patients had an enhanced production rate of both VLDL and LDL apoB, but LDL apoB fractional catabolic rates were normal. These studies demonstrate a great variation in terms of plasma concentration of LDL apoB, synthetic rate, and fractional catabolic rate in patients with coronary artery disease.

APOLIPOPROTEIN B (APOB)

ApoB is an obligatory structural component of chylomicrons, VLDL, IDL, and LDL. It also acts as a ligand in cellular recognition of lipoproteins by receptors. ApoB exists in two forms: apo B-100 and apo B-48 (295,296). Definitive evidence has not yet been reported as to whether these two forms of apoB are organ-specific. In humans, recent work with short-term organ cultures of normal adult liver (297) suggest that it synthesizes apo B-100 but not apo B-48. In addition, a human hepatoma cell line (HepG2), which secretes a number of apolipoproteins, secreted only the apo B-100 form of apoB (298). Recent work by Law et al. (313) using a complementary DNA (cDNA) of apo B-100, suggested that apo B-48 and apo B-100 have a common nuclear RNA, which then undergoes transcription to two different mRNA, B-100 mRNA and B-48 mRNA. This view is opposed by findings from Glickman et al. (314), whose data suggest a single apoB mRNA with organ-specific factors responsible for translational control of

synthesis. Isolated liver and cultured hepatocytes from rats showed that it produces both apo B-48 and apo B-100 (299,300).

The ability to fully characterize the physicochemical structure of apoB was hindered by its insolubility (301), its susceptibility to degradation by proteases (302) and to oxidative cleavage (303,304), and its tendency to aggregate after delipidation. Therefore, progress in determining the primary and secondary structure of apoB was slow until the recent use of molecular biology techniques; 30% of the carboxyl-terminal end of the primary structure has been delineated from nucleotide sequence of complementary DNA (305). The data indicate that the carboxyl terminus has a more hydrophilic than hydrophobic character. The hydrophilic regions possess more alpha than beta structure, while the hydrophobic regions have more beta structure. There are frequent cross-overs from hydrophobic to hydrophilic character; therefore apo B-100 polypeptide may be woven in and out of the lipid environment of the lipoprotein particle at irregular intervals and apo B-100 may then have more than one lipid-binding domain.

Using monoclonal antibodies, it has been demonstrated that the spatial relationship between lipids and apoB antigenic determinent might change when the lipoprotein particle size changes (206). This, in turn, might result in the alteration of apoB conformation and so might affect the interaction of apoB binding domain to its cellular receptor.

The molecular weight of apoB is also under debate. A number of studies have suggested that apo B-100 is composed of two or more subunits per lipoprotein particle, with an estimated

molecular weight around 250,000 kD (306-308). However, several investigators now agree that apoB is most likely to be a single, large polypeptide with a molecular weight around 500,000 kD (309,310).

In 1962, immunochemical polymorphism of human apoB was shown to exist (311). With the availability of monoclonal antibodies, Schumaker et al. (312) identified three different phenotypes among human LDL; the new data fit a model consisting of two co-dominant apoB alleles. Recently, with cDNA probes, a common DNA polymorphism within the apoB gene coding sequence, which is associated with altered triglyceride and cholesterol levels, has been described (315).

APOB REGULATION

ApoB is essential for hepatic VLDL formation and lipid export. This section will discuss factors such as fatty acids, carbohydrate, cholesterol, and hormones that affect hepatic apoB and lipid synthesis and secretion of VLDL by the liver.

Fatty acids have a pronounced positive effect on triglyceride synthesis and secretion in perfused rat livers (316), in cultured rat hepatocytes (317,318), and in humans (99,118-121). Davis and Boogaerts (317) used ^{3}H-glycerol and ^{14}C-leucine as markers for triglyceride and apoprotein synthesis, respectively. They showed, <u>in vitro</u>, that triglyceride and phospholipid secretion from rat hepatocytes increased within fifteen minutes after addition of oleic acid to the medium, but the secretion of VLDL cholesterol and cholesteryl ester was not affected. Further-

more, neither the ratio of apo B-100 and apoB-48 nor the content of either apoB in VLDL was affected by oleic acid. Thus they concluded that fatty acid stimulation did not result in an increase in VLDL apoB secretion, but rather an increased triglyceride secretion per VLDL particle. These findings are supported by those of Patsch et al. (318). When fatty acid availability was increased, the rate of triglyceride secretion reached a plateau where esterified fatty acids began to accumulate intracellularly, but the levels of apoprotein remained unchanged. Recent work by Borchardt et al. (319), using a pulse-chase experiment with ^{25}S-methionine to study the nature of apoB with intracellular membranes, showed that adding oleic acid to stimulate lipogenesis did not affect apoB secretion. They suggested that lipogenesis may drive the process of moving apoB from rough to smooth microsomes. In contrast to these findings, two groups present different outcomes: Dashti et al. (320) studied the effects of oleic acid and insulin on production of lipoproteins. They showed that oleic acid increased the total lipoprotein production by 66%, predominantly in VLDL (23-40% of the total). The most pronounced effect was on triglyceride and apoB, which were increased by 100% and 40%, respectively. Insulin had the opposite effect; it inhibited the secretion of neutral lipids and decreased the total lipoprotein production. Salam et al. (321) studied oleic acid stimulation of VLDL apoprotein in the perfused rat liver using ^{3}H-leucine as a marker to study VLDL apoprotein synthesis. The net secretion of total VLDL protein mass was increased by oleate (64% in the fed rat and 150% in the fasted rat). Apoprotein

secretion was accompanied by a proportional increase in VLDL triglyceride. This study, however, only examined total protein mass, not the mass of different apoproteins. In vivo, we and others (102,322-324) have shown that following oral fat intake both plasma triglyceride and VLDL triglyceride level rises. We also have shown, however, that apo B-100 in the VLDL fraction increased (322). Redgrave and Carlson also observed an increase in apoB in large VLDL fraction (323). Therefore it appears that in vivo fatty acid increases triglyceride synthesis and probably apoB synthesis as well.

The effect of carbohydrate on hepatic VLDL synthesis and secretion has also been studied (325-327). Rats fed a sucrose-enriched diet (325) showed increased apoprotein synthesis and VLDL triglyceride and cholesteryl ester both accumulated faster than in control rats. Opposite results were shown from studies in humans. Hollenbeck et al. (326) gave high carbohydrate diets to insulin-dependent diabetics. After 20 days, VLDL triglycide and cholesterol were elevated, whereas the apoB level had dropped. Le et al. (327) studied VLDL-triglyceride and VLDL apoB kinetics after a high carbohydrate diet. They concluded that the production rate of VLDL-triglyceride increased, while that of VLDL apoB did not. Relatively fewer triglyceride-rich VLDL particles were converted to LDL than in controls and were more likely to be removed irreversibly. This might explain the previous study (326) in which apoB level actually dropped after carbohydrate perturbation.

A high cholesterol diet in experimental animals causes severe atherosclerosis. Perfused liver studies in hypercholestro-

lemic rats (328) have shown that the liver secretes both cholesteryl ester-enriched, apoC-deficient VLDL particles and cholesteryl ester-enriched, apoE-enriched LDL-like particles. Both cholesteryl ester and apoB levels are increased. This was also demonstrated in perfused liver of cholesterol-fed guinea pigs (151). In cholesterol-fed monkeys (205), perfused liver also secretes increased amounts of newly-synthesized LDL-like particles which are cholesteryl ester-enriched. In humans, a diet high in cholesterol and saturated fat (P/S = 0.25 - 0.4) increased the plasma apoB and LDL cholesterol levels; however, a diet high in polyunsaturated fat (P/S = 2.5), with even 1,500 mg cholesterol, produced no significant change in apoB and LDL cholesterol levels (329). This intriguing finding seemed to be related to the composition of the fat. Mevinolin, a competitive inhibitor of HMG-CoA reductase, has been used to treat patients with hypercholestrolemia. Kinetic study of these patients after mevinolin treatment showed that the input rate of LDL apoB decreased (330). It has also been demonstrated in animals that, after mevinolin treatment, LDL apoB direct synthetic pathways have been selectively inhibited by up to 90% (331).

The effect of estrogen on apoprotein synthesis has been studied most thoroughly in avian species. Estrogen treatment of the cockerel increases plasma VLDL cholesterol and triglyceride production and stimulates apoB synthesis (332). The stimulation of apoprotein synthesis is believed to be mediated at a pretranslational level. The effect of insulin on hepatic VLDL secretion has been demonstrated in cultured rat hepatocytes (318) and in

HepG2 cells (320). Insulin inhibited VLDL secretion but had no effect on apoB synthesis (318,320).

BASIS OF THE PRESENT STUDIES

Hyperapobetalipoproteinemia (HyperapoB) was first described in 1980 and defined as the combination of a normal, or near-normal, LDL cholesterol in the face of an elevated plasma LDL apoB level (47). This disorder is often associated with coronary artery disease. It has since been recognized in patients after myocardial infarction (51), in patients with hypertriglyceridemia (51), in normolipidemic patients with xanthelasma (333), and in a large Amish kindred in which a rare disorder, phytosterolemia, coexisted (334). The work which I will present in this thesis elucidates the compositional and metabolic abnormalities in HyperapoB.

Before I started my graduate training, I worked in Dr. Sniderman's laboratory. In order to examine the potential role of LDL in cholesterol transport to the liver, we first developed a method to measure LDL apoB in plasma by the radial immunodiffusion technique (RID) (335). This method is simple and enabled us to calculate the recovery of LDL particles after the conventional ultracentrifugation separation of LDL from plasma. LDL compositional changes across the splanchnic bed in humans were then demonstrated (134). This study showed that during passage through the splanchnic bed cholesteryl ester is taken up from apparently intact LDL, which is then enriched with triglyceride. This uptake in LDL cholesterol was inversely related to

cholesterol output in VLDL. This data raised the possibility of cholesteryl ester and triglyceride exchange between LDL and VLDL. We then collaborated with Dr. Y. Marcel to demonstrate, in vitro, that cholesteryl ester can be exchanged or transferred among lipoproteins facilitated by a protein factor in the d > 1.25 g/ml infranant of human plasma (182). These two concepts, variable LDL composition across the splanchnic bed and cholesteryl ester and triglyceride exchange among lipoproteins, are the main bases to explain the physiochemical basis of HyperapoB and to build a multicompartmental model for LDL apoB metabolism.

With regard to LDL composition in HyperapoB, I then modified Shen's discontinuous salt gradient technique to study LDL composition in patients with HyperapoB and compare these with normolipidemic control subjects and contrast them with patients with familial hypercholesterolemia. The results are presented in Chapter 2. Briefly, using equilibrium density gradient ultracentrifugation, LDL can be separated into two main subfractions: Buoyant LDL (B-LDL) and Dense LDL (D-LDL). D-LDL is smaller and has a lower cholesterol-to-protein ratio than B-LDL. HyperapoB patients are then characterized by the presence of D-LDL which are smaller, denser, cholesteryl ester-depleted, and relatively protein-enriched compared with that of controls. By contrast, LDL in patients with FH are characterized by the presence of B-LDL which are larger, more buoyant, cholesteryl ester-enriched and relatively protein-depleted at compared to controls and HyperapoB.

The effect of LDL heterogeneity on the possible structure change in relation to apoB and lipids was then investigated. This

study is reported in Chapter 3. The immunoreactivities of LDL subfractions with six monoclonal antibodies (kindly supplied from Dr. Y. Marcel's lab) were studied. Briefly, B-LDL is more immunoreactive than D-LDL with three of the monoclonal antibodies studied. As the particle becomes smaller, the cholesterol-to-protein ratio decreased and the immunoreactivity decreased also. These results led us to predict that LDL subfractions might have different physiological roles and decreased particle size might alter apoB conformation.

At the same time, under the supervision of Doctors Sniderman and Thompson, I studied apoB kinetics in HyperapoB patients to determine the possible fault responsible for the disorder. The study is reported in Chapter 4. In brief, HyperapoB is characterized by the elevation of LDL apoB, which is secondary to oversynthesis of VLDL apoB. This result lead to two possible speculations: first, there may be a primary fault in protein structure, a fault which seems unlikely because there is no defect in LDL catabolism, or a primary fault in the regulation of apoB gene exprssion. Second, there may be secondary abnormal regulation of apoB synthesis. As we know, apoB production is regulated by a wide variety of factors. Abnormal clearance of chylomicron lipid after oral fat load to HyperapoB patients led us to search for a possible peripheral tissue marker in this disorder. To do so, I have studied fatty acid uptake and esterification in adipose tissue from patients with HyperapoB. The study is presented in Chapter 6. The results indicate that there might be an intracellular defect responsible for this disorder.

Due to the heterogeneity in LDL subfractional turnover, the classical model to analyze LDL kinetics is no longer sufficient. In collaboration with Dr. L. Zech, I then attempted to build a new multicompartmental model to study LDL apoB metabolism. The study is reported in Chapter 5. This model presents novel insights into LDL apoB metabolism and underscores the physiologic importance of cholesteryl ester and triglyceride exchange processes.

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CHAPTER 2

COMPOSITION AND DISTRIBUTION OF LOW DENSITY LIPOPROTEIN SUBFRACTIONS IN HYPERAPOBETALIPOPROTEINEMIA, NORMOLIPIDEMIA, AND FAMILIAL HYPERCHOLESTEROLEMIA

ABSTRACT

Hyperapobetalipoproteinemia is defined as the combination of a normal cholesterol level of low density lipoprotein (LDL) in the face of an increased LDL apolipoprotein B (apoB). To examine the physical and chemical basis for the apparent disproportion between LDL cholesterol and apoB, so characteristic of this syndrome, we used density gradient ultracentrifugation to separate LDL into two major subfractions: fraction 1 or buoyant-LDL and fraction 2 or dense-LDL from 10 normal subjects, 20 patients with hyperapobetalipoproteinemia (10 normotriglyceridemic and 10 hypertriglyceridemic), and 9 patients with familial hypercholestrolemia. In familial hypercholesterolemia, more LDL was in fraction 1 and this LDL subfraction was relatively enriched in cholesterol and poor in protein. By contrast, it was fraction 2 that differed in hyperapobetalipoproteinemia, being denser, depleted of cholesterol (particularly cholesteryl ester), and relatively enriched in protein. These findings were more pronounced in the hypertriglyceridemic patients than in the normotriglyceridemic patients with hyperapobetalipoproteinemia. Thus this study confirms that considerable heterogeneity exists between LDL subfractions within individuals but, in addition, it indicates there are also marked -- and apparently characteristic -- differences in LDL composition amongst normal subjects and patients with hyperapobetalipoproteinemia and familial hypercholesterolemia.

INTRODUCTION

The risk of coronary heart disease in the Framingham study was initially shown to be related to plasma total cholesterol (1) and subsequently to the levels of cholesterol in very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoproteins (HDL) (2,3). Recently, several studies have suggested that quantitation of the apoprotein moieties of these lipoproteins can provide additional information in this respect (4-12). In particular, the plasma concentration of the major apoprotein of LDL, apolipoprotein B (apoB), may frequently be increased in patients with coronary artery disease despite their having an LDL cholesterol within the normal range, a combination we termed hyperapobetalipoproteinemia (HyperapoB) (4,10). By contrast, patients with familial hypercholesterolemia (FH), in whom the risk of coronary heart disease is also very high, exhibit increases in both LDL cholesterol and LDL apoB.

LDL are spherical particles that range in diameter between 21.0 and 29.0 nm and in density beteween 1.019 and 1.063 g/ml. Not surprisingly, there is mounting evidence that considerable heterogeneity exists among these LDL particles (13-19). For example, Shen et al. (16), using density gradient ultracentrifugation, demonstrated marked diversity of LDL in terms of both size and composition in normal individuals, the larger particles being enriched in cholesteryl ester compared to the smaller protein-enriched particles. In addition, Fisher and his colleagues have shown in a series of studies that in some individuals LDL is monodisperse, whereas in others it is polydisperse (19). A possible explanation for the decreased cholesterol to

protein ratio in HyperapoB patients might be a shift in the spectrum of LDL towards smaller, denser particles. In contrast, LDL from patients with FH has a higher than normal cholesterol to protein ratio (20), suggestive of a shift towards larger, less dense particles. To test this hypothesis, we investigated the pattern of distribution of LDL in normal subjects, as well as patients with HyperapoB and FH, by means of density gradient ultracentrifugation.

SUBJECTS AND METHODS

Normal Subjects: Ten physicians (9 males, 1 female) served as healthy controls; their age was 46±11 years. None had a history of coronary heart disease and all had plasma levels of total and LDL cholesterol, total triglyceride, and LDL apoB within the normal range (4) (Table 1). The value for upper limits of LDL cholesterol, total triglyceride and LDL apoB were 200, 200, and 120 mg/dl, respectively.

Patients with HyperapoB: Twenty patients with HyperapoB were selected. All had suffered a documented myocardial infarction at least 3 months before the study and all had normal plasma total and LDL cholesterol levels but increased values of plasma LDL apoB (Table 1). They were divided into two subgroups: 10 (9 males, 1 female) had a plasma total triglyceride level $\leq$ 200 mg/dl; their average age was 51±8 years; the other 10 (8 males, 2 females) had plasma total triglyceride levels > 200 mg/dl; their average age was 46±11 years. None were on special diets or exercise programs, and none were being treated with

lipid-lowering agents. Thirteen were, however, receiving ß blockers, 4 diuretics, 3 digitalis, and 10 long-acting nitrates. All were fully ambulatory.

Patients with FH: Blood was obtained from nine FH patients attending the Hammersmith Hospital Lipid Clinic, 3 homozygotes (all males) and 6 heterozygotes (2 males, 4 females). The plasma was separated and kept at 4°C during transportion by air to Montreal. Two homozygotes were undergoing regular plasma exchange. All the heterozygotes were on a lipid-lowering diet supplemented in one by cholestryramine, in another by cholestryramine and nicotinic acid, and in a third by cholestryramine and probucol.

Isolation and Fractionation of LDL: After a 12 hr fast, blood samples were collected into tubes containing EDTA (0.1 mg/ml) and plasma was separated by centrifugation at 2,500 rpm at 4°C. LDL were then isolated by preparative ultracentrifugation (21). The density of plasma was first adjusted to 1.019 g/ml by addition of a solution of NaCl/KBr and the plasma was centrifuged at 4°C for 16 hr in a Beckman 50 Ti rotor at 40,000 rpm. The very low density and intermediate density lipoproteins were removed by tube slicing, the density of the infranate was then increased to 1.071 g/ml by addition of a solution of NaCl/KBr, and the sample was subjected to centrifugation in a Beckman SW 50.1 rotor at 40,000 rpm for 16 hr at 4°C. Due to salt redistribution, the final density of the top milliliter after this procedure was 1.063 g/ml. The supernatant LDL was aspirated and then dialyzed

for 6 hr against two changes of a salt density 1.0500 g/ml solution of NaCl/KBr. Discontinuous gradient ultracentrifugation was then performed as follows: to a 2 inch (5 cm) cellulose nitrate tube, 1 ml of the following solutions of NaCl/KBr were added in succession: 1.1300 g/ml, 1.0645 g/ml, LDL (1.0500 g/ml), 1.0410 g/ml, and finally 1.0286 g/ml. The gradient was then centrifuged in an SW 50.1 rotor at 40,000 rpm for 40 hr at 10°C. At the conclusion, the tubes were removed and inspected visually. Except occasionally in FH, two yellowish bands were always observed. They were most distinct and most separated in HyperapoB patients, with the upper band at the meniscus and the lower one separated from it by a colorless interface at or more frequently more than 1 cm from the meniscus. The separation of the two bands was less pronounced in normal subjects and in patients with FH, with the lower band closer to the upper band than in HyperapoB patients, and occasionally as noted, particularly with FH, the two bands virtually overlapped.

Fractions were collected by piercing the bottom of the tube using a Beckman fraction recovery system. The first three fractions consisted of 1 ml each, the last two contained the lower and upper bands -- fractions 2 and 1, respectively, which were separated visually. Tne salt density profile of the discontinuous gradient was determined by refractometry of successive 0.5-ml aliquots withdrawn by pipetting from the top or piercing from the bottom of the gradient from control tubes to which LDL had not been added.

Sample Analysis: Cholesterol and triglyceride were measured enzymatically (Cholesterol and triglyceride Kits, Beckman Instrument Corp., Calif.). LDL apoB in plasma and in LDL subfractions was determined by radial immunodiffusion (22) and the lipid composition of LDL subfractions was determined by thin-layer chromatography (23). The total protein concentrations in LDL and LDL subfractions before and after dialysis were measured by radial immunodiffusion (22) and by the Lowry method (31). LDL particle size was measured by electron microscopy in which the lipoproteins were negatively stained with 2% sodium phosphotungstate after dialysis against 0.13 M ammonium acetate buffer, pH 7.4. Diameters of 200 free-standing particles were computed from each lipoprotein fraction by using a sonic digitizer and a computer program, as described (24,25). Lipoproteins of d 1.006-1.063 g/ml were also studied by analytic ultracentrifugation. The concentration of lipoproteins and their peak flotation rates were computed from the Schlieren patterns (26). Statistical significance was assessed by two-tailed Student's t test.

RESULTS

The concentrations in plasma of total cholesterol, triglyceride, LDL apoB, and LDL cholesterol of the subjects studied are shown in Table 1. Plasma cholesterol levels were much higher in FH patients than in those with HyperapoB, in whom the mean value was only slightly higher than in the normal subjects. In contrast, plasma LDL apoB levels were much higher in both FH and HyperapoB patients than in the normal subjects.

Distribution of LDL Subfractions: The distribution of apoB amongst the fractions obtained by density gradient ultracentrifugation is shown in Table 2. Over 95% of the LDL apoB centrifuged on the gradient was recovered from the upper three fractions, fraction 1 being the most buoyant. In normal subjects and patients with HyperapoB 30% of LDL apoB is distributed in fraction 1, 60% of it is in fraction 2, and the rest (10% of it) is in fraction 3. However, in patients with FH the LDL apoB was found to a greater extent in fraction 1 in heterozygotes compared with either normal subjects or HyperapoB patients, a finding that was even more noticeable in FH homozygotes.

Cholesterol to apoB Ratios of LDL Subfractions: The small amount of LDL present in fraction 3 was precluded from the analysis. Table 2 shows the cholesterol to apoB ratios of subfractions 1 and 2 of the subject studied. This ratio in fraction 2 is significantly lower than in fraction 1 in all groups of subjects studied ($p < 0.01$). Furthermore, the cholesterol to apoB ratio in fraction 2 was significantly lower in the normotriglyceridemic HyperapoB patients compared to the normal subjects (1.16 ± 0.16 vs. 1.33 ± 0.11, $p < 0.02$). Indeed, this ratio was even markedly decreased in the hypertriglyceridemic HyperapoB group compared to the normal subjects (0.88 ± 0.11 vs. 1.33 ± 0.11, $p < 0.001$), whereas there was no significant difference in the cholesterol to apoB ratio of fraction 2 in the heterozygotic FH patients compared to that in normal subjects (1.23 ± 0.09 vs. 1.33 ± 0.11, $p < 0.2$). In LDL fraction 1 the cholesterol to apoB ratio is similar in the first three groups -- normal, normotriglyceridemic HyperapoB, and

hypertriglyceridemic HyperapoB, but this ratio was significantly higher in FH heterozygote than in the normal subjects (1.81±0.29 vs. 1.53±0.04, $p < 0.01$). This difference in ratio is even more pronounced in FH homozygote compared to that in normal subjects (2.14 vs. 1.53).

Chemical Composition of LDL subfractions: The composition of LDL subfractions 1 and 2 is shown in Table 3.

Intragroup comparison: In normal subjects, LDL fraction 2 contained significantly more protein and less phospholipids than fraction 1 (24±3% vs. 20±3%, $p < 0.02$ and 22±2% vs. 24±3, $p < 0.05$, respectively). In FH, LDL fraction 2 was also significantly enriched in protein compared to fraction 1 (22±2% vs. 17±4%, $p < 0.01$). LDL subfractions 1 and 2 also differed in the patients with HyperapoB. Once again, LDL fraction 2 was protein enriched compared to fraction 1 (34±8% vs. 24±7%, $p < 0.005$). In this instance, the proportions of cholesteryl ester and free cholesterol were reduced in fraction 2 compared to fraction 1 (36±4% vs. 44±6%, $p < 0.0025$, and 7±2% vs. 8±2%, $p < 0.05$, respectively). Therefore, in all three instances LDL fraction 2 contained significantly more protein than did fraction 1.

Intergroup comparison: There were also significant differences in LDL subfractions composition amongst normal, FH, and HyperapoB subjects. LDL fraction 2 in HyperapoB group contained substantially more protein and less cholesteryl ester than did fraction 2 in normal subjects (34±8% vs. 24±3%, $p < 0.025$ and 36±4% vs. 42±4%, $p < 0.01$, respectively) while fraction 1 in HyperapoB group also contained less phospholipids than did the

fraction 1 in normal subjects (20±4% vs. 24±3%, $p < 0.05$).

On the other hand, LDL fraction 1 in FH contained less protein than did fraction 1 in the normal subjects (17±4% vs. 20±3%, $p < 0.05$) but more cholesteryl ester and free cholesterol (48±2% vs. 43±3%, $p < 0.025$ and 11±2% vs. 8±2%, $< p$ 0.01, respectively). By contrast, the composition of LDL fraction 2, except free cholesterol, did not differ significantly between FH patients and normal subjects (free cholesterol: FH vs. normal subjects, 11±3% vs. 7±3%, $p < 0.05$).

Density of LDL subfractions: The mean density of LDL fraction 1 in all the subjects studied was 1.0405 g/ml, usually recovered in 0.75 ml, fraction 2 was 1.0480 g/ml, usually recovered in 1.25 ml, and fraction 3 was 1.0600 g/ml, usually recovered in 1.00 ml. The locations of the two yellowish bands in the equilibrium density gradient varied among each individual. The top band was always observed at the meniscus and the mean density is 1.0381 g/ml in all the subjects studied. However, the second observable yellowish band was located at a different position in each subject; in normal subjects it was usually located at density 1.0407 g/ml, in patients with FH it was between 1.0381 and 1.0407 g/ml, and in HyperapoB patients with normotriglyceridemia it was 1.0470 g/ml and 1.0550 g/ml in those with hypertriglyceridemia.

Electron Microscopy Results: Electron micrographs of LDL fractions 1 and 2 are shown in Fig. 1. Fig. 1A shows fraction 1 from a normal subject: these particles show typical LDL morphology -- i.e., free-standing particles are round while contiguous

ones are deformed and often polygonal. Fig. 1B is a micrograph of fraction 2 material from a normotriglyceridemic HyperapoB patient and demonstrates that the LDL particles are round and fairly homogeneous in size. Fig. 1C shows that micrograph of fraction 2 material from a hypertriglyceridemic HyperapoB patient. These particles represent unusually small LDL structures; they are extremely uniform in size and have a tendency toward hexagonal packing. Such particles were not encountered in the normal subjects. The diameters of LDL particles (mean±SD) were also computed. In LDL fraction 1 there was no difference between the 6 normal subjects and 5 HyperapoB patients studied (26.6±0.7 vs. 26.1±1.0 nm, respectively) and there was also no difference in LDL fraction 2 between the 6 normal subjects and 4 normotriglyceridemic HyperapoB patients studied (24.5±1.1 vs. 24.2±0.4 nm, respectively). However, in comparison with the diameter of LDL fractions 1 and 2 in the subjects studied, the LDL particle is significantly larger in fraction 1 than in fraction 2 ($p < 0.01$). Furthermore, the diameter of the LDL fraction 2 particles in the 2 hypertriglyceridemic HyperapoB patients was rather small; 21.7±2.7 nm in one instance, and 21.9±2.3 nm in the other.

Analytical Ultracentrifugation of LDL: The distribution of mass within LDL in normal and HyperapoB subjects was also assessed by analytical ultracentrifugation (Fig. 2). Results are shown for one female and one male patient with normotriglyceridemic HyperapoB (Fig. 2A) and three normal males (Fig. 2B). The total mass of S_f^0 0-20 lipoproteins is higher in HyperapoB patients than in normal subjects (523 vs. 258 mg/dl), and the

peak LDL distribution shifted toward slower flotation rates in the HyperapoB patients (normal vs. HyperapoB; S_f^o, 7.1 vs. 6.0).

DISCUSSION

The findings of this study provide a plausible explanation for the disproportionate increase in LDL apoB over LDL cholesterol in plasma that we previously reported in a group of patients with coronary heart disease and HyperapoB (4). The study demonstrated that the increased concentration of LDL apoB in these patients is present mostly in LDL subfraction 2 particles which are denser, relatively protein enriched, and cholesteryl ester depleted as compared with LDL particles from normal subjects. By contrast, in patients with FH there is an increase in the more buoyant LDL particles, which are relatively cholesterol enriched and protein depleted. The present findings are consistent with the previous reports of altered LDL composition in hypertriglyceridemic patients (8,19) and FH patients (20,27-29).

LDL has usually been regarded as a homogeneous entity for clinical and experimental purposes despite long-standing evidence to the contrary (13-19,30). The likelihood that different fractions of LDL existed had been suggested in earlier studies (13-14), and recently confirmed in normal subjects (15-18). The different composition and density of LDL subfractions observed in these studies are compatible with a spherical model for LDL in which particle size will diminish if core lipids, cholesteryl ester or triglyceride, and surface polar lipids decrease while the particle becomes denser due to the changes of lipids to protein

ratio. Our findings in HyperapoB are consistent with this model, and the concept of LDL heterogeneity is further strengthened by the contrasting findings in terms of chemical composition and distribution of LDL fractions in FH.

The above line of reasoning is dependent upon establishing that the subfractions obtained by ultracentrifugation are not experimentally induced artifacts and upon the validity of the apoB immunoassay as a measurement of LDL protein concentration. Krauss and Burke (18) demonstrated the existence of several LDL subclasses by gradient gel electrophoresis in fresh unfractionated plasma which are comparable with LDL subfractions by ultracentrifugation, thus confirming the validity of observations based on density gradient ultracentrifugation. The method used to measure apoB in the present study was radial immunodiffusion, the results of which were compared with measurements of protein obtained by the Lowry method. The two methods agreed well ($r=0.92$) both before and after separation of LDL into its subfractions, as long as the associated salt was first removed by dialysis.

Myocardial infarction perturbs plasma LDL apoB levels (32), but Avogaro et al. have shown that, by 3 weeks after infarction, these have regained their day 1 values (33). The interval of at least 3 months in the present study was chosen to correspond to previous works (10,34); even so, our patients had not all resumed entirely normal lives and equally important, many were receiving cardioactive drugs whose effects on plasma LDL apoB levels are not known. In the case of the homozygous FH

patients treated with plasma exchange, the sample analyzed was taken more than 2 weeks after the last exchange, an interval sufficient for LDL composition to revert to its preexchange state (20); although cholestryramine may change LDL composition in the heterozygotes by reducing LDL cholesterol to apoB ratios (35), this effect would, if anything, diminish the differences observed between FH patients and the other two groups studied.

The lipid hypothesis stipulates that the risk of coronary heart disease is related, at least in part, to the lipid levels of various lipoprotein fractions. And indeed, in clinical practice, both abnormality and adequacy of therapy are defined exclusively by lipid levels. However, because LDL is heterogeneous in composition in normal subjects, and because, as shown in this study, there are substantial and apparently characteristic differences in LDL composition in particular pathologic states, it appears that lipid levels incompletely characterize lipoproteins. In the case of LDL, important differences in composition and plasma particle number may pass unrecognized if only LDL cholesterol is measured. Indeed, the present findings suggest that the relationship might be even more powerful than that between lipids and the chance of disease.

The association between HyperapoB and coronary artery heart disease is due presumably to accelarated atherosclerosis, and several possible mechanisms for this can be considered. The relatively delipidated LDL particles might be associated with structural changes in LDL resulting in increased interaction between LDL and glycosaminoglycans which make up the structural matrix of the artery well (36,37). Alternatively, the increased

number of LDL particles in plasma as well as their smaller particle size might produce an increased entry rate into the arterial wall, saturating the catabolic capacity of phagocytic cells in the vicinity of damaged intima (38,39). Smith and Slater showed that 50-75% of the lipid in the advanced lesions is in intact LDL form (38). This is also supported by Scott and Hurley (39), who demonstrated the intact LDL molecule in the developing atherosclerotic plaque. The results of the present study do not establish the association between HyperapoB and coronary heart disease as being causal, they do illustrate that potentially pathogenic variations in the spectrum of LDL particles can occur despite normal serum lipids.

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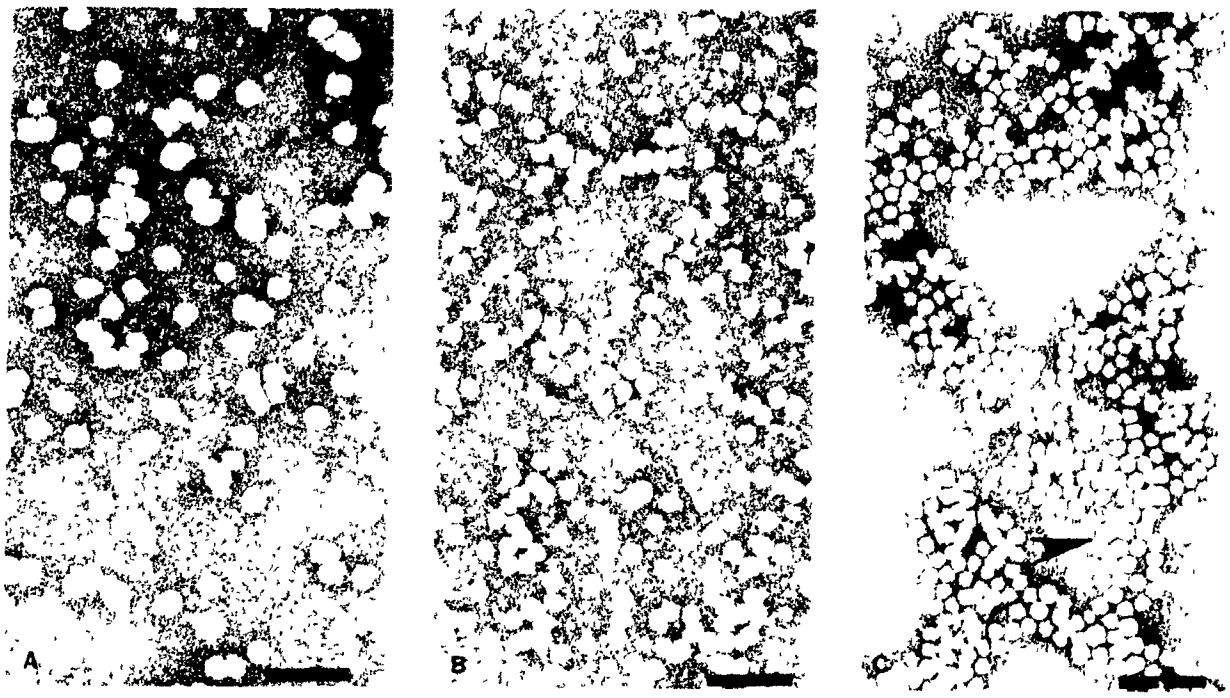

Figure 1. Electron micrographs of LDL subfractions. Bars represent 100 nm. (A) subfraction 1 in a normal subject; (B) subfraction 2 in a normotriglyceridemic patient with HyperapoB; (C) subfraction 2 in a hypertriglyceridemic patient with HyperapoB. Arrow indicates area where hexagonal packing is evident.

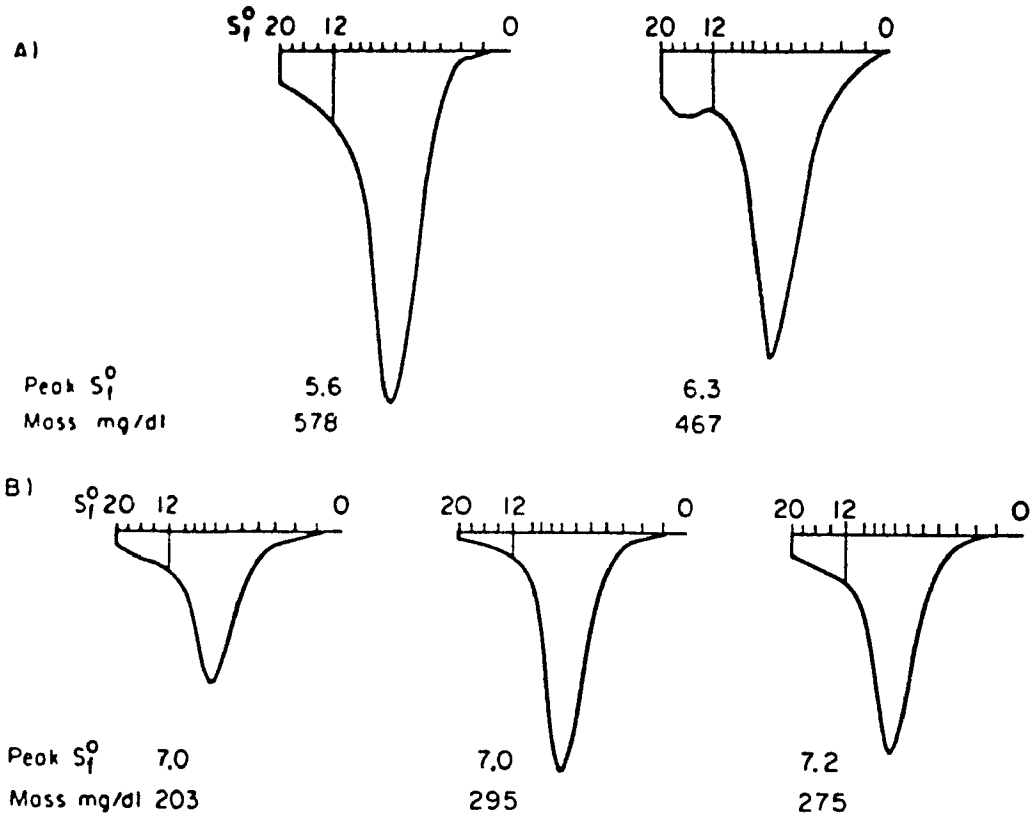

Figure 2. Analytical ultracentrifugation of S_f^0 0-20 lipoproteins. The concentrations of the lipoproteins and their distribution as a function of flotation rate (S_f^0) were determined by a computerized analysis of data from the Schlieren patterns of ultracentrifugal spins at 52,640 rpm of isolated total low density lipoproteins in a solution of density 1.063 g/ml (26). The flotation rates are expressed in Svedberg units (10^{-13} sec) corrected for concentration dependence and to standard conditions (26°C). $S_f^0$0-12=low density lipoproteins; $S_f^0$12-20=intermediate density lipoproteins. (A) Patients with normotriglyceridemic HyperapoB (left, female; right, male); (B) male normal subjects.

Table 1. Plasma lipid and LDL apoB concentrations

Subjects	n	TC	TG	LDL-C	LDL apoB
		mg/dl plasma			
Normal	10	172± 20	90±31	106± 13	80±11
NTG HyperapoB	10	233± 14	150±24	170± 11	150±18
HTG HyperapoB	10	247± 14	332±86	158± 17	164±18
FH heterozygotes	6	420±131	165±11	350±145	223±60
FH homogygotes	3	513	186	397	239

Results are presented as mean±SD for n subjects.
NTG; normotriglyceridemic;
HTG; hypertriglyceridemic.
HyperapoB; hyperapobetalipoproteinemia
TC; total plasma cholesterol
TG; total plasma triglyceride
LDL-C; low density lipoprotein cholesterol
LDL apoB; LDL apolipoprotein B

Table 2. LDL fractions 1 and 2: Distribution of apoB and cholesterol-to-apoB ratio

Subjects	n	% of total apoB		cholesterol/apoB ratio	
		1*	2	1	2
Normal	10	34± 8	54±11	1.53±0.04	1.33±0.11
NTG HyperapoB	10	30±12	62±11	1.52±0.18	1.16±0.16
HTG HyperapoB	10	30±10	59±11	1.44±0.15	0.88±0.11
FH heterozygote	6	45±26	46±24	1.81±0.29	1.23±0.09
FH homozygote	3	69	22	2.14	1.13

Results are presented as mean±SD for n subjects.
NTG; normotriglyceridemic
HTG; hypertriglyceridemic

1*; LDL subfraction 1
2 ; LDL subfraction 2

Table 3. Composition of LDL in fractions 1 and 2 in normal subjects and patients with either HyperapoB or FH

Subjects	n	subfraction	apoB	CE	FC	TG	PL
Normal	7	1	20±3	43±4	8±2	5±3	24±3
		2	25±3	42±4	7±3	3±2	22±2
HyperapoB	10	1	24±7	44±6	8±2	4±2	20±4
		2	34±8	36±4	7±2	3±1	20±4
FH	5	1	17±4	48±2	11±2	5±1	20±1
		2	22±2	43±7	11±3	5±1	19±3

Results are presented as mean±SD for n subjects.
CE; cholesteryl ester
FC; free cholesterol
TG; triglyceride
PL; phosphlipid

CHAPTER 3

MODULATION OF APOLIPOPROTEIN B ANTIGENIC DETERMINANTS IN HUMAN LOW DENSITY LIPOPROTEIN SUBFRACTIONS

ABSTRACT

To investigate the effect of low density lipoprotein (LDL) heterogeneity on the conformation of LDL apolipoprotein B (apoB), the immunoreactivities of 6 monoclonal antibodies against LDL apoB were measured in 3 LDL subfractions isolated by equilibrium density gradient ultracentrifugation. To ensure a broad range of LDL particles, the LDL subfractions were prepared from normal subjects and patients with hyperapobetalipoproteinemia. With 3 of the antibodies, 1D1, 5E11, and 3A10, LDL subfractions 1, 2, and 3 (the buoyant, the dense, and the very dense LDL respectively) were equally immmunoreactive and competed similarly with reference whole LDL. By contrast, with 3 other antibodies, 2D8, 3F5, and 4G3, fraction 1 was significantly more reactive than fraction 3; that is for each in turn, 190, 179, and 47% more of the very dense LDL protein was required to achieve the same displacement as with fraction 1. Further, the immunoreactivities of the 3 LDL subfractions with antibodies 2D8, 3F5, and 4G3 were correlated with their LDL cholesterol to LDL protein ratio with r values of 0.727, 0.898, and 0.870, respectively, suggesting that as LDL particle size decreases, the conformation of the LDL apoB changes progressively. It is of interest that the antigenic determinants recognized by 3F5 and 4G3 are close to the LDL receptor recognition site on LDL apoB. Therefore, it is possible that the reduced immunoreactivity of these determinants in dense LDL may be the in vitro correlate of the reduced fractional catabolic rate of dense LDL compared to buoyant LDL previously observed in vivo.

INTRODUCTION

Low density lipoprotein (LDL) is the main cholesterol carrier in plasma. However, these cholesterol-rich particles are not made up uniformly but differ in size, hydrated density, and chemical composition. Thus, equilibrium density gradient ultracentrifugation studies in normal subjects (1,2) have shown that as LDL particle density increases a series of related changes occur: peak flotation rate decreases, mean particle diameter decreases, and phospholipid to protein ratio decreases while, at the same time, core lipid to protein ratio also decreases. These studies in normal subjects have been extended by the demonstration that disease may alter the composition of LDL subfractions; for example, in familial hypercholesterolemia (FH), LDL mass is increased and a cholesteryl ester-enriched subfraction is present (3) whereas in hyperapobetalipoproteinemia (HyperapoB) and familial combined hyperlipoproteinemia (FCH), LDL mass is also increased but a LDL subfraction is present that is smaller, denser, depleted in cholesteryl ester, and relatively enriched in protein (3-5).

All these observations are consistent with a spherical model of LDL in which cholesteryl ester and triglyceride are confined principally to the core with phospholipid, free cholesterol, and apoB forming a surface coat of constant thickness (6). We speculated that this plus the decreasing diameter of the particle and possibly changes in core lipid as well might alter the conformation of the apoB. Accordingly the present study examines whether the immunoreactivity of a series of apoB antigenic determinants is altered predictably in LDL particles of

different composition and density.

SUBJECTS AND METHODS

Subjects Studied: LDL was isolated from 14 adults (13 males, 1 female, average age 57±7 years). None had suffered a documented myocardial infarction at least 3 months previously while 4 had no history of coronary heart disease. Plasma lipid, lipoprotein lipid, plasma LDL apoB levels and apoE phenotype are given in Table 1. Using the criterion for HyperapoB, patients 1, 5, 7, 8, and 10 had combination of LDL cholesterol $\leq$ 200 mg/dl and plasma LDL apoB $\geq$ 120 mg/dl. On the basis of laboratory and clinical evaluations, none of the subjects presented with secondary causes of dyslipoproteinemia, such as hypothyroidism or nephrosis, or with the E2/E2 phenotype.

Isolation and Fractionation of LDL: After a 12 h fast, blood samples were collected into tubes containing EDTA (1 mg/ml). Plasma was then separated by centrifugation at 2500 rpm at 4°C and kept in tubes containing EDTA (1 mM) and NaN_3 (0.02%). LDL was then isolated by preparative ultracentrifugation; the density of plasma was adjusted to 1.019 g/ml by addition of NaCl/KBr solution, and the sample was centrifuged at 4°C for 16 h in a Beckman 50 Ti rotor at 40,000 rpm after which the supernatant containing very low density lipoproteins and intermediate density lipoproteins was removed by tube slicing. The density of the infranate was then increased to 1.071 g/ml by addition of NaCl/KBr solution, and the infranate was further centrifuged at

4°C for 16 h in a Beckman SW 50.1 rotor at 40,000 rpm. Because of salt redistribution, the final density of the top ml after this procedure is 1.063 g/ml. The supernatant LDL was aspirated and dialysed for at least 6 h against two changes of NaCl/KBr solution (d 1.050 g/ml).

Discontinuous density gradient ultracentrifugation was then performed as follows. To a 2-inch (5-cm) cellulose nitrate tube, 1 ml of the following solutions was added in succession: 1.1300 g/ml, 1.0645 g/ml, LDL 1.0500 g/ml, 1.0410 g/ml, and 1.0286 g/ml. The gradient was centrifuged in a Beckman SW 50.1 rotor at 40,000 rpm for 40 h at 10°C after which the tubes were removed and the fractions collected by piercing the bottom of the tube using a Beckman fraction recovery system. The initial 3 fractions removed were 1 ml each; the last 2 fractions contained the lower and upper bands -- Fractions 2 and 1, respectively, which were separated visually. For each subject, the last 3 fractions (that is, the least dense fractions; fractions 1, 2, and 3) were then dialysed against a phosphate-buffered saline (PBS) solution and subsequently studied with monoclonal antibodies.

A reference preparation of LDL used for radioimmunoassays was obtained from normolipemic plasma by sequential ultracentrifugation between densities 1.020 and 1.050 g/ml.

Monoclonal Antibodies against apoB: The production and characterization of monoclonal antibodies against apoB from mice immunized by human LDL has been described earlier (7,8). Ascitic fluid obtained from mice injected with selected clones was used

as sources of antibodies. Those employed in this study have been identified as follows: 1D1 and 2D8, both of which react against apoB 48 and apoB 100, and 3F5, 4G3, 5E11, and 3A10 which react only with apoB100 and inhibit the interaction of LDL with its receptor (7,8).

Competitive Radioimmunoassays of apoB: Solid phase radioimmunoassay was performed in Removawells (Dynatech Laboratories, Alexandria, VA) as follows. The wells were coated by an overnight incubation with 200 ul of reference LDL (30 ug/ml in 5 mM glycine buffer, pH 9.2) and subsequently saturated by incubation for 1 h with 250 ul of 1% bovine serum albumine (BSA) in PBS, pH 7.4. Each antibody appropriately diluted in BSA-PBS solution was incubated overnight with dilutions of either the standard reference LDL or the various LDL subfractions at final concentrations ranging from 0.1 to 10 ug of LDL protein/ml in BSA-PBS in disposable culture tubes. 200 ul aliquots of these mixtures were then added to the wells which had been washed with 0.15 M NaCl contained 0.025% Tween 20. The wells were incubated overnight and again washed with the Tween-saline solution as above. Rabbit anti-mouse IgG was labeled with ^{125}I, purified as described earlier (7), and diluted in BSA-PBS before use. 200 ul of this solution representing about 60,000 cpm was added to each well and incubated overnight. The wells were washed with the Tween-saline solution as above and counted for radioactivity. The intra-assay coefficient of variation for the measurement of LDL apoB was 10% or less regardless of the antibody used.

Analyses: Protein concentration of the LDL subfraction was measured by the method of Lowry et al. (9) using bovine serum albumin as a standard. Triglyceride and cholesterol concentrations were measured by enzymatic methods (Triglyceride and Cholesterol Kits, Beckman Instrument Corp., Calif.) High density lipoprotein (HDL) cholesterol was determined after heparin-manganese precipitation of plasma (10), adapted for enzyme assay by the addition of 8 mmol/litre EDTA to the reagent. LDL cholesterol was calculated by substrating HDL cholesterol from d > 1.006 g/ml infranate cholesterol (11). Plasma LDL apoB was determined by radial immunodiffusion method (12). SDS-polyacrylamide gel electrophoresis was carried out according to Kane et al. (13).

RESULTS

In each subject, LDL was recovered over 98% in the last three fractions after equilibrium density gradient ultracentrifugation. These were labeled as fraction 1 (the buoyant LDL and located just at the meniscus), fraction 2 (the dense LDL), and fraction 3 (the very dense LDL). The cholesterol to apoB ratio was determined for each LDL subfraction in each subject (Table 2) and was highest in fraction 1, lowest in fraction 3, and intermediate in fraction 2. That is, as the density of the LDL subfraction increased within a subject, the LDL cholesterol to apoB ratio decreased. The average density of the solution in fraction 1 after centrifugation was approximately 1.040 g/ml, that in fraction 2 aproximately 1.050 g/ml, and in fraction 3 approximately 1.060 g/ml.

Next, the immunoreactivities of the 3 LDL subfractions in each subject were examined with each of the 6 monoclonal antibodies. Typical binding curves in a single subject are shown in Fig. 1. Note that in each instance the displacement curves of the 3 LDL subfractions interacting with any particular monoclonal antibody are parallel. However, in some instances, most obvious with 3F5 in this subject, there is considerable difference in the displacement obtained with the 3 LDL subfractions whereas with other antibodies, for example 1D1 and 3A10, there is little difference.

From these displacement curves, the immunoreactivity of the LDL subfractions to the 6 monoclonal antibodies was calculated as the concentration of protein necessary for 66% displacement of the maximum binding of the monoclonal antibody to the immobilized reference control LDL. These results are summarized in Table 3. Immunoreactivity of the 3 LDL subfractions differed little with antibodies 1D1, 5E11, and 3A10 but differed substantially with 2D8, 3F5, and 4G3. With the last 3 antibodies, fraction 1, the buoyant LDL, was significantly more immunoreactive than fraction 3, the very dense LDL. Indeed, about 190, 179, and 47% more LDL protein was required to obtain the same displacement with fraction 3 as fraction 1 using 2D8, 3F5, and 4G3, respectively. Fraction 2 tended to be less immunoreactive than fraction 1 (for 2D8 and 3F5 but not for 4G3) but more immunoreactive than fraction 3 (for 3F5 and 4G3 but not 2D8).

This type of analysis presumes each of the 3 subfractions is the same in each of the 14 individuals, but as the data in Table 2 indicate this is clearly not the case. That is, while LDL

particles differ within an individual, there are differences among individuals as well. Therefore, in Fig. 2, the LDL cholesterol to apoB ratio is plotted against the amount of LDL protein necessary for 66% displacement. For 1D1, 5E11, and 3A10, no strong correlation is apparent and the p values are > 0.05. But for 2D8, and particularly for 3F5 and 4G3, there are strong significant correlations ($p < 0.05$) between LDL immunoreactivity and LDL cholesterol to apoB ratio, indicating that as the LDL particles become denser, these specific determinants become proportionately less immunoreactive. These differences in immunoreactivity could not be ascribed to any apparent differences among the LDL subfractions of a given subject in their respective apoprotein compositions as judged by SDS-polyacrylamide gel electrophoresis and densitometric scanning of the stained gels. When in some subjects, apoB74 and apoB26 were noted in addition tc apoB100 (13), these same fragments of apoB were present in all of the 3 LDL subfractions (results not shown). Also, the presence of apoB fragments was not associated with any lipoprotein phenotype of the patients, that is type IV and/or hyperapobetalipoproteinemia, and, therefore, was not more prevalent in subjects with the dense LDL. In addition, these electrophoreses demonstrated that none of the LDL subfractions were contaminated with lower molecular weight proteins.

DISCUSSION

LDL particles are heterogeneous, differing in lipid content and, therefore, differing in size and density. The present

study indicates that the immunoreactivity of certain specific apoB antigenic determinants varies as a function of LDL composition and, therefore, suggests a possible linkage between altered LDL composition and LDL metabolism.

We chose LDL cholesterol to apoB ratio as our index of LDL heterogeneity since there is general agreement that this ratio decreases when LDL particle size and density increase (1-3). If apoB content/LDL particle is constant, as most evidence indicates (15), then as LDL size decreases, the conformation of the protein would be expected to change. In addition, because surface lipid to protein ratios decrease (1-3), the spatial relationship of the phospholipids and free cholesterol to apoB antigenic determinants must also change. The recent published human apoB structure suggests that apoB100 polypeptide may be in and out of the lipid environment of the lipoprotein particle at irregular intervals, which indicated the possibility of numerous lipid anchoring sites (14). This supports our speculation that the changes of lipid to protein relationship must mask or unmask the apoB antigenic determinants or change the apoB conformation.

Among the 6 antigenic determinants studied, the strength of the relationship between immunoreactivity and LDL composition varied, we speculated that this could indicate the specific conformational changes occurred which differentially affected certain determinants as particle size diminished. Two of the determinants showing the strongest relations (3F5 and 4G3) have been shown to be adjacent to one another in cotitration experiments (7) and on tryptic LDL fragments (16), while 2D8 and 4G3

have been found together on small fragments of soluble apoB obtained by CNBr cleavage (32), thus demonstrating the proximity of these 3 determinants on LDL apoB. Furthermore, these 3 determinants in general have exhibited the same requirements for lipids when solubilized apoB was incorporated into recombinant lipid vesicles (17). The antibodies 2D8, 3F5, and 4G3 were all antigenically active upon incorporation of apoB into lecithin-cholesteryl oleate microemulsions (17), which are characterized by a hydrophobic core and so mimic LDL structure (18,19). Thus it appears that it is the conformation of a specific region of apoB that changes as the LDL particles become smaller and denser.

It is possible that changes in lipoprotein or apolipoprotein structure associated with preparation of the lipoprotein fractions could have contributed to the differences in immunoreactivity shown here. However, with SDS electrophoresis we have not noted differences in the major apolipoprotein B components among the fractions and have found no peptide fragments indicative of possible proteolysis. While the denser LDL fraction may have been contaminated with lipoprotein Lp(a), previous studies have shown that it is unlikely to be a significant component in fractions of $d < 1.050$ g/ml (2), and, therefore, such contamination could not account for the differences in immunoreactivity between subfractions 1 and 2 or the linear relationship of immunoreactivity with cholesterol to apoB ratio across the particle spectrum.

Because the antibodies directed against 3F5 and 4G3 are also capable of interfering with the binding of LDL to the apoB/E receptor of fibroblasts (7), this region may also be important in

regulating LDL catabolism. Turnover studies in humans using ^{125}I-VLDL and ^{131}I-LDL have shown a precursor-product relationship between VLDL and IDL, between IDL and buoyant LDL, and between buoyant LDL and dense LDL (20). This is the case both in normal subjects and in patients with hyperapobetalipoproteinemia. In both situations as well, the fractional catabolic rate of dense LDL is significantly less than that of buoyant LDL, it would, therefore, appear that as LDL particles become lipid depleted and denser, there are conformational modifications of certain determinants of apoB, especially 2D8, 3F5, and 4G3, which consequently may decrease the fractional catabolic rate of the dense LDL.

The antigenic determinants recognized by antibodies 5E11 and 3A10 are also located close to the receptor recognition site on the LDL apoB (7); however, the reactivities of these determinants show no correlation with the cholesterol to apoB ratio of LDL. In previous studies designed to characterize apoB antigenic determinants, these 2 determinants were found to be poorly immunoreceptive upon delipidation of apoB (8). Upon equilibriation of apoB with lecithin-cholesteryl oleate microemulsions, 5E11 regained only partial activity while 3A10 remained inactive (17). It appears, therefore, that these determinants are highly susceptible to modifications of the LDL apoB conformation, and it may be that the labile nature of the immunoreactivity does not allow observation of correlations with LDL size and density.

This modulation of apoB antigenic determinants in LDL subfractions may be a consequence of either conformational modification of apoB by lipoprotein structure and composition or due

to genetic polymorphism of apoB. Several reports link apoB immunoreactivity to the composition of apoB-containing lipoproteins. Polyclonal antisera directed against apoB were first reported able to differentiate VLDL of various sizes (21), and certain monoclonal antibodies can also distinguish VLDL, IDL, and LDL (22-25). Most of the determinants studied by Tikkanen et al. exhibited increased immunoreactivities with decreased VLDL flotation rates and the determinants were more active with LDL and with VLDL (22). In addition, the immunoreactivities were influenced by LDL lipid compositions also (33). Tsao et al. (24), using a different battery of monoclonal antibodies, defined distinct patterns of antigenic determinant expression in VLDL, IDL, and LDL. These observations suggest that apoB conformation in VLDL, IDL, and LDL is similar but not identical. Marcel et al. observed earlier that the determinants recognized by antibodies 1D1, 3F5, 4G3, 5E11, and 3A10 reacted less with VLDL than with LDL and that their immunoreactivity could be increased by partial delipidation (25). Therefore, it appears that a number of apoB antigenic determinats are either masked or structurally modified in VLDL and that their immunoreactivity increass as VLDL particles are transformed into LDL through the normal metabolic sequence. In keeping with the data presented here, the immunoreactivity of determinants such as 3F5 and 4G3 reaches a maximum with LDL particles of a defined hydrated density and thereafter decreases as these particles become denser, possibly as a result of the conformation constraints exerted on apoB. This interpretation is supported by the results of Mao et al. (26) who found LDL immunoreactivity to vary as a function of temperature,

an observation compatible with modulation of LDL immunoreactivity by lipid composition and content, since temperature changes should result in modification of lipoprotein conformation and fluidity. Finally it is unlikely that partial proteolysis may be the cause of the decreased immunoreactivity of 2D8, 3F5, and 4G3 with increasing LDL density since apoB74 and apoB26, when present in a given subject LDL, were equally distributed in the 3 LDL subfractions. In addition, the determinants recognized by our antibodies were not found to be susceptible to proteolysis (16).

Alternatively, or simultaneously, we must also consider that this modulation of apoB antigenic determinants could be related to genetic polymorphism of apoB. Immunochemical polymorphism of human apoB was demonstrated by Blumberg et al. (27) and by Butler and Brunner (28) while Fisher et al. (29) reported that LDL sizes are determined genetically. More recently immunochemical polymorphism of LDL apoB was demonstrated by the reduced binding of 3 monoclonal antibodies which allowed LDL donors from different families to be separated (30). With genomic restriction fragment a common DNA polymorphism within the apoB gene coding sequence is reported to be associated with altered triglyceride and cholesterol levels (31). Therefore, genetic polymorphism must also be included as a possible interpretation of the reduced immunoreactivity of antibodies 2D8, 3F5, and 4G3 with dense LDL, especially in the dense LDL found in hyperapobetalipoproteinemia (3).

In conclusion, the present study has shown that the immunoreactivity of certain specific antigenic determinants var-

ies directly with LDL composition and more specificially with a parameter proportional to LDL size and density. Of special interest is the fact that two of these determinants which are predictably affected by LDL apoB conformation are located near the receptor recognition site on LDL apoB (7). This observation together with the differential fractional clearance rates from plasma which have been demonstrated for buoyant versus dense LDL (20) leads to the hypothesis that LDL affinity for the apoB/E receptor may be affected by the density and size of LDL particles. Future experiments must be designed to test this hypothesis and to verify whether a correlation may be found between the immunoreactivity of the determinants 3F5 and 4G3 and LDL affinity for its cellular receptors.

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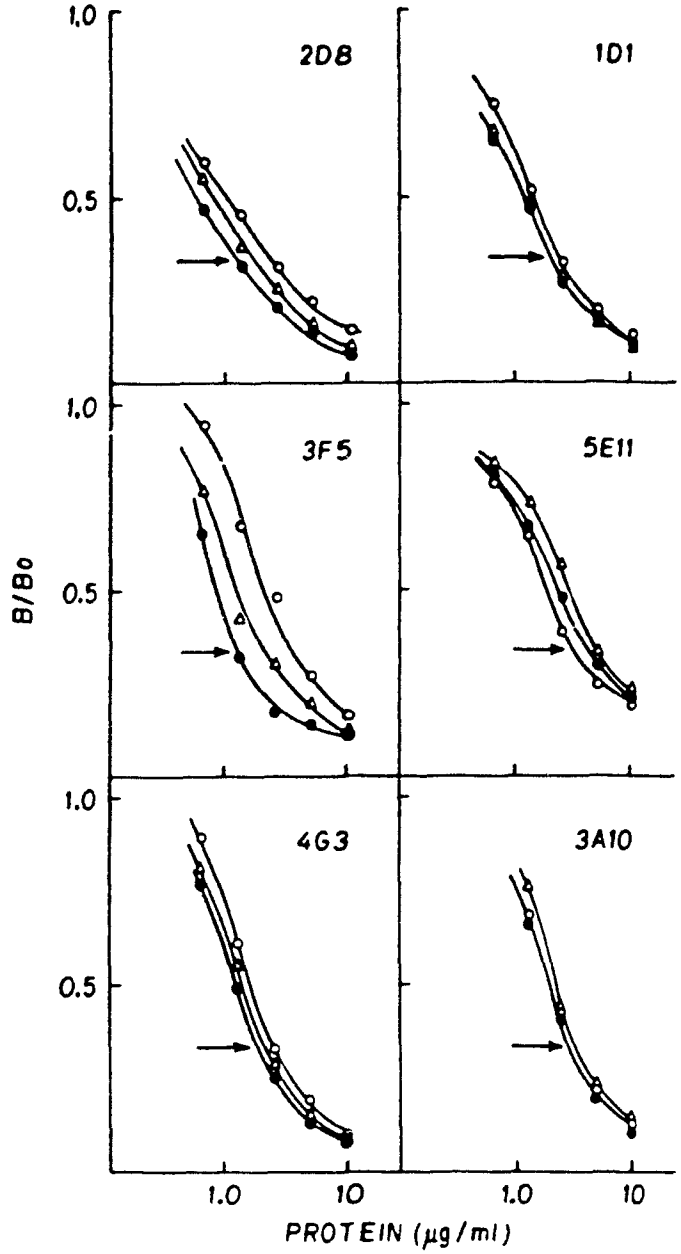

Figure 1. Comparison of the displacement curves obtained in competitive radioimmunoassays with LDL subfractions 1 (o---o), 2 (Δ---Δ), and 3 (•---•) from a single subject with the different antibodies as noted. The reference LDL were fixed on the wells, and the arrow indicates the position of the 66% displacement which was used as an expression of the relative antigenicity of each LDL fraction in Fig. 2.

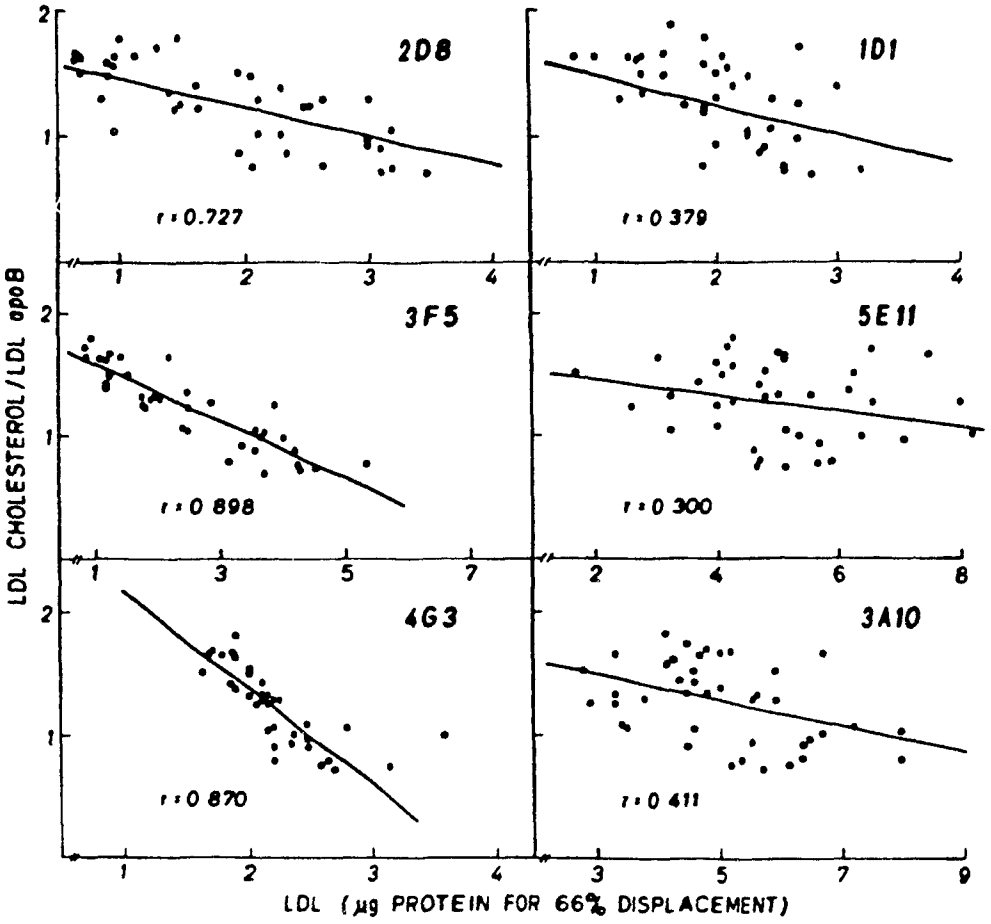

Figure 2. Correlations between the LDL cholesterol to LDL apoB ratio of each LDL subfraction of each subject and their respective immunoreactivity with the different antibodies expressed as the amount of protein of each LDL subfraction necessary for 66% displacement of a given antibody to the immobilized reference LDL.

Table 1. Clinical and biochemical data of the subjects studied

Patient no.	Age	Sex	Clinical status	ApoE pheno-type	TC	TG	HDL-C	LDL-C	LDL apoB
					mg/dl plasma				
1	53	M	CAD	E4/3	218	201	35	139*	142
2	49	M	N	E3/2	216	135	47	148	110
3	64	F	CAD	E3/3	179	108	55	114	108
4	63	M	CAD	E3/2	176	109	46	121	89
5	57	M	CAD	--	218	326	18	135	163
6	59	M	CAD	E3/3	202	110	38	150	114
7	59	M	CAD	E3/3	284	334	20	197	146
8	60	M	CAD	E4/3	266	130	70	186	142
9	62	M	CAD	E3/3	195	146	48	132	104
10	61	M	CAD	E3/2	234	245	35	159	135
11	65	M	CAD	E3/3	214	93	45	152	101
12	47	M	N	E4/3	187	101	44	125	85
13	63	M	N	E3/2	179	123	69	93	77
14	42	M	N	E3/3	204	113	41	140	85

* The LDL cholesterol values are calculated from d > 1.006 g/ml infranate cholesterol minus HDL cholesterol.

CAD, Clinical coronary artery disease; N, no clinical coronary artery disease; TC, total cholesterol; TC, triglyceride; HDL-C, High density lipoprotein cholesterol; LDL-C, Low density lipoprotein cholesterol; LDL apoB, Low density lipoprotein apolipoprotein B; ApoE, apolipoprotein E

Table 2. Cholesterol to apoB ratio in LDL subfractions

Cholesterol to apoB ratio is calculated from the cholesterol and protein values which are measured directly on the LDL subfractions.

Patient no.	cholesterol/apoB ratio		
	1*	2	3
1	1.51	1.27	0.88
2	1.66	1.37	1.00
3	1.06	0.89	0.78
4	1.68	1.33	0.80
5	1.52	0.93	0.75
6	1.63	1.28	1.00
7	1.60	1.25	0.74
8	1.56	1.41	1.08
9	1.65	1.28	0.78
10	1.53	1.14	0.71
11	1.73	1.41	1.04
12	1.57	1.32	1.05
13	1.66	1.43	0.89
14	1.64	1.32	1.24

* The numbers 1, 2, and 3 represent LDL subfractions 1, 2, and 3.

Table 3. Summary of the mean immunoreactivities of the LDL subfractions from normal subjects and hyperapobetalipoproteinemic patients

The mean of the concentrations of individual LDL subfractions is expessed in ug protein/ml from the different subjects necessary for 66% displacement of maximal binding of the monoclonal antibody to the immobilized control LDL.

	Antibody								
	2D8			3F5			4G3		
	1*	2	3	1	2	3	1	2	3
Mean	1.0	2.1	2.9	1.4	2.2	3.9	1.7	2.1	2.5
SD	0.4	0.6	1.0	0.7	1.0	1.3	0.3	0.1	0.5
Paired t test	0.05	NS		0.05	0.02		NS	0.05	
	0.02			0.02			0.05		

	1D1			5E11			3A10		
	1	2	3	1	2	3	1	2	3
Mean	1.8	2.1	2.3	4.7	5.5	5.1	5.0	4.9	5.8
SD	0.4	0.5	0.6	1.5	1.8	1.2	1.3	1.0	1.6
Paired t test	NS	NS		NS	NS		NS	NS	
	NS			NS			NS		

* The numbers 1, 2, and 3 indicate LDL subfractions.
NS, not significant.

CHAPTER 4

METABOLIC BASIS OF HYPERAPOBETALIPOPROTEINEMIA: TURNOVER OF APOLIPOPROTEIN B IN LOW DENSITY LIPOPROTEIN AND ITS PRECURSORS AND SUBFRACTIONS COMPARED WITH NORMAL AND FAMILIAL HYPERCHOLESTEROLEMIA

ABSTRACT

The turnover of apolipoprotein B (apoB) in very low density, intermediate density, and low density lipoproteins (VLDL, IDL, and LDL) and in the buoyant and dense fractions of LDL was determined in 7 patients with hyperapobetalipoproteinemia (HyperapoB), 6 normolipidemic subjects, and 5 patients with heterozygous familial hypercholesterolemia (FH). After receiving an injection of ^{125}I-VLDL, HyperapoB patients were found to have a higher rate of synthesis of VLDL-apoB than controls (40.1 vs. 21.5 mg/kg per d, $p < 0.05$) but a reduced fractional catabolic rate (FCR) (0.230 vs. 0.366/h, $p < 0.01$). After receiving an injection of ^{131}I-LDL, HyperapoB patients had higher rates of LDL-apoB synthesis than controls (23.1 vs. 13.0 mg/kg per d, $p < 0.001$), as did FH patients (22.7 mg/kg per d). The FCR of LDL was similar in HyperapoB patients and controls (0.386 vs. 0.366/d) but was markedly decreased in FH patients (0.192/d). Most subjects exhibited precursor-product relationships between VLDL and IDL, and all did between IDL and buoyant LDL; an analogous relationship between buoyant and dense LDL was evident in most HyperapoB patients and controls but not in FH patients. Simultaneous injection of differentially labeled LDL fractions and deconvolution analysis showed increased buoyant LDL synthesis with normal conversion into dense LDL in HyperapoB, whereas in FH conversion of buoyant LDL was reduced and there was independent synthesis of dense LDL. These data show that the increased concentration of LDL-apoB in HyperapoB is solely due to increased concentration of LDL synthesis, which is secondary to increased VLDL synthesis; in contrast, in FH there is both an increase in

synthesis of LDL (which is partly VLDL-independent) and reduced catabolism.

INTRODUCTION

Evidence that low density lipoprotein (LDL) is heterogenous in terms of particle size and composition has been published by several groups of workers, using a variety of methods of separation (1-6). These studies have shown that LDL can be separated into 3 to 5 subfractions in normal subjects (1,3-6) and patients with type IV hyperlipoproteinemia (2) in whom, as pointed out by Fisher (7), heterogeneity seems especially marked. Incubation of LDL with postheparin plasma results in a shift in the distribution of LDL from less dense into denser subfractions (1), which suggests the conversion of larger into smaller particles consequent on lipolysis (3,5). Kinetic studies by Phair et al. (8) provide additional support for the existence of precursor-product relationships within the spectrum of LDL particles.

Recently we described a simple method of separating LDL into two major subfractions, fraction 1 or buoyant LDL, and fraction 2 or dense LDL, using discontinuous density gradient ultracentrifugation, and we documented differences in the densities and composition of these fractions between normal subjects and patients with increased plasma levels of LDL-apolipoprotein B (apoB) (6). The latter fell into two categories: patients with familial hypercholesterolemia (FH), in whom fraction 1 was cholesteryl ester-enriched and less dense than normal, and patients with hyperapobetalipoproteinemia (HyperapoB), in whom fraction 2 was cholesteryl ester-depleted and denser than normal. As defined previously (9,10), the term HyperapoB denotes a disproportionately increased concentration of LDL-apoB in

plasma in the face of a normal concentration of LDL cholesterol, often accompanied by hypertriglyceridemia and associated with coronary heart disease (CHD). An increase in LDL-apoB, hypertriglyceridemia, and a predisposition to CHD are also features of familial combined hyperlipidemia (11), with which HyperapoB undoubtedly overlaps in some instances. The relationship between these two entities and the possibility that an abnormality of apoB metabolism is common to both has recently been discussed in detail elsewhere (12).

In the present study the turnover of apoB within the main subfractions of LDL and in its precursors, very low density lipoprotein (VLDL) and intermediate density lipoprotein (IDL), has been investigated in normal subjects, patients with HyperapoB, and patients with heterozygous FH; a major objective was to determine the mechanisms responsible for the increased levels of LDL-apoB and contrasting changes in LDL composition, which distinguish these two disorders.

SUBJECTS AND METHODS

Studies were performed in 17 male subjects, 7 patients with HyperapoB, 4 FH heterozygotes, 6 normal controls, and 1 female FH heterozygote (patient 18). Subjects 7, 11, and 14-18 were studied in London and the remainder were studied in Montreal, but the analyses of all the subjects were determined in Montreal. Their clinical and biochemical details are shown in Table 1. The cholesterol and triglyceride content of samples was determined enzymatically, each value representing the mean of

triplicate analyses of single samples or the mean of single estimates of two or more samples. LDL cholesterol was calculated by subtracting VLDL and high density lipoprotein (HDL) cholesterol from total cholesterol (13), except in the FH patients whose LDL cholesterol was calculated as described by Friedewald et al. (14) (see Table 1). HDL cholesterol was determined after heparin-manganese precipitation of plasma (15), adapted for enzyme assay by the addition of 8 mmol/liter EDTA to the reagent. LDL-apoB was assayed by radial immunodiffusion, using a method designed to minimize any contribution from VLDL apoB (16), as validated below. Upper limits of normality for serum triglyceride, LDL cholesterol, and LDL-apoB upon which lipoprotein phenotyping and the diagnosis of HyperapoB have been based were 200, 200, and 120 mg/dl, respectively, as defined previously (6,10). All subjects consumed their usual diets except during studies of VLDL turnover when their fat intake was restricted to < 5 g/d; meals were supplied by the diet kitchen during this 2-d period. Dietary intake was not assessed routinely but both groups of patients had received advice in the past aimed at achieving or maintaining ideal body weight and restricting the intake of saturated fat and cholesterol. None of the patients was on any lipid-lowering drug during the course of these studies with tne exception of FH patient 18, who was on cholestyramine during one LDL turnover study. HyperapoB patients 2 and 3 were on hydrochlorothiazide and patient 3 was also on propranalol but none of the other patients were on diuretics or ß-blockers. All were ambulant and maintained their customary exercise habits. Relative

body weight was within the normal range in all but two subjects, the exceptions being one HyperapoB and one FH patient, who were slightly obese. These studies were sanctioned by the Research Ethics Committees of the Royal Postgraduate Medical School and Hammersmith Hospital, London, and the Royal Victoria Hospital and McGill University, Montreal, and all subjects gave informed consent.

Measurement of LDL-apoB: This was performed as previously described (16); the samples were allowed to diffuse radially for 18 h into a 1.5% agarose gel containing a rabbit antibody against human LDL of d 1.025-1.050 g/ml. To ascertain whether a significant contribution to the diameter of the rings was made by the apoB in VLDL and IDL, as has been claimed to occur with hypertriglyceridemic samples (17), two approaches have been followed: first, in Montreal, retrospective analysis of all the previous results that have records of patients' total cholesterol, triglyceride, plasma LDL apoB, and d > 1.019 g/ml LDL apoB; secondly, in London, blood samples were obtained from 50 fasting subjects undergoing health screening in London.

All subjects from the latter study had a serum cholesterol of < 260 mg/dl, 31 of which had a serum triglyceride of < 177 mg/dl, and 19 of which had a triglyceride of > 220 mg/dl, the highest value being 526 mg/dl. A 4-ml aliquot of each subject's serum was adjusted to d 1.019 g/ml and ultracentifuged. The concentration of LDL-apoB was compared in each serum and its corresponding d > 1.019 g/ml fraction; the respective values were 106±19 vs. 100±19 mg/dl in normotriglyceridemic samples and

120±23 vs. 113±26 mg/dl in hypertriglyceridemic samples. In each group 94% of the apoB in serum was accounted for by apoB in the corresponding d > 1.019 g/ml fraction. Using an LDL apoB of 120 mg/dl or above as a criterion, four of the normotriglyceridemic group had HyperapoB when serum was assayed, vs. three who had HyperapoB when the d > 1.019 g/ml fraction was assayed; in the hypertriglyceridemic group the corresponding numbers were 11 vs. 8. Similar results were found from the former retrospective analysis; all subjects (n=66) had total cholesterol ranging from 130-320 mg/dl, 19 had triglyceride level which varied from 210-600 mg/dl, and 47 had triglyceride levels ≤ 200 mg/dl. The concentration of LDL apoB in plasma and its corresponding d > 1.019 g/ml fraction were 111±34 vs. 116±33 mg/dl in normotriglyceridemic samples and 137±25 vs. 140±26 mg/dl in hypertriglyceridemic samples. In each group over 95% of the apoB in plasma was accounted for apoB in the corresponding d > 1.019 g/ml fraction. These data from two laboratories and two geographic sources confirm that the contribution of VLDL and IDL-apoB to the measurement of LDL-apoB in plasma by RID is slight in our hands and is no greater in hypertriglyceridemic than in normotriglyceridemic samples. It is possible, however, that the LDL-apoB in plasma or serum might be significantly overestimated by RID at triglyceride levels of > 1,000 mg/dl (17).

Family studies: Data was obtained on other family members of three of the seven HyperapoB patients. Patient 1, who had transiently exhibited a type IV phenotype in the past, had a mother and brother with elevated LDL cholesterols and type IIa

phenotypes, a daugher age 4 yr with a plasma LDL-apoB of 137 mg/dl, and a son age 7 yr with a plasma LDL-apoB of 98 mg/dl, both with normal LDL cholesterols and lipoprotein phenotypes. LDL-apoB levels were 138-205 mg/dl in one son and three other daughters, age 19-26 yr, of patient 3 and were 89-111 mg/dl in three other daughters age 14-16 yr. This patient's wife had a value of 197 mg/dl. All these individuals had normal LDL cholesterols and lipoprotein phenotypes. Patient 5 had three adult first degree relatives with LDL-apoB levels of 122-129 mg/dl, a daughter age 14 yr with a value of 155 mg/dl, and a wife with a value of 172 mg/dl, all with normal LDL cholesterols and lipoprotein phenotypes. Thus, three families showed evidence of HyperapoB in first degree relatives, both adults and children; and in two families HyperapoB was present in a spouse.

Of the FH heterozygotes, patient 16 had three hypercholesterolemic first degree relatives and the other four patients had tendon xanthomata, including patient 15, who had a type IIb phenotype.

Turnover of apoB: The turnover of apoB in VLDL, IDL, and LDL was determined by simultaneously injecting ^{125}I-VLDL and ^{131}I-LDL, using standard techniques to isolate and label the lipoprotein. After an overnight fast, blood (50-60 ml) was obtained in EDTA tubes (1 mg/ml) from each subject for isolation of VLDL and LDL. VLDL ($d < 1.006$ g/ml) was isolated from the plasma by sequential ultracentrifugation, using a Beckman Ti 50 rotor at 40,000 rpm for 18 h. The VLDL collected was then washed and concentrated by centrifugation in a Beckman SW50.1 rotor at

40,000 rpm for 16 h. The concentrated VLDL was then ready for radioiodination. After removing the IDL fraction (d 1.006-1.019 g/ml) by ultracentrifugation, the density of d > 1.019 g/ml infranate was adjusted to d 1.071 g/ml by addition of NaCl/KBr solution, and the infranate was centrifuged for 16 h in a Beckman SW50.1 rotor at 40,000 rpm. The supernatant LDL was collected, washed, and concentrated as described above. The VLDL and LDL fraction prepared as above were used for labelling with ^{125}I and ^{131}I, respectively, by the iodine monochloride method (18). Free iodide was removed by chromatography on a Sephadex G-25 column and exhaustively dialysed against 0.01% EDTA/0.15 M NaCl solution. The amounts of ^{125}I and ^{131}I injected averaged 79 and 58 uCi, respectively. Lipid labeling was 20% for VLDL and < 5% for LDL. All patients received oral potassium iodide, 180 mg/day throughout the 2 weeks of the study period. Those undergoing VLDL turnovers were placed on an isocaloric, low fat diet for 48 h after receiving ^{125}I-VLDL. As a rule ^{131}I-LDL was injected 10 min after ^{125}I-VLDL, after an overnight fast. In some instances buoyant and dense fractions of LDL were isolated, as described below, labeled with ^{125}I and ^{131}I, respectively, and then re-injected.

Blood samples were taken into tubes with EDTA (1 mg/ml) at 2-4 hourly intervals during the first 24 h after the injection of labeled lipoproteins and at 12-24 hourly intervals for the next 9 days. Plasma was separated, stored at 4°C for 5-10 days, and then successively ultracentrifuged at d < 1.006 and d 1.006-1.019 g/ml in a Beckman Ti 50 rotor at 40,000 rpm for 18 h at 4°C. The VLDL and IDL were recovered after tube slicing for

analysis of apoB specific activities. This involved delipidation of samples with ether/methanol (3/1, V/V), followed by column chromatography on Sephadex G-150 and assay of the leading edge of the apoB peak, which elutes with the void volume, for radioactivity and protein content (19), as originally described by Sigurdsson et al. (20). Sample count rates ranged from 100 to 550,000 cpm above background. The fractional catabolic rate (FCR) of apoB in VLDL was calculated from area under the VLDL specific activity/time curve by multiexponential curve-fitting technique, computed graphically, and the absolute catabolic rate (ACR) was calculated as FCR x pool size and since these were steady state studies synthesis was taken to equal ACR (21). Wherever possible the proportion of VLDL converted to LDL was determined by deconvolution analysis (20). The turnover of apoB in IDL derived from VLDL was calculated by the method of Zilversmit (22) where the FCR of a product is the increase in its specific activity over any given period of time, divided by the corresponding area between the precursor and product specific activity/time curves. This method can be used only where a precursor/product relationship exists and was applied only in those instances where the IDL specific activity curve intersected the VLDL specific activity curve at or not more than 2 h before the peak of IDL specific activity was reached. The sizes of VLDL- and IDL-apoB pools were calculated as plasma volume x concentration of tetramethylurea-insoluble protein (23) in the d < 1.006 and d 1.006-1.019 g/ml fractions, respectively; protein was measured by the method of Lowry et al. (24). Plasma volume was estimated at 4.5% of body

weight rather than using the values obtained from the 10-min blood sample (20).

In two FH patients 'broad spectrum' IDL (S_f 12-60) rather than the 'narrow spectrum' IDL (S_f 12-20) was isolated. $S_f > 60$ was removed from plasma by ultracentrifugation in a Beckman Ti 50 rotor at 40,000 rmp for 2 h. The density of the infranate was then adjusted to d 1.019 g/ml with NaCl/KBr solution and subjected to centrifugation for 20 h at 40,000 rpm in a Beckman Ti 50 rotor (25). The supernatant (S_f 12-60) was recovered, washed, and concentrated as described above. S_f 12-60 was then labeled with ^{125}I (18) and injected into the patient. Its FCR was calculated by monoexponential analysis.

The FCR of LDL was calculated by two compartmental analysis of the plasma radioactivity/time curve, as described by Matthews (26), with the pool size of LDL being calculated as the mean LDL-apoB concentration of multiple samples of the d > 1.019 g/ml fraction x plasma volume, using radial immunodiffusion to determine LDL-apoB (16). ACR was calculated as FCR x pool size and since these were steady state studies synthesis was taken to equal ACR.

To obtain LDL subfractions, d > 1.019 g/ml samples were adjusted to d 1.071 g/ml with NaCl/KBr and centrifuged in a SW 50.1 rotor to isolate LDL of d 1.019-1.063 g/ml; a portion of this sample was then dialyzed to d 1.050 g/ml and 1 ml was centrifuged on a discontinuous density gradient in a SW50.1 rotor at 40,000 rpm for 40 h at 10°C (the temperature is critical since this influences the time taken for the gradient to reach equilibrium), as described in detail in Chapter 2 (6). At the

conclusion the gradient was fractionated, using a tube piercer, into three 1-ml fractions (fractions 3-5) followed by visual separation of dense LDL (fraction 2) usually recovered in approximately 1.25 ml (mean density, 1.0480 g/ml), and buoyant LDL (fraction 1), usually recovered in approximately 0.75 ml (mean density, 1.0405 g/ml). The ^{125}I and ^{131}I in fractions 1, 2, and 3 were then counted in a dual channel gamma counter, appropriately calibrated to minimize spillover, and their apoB and cholesterol content were assayed, as described above. The 5-10% of counts and apoB recovered in fraction 3 (mean density, 1.063 g/ml) were included with dense LDL. Quenching of ^{125}I in these fractions was assessed by an internal standard and seldom exceeded 10%.

After injection of LDL subfractions the FCR of buoyant and dense LDL were calculated by two compartmental analysis (26) of the radioactivity/time curves of apoB in the buoyant or dense fraction of LDL, pool sizes were calculated by multiplying the total LDL pool by the mean percentage of apoB recovered in buoyant or dense LDL, and turnover (ACR) was calculated as FCR x pool size of buoyant or dense LDL. The proportion of buoyant LDL converted into dense LDL was determined by deconvolution analysis (20). The independent input of dense LDL was calculated as turnover of dense LDL minus turnover of buoyant LDL x percent converted to dense LDL. These calculations were based on the premise that buoyant LDL can be converted into dense LDL but that the reverse does not occur. The statistical significance of differences between means was calculated by the two-tailed t test (Hewlett-Packard 65 Statistical Program; Hewlett-Packard Co.,

Palo Alto, CA).

RESULTS

The clinical and biochemical characteristics of the three groups of subjects are detailed in Table 1. The HyperapoB patients did not differ greatly from the normal controls with respect to age and relative body weight but all had CHD, documented angiographically. They had significantly higher plasma levels of total cholesterol, triglyceride (five having a type IV phenotype), and VLDL cholesterol than the controls ($p < 0.05$, < 0.05, and < 0.05, respectively) but there was no significant difference between the two groups with respect to LDL cholesterol and HDL cholesterol. However, the HyperapoB patients had significantly higher LDL-apoB levels than normal controls ($p < 0.001$). All but one (No. 17) of the FH patients had CHD, for which three had previously undergone coronary artery bypass grafting. Their total and LDL cholesterol levels were greatly increased, as was the concentration of LDL-apoB in plasma, which were significantly higher than normal control ($p < 0.001$, < 0.001, and < 0.001, respectively) but their HDL cholesterol values were within the normal range. VLDL cholesterol was not determined in FH patients but one of them had a raised triglyceride and type IIb phenotype.

Table 2 gives the distribution of apoB in the three fractions of LDL after density gradient ultracentrifugation and the cholesterol/apoB ratio of the two main fractions. The chief differences between the three groups are the reduced choles-

terol/apoB ratio of dense LDL (fraction 2) in HyperapoB patients which is significantly lower than the normal controls ($p < 0.01$) and the increased cholesterol/apoB ratio of buoyant LDL (fraction 1) in FH, which is significantly higher than the normal control ($p < 0.001$). In control subjects and HyperapoB patients, approximately two-thirds of LDL-apoB was found in fraction 2 and approximately one-fourth in fraction 1; the remaining 5-10% was recovered in fraction 3. Significantly more apoB was present in fraction 1, and less was present in fractions 2 and 3 in FH patients compared with the other two groups (FH vs. control of fractions 1 and 2, $p < 0.01$ and < 0.05, respectively). Only trace amounts of apoB were detectable in the bottom 2 ml of the gradient.

Kinetics of apoB: Quantitative data on apoB turnover in VLDL, IDL, and unfractionated LDL are shown in Table 3. Examples of turnover studies in individuals from each of the three groups of subjects are illustrated in Figs. 1-4.

VLDL-apoB turnover: HyperapoB patients had significantly higher rates of VLDL synthesis than normal subjects, a lower FCR, and a larger VLDL-apoB pool ($p < 0.05$, < 0.01, and < 0.01, respectively); these abnormalities were especially marked in the hypertriglyceridemic patients. VLDL turnover was measured in only one of the FH patients (No. 14) but his values were comparable with those obtained in normal subjects.

IDL-apoB turnover: The rate of synthesis of IDL-apoB (Sf 12-20) from VLDL-apoB could be calculated in only three HyperapoB

patients, three controls, and one FH patient, for reasons discussed below. In two other FH patients, patients 15 and 16, direct estimates of IDL turnover were obtained by injecting 'broad spectrum' IDL of S_f 12-60. Overall, the rates of synthesis, FCR, and pool size of IDL-apoB did not differ greatly between the three groups (Table 3). However, unlike the controls, IDL synthesis rates were considerably lower than the corresonding LDL synthesis rates in both groups of patients, irrespective of whether indirect estimates of S_f 12-20 or direct estimates of S_f 12-60 apoB turnover were calculated.

LDL-apoB turnover: The rate of synthesis of LDL-apoB, analyzed in the conventional two compartments after injection of labeled LDL into all subjects, was significantly higher in HyperapoB and FH patients than in controls ($p < 0.001$ and < 0.001, respectively); the FCR of HyperapoB patients was similar to controls, whereas that of FH patients was markedly reduced (FH vs. control, $p < 0.001$) (Table 3). The pool size of LDL apoB was even higher in FH than in HyperapoB patients, both being significantly greater than normal control ($p < 0.001$ and <0.01, respectively).

Precursor-product relationships between VLDL, IDL, and LDL subfractions: Fig. 1 shows the changes in specific activity of apoB in the plasma of a control subject after injection of ^{125}I-VLDL. The classical precursor-product relationship between specific activity/time curves, where the precursor (VLDL) should intersect the product (IDL) at or just before the latter reaches its maximum, was not observed in this subject nor in another

control and two HyperapoB patients, in all of whom the cross-over occurred 3 h or more before IDL specific activity had reached its peak. However, in the remainder (see Fig. 2, left), where the delay was less than 2 h, an attempt was made to calculate the synthesis of IDL from VLDL, using the method of Zilversmit (22) but accepting that the values obtained (Table 3) are only approximate estimates.

The proportion of VLDL-apoB converted into LDL-apoB, calculated by deconvolution analysis, averaged 58% (22-80%) in three control subjects compared with 49% (35-69%) in four HyperapoB patients. In one of the control subjects and two of the HyperapoB patients only 40-60% of the LDL synthesized could be accounted for on the basis of conversion of VLDL, but in the remainder all LDL was derived from VLDL.

Relationships between the specific activity/time curves of IDL and buoyant LDL are shown in Figs. 1 and 2 (left) and of buoyant and dense LDL in Fig. 1 (right). All subjects studied exhibited precursor-product relationships between IDL and buoyant LDL including the FH patients given ^{125}I-VLDL (Fig. 3) and ^{125}I-IDL (Fig. 4). However, in one control subject (No. 10) and one HyperapoB patient (No. 4) there was no precursor-product relationship between buoyant and dense LDL, as exemplified in Fig. 2 (right); in both these individuals LDL synthesis was only partially accounted for by conversion of VLDL, as noted above. The lack of any such relationship between buoyant and dense LDL was even more evident in the three FH patients in whom this was studied, irrespective of whether the buoyant LDL was originally

derived from injected VLDL (Fig. 3) or from injected IDL of S_f 12-60 (Fig. 4).

Turnover of LDL subfractions: The turnover of apoB in the buoyant and dense fractions of LDL was qualitatively assessed in nine subjects after an injection of unfractionated, singly labeled LDL, which was given primarily to measure total LDL turnover (see Table 3). As illustrated in Fig. 5, the early part of the specific activity/time curve for buoyant LDL decays faster and is more curvilinear than that of dense LDL in a control subject. These differences are accentuated in the HyperapoB patient but are much less evident in the FH patient. Quantitation of buoyant and dense LDL turnover was not possible during these studies because the proportion of buoyant LDL converted to dense LDL was not determined.

The turnover and interconversion of isolated LDL fractions was studied in six subjects who were injected with differentially labeled buoyant and dense LDL. As illustrated in Fig. 6 (left), there was a precursor-product relationship between buoyant and dense LDL when buoyant LDL was injected into a HyperapoB subject but the reverse did not occur when dense LDL was injected (Fig. 6, right). Similar findings were observed in a control subject in whom administration of intravenous heparin to stimulate lipolysis caused a transient dip in the specific activity/ time curve of buoyant LDL (Fig. 7, left), presumably reflecting conversion of unlabeled IDL into buoyant LDL, without affecting dense LDL turnover (Fig. 7, right). In FH, no precursor-product relationship was evident between buoyant and dense

LDL, confirming earlier studies in which VLDL or IDL had been injected (Fig. 8, left). Indeed, the cross-over of specific activities 4 days after injection of dense LDL (Fig. 8, right) suggests that some of the latter may be converted back to buoyant LDL in this disorder, though at a slow rate. Alternatively, it may have been due to an influx of unlabeled dense LDL.

Turnover rates of buoyant and dense LDL in the six subjects were determined by analysis of the respective radioactivity/time curves and the proportion of buoyant LDL converted to dense LDL was calculated by deconvolution analysis of the injected dense LDL and the dense LDL derived from injected buoyant LDL. Total LDL turnover was then derived, as shown under method A, Table 4. Four of these subjects also had total LDL turnover measured in the conventional method on a different occasion (method B), three under similar conditions to those of the previous study, whereas the fourth was on cholestyramine during study A but not during study B. In general, values of FCR and ACR for total LDL were lower when calculated by method B, despite similar pool sizes during the two sets of studies.

Synthesis (ACR), FCR, and conversion rate of buoyant LDL were highest in HyperapoB subjects whereas FCR and percent conversion were reduced in FH. FCR of dense LDL was also reduced in FH but synthesis (ACR) of dense LDL was increased as it also was in HyperapoB, although in FH the greater proportion was not derived from buoyant LDL. In one FH patient administration of cholestyramine (Questran) for clinical reasons resulted in a decrease in the size of the buoyant LDL pool.

DISCUSSION

The HyperapoB subjects in this study were characterized by plasma levels of LDL-apoB in excess of 120 mg/dl in the face of LDL cholesterol levels of 200 mg/dl or less. This combination was due to an increased concentration of dense LDL particles, denser than normal and with a reduced content of cholesteryl ester (6). These features and a decrease in the particle size tend to be especially marked in hypertriglyceridemic HyperapoB patients; five of our seven HyperapoB patients, all of whom had CHD, fell into that category. It was recently suggested that the RID assay we used over-estimates the concentration of LDL-apoB in hypertriglyceridemic plasma because of the diffusion into the gel of small VLDL and IDL particles containing apoB (17). However, we could not find any evidence of this since 94% of the apoB in 136 sera or plasma that we tested was attributable to LDL, both in normolipidemic and hypertriglyceridemic samples (see Methods). Another potential drawback to this method is the possibility that small LDL particles might diffuse faster into the gel than larger LDL particles, thus giving a spuriously high value in hypertriglyceridemic samples. However, comparison of the Lowry and RID methods of quantitating protein in buoyant and dense LDL, which differ in size, failed to demonstrate any bias of the RID assay toward dense LDL (6). Thus, over-estimatation of LDL-apoB by RID of plasma, at least in our hands, is relatively slight. Nevertheless, all the HyperapoB patients in this study had an LDL apoB of > 120 mg/dl as measured both in plasma and in its d > 1.019 g/ml fraction.

Although each of the three HyperapoB families we studied contained first degree relatives with raised LDL-apoB levels, only one family exhibited the pattern of multiple lipoprotein phenotypes, which is characteristic of familial combined hyperlipidemia (11). The latter disorder includes patients with increased levels of LDL cholesterol and type IIa and IIb phenotypes, whereas such individuals were, by definition, excluded from the present study. Furthermore, LDL-apoB levels are often but not invariably raised (> 120 mg/dl) in familial combined hyperlipidemia (11). VLDL-apoB synthesis is known to be increased in patients with the latter disorder (27-29), and a similar increase was evident in most of our HyperapoB patients, especially if they were hypertriglyceridemic. The area under the curve method of calculating VLDL-apoB turnover, which we used, gives a lower but more accurate estimate of synthesis than monoexponential analysis, which fails to take into account the slow turnover tail of the specific activity/time curve (30). The nearly twofold increase in VLDL-apoB synthesis in HyperapoB patients was accompanied by a marked decrease in FCR and an expanded VLDL pool.

LDL-apoB synthesis was also nearly twice normal in the HyperapoB patients as was the size of the LDL pool. Similar results were obtained by Kesaniemi and Grundy (31), who found a strong correlation between LDL synthesis and the concentration of LDL-apoB in plasma. In both studies the FCR of LDL was normal in HyperapoB subjects. The same authors recently described another group of patients with CHD and overproduction of LDL, whose LDL-apoB levels in plasma remained normal because of an increase in

FCR; these, they suggest, might represent normolipidemic variants of familial combined hyperlipidemia (32). They then reported another group of patients with CHD and overproduction of LDL. The LDL apoB levels in plasma in these patients were normal although the FCR of LDL was similar to normal controls (33). Thus it would seem that oversynthesis of apoB with normal or even increased LDL catabolism is common to both familial combined hyperlipidemia and HyperapoB. This contrasts with FH where increased synthesis of LDL is accompanied by a decrease in FCR, as observed in this and previous studies (18,34).

Since HyperapoB exhibits similar phenotypic features to familial combined hyperlipidemia and both disorders seem to have the same metabolic defect, namely overproduction of apoB, this suggests that they may be identical. However, familial combined hyperlipidemia is considered to be a dominantly inherited disorder (35), whereas the patttern of distribution of HyperapoB within families is compatible with polygenic inheritance (36). One large Amish pedigree with HyperapoB and sitosterolemia was identified through a single gene factor that accounted for the ratio of LDL cholesterol to LDL apoB (37). Therefore, until such time as the genetic defect or defects responsible have been identified it seems reasonable to keep an open mind as to whether HyperapoB represents a subgroup of familial combined hyperlipidemia or whether it represents a phenotypically similar but genetically distinct disorder.

The increase in LDL synthesis in HyperapoB is accompanied in most instances by an increase in VLDL apoB synthesis, as

discussed above. Based on the estimate that almost 50% of VLDL was converted into LDL, this increase in VLDL synthesis accounted for most of the increase in LDL synthesis in HyperapoB. The absence of any increase in IDL synthesis in those HyperapoB patients in whom this could be calculated suggests that increased synthesis of LDL in this disorder seemingly involves direct conversion of VLDL to LDL, as has been postulated in hypertriglyceridemic subjects (38) and suggested by multicompartmental analysis for the existing of a sequestered IDL pool not detected in plasma (39). In the former study approximately 20-30% of labeled VLDL was converted directly tc LDL without appearing in IDL, while a similar proportion was converted to LDL via IDL, the remainder being completely catabolized (38). These several fates of VLDL presumably reflect its metabolic heterogeneity in hyperglyceridemic subjects, as has also been shown by others (40). However, the possibility of a significant contribution to LDL-apoB levels being derived from a VLDL-independent synthetic pathway in HyperapoB cannot be excluded, especially in those subjects in whom a precursor-product relationship between buoyant and dense LDL was not demonstrable and in whom total LDL synthesis exceeded that derived from VLDL. Finally, the specific activity/time curves of IDL provide useful qualitative information, but accurate quantitation of IDL turnover is dependent upon a valid estimate of pool size, which may be underestimated if S_f 12-20 concentrations of apoB are low or losses are high due to the methodology limitation. These are the possible explanations of why IDL synthesis rates were often lower than those of LDL.

The discrepancy between IDL and LDL synthesis persists even when 'broad spectrum' IDL (S_f 12-60) was directly injected into two FH patients. This observation, together with the lack of any precursor-product relationship between buoyant and dense LDL in all five FH patients, supports the concept of a VLDL- and IDL-independent source of LDL in this disorder (34). Janus et al. (41) studied FH heterozygotes, in addition quantitating the proportion of VLDL converted to LDL. Their results suggested that 20-72% of LDL was synthesized independently of VLDL. More recently Soutar et al. showed reduced clearance of IDL in FH (42) and a similar finding was later reported in Watanabe Heritable Hyperlipidemic (WHHL) rabbits by Kita et al. (43). The latter study showed that reduced clearance of IDL resulted in increased formation of LDL which, they considered, was secondary to the LDL receptor deficiency that characterizes the WHHL rabbit as well as its human counterpart. There seems little doubt that a similar mechanism could contribute to increased LDL synthesis in FH patients but this does not exclude the possibility that 'direct' secretion of LDL also occurs. Eaton et al. (44) demonstrated a major 54% of LDL apoB in heterozygotic FH is derived from non-IDL precursors, the remaining 46% is derived from IDL. They then suggest two pathways of production of LDL apoB in FH and support the concept of direct synthesis of LDL apoB independent of IDL catabolism. Furthermore, liver perfusion studies in pigs (60) and nonhuman primates (61) also demonstrated direct hepatic secretion of LDL particles.

Our studies not only explain the increase in LDL-apoB levels in HyperapoB but also have some bearing on the mechanism

whereby buoyant LDL is converted to dense LDL. This process is accentuated in HyperapoB, especially in hypertriglyceridemic subjects in whom dense LDL is denser, smaller, and more depleted of cholesteryl ester than in control subjects or normotriglyceridemic HyperapoB patients (6,45). In vitro, the exchange process of triglyceride and cholesteryl ester between lipoproteins in the presence of a factor has been shown by us and others (46,48). A reciprocal relationship exists between the decrement in LDL cholesterol and increment in VLDL plus HDL cholesterol that occurs in vivo during the transit of these lipoproteins through the liver (49). Reanalyis of those data, after excluding the contribution made by HDL cholesterol, gave almost as good a correlation between the decrease in LDL cholesterol and increase in VLDL cholesterol ($r = 0.72$, $p < 0.005$), the differences being confined to cholesteryl ester. Recently, Barter et al. (50) proposed that exchange of cholesteryl ester between lipoproteins is a function of the pool size of the individual lipoprotein classes. Morton and Zilversmit (48) proposed that the transfer of cholesteryl ester and triglyceride is dependent on the composition of donor and acceptor. That is, there is net transfer of core lipids between VLDL and LDL or VLDL and HDL but only exchange between LDL and HDL with which both have nearly identical cholesteryl ester to triglyceride ratio. These being so, an increase in the size of the VLDL pool relative to LDL, as occurs in HyperapoB, and the difference in composition of VLDL and LDL would accentuate movement of cholesteryl ester from LDL to VLDL and of triglyceride from VLDL to LDL. The consequence after lipolysis

either by hepatic triglyceride lipase (51,52) or lipoprotein lipase (53,54) would generate the dense LDL. In FH pateints who are deficient in LDL receptors, the finding that buoyant LDL is enriched in cholesteryl ester and accumulated in plasma implies that the conversion of buoyant to dense LDL may depend on efficient functioning of the LDL receptor. It is also possible that buoyant LDL has a higher affinity for the LDL receptor than does dense LDL, as suggested recently (55). The decrease in the cholesterol/apoB ratio of LDL and increase in FCR, especially of buoyant LDL, induced by therapy known to stimulate receptor-mediated LDL catabolism in FH support these conclusions (56).

Lastly, the question arises as to which is the best method of analyzing LDL turnover. Berman (57) postulated the existence of at least two intravascular populations of LDL particles in equilibrium with an extravascular pool to explain apoB kinetics in humans and this concept is supported by our data. The method we have used to derive total LDL turnover (method A, Table 4) is to determine the turnover of each major fraction by the conventional two-compartmental model and to calculate the proportion of buoyant LDL converted into dense LDL by deconvolution analysis. In essence this is a twin two-compartmental model that allows for independent input into each intravascular pool and independent catabolic pathway of these LDL particles as well as conversion of buoyant into dense LDL. Analysis of LDL turnover by such a model would be expected to give higher values for FCR and thus ACR than the conventional two-compartmental model, which is in accord with our observations. Conversion of less dense into denser LDL particles has been well documented in hypertri-

glyceridemic subjects (58) but our data suggest the possibility that retroconversion of dense to buoyant LDL may occur in FH, which complicates the mathematical analysis. Computer modelling can resolve such problems and for this and other reasons may prove to be the better means of quantitating apoB turnover in LDL as well as in VLDL, as discussed by Fisher (59) and, more recently, by Beltz et al. (39).

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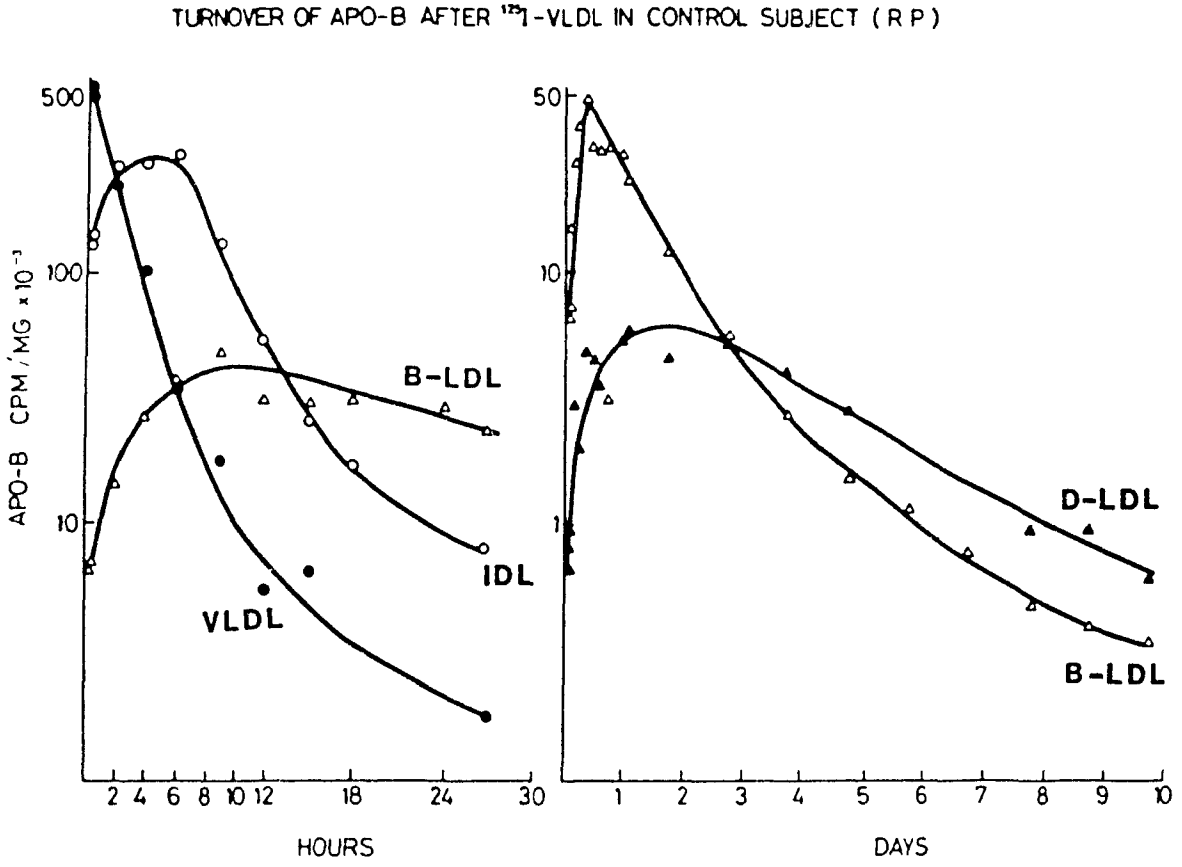


Figure 1. Specific activity of apoB in plasma lipoproteins of a control subject (No. 10) during the initial 27 h (left) and over the course of 10 d (right) after administration of ^{125}I-VLDL.

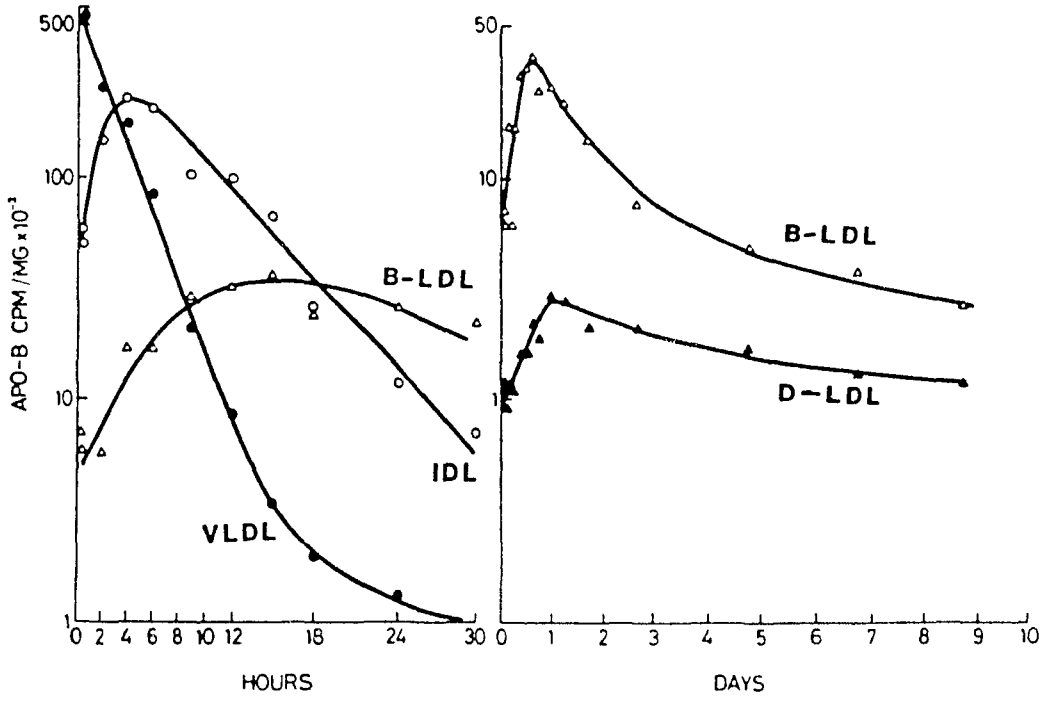


Figure 2. Specific activity of apoB in plasma lipoproteins of a HyperapoB patient (No. 4) during the initial 30 h (left) and over the course of 9 d (right) after administration of ^{125}I-VLDL. A similar pattern was observed in control subject No. 12.

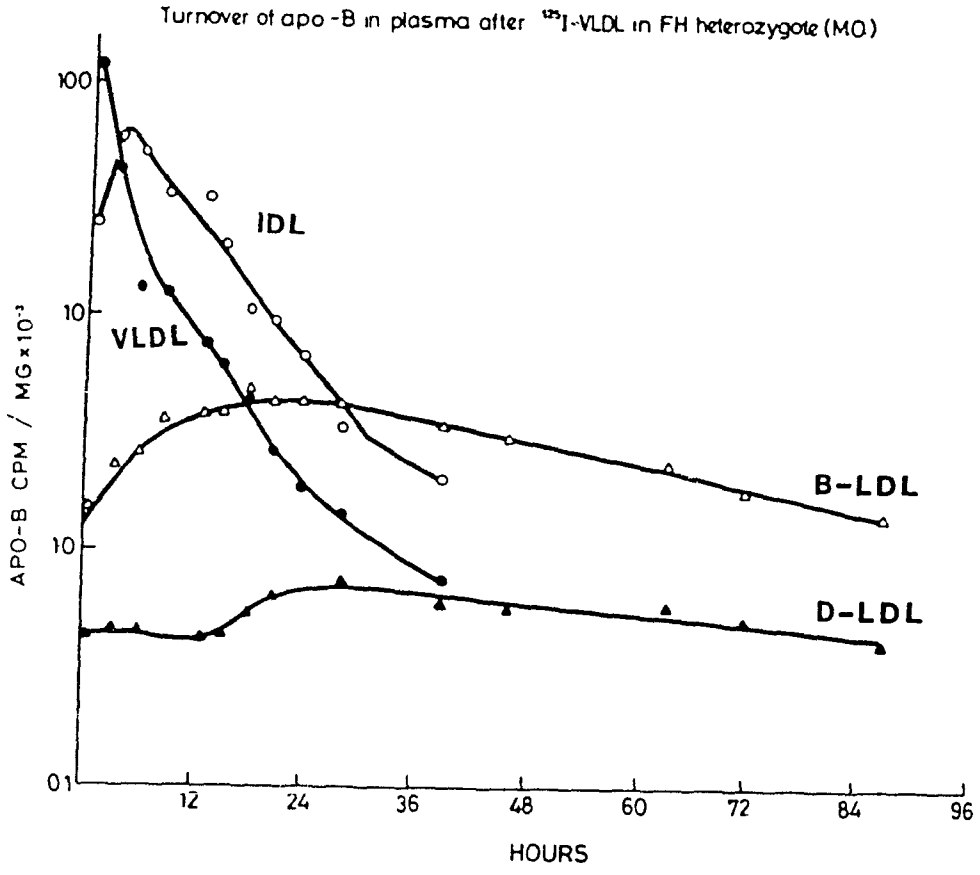


Figure 3. Specific activity of apoB in plasma lipoproteins of an FH patient (No. 14) during the 4 d after an injection of ^{125}I-VLDL.

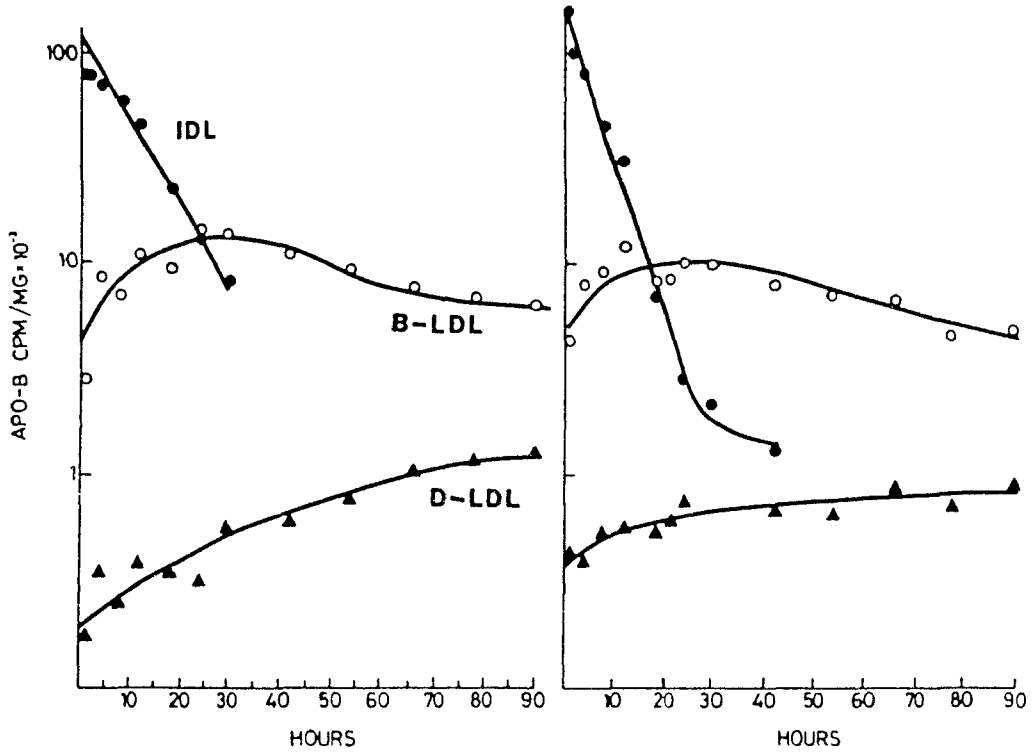


Figure 4. Specific activity of apoB in plasma lipoproteins of two FH patients (No. 15, left, and No. 16, right) during the 4 d after injection of ^{125}I-IDL (S_f 12-60).

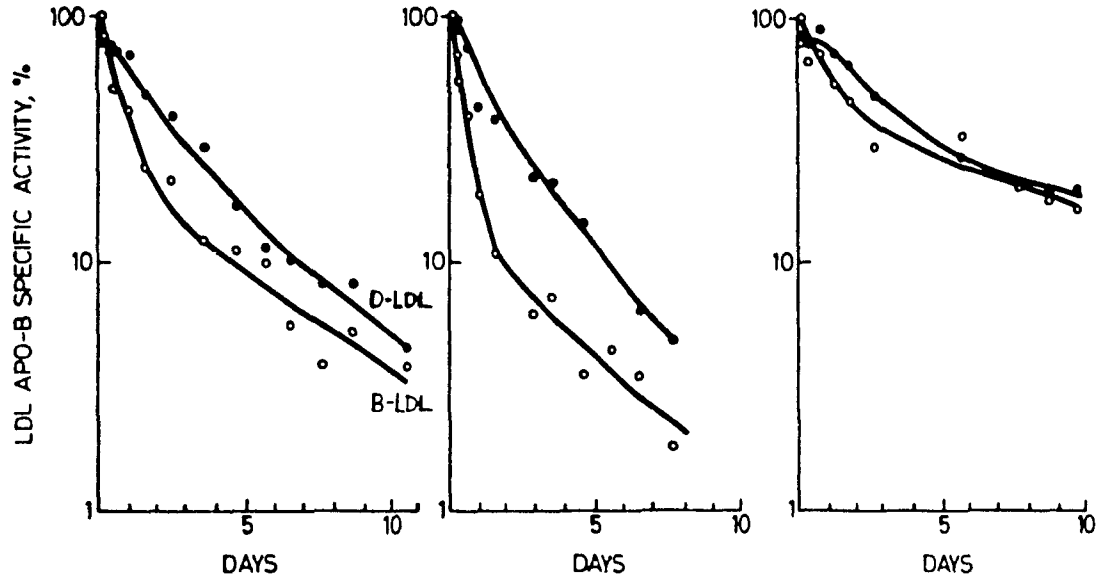


Figure 5. Specific activity of apoB in buoyant (o) and dense (•) LDL, expressed as percent of the specific activity of buoyant LDL at 10 min after injection of ^{125}I- or ^{131}I-LDL, in a control subject (No. 9, left), a HyperapoB patient (No. 2, centre), and an FH heterozygote (No. 17, right).

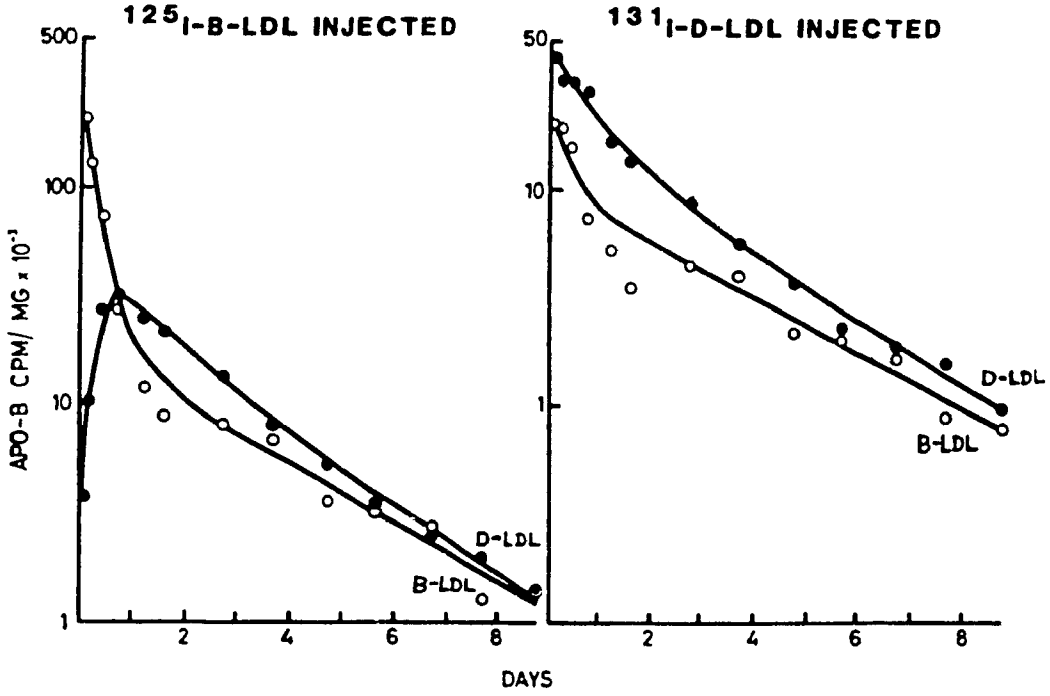


Figure 6. Specific activity of apoB in buoyant (o) and dense (●) LDL in a HyperapoB patient (No. 6) after administration of ^{125}I-buoyant LDL (left) and ^{131}I-dense LDL (right).

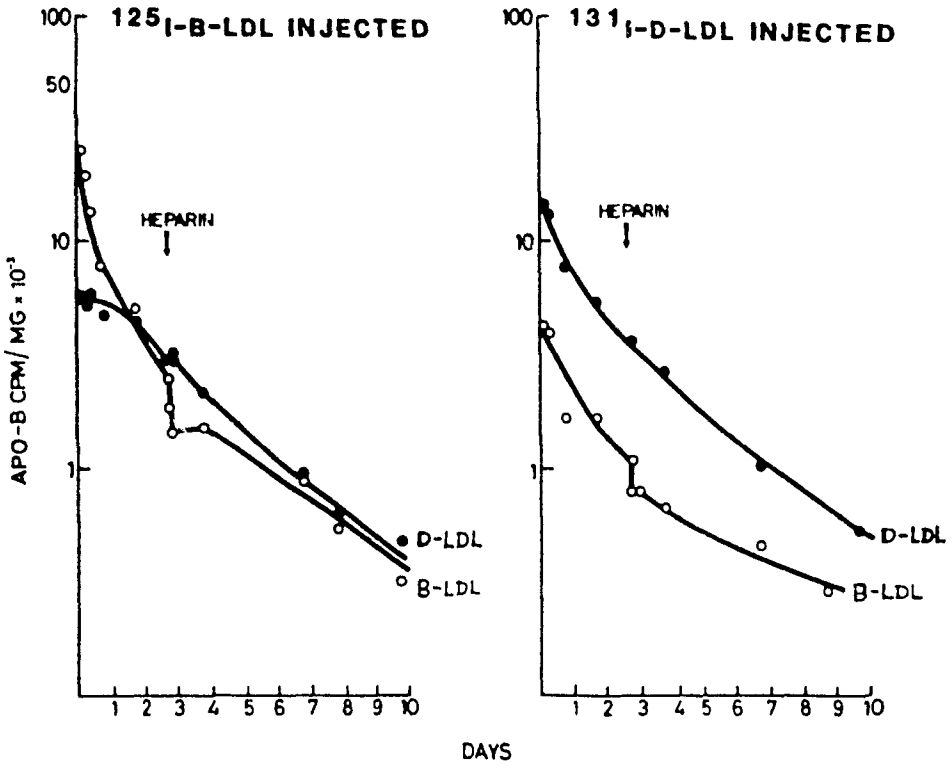


Figure 7. Specific activity of apoB in buoyant (o) and dense (●) LDL in plasma of a control subject (No. 11) after administration of ^{125}I-buoyant LDL (left) and ^{131}I-dense LDL (right). An intravenous injection of heparin 100 i.u./kg body weight was given at 2.75 d, as indicated by the arrow; blood samples were taken immediately before and 15 and 60 min later. Deconcolution analysis showed virtually complete conversion of buoyant to dense LDL before heparin was injected (77.0% at 2.75 compared with 77.1% at 10d) but both postheparin samples have been excluded from the turnover analysis.

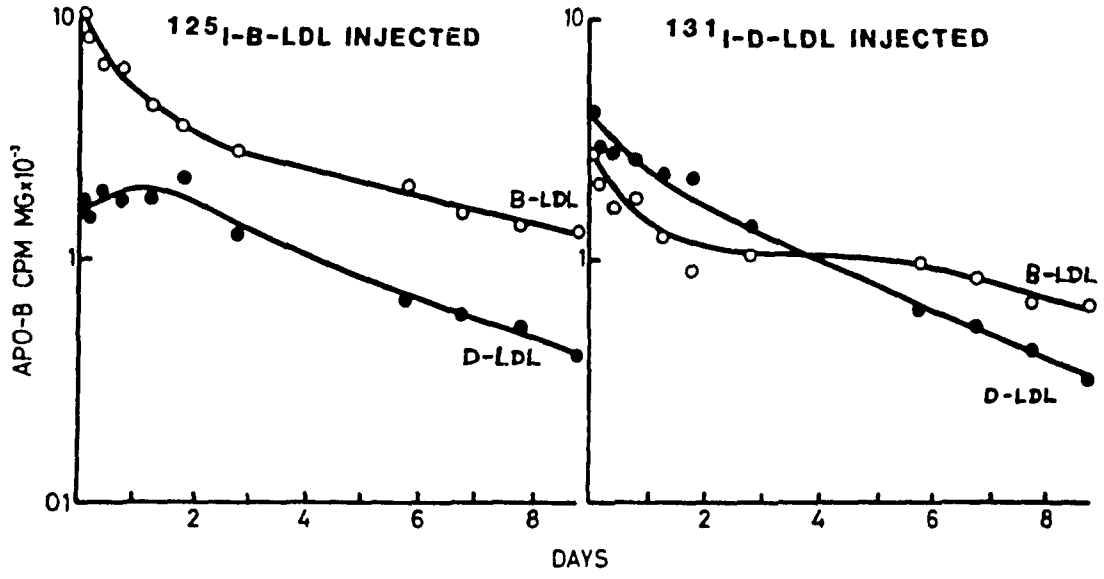


Figure 8. Specific activity of apoB in buoyant (o) and dense (●) LDL in an FH patient (No. 17) after administration of ^{125}I-buoyant LDL (left) and ^{131}I-dense LDL (right).

Table 1. Clinical and biochemical data.

Subject	Age	RBW	TC	TG	VLDL-C	LDL-C	HDL-C	LDL-B	WHO type
	yr.	%	mg/dl plasma						
HyperapoB									
1	33	107	280	198	40	200	40	147	N
2	57	127	309	533	96	162	51	143	IV
3	61	105	231	584	35	149	47	134	IV
4	53	89	183	113	23	111	49	124	N
5	56	116	261	236	47	162	52	146	IV
6	38	94	135	318	24	94	17	121	IV
7	61	95	250	226	--	186*	19	150	IV
Mean±SD	51±11	105±13	236±59	315±177	44±27	152±38	39±15	138±11	
Control									
8	40	95	204	113	23	139	41	85	N
9	46	105	191	84	9	131	51	94	N
10	50	105	216	136	27	147	42	95	N
11	47	96	182	72	3	129	49	98	N
12	63	104	152	54	6	100	46	77	N
13	40	97	160	127	5	105	30	73	N
Mean±SD	48± 9	100±5	184±25	98±33	12±10	129±18	43±8	87±10	
FH									
14	27	102	550	142	-	489*	33	283	IIa
15	64	102	476	290	-	385*	33	242	IIb
16	52	92	491	139	-	422*	41	258	IIa
17	38	101	480	164	-	402*	45	270	IIa
18	62	128	526	186	-	398*	43	234	IIa
Mean±SD	49±16	105±14	505±32	184±62	-	419±41	39±6	257±20	

* calculated from formula of Friedwald et al. (14).

Abbreviations used in this table:
TC, total cholesterol; TG, triglyceride; VLDL-C, LDL-C, and HDL-C, VLDL-, LDL- and HDL-cholesterol; LDL-B, LDL-apoB in d > 1.019 fraction of plasma; RBW, relative body weight, calculated as kg/(cm-100) x 100%.

Table 2. Distribution of apoB and Cholesterol/apoB Ratios in LDL Subfractions

		ApoB, % of total apoB			Cholesterol/apoB	
Subject	n	1**	2	3	1	2
HyperapoB*	6	24.6±6.2	65.9±10.6	9.4±6.4	1.47±0.16	0.98±0.11
Control	6	26.8±9.8	68.4±10.9	4.6±5.8	1.55±0.14	1.23±0.12
FH	5	43.2±6.3	55.0± 6.6	1.8±2.5	2.05±0.23	1.37±0.16

* Samples from patient 7 were lost and mean values were used to calculate his pool sizes in Table 4.

** The numbers 1, 2, and 3 represent LDL subfractions 1, 2, and 3.

Table 3. Kinetics of apoB Turnover in VLDL, IDL, and LDL

	VLDL-apoB			IDL-apoB			LDL-apoB		
	ACR mg/kg-d	FCR /hr	Pool mg	ACR mg/kg-d	FCR /hr	Pool mg	ACR mg/kg-d	FCR /d	Pool mg
HyperapoB									
1	46.0	0.261	615	-	-	123	20.8	0.343	5538
2	50.2	0.168	822	19.3	0.244	217	32.1	0.521	4063
3	32.7	0.127	810	-	-	172	20.8	0.355	4002
4	28.4	0.355	199	7.6	0.258	81	20.3	0.362	3487
5	43.3	0.237	543	4.8	0.095	148	22.1	0.350	4430
Mean±SD	40.1±9.2	0.230±0.088	598±254	10.6	0.199	148±51	23.1±5.1	0.386±0.076	4304±767
Control									
8	25.9	0.350	204	-	-	176	15.9	0.358	2952
9	17.5	0.351	165	14.3	0.404	124	13.8	0.327	3538
10	23.2	0.388	209	-	-	84	12.7	0.376	2973
11	19.5	0.380	164	5.8	0.190	99	11.2	0.346	2973
12	21.6	0.362	214	19.1	0.516	129	12.8	0.414	2460
13	-	-	-	-	-	-	11.7	0.374	2056
Mean±SD	21.5±3.3	0.366±0.017	191± 25	13.1	0.370	122±35	13.0±1.7	0.366±0.030	2752±515
FH									
14	27.6	0.383	316	6.5	0.203	97	33.0	0.259	9233
15	-	-	-	11.44*	0.088*	381*	22.1	0.192	8095
16	-	-	-	5.92*	0.167*	91*	19.7	0.168	7203
17	-	-	-	-	-	-	21.0	0.173	9161
18	-	-	-	-	-	-	17.8	0.169	6308
Mean±SD							22.7±6.0	0.192±0.039	8000±1262

* S_f 12-60

Table 4. Quantitation of turnover of apoB in buoyant (B) and dense (D) fractions of LDL

Subject	Method of analysis	B-LDL				D-LDL				total LDL		
		Pool	FCR	ACR	Conversion	Pool	FCR	ACR	Direct synthesis	Pool	FCR	ACR
		mg	/d	mg/kg-d	%	mg	/d	mg/kg-d	%	mg	/d	mg/kg-d
HyperapoB												
6	A	1174	1.72	25.4	77.8	2764	0.495	17.2	0	3938	(0.513	25.4)
7	A	1257	0.475	8.76	100	3837	0.307	17.3	49.0	5094	(0.231	17.3)
Control												
11	A	619	0.80	6.4	77.1	2247	0.445	13.0	61.5	2866	(0.386	14.4)
	B	-	-	-	-	-	-	-	-	2531	0.346	11.2
13	A	563	0.628	5.34	73.8	1522	0.55	12.65	69.2	2085	(0.447	14.1)
	B	512	-	-	-	1544	-	-	-	2056	0.376	11.7
FH												
17	A	3331	0.224	10.2	49.4	4317	0.273	16.2	68.5	7648	(0.203	21.3)
	B	3023	-	-	-	6138	-	-	-	9161	0.173	21.0
18	A Q	2047	0.239	8.0	39.4	3034	0.240	11.9	73.9	5081	(0.203	16.8)
	B	3116	-	-	-	3192	-	-	-	6308	0.169	17.8

A. Buoyant and dense fractions were injected and turnover of each analysed by the method of Matthews (27); ACR of total LDL was derived as ACR of B-LDL plus direct synthesis of D-LDL, the latter being calculated as ACR of D-LDL minus ACR of B-LDL x percent conversion. Derived data are shown in parenthesis.

B. Unfractionated LDL injected and specific activity/time curves of whole LDL were analyzed by the method of Matthews (27); pool sizes of buoyant and dense DL were measured after density gradient ultracentrifugation, as in Methods.

Q. On Questran 16 g/d.

CHAPTER 5

PHYSIOLOGIC INSIGHTS FROM A MULTICOMPARTMENTAL MODEL OF LDL METABOLISM

ABSTRACT

LDL apoB turnover has generally been analyzed by the two-compartment model of Mathews, the presumption being that all plasma LDL particles have an equal probability of being catabolized. But LDL has been shown to be both structurally and kinetically heterogeneous. Accordingly, a new model of LDL metabolism was developed using multicompartmental analysis. One normal and three patients with hyperapobetalipoproteinemia were studied. All received simultaneous injection of ^{125}I-buoyant LDL (B-LDL) and ^{131}I-dense LDL (D-LDL). The model contains: 1) a B-LDL delipidation cascade with a slowly catabolized pool that derived from this cascade; 2) not only is B-LDL converted to D-LDL but also D-LDL can return to the liver and re-enter the plasma space; 3) B-LDL and D-LDL can each be directly catabolized.

Quantitative analysis showed that the apoB transport rate of B-LDL and D-LDL is increased in HyperapoB compared to normal (22.6 vs. 6.58, 18.7 vs. 8.70 mg/kg-d, respectively), the fractional catabolic rate of B-LDL apoB is three times faster than the D-LDL apo3 (1.287 vs. 0.421/d), and an average of 54% (36 to 71%) of B-LDL apoB goes through the cascade to be converted to D-LDL apoB. The model underscores the physiologic importance of cholesteryl ester-triglyceride exchanges in the production of the D-LDL in lipoprotein metabolism.

INTRODUCTION

Apolipoprotein B-100 (apoB) is essentially the only apoprotein of low density lipoproteins (LDL) (1,2); it is also a major constitutent of very low density lipoproteins (VLDL). In the circulation, VLDL undergoes lipolytic degradation to smaller lipoproteins: intermediate density lipoproteins (IDL) and low density lipoproteins (LDL) (3). In this lipolytic process apoB-100 remains in the lipoprotein particles. The final product, LDL, is removed from the circulation mainly by LDL receptors (4). Therefore, understanding the metabolism of apoB-containing lipoproteins is vital to understanding the overall metabolism of these lipoproteins.

Several groups of workers, using a variety of separation methods, have demonstrated that LDL is heterogeneous in terms of particle size and composition (5-10). These studies have shown that LDL can be separated into 3-5 subfractions with distinct physical and chemical characteristics (5,7-10) and that heterogeneity seems especially marked in patients with type IV hyperlipoproteinemia (11). Recently we described a simple method, discontinuous density gradient ultracentrifugation (10), to separate LDL into three major subfractions: fraction 1 or buoyant LDL (B-LDL), fraction 2 or dense LDL (D-LDL), and fraction 3 or very dense LDL. We documented differences in the densities and composition of the two major subfractions, fraction 1 and fraction 2, among normal subjects, patients with hyperapobetalipoproteinemia (HyperapoB) and patients with familial hypercholesterolemia (FH). In patients with FH, fraction 1 was cholesteryl ester-enriched and less dense than normal, and in patients

with HyperapoB fraction 2 was relatively cholesteryl-ester depleted and denser than normal. Kinetic study of apoB in VLDL, IDL, LDL, and the buoyant and dense fractions of LDL (12) showed that the increased concentration of LDL apoB in HyperapoB was due solely to increased LDL synthesis, which in turn was secondary to increased VLDL synthesis; by contrast, in FH there was both an increased synthesis and reduced catabolism of LDL. With regard to the LDL-subfractions there was a precursor-product relationship between buoyant and dense LDL in most HyperapoB patients and controls but not in FH patients. Kinetic studies by Phair et al. (13) also supported the existence of precursor-product relationships within the spectrum of LDL particles.

Three different models have been proposed to analyze LDL apoB metabolism (14-16). Although there is no doubt that LDL is heterogenous, kinetic analyses of LDL apoB have generally used a two-compartment model (14). This model hypothesizes a single intravascular pool in equilibrium with an extravascular pool. It stringently limits the analysis of LDL kinetic data since it presumes LDL is homogeneous with one output whereas the heterogeneity of LDL suggests that there may be several catabolic routes for LDL. Therefore it is apparent that a more sensitive system of metabolic study for LDL apoB is needed to elucidate the mechanism of LDL turnover and to quantify more precisely the kinetics of LDL apoB.

In the present study the turnover of LDL apoB in LDL subfractions has been investigated in one normal subject and three patients with HyperapoB; the major objective was to eval-

uate LDL apoB metabolism with a multicompartmental model developed to accomodate the heterogeneity of LDL.

SUBJECTS AND METHODS

Studies were performed in 4 male subjects: 3 male patients with HyperapoB, and 1 male normal control. The cholesterol and triglyceride concentration of plasma samples were determined enzymatically (Beckman cholesterol kit no. 683197; Beckman triglyceride kit no. 683248), each value representing the mean of triplicate analyses of single samples. LDL cholesterol was calculated by subtracting VLDL and HDL cholesterol from total cholesterol (17). HDL cholesterol was determined after heparin-manganese precipitation of plasma (18), adapted for enzyme assay by the addition of 8 mmol/l EDTA to the reagent. LDL apoB was assayed by radial immunodifffusion, using a method designed to minimize any contribution from VLDL apoB (19).

The upper limits of normal for serum triglyceride, LDL cholesterol, and LDL apoB upon which lipoprotein phenotyping and the diagnosis of HyperapoB have been based, were 200, 200, and 120 mg/dl respectively. All subjects consumed their usual diets during the studies. None of the subjects was on any lipid-lowering drug. All were ambulant and maintained their customary exercise habits. Relative body weight was within the normal range in all subjects. These studies were sanctioned by a research ethics committee of the Royal Victoria Hospital and all subjects gave informed consent.

The study was designed to evaluate the kinetics of apoB in LDL subfractions; fraction-1, fraction-2, and fraction-3 being respectively buoyant LDL, dense LDL, and very dense LDL. To obtain LDL subfractions, LDL was first isolated ultracentrifugally. The d > 1.019 g/ml infranant was adjusted to d 1.071 g/ml with NaCl/KBr solution and centrifuged in a SW 50.1 rotor to collect LDL of d 1.019-1.063 g/ml; LDL was then dialysed against d 1.050 g/ml and 1 ml of it was loaded on a discontinuous density gradient and centrifuged in a SW 50.1 rotor at 40,000 rpm for 40 hours at 10°C (10), as described in detail in Chapter 2, methods section. At equilibrium the gradient was fractionated, using a tube piercer, into three 1 ml fractions (fractions 3, 4, 5) from the bottom of the tube. Fraction 3 was the very dense LDL fraction. Next, by visual separation, dense LDL (fraction 2) was usually recovered in approximately 1.25 ml, and buoyant LDL (fraction 1) usually recovered in approximately 0.75 ml. Fractions 1 and 2, collected as above, were then concentrated by centrifugation in a SW50.1 rotor at 40,000 rpm for 20 hours. The two fractions were then dialysed exhaustively against saline/EDTA solution before the radioiodination.

Buoyant and dense LDL (fractions 1 and 2) were radioiodinated with ^{125}I or ^{131}I by a modification of the iodine monochloride method of McFarlane (20). The radiolabeled LDL subfractions were then injected intravenously into the subject (10 minutes apart). Blood samples were obtained as follows: 10 minutes after the injection, 4 hr, 9 hr, 18 hr, 30 hr, and then daily for the next 10 days. Twenty-four hour urine collections were made for each day of the study.

Plasma samples collected as above were first subjected to ultracentrifugation to remove d < 1.019 g/ml supernatant. LDL d 1.019-1.063 g/ml was then collected by SW50.1 rotor as described above. To avoid possible loss due to dialysing the LDL fraction, the LDL (d 1.019-1.063 g/ml) was then loaded directly on the discontinuous density gradient at a salt density of 1.063 g/ml. The discontinuous density gradient was then centrifuged in a SW50.1 rotor at 40,000 rpm for 40 hours at 10°C. At equilibrium, the gradient was fractionated as described above. The volume of each fraction was calculated according to its weight and density. The radioactivity was then counted in a dual channel gamma counter, appropriately calibrated to minimize spillover, and then apoB and cholesterol content were assayed, as described above. The data of the plasma samples, the urine samples, and each LDL subfraction were expressed as percent of injected dose. Plasma volume was calculated as 4.5% of the body weight rather than using the values obtained from the 10 min blood sample (21).

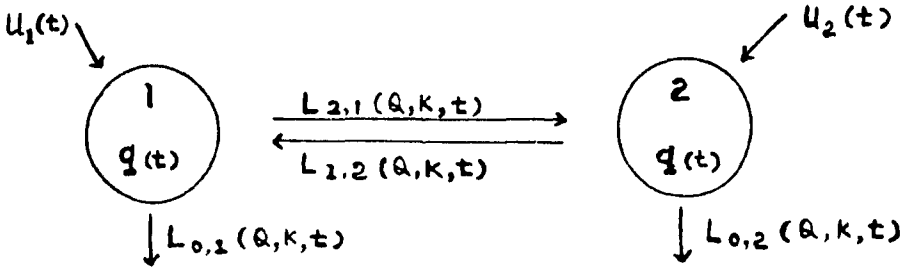
Each subject had approximately 150 data points with the mass values of LDL apoB subfractions which were analysed on a Vax 11-750 computer using the SAAM computer program (version 27) of Berman and Weiss (22,23). The development of the multicompartmental model to account for the tracer and tracee (nonlabeled) kinetic data is discussed in the next section. The model has to simultaneously fit all the 150 data points plus the initial condition of the system. Urinary excretion of labeled iodide served as an additional independent constraint on the model by requiring that all the label be accounted for. Best fits were

obtained by using nonlinear regression to minimize the weighted sum of squares (23).

CONSTRUCTION OF THE MODEL

Models are a schematic for the physiologic and biochemical processes involved in a system. As stated by Phair (24), "Modeling is quantitative formulation and testing of hypotheses. The power of models is to predict with precision of a complex hypothesis and it allows rapid rejection of inadequate theories. The power of models is also to extract information distributed over large data bases which can be used to estimate parameters and variables that are inaccessible to direct experimental measurement". Ultimately, models should be fully compatible with knowledge of physiology and biochemistry and their components should be individually identified with physiological or biochemical entities.

Most biological systems are nonlinear and the dynamic system can be simulated by compartmental models. The model can be described by ordinary differential equations which are usually nonlinear. For example, in a two-compartment model



q(t) is the mass of material in compartment 1 and 2 at time t;

u is the rate of entry of new material into the compartment from outside the system;

$L_{2,1}(Q,K,t)$ and $L_{1,2}(Q,K,t)$ are the rate constant describing the exchange of material between the compartments;

$L_{0,1}(Q,K,t)$ and $L_{0,2}(Q,K,t)$ are the rate constants of material which are irreversibly lost from the system;

The vector $Q = Q(t) = (q_1(t), q_2(t))$.

The vector K is a vector of parameters upon which the rate constant depends.

The equations are as follow

$$dq_1/dt = -(L_{0,1} + L_{2,1})\, q_1 + L_{1,2}\, q_2 + U_1$$

$$dq_2/dt = L_{2,1}\, q_1 - (L_{0,2} + L_{1,2})\, q_2 + U_2$$

These set of differential equations are nonlinear when the rate constants L are functions of the mass q. What can one then do to learn something about nonlinear systems? One experimental constraint that can be imposed is that the experiment be carried out in the steady state. This means that K is a constant vector and q is constant also. Therefore the Lij, the rate constant, if not constant, depends only upon time, t. The set of differential equations are then linear. When the rate constant, Lij, and Ui are constant, i.e., where they do not depend explicitly upon time, they are referred to as linear, constant coefficient differential equations. To solve these equations, linear and nonlinear differential equations can be done with the aid of a computer. Solutions of the differential equations can estimate the parameter values of a model and their confidence ranges (covariance matrix). This is done by the method of least squares data fitting.

SAAM 27 (Simulation, Analysis, and Modeling): SAAM is the most widely used compartmental model to study apoB kinetics in human subjects. SAAM contains both simulations; the solution of a set of equations to study the behaviour of a system in mathematical terms, the biological implications of the model, and the fitting of parameters to experimental data, where the equations describing the model may be differential, integral, algebraic, or any set of functions as long as an analytical or numerical procedure exists for its solution. There are four stages of computation in model-building:

1) solving the set of equations with numerical solutions developed by Chu and Berman (25);
2) comparing the model solution with the experimental data;
3) adjusting the parameters to obtain a "best" fit (this involves both linear and nonlinear data);
4) deriving statistical information. This involves approximation to give estimates of the uncertainties of the derived measures.

We used this program to develop the new LDL apoB multicompartmental model. The process of developing the model is described next.

Model Development: Figures 1A and 1C present percent injected dose/time curves in plasma and urine from the control after injection of radioiodinated B-LDL and D-LDL respectively. In both instances, the plasma curve always showed the typical biphasic decay. The envelope of the urine curve peaked at approximately 2 days, and then decreased in parallel with the

plasma curve. Also note that the decay of the plasma curve after injection of either radioiodinated B-LDL or D-LDL was almost the same as shown in the figures. This was true in both for the control and patients with HyperapoB.

Figure 1B shows the changes in radioactivity of LDL subfractions of the control subject after injection of ^{125}I-B-LDL. There was a precursor-product relationship between the percent of injected dose/time curves, where the precursor (B-LDL) intersects the product (D-LDL) at or just before the latter reaches its maximum. This relationship was observed in all the subjects studied. Note that the decay of B-LDL is curvilinear and that there is a slow turnover tail. Also, in this subject, the radioactivity in fraction-3, very dense LDL, was minimal. When D-LDL was injected (Fig. 1D), the B-LDL did not decay rapidly; instead there was an initial upswing (shoulders). This was the case in all the subjects studied. It was also observed that the decay of B-LDL and D-LDL in the percent of injected dose/time curves were different. This is illustrated more clearly in Figure 2, which shows the radioactivity decay of percent of injected dose/time curve of B-LDL when B-LDL was injected and of D-LDL when D-LDL was injected. Figure 2A are the data from the control subject studied. The early part of the percent injected dose/time curve for B-LDL decays faster and is more curvilinear than that of D-LDL, and these differences are accentuated in the HyperapoB patients (Fig. 2B). The B-LDL and D-LDL apparently have different kinetic curves with B-LDL turnover faster than D-LDL and therefore two intravascular compartments of LDL are apparently

required to explain the data.

New LDL apoB model: From the observation of radioactivity/time curves in the plasma, urine, and LDL subfractions after injection with both the radioiodinated B-LDL and D-LDL as well as the data from our previous studies (12,31,33), a new model was developed as shown in Figure 3.

B-LDL subsystem (Fig. 3 Fraction-1): Phaire (13) proposed the concept of a delipidation chain within the LDL particle spectrum. We observed that LDL particles change in composition across the splanchnic bed (31) and we also noted B-LDL gradually becomes denser with time (12). From the radioactivity/time curve, the decay of B-LDL always demonstrated a slow turnover tail. Therefore a delipidation chain was added to the model consisting of four compartments with a single compartment that turns over slowly which is derived from the delipidation chain. The mass of B-LDL apoB in the chain is gradually decreased by a small amount in each compartment and the rate constants are the same for all four compartments. This delipidation chain pathway accounts for the shoulder before the rapid phase of the B-LDL decay curve. The slow turnover pool which derived from the B-LDL delipidation chain accounts for the slow turnover tail. These LDL are then either removed from this system by LDL receptors or scavenger pathways (4), or they are eventually converted to D-LDL.

D-LDL subsystem (Fig. 3 Fraction-2): The B-LDL particles that pass through the delipidation chain become smaller and denser; these particles are the D-LDL particles. In this sub-

system it was predicted that there are two compartments. One compartment is generated from the B-LDL delipidation chain, while the other is mainly generated from the slow turnover pool of B-LDL. Both compartments will be either metabolized further to fraction-3 (the very dense LDL) which accounts for less than 5% of the total radioactivity or more likely will be removed irreversibly in plasma by LDL receptors or scavenger pathway (4). In this subsystem there is another pathway which is important to the integration of the system. This is the return of D-LDL to the extravascular space (almost certainly the liver) where most of it will re-enter the vascular system to be hydrolyzed again. This pathway could explain the observation of the initial shoulder in B-LDL decay when D-LDL was injected and the observation of the two plasma decay curves which are the same or very similar after injection with either B-LDL or D-LDL. When this pathway is removed, the shoulder of the B-LDL decay curve will disappear, and instead the B-LDL will decay very fast, as illustrated in Figure 4.

Very dense LDL subsystem (Fig. 3 Fraction-3): This is the final product of the LDL lipolysis system and the radioactivity within it varies from 10 to less than 1% of the total. There are two compartments in this system: one is derived from continued lipolysis of the delipidation chain system, the other from the continuation of the slow turnover compartment system. The LDL particles in this subsystem will be removed irreversibly by LDL receptors or the scavenger pathways (4).

Subsystem Integration: The model integrating the subsystems for B-LDL, D-LDL, and very dense LDL is presented in Figure 3. This model assumes that most apoB enters this system as B-LDL (compartment 1) which is mainly derived from VLDL apoB (12). This newly produced B-LDL will first equilibrate with the extravascular space (the liver) where B-LDL composition will be changed by interacting with nascent VLDL (31). The B-LDL is then transported down the delipidation chain to the D-LDL pool. The apoB in B-LDL can have several fates:

a) irreversible removal from the plasma before equilibration with the extravascular space;
b) irreversible removal by the liver while in the principal extravascular space;
c) conversion to D-LDL apoB through the delipidation chain;
d) entry into the slow turnover pool which will then be converted to D-LDL or irreversibly removed from the plasma.

Finally, D-LDL, fraction-2, can be derived from the B-LDL delipidation pool or from the B-LDL slow-turnover pool or can be synthesized "directly". D-LDL apoB can either return to the extravascular space (the liver) and re-enter the vascular space again and/or be hydrolyzed further to the very dense LDL, or irreversibly removed from the plasma. The very dense LDL apoB, fraction-3, has only one fate -- to be irreversibly removed from plasma.

Slow turnover pool: Most of the B-LDL apoB passes through the delipidation chain to be converted to D-LDL, but a very small

portion can be converted to the slow turnover pool, perhaps due to incomplete hydrolysis. The condition of this pool may vary depending upon the subject studied. Estimation of the production rate in this pool (mass x rate constant) can vary from 3 mg/d in the control to 13 mg/d in the HyperapoB patients. When it was compared, however, with the total transport rate, this pool was very small (< 1%). However, this pool cannot be treated lightly. Without the slow turnover pool, the tail of the B-LDL apoB would decay very quickly rather than be flat as was observed in all subjects studied. This is shown in Figure 5. Therefore, a slow turnover pool is essential to fit of the data.

Conversion of B-LDL to D-LDL: When the radioactivity curves for ^{125}I-D-LDL and ^{131}I-D-LDL were normalized to percent of the injected dose, the area under the ^{125}I-D-LDL apoB curve (derived from B-LDL apoB) was almost the same as that of ^{131}I-D-LDL apoB (from direct D-LDL injection) (Fig. 6). This indicated that most of the B-LDL apoB did reach D-LDL apoB. This was the case in the control subject and the three HyperapoB patients (KB, EB, and AB) studied (Figs. 6A, 6B, 6C, and 6D, respectively).

RESULTS DERIVED FROM THE NEW MODEL

The biochemical and clinical data of the subjects studied are presented in Table 1. The relative body weight in each subject studied is similar. These four subjects all had total cholesterol level less than 250 mg/dl. Using the criteria for

HyperapoB: LDL cholesterol $\leq$ 200 mg/dl and LDL apoB $\geq$ 120 mg/dl, the three HyperapoB patients all had normal levels of LDL cholesterol with disproportionatly elevated levels of LDL apoB. Two of them had type IV phenotype. The level of HDL cholesterol in the HyperapoB patients was less than the fifth percentile of normal. Table 2 gives the distribution of apoB in the three fractions of LDL after density gradient ultracentrifugation and the cholesterol/apoB ratio of the two main fractions, buoyant and dense LDL. In control and HyperapoB patients, approximately two-thirds of LDL apoB was found in fraction 2 and approximately one-fourth in fraction 1; the remaining 0-10% was recovered in fraction 3. In the HyperapoB patients, the cholesterol/apoB ratio in fraction 2 was reduced as is characteristic of HyperapoB. However, patient KB had a low cholesterol/apoB ratio even in fraction 1, an unusual finding in our experience.

Model Fitting: The preliminary model described above was used to fit the data of the one control and three HyperapoB patients. The best fitted lines generated by computer from the model using data from plasma, urine, and LDL subfractions of control, HyperapoB patients KB, AB, and EB are shown in Figures 7, 8, 9, and 10, respectively. The symbols represent the data points and the lines the best fit generated by the present model.

After injection of B-LDL, the model generated line of percent of injected dose of B-LDL/time of plasma decay and accumulation of urine as shown in Figure "A". That for LDL subfractions, fraction-1, -2, and -3 are shown in Figure "B". After injection of D-LDL, the model generated line of percent of in-

jected dose of D-LDL/time of plasma decay and accumulation of urine are shown in Figure "C"; that of the LDL subfractions, fraction -1, -2, and -3 are shown in Figure "D".

The model generated lines for the control are shown in Figure 7. In Figures 7A and 7B the model fits plasma decay and LDL subfractions-1, -2, and -3 curves well, whereas the urine output by the model is not enough. In Figures 7C and 7D the model also fits the plasma decay curve and the subfraction -2 curve well whereas the output of accumulated urine by the model is too high and the shoulder of subfraction-1 does not yet fit. The model generated lines of HyperapoB patient KB are shown in Figure 8. From Figures 8A and 8B the model again fits all the data of plasma, accumulated urine, and LDL subfractions well. However, after D-LDL injection, the decay of subfraction-1 is too fast in this fitting (Fig. 8D). The model generated lines of HyperapoB patient AB are shown in Figure 9. In Figures 9A and 9B the model fits the plasma decay, accumulated urine, LDL subfractions-1, -2 and -3 curves quite well. In Figures 9C and 9D the model also fits the plasma decay, accumulated urine, LDL subfractions-2 and -3 curves quite well except for LDL subfraction-1 curve, in which the initial shoulder of the decay curve needs to be better fitted. The model fitted lines of patient EB are shown in Figure 10. Patient EB does not have accumulated urine data due to the two missing data points on the weekend of the study; instead, the envelope of urine collection of every 24 hours is shown. The model generated lines by computer fit all the data points of either B-LDL injected (Fig. 10A and 10B) or D-LDL injected (Figs. 10C and 10D) very well.

Kinetics of LDL apoB subfractions: The kinetic results of LDL apoB and LDL subfractions generated from this model are listed in Tables 3, 4, 5, and 6. Table 3 shows the production rate of LDL. Although there is only one control in this study, this subject has been studied in the previous apoB turnover and the results are comparable with the other normal subjects (12). The production rate of total LDL apoB in patients with HyperapoB is two times higher than the control. The production rates of B-LDL and D-LDL apoB in HyperapoB patients are also at least two-fold higher than the control. Therefore, patients with HyperapoB overproduce B-LDL and consequently D-LDL.

The fate and transport rate of LDL apoB in this system is tabulated in Table 4. Most of the B-LDL (43 to 100%) will equilibrate in the liver before entering the delipidation chain. In the liver, the fraction of LDL from either ne novo B-LDL or from recirculating D-LDL varied among the subjects studied. Thus 71% of LDL transported through the liver in patient AB is from recirculating D-LDL whereas patient EB has only 9% with the rest, 91% of the LDL, from B-LDL. However, not all of the LDL that equilibrated in the liver will enter the delipidation chain; in patient KB only about 36% of the LDL which equilibrated in the liver entered the delipidation chain while the rest, 64% of the LDL, was irreversibly removed by the liver. After LDL passes through the delipidation chain, over 99% of LDL in each subject studied converted to D-LDL and less than 1.2% of the LDL entered the slow turnover pool. It was than converted to D-LDL or irreversibly removed from the plasma. The fate and transport rate of LDL apoB in each fraction, fraction 1, fraction 2, and fract-

ion 3, is tabulated in Table 5. The transport rate through each fraction is again higher in HyperapoB patients than in control. The total fractional conversion of fraction 1 to fraction 2, which originated from de novo B-LDL or recirculating D-LDL, varied from 36% to 86%. The LDL apoB transport through fraction 2 is interesting. In control DF most of the LDL through this fraction is from de novo D-LDL synthesis (56%) whereas in the HyperapoB patients it is mainly from conversion of B-LDL (mean = 70%) which can come from either the conversion of de novo B-LDL or the recirculating D-LDL. In all the subjects studied, the majority of LDL in this fraction is degraded irreversibly. LDL transported through fraction 3 was all degraded irreversibly.

There are four irreversible removal sites for LDL apoB: it can be irreversibly removed either from liver or from plasma as B-LDL, D-LDL, or very dense LDL (Table 6). In the subjects studied most of the LDL was removed from fraction 2 except in HyperapoB patient KB. The percentage of LDL catabolized from these three subfractions varied among each individual. For example, 61% of the LDL in the control is catabolized in the fraction 2 and 71% of the LDL in patient AB is also catabolized in the fraction 2 whereas 66% of the LDL in HyperapoB patient KB is presumably removed from the liver, which is compartment 2. The removal of LDL in patient EB is distributed quite equally among the three subfractions with 21% from fraction 1, 41% from fraction 2 and 38% from fraction 3.

The FCR of LDL and LDL subfractions are listed in Table 7. The FCR of total LDL of the three HyperapoB patients on aver-

age was faster than the control DF (0.539 vs. 0.376/d, respectively). Also, the FCR of fraction 1 of HyperapoB patients was twice as fast as the control DF (1.465 vs. 0.751/d, respectively) whereas that of fraction 2 is similar in the patients and the control DF (0.471 vs. 0.367/d, respectively). Furthermore, the FCR of fraction 1 of each subject studied was always faster than that of fraction 2, averaging 1.286 per day for fraction 1 vs. 0.445 per day for fraction 2.

DISCUSSION

The model for LDL apoB metabolism presented here was compatible with the kinetics of normal control and HyperapoB patients. The model shows 1) after the equilibrium of B-LDL with the liver, there is an LDL lipolytic cascade with a slow turnover pool derived from the delipidation chain, 2) a return pathway of D-LDL to the liver with re-entry to the delipidation chain, and 3) the B-LDL, D-LDL, and very dense LDL have their own irreversible removal pathways. The kinetic data were also examined with this model in detail in one normolipidemic control and three HyperapoB patients. Although the number of subjects studied was small, the kinetic data obtained was consistent with our previous work (12). The results demonstrated that the production rate of total LDL and its subfractions, B-LDL and D-LDL, are increased in HyperapoB patients in comparison with the control and the FCR of B-LDL in HyperapoB patients is greater than the control whereas D-LDL is relatively similar to the normal control. Finally, the FCR of B-LDL in all subjects studied is always greater than that

of D-LDL in the same subject.

Three models have been suggested for LDL apoB kinetics. A two-compartmental model (14) hypothesized a single intravascular pool in equilibrium with an extravascular pool. This model assumes the LDL particles to be a homogenous pool and each particle thus has equal capacity to be catabolized. However, as already noted, more recent data suggest that LDL is structurally (5-11) and kinetically (11-12) heterogeneous. Therefore the two compartment model limits one's ability to analyze LDL kinetic data. The second model was proposed by Fisher et al. (15). This model was developed with endogenous ^{3}H-leucine as the precursor. They suggested a stepwise conversion of S_f 20 to S_f 10 to S_f 4. Only when polydisperse LDL is present are two intravascular compartments of LDL needed and only the compartment S_f4 was assumed to equilibrate with an extravascular pool. The third model was suggested by Goebel et al. (16). They proposed a two plasma compartment model of LDL with different turnover rates. The current model proposes a much more complex pathway which however points to the mechanism of conversion, synthesis, the catabolism of the LDL particle spectrum. All the pathways created from this model conform to our present knowledge of the metabolism of LDL.

In vivo, the B-LDL has a higher fractional catabolic rate than the D-LDL. With regard to immunoaffinity, B-LDL is also more reactive to certain antibodies than D-LDL (26). However, these results were not consistent with one in vitro LDL-receptor-pathway study (27), which demonstrated B-LDL and D-LDL were bound to the same extent by the LDL receptors of cultured human fibroblasts. This discrepancy can be explained after analyzing LDL

kinetics by the model. The fractional catabolic rate of B-LDL is calculated as input rate U (1) divided by the mass of fraction 1. Therefore the FCR is influenced both by the rates of direct removal of B-LDL by the receptor-mediated or the receptor-independent pathways and the rates of the conversion of B-LDL to D-LDL particles. Three of the subjects studied converted more than 55% (55-86%) of B-LDL into D-LDL whereas patient KB converted 36% of B-LDL to D-LDL and degraded 64% of B-LDL in the liver. Therefore the FCR of B-LDL in the above three subjects (DF, AB, and EB) may represent mainly the fast conversion rate of B-LDL to D-LDL rather than the direct removal pathways by the peripheral tissue, whereas in patient KB the FCR of B-LDL may represent both the conversion of B-LDL to D-LDL and the direct removal of B-LDL by the liver. Recently, Hoeg et al. (28) and Edge et al. (29) showed that, in man, the hepatic recognition site for LDL may differ from the LDL receptor of peripheral tissue and this may also explain the apparent inconsistencies between the *in vitro* (27) and *in vivo* studies.

The mechanism by which buoyant LDL is converted to dense LDL is the main feature of this model. In man, the plasma decay curve of ^{125}I-LDL is biexponential, indicating that plasma LDL exists in a rapidly reversible equilibrium with a large extravascular pool. In swine, the major portion of this extravascular pool can anatomically be assigned to the liver (30). Study of LDL composition in man indicated that during the transit of LDL through the liver there was a decrease in LDL cholesteryl ester with an apparent reciprocal increase in LDL triglyceride, whereas

by contrast the VLDL and HDL cholesterols were higher crossing the splanchnic bed (31). In fact, the decrease in LDL cholesterol correlated significantly with the increase in VLDL cholesterol (12). Furthermore, observation by others showed that in man the rate of cholesterol turnover and its esterification is related to VLDL triglyceride production (32). Also, the exchange of cholesteryl ester and triglycerides between lipoprotein has been shown in vitro by many investigators (33-35). Therefore, in this model, it is suggested that most of the B-LDL enters the liver or the splanchnic bed where the cholesteryl ester in the B-LDL is exchanged for triglyceride in VLDL. Nascent VLDL is particularly rich in triglyceride since it contains almost no cholesteryl ester (3). This exchanged product, the triglyceride-rich B-LDL, will then be subjected to lipolysis. This lipolytic process can happen either in the liver (36,37) or in the plasma (38). Further, the relative content of triglyceride in the B-LDL particles after the exchange process can vary. Therefore, the hydrolytic process which drives the B-LDL particles through the lipolytic cascade can vary also. The particle with relatively more triglyceride will pass through each compartment until it appears in the density range of D-LDL. The one with relatively less triglyceride may bypass one or two of the lipolytic compartments to reach the D-LDL region. Beltz et al. (39) have determined that the delipidation cascade of VLDL apoB can vary among each individual. However, for this preliminary model we only tested a four compartment delipidation chain. The recent study of apoB and triglyceride kinetics in CHD patients demonstrated that CHD patients had increased secretion of VLDL apoB which were

poor in triglyceride and these patients also have increased transport rate of LDL apoB (39,40). This observation could indicate that the relatively triglyceride-poor VLDL particles occur because of the exchange of triglyceride with the LDL particles before secretion into the plasma.

The tail of the buoyant LDL curve signified a pool of slowly catabolized LDL. It is probably formed by the incomplete hydrolysis of B-LDL which will eventually be hydrolyzed to reach the D-LDL density range or be removed irreversibly. This pool is small in the subjects studied. However, it can be large, as in one FH patient studied where this pool accounted for more than 20% of the LDL production rate (unpublished observations). Therefore the metabolism of the slow turnover LDL pool will not be identical in all subjects and without this pool the kinetic data of LDL will be underestimated.

Finally, there is significant quantitative direct synthesis of D-LDL, as shown in the kinetic data in one HyperapoB patient and one control. In our view, this may happen where there is rapid hydrolysis of LDL by hepatic triglyceride lipase so that the triglyceride-rich B-LDL is hydrolyzed quickly to reach the D-LDL density range all within the liver. In this case, the radiolabeling of these particles is very difficult, and this might cause the apparent direct synthesis of D-LDL. That is, the buoyant LDL particle goes down the complete cascade before entering plasma and so there is in reality no direct synthesis of D-LDL only the appearance of it.

This current model undoubtedly needs to be validated extensively. The model has to fit all the data in each subject better, and it needs to be verified with more subjects. To build a model is to demonstrate a hypothesis and to suggest new studies to test the hypothesis. Therefore, we think the model needs to be validated with further kinetic studies of LDL apoB and cholesteryl ester to demonstrate the mechanism of conversion of B-LDL to D-LDL and the theory for the recycled D-LDL.

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Fig. 1. LDL apoB turnover from the control subject DF after ^{125}I-B-LDL injected (A and B) and after ^{131}I-D-LDL injected (C and D). The symbols represent the observed data points.

Fig. 1A. The observed radioactivity values of plasma decay curve (● ●) and envelop of urine radioactivities every 24 hours (▼ ▼) after ^{125}I-B-LDL injected.

Fig. 1B. The observed radioactivity values of LDL subfractions, fraction-1 (▲ ▲), fraction-2 (■ ■), and fraction-3 (◇ ◇) after ^{125}I-B-LDL injected.

Fig. 1C. The observed radioactivity values of plasma decay curve (● ●) and envelop of urine radioactivities every 24 hours (▼ ▼) after ^{131}I-D-LDL injected.

Fig. 1D. The observed radioactivity values of LDL subfractions, fraction-1 (▲ ▲), fraction-2 (■ ■), and fraction-3 (◇ ◇) after ^{131}I-D-LDL injected.

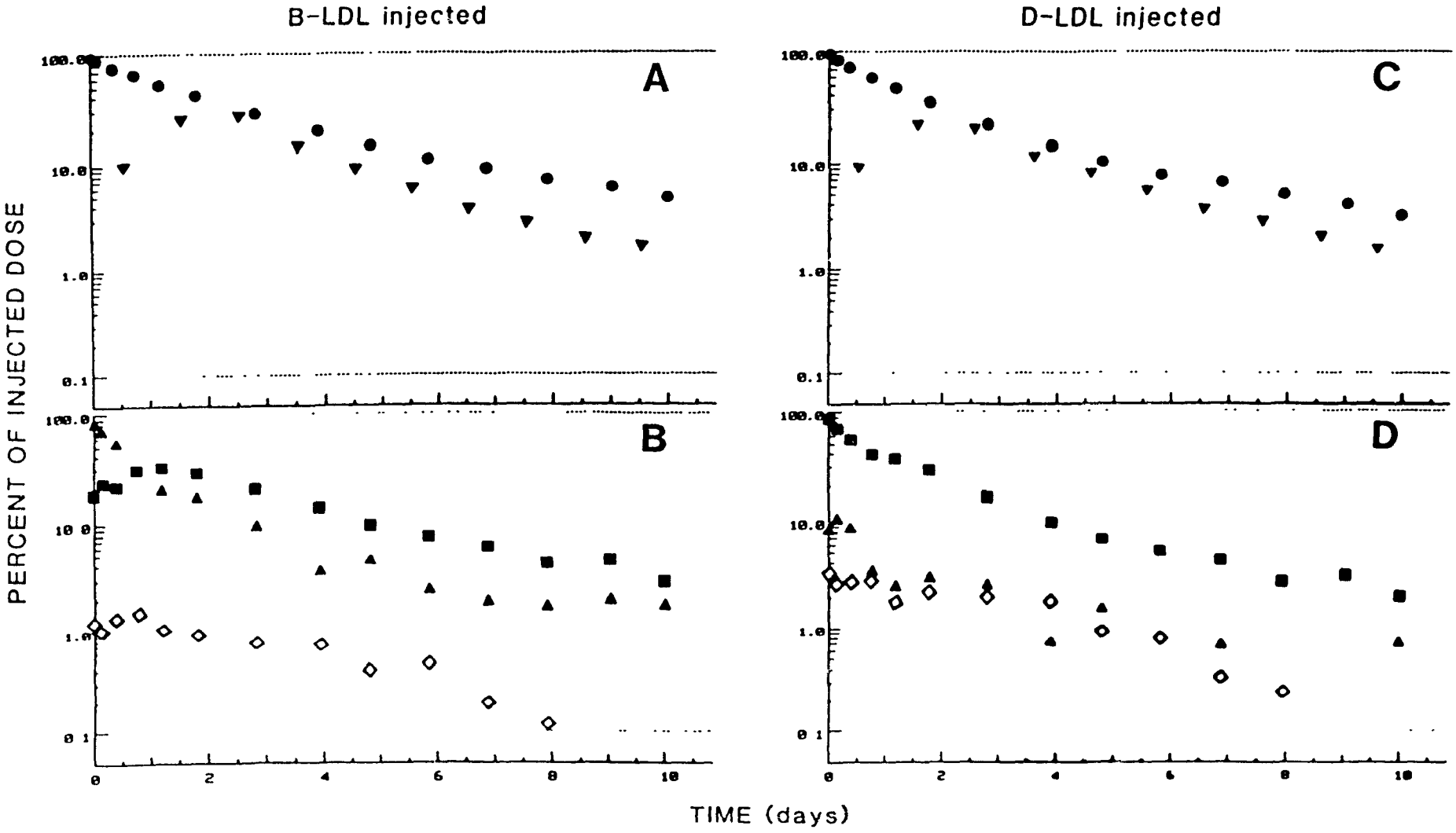
B-LDL injected
D-LDL injected
A
B
C
D
PERCENT OF INJECTED DOSE
TIME (days)

Fig. 2A. Tne observed radioactivity values from the control DF of LDL subfraction-1 (▲ ▲) after ^{125}I-B-LDL injected and of LDL subfraction-2 (□ □) after ^{131}I-D-LDL injected.

Fig. 2B. The observed radioactivity values from HyperapoB patient KB of LDL subfraction-1 (▲ ▲) after ^{125}I-B-LDL injected and of LDL subfraction-2 (□ □) after ^{131}I-D-LDL injected.

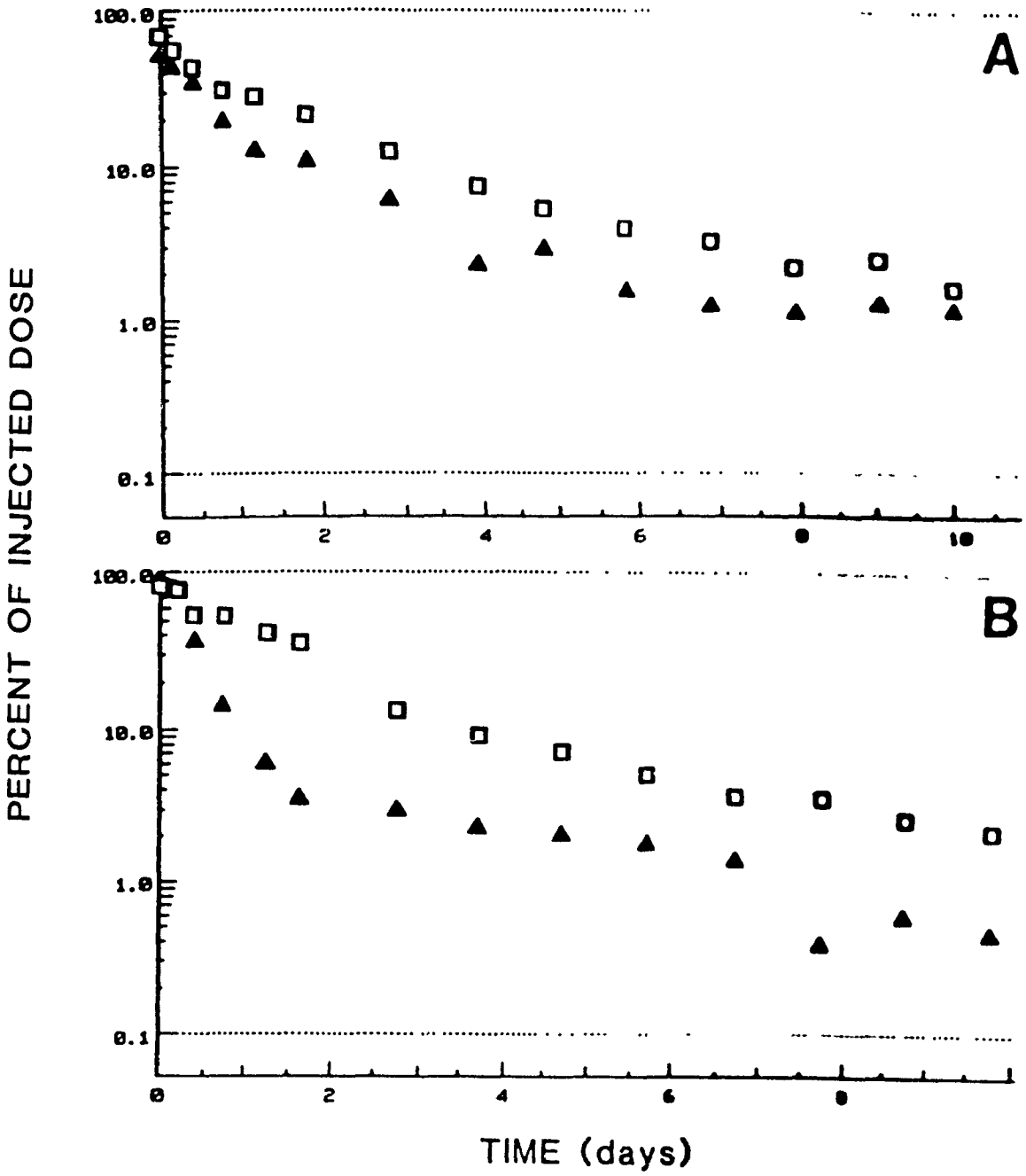
A
B
100.0
10.0
1.0
0.1
0
2
4
6
8
10
PERCENT OF INJECTED DOSE
TIME (days)

Fig. 3. Schematic diagram of the compartments of the new multi-compartmental model for LDL apoB metabolism.

1, Compartment 1 of B-LDL before equilibration with extravascular space (the liver).

2, Compartment 2, extravascular space; the liver (L).

12, Compartment 12, the first compartment of the LDL delipidation chain.

16, Compartment 16, the slow-turnover pool derived from delipidation chain.

23, Compartment 23, the compartment of D-LDL.

EV, extravascular space.

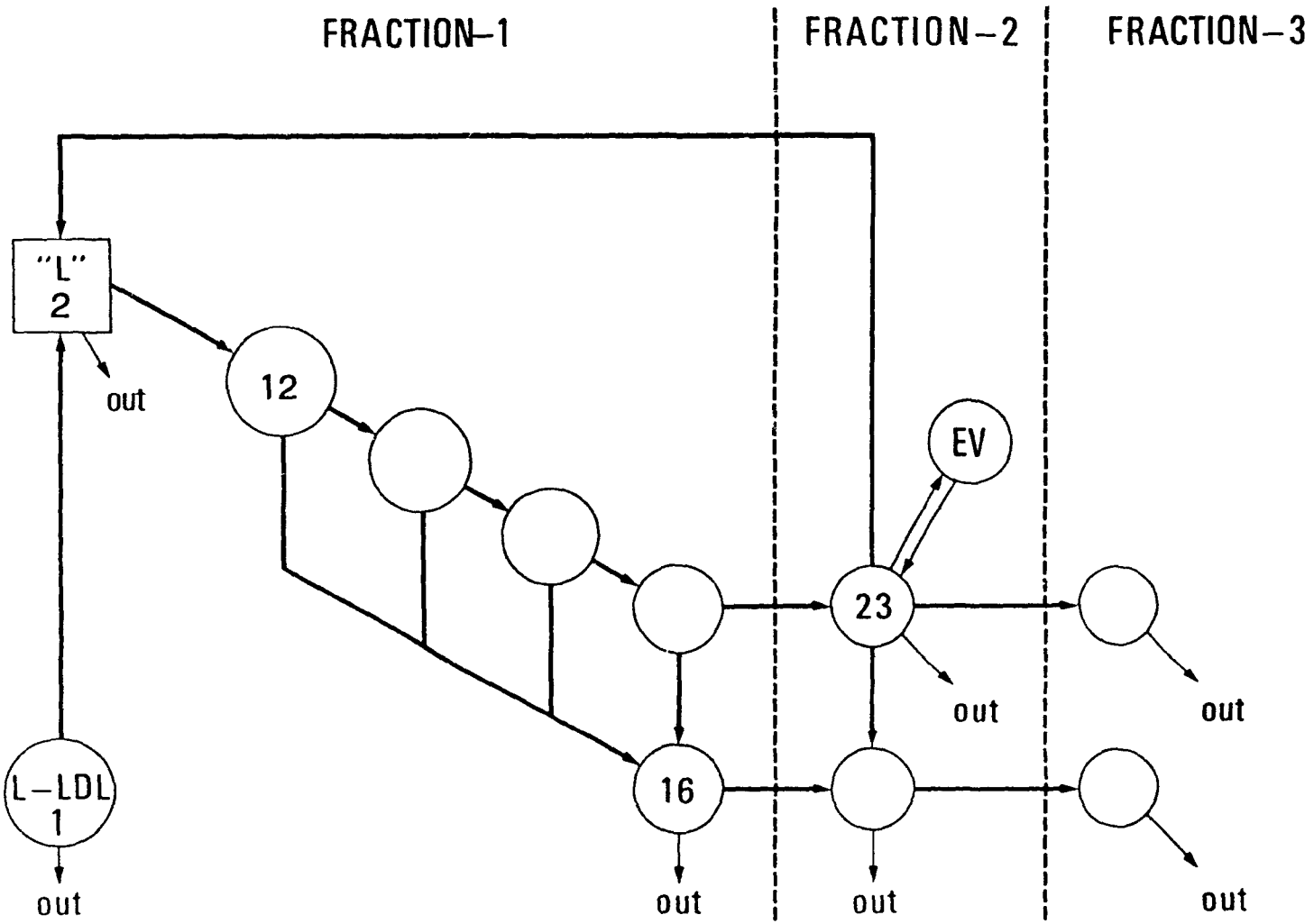
FRACTION–1
FRACTION–2
FRACTION–3
"L"
2
out
12
EV
23
out
out
L–LDL
1
out
16
out
out
out
out

Fig. 4. The observed radioactivity values of LDL subfraction-1 from HyperapoB patient AB after injection of ^{131}I-D-LDL. The observed data are expressed as open symbol (□). The simultaneous model generated line is expressed as dotted line (---). When the rate ccefficient of the D-LDL return pathway to liver is decreased to 1/10 of the actual rate, the simultaneous model generated line is expressed as solid line (—).

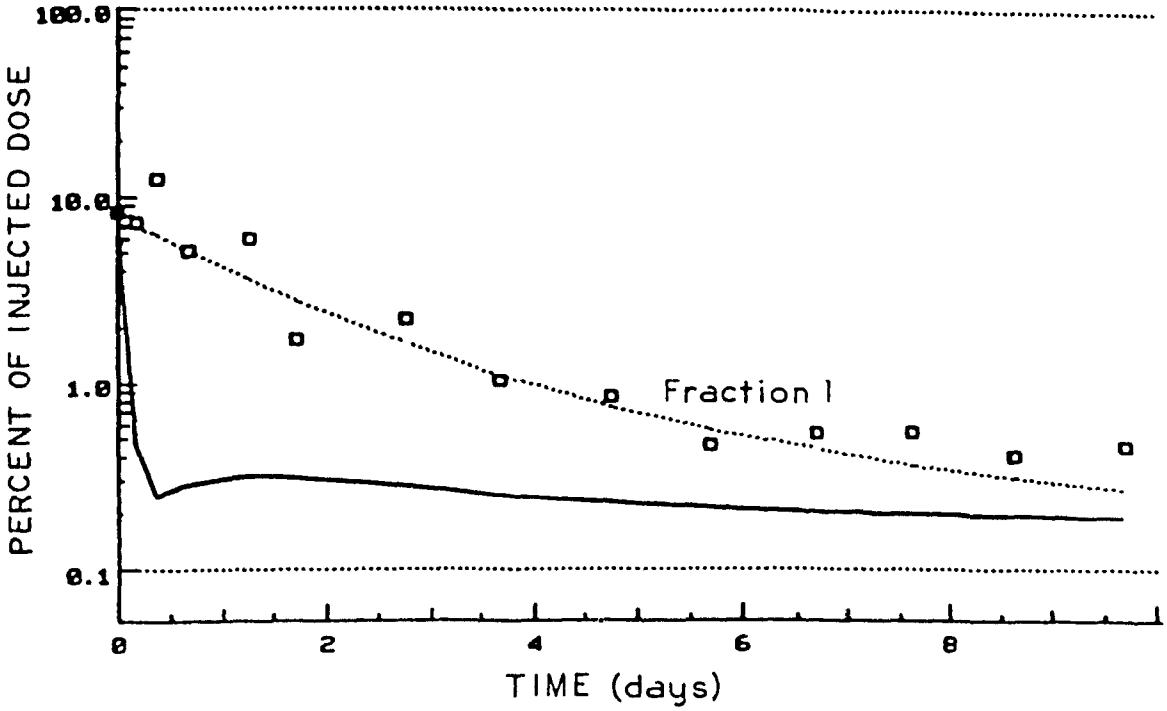
100.0
10.0
1.0
0.1
PERCENT OF INJECTED DOSE
Fraction I
0
2
4
6
8
TIME (days)

Fig. 5. The observed radioactivity values of LDL subfraction-1 from HyperapoB patient EB after injection of ^{131}I-D-LDL. The observed data are expressed as open symbol (□). The simultaneous model generated line is expressed as a dotted line (---). When the slow turnover pool was suppressed to zero, the simultaneous model generated line is expressed as a solid line (—).

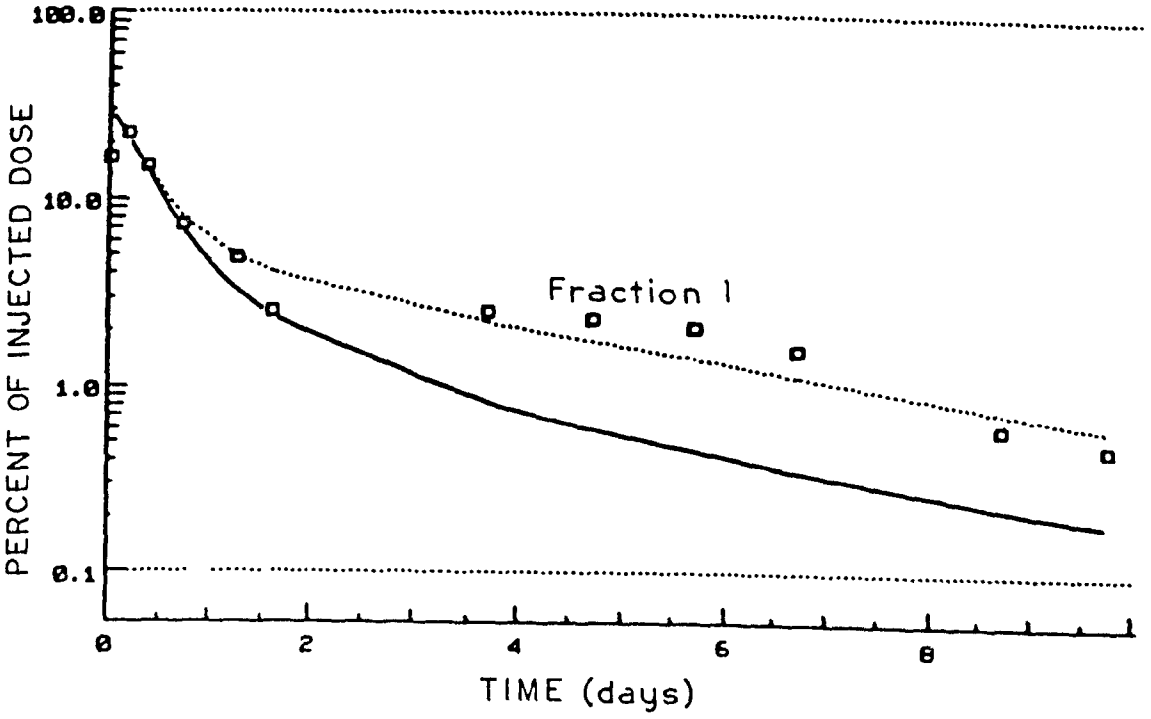
100.0
10.0
1.0
0.1
PERCENT OF INJECTED DOSE
Fraction 1
0
2
4
6
8
TIME (days)

Fig. 6. The observed radioactivity value of ^{131}I-LDL subfraction-2 (□) after injection of ^{131}I-D-LDL and ^{125}I-LDL subfraction-2 (▲) derived from ^{125}I-LDL subfraction-1 after injection of ^{125}I-B-LDL in the control DF (Fig. 6A), HyperapoB patient KB (Fig. 6B), HyperapoB patient EB (Fig. 6C), and HyperapoB patient AB (Fig. 6D).

The dotted line (□ --- □) and solid line (▲ —— ▲) are simultaneous model generated lines. All data are normalized to the fraction of the injected labeled-apoB dose. If all B-LDL were converted to D-LDL, the areas under these two curves would be equal.

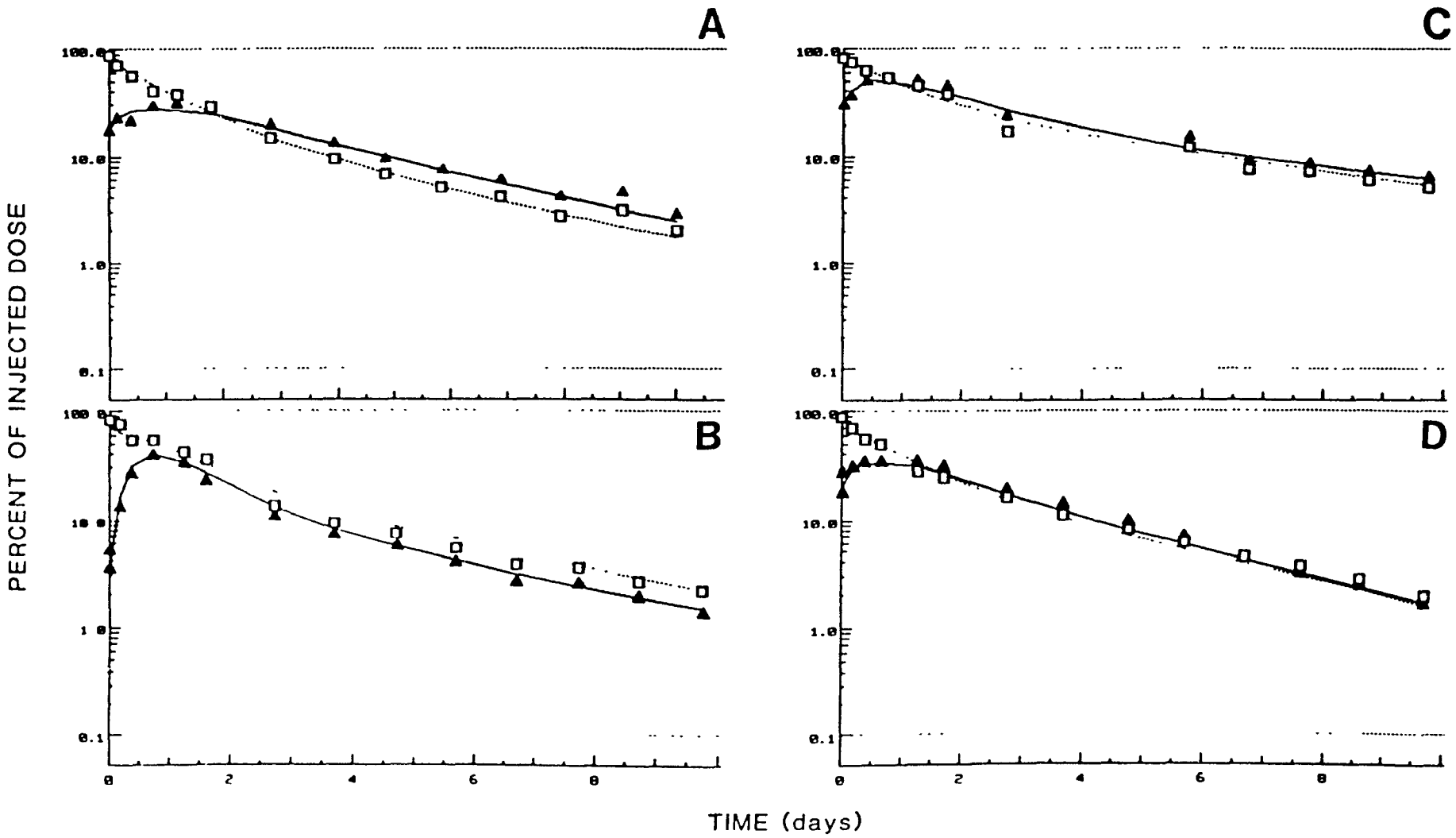
A
B
C
D
PERCENT OF INJECTED DOSE
TIME (days)

Fig. 7. The simultaneous model generated best fitted line of the control subject DF. The symbols are the observed values and the lines are predicted by the model.

Figs. 7A and 7B are data after injection of radiolabeled B-LDL whereas Figs. 7C and 7D are data after injection of radiolabeled D-LDL.

The plasma decay curve (● ——— ●) and the accumulated urine curve (▼ - - - ▼) are shown in Figs. 7A and 7C after B-LDL and D-LDL injection respectively. The LDL subfractions curve, fraction 1 (▲—— ▲), fraction 2 (■·····■), and fraction 3 (◇•—•◇) are shown in Figs. 7B and 7D after B-LDL and D-LDL injection respectively.

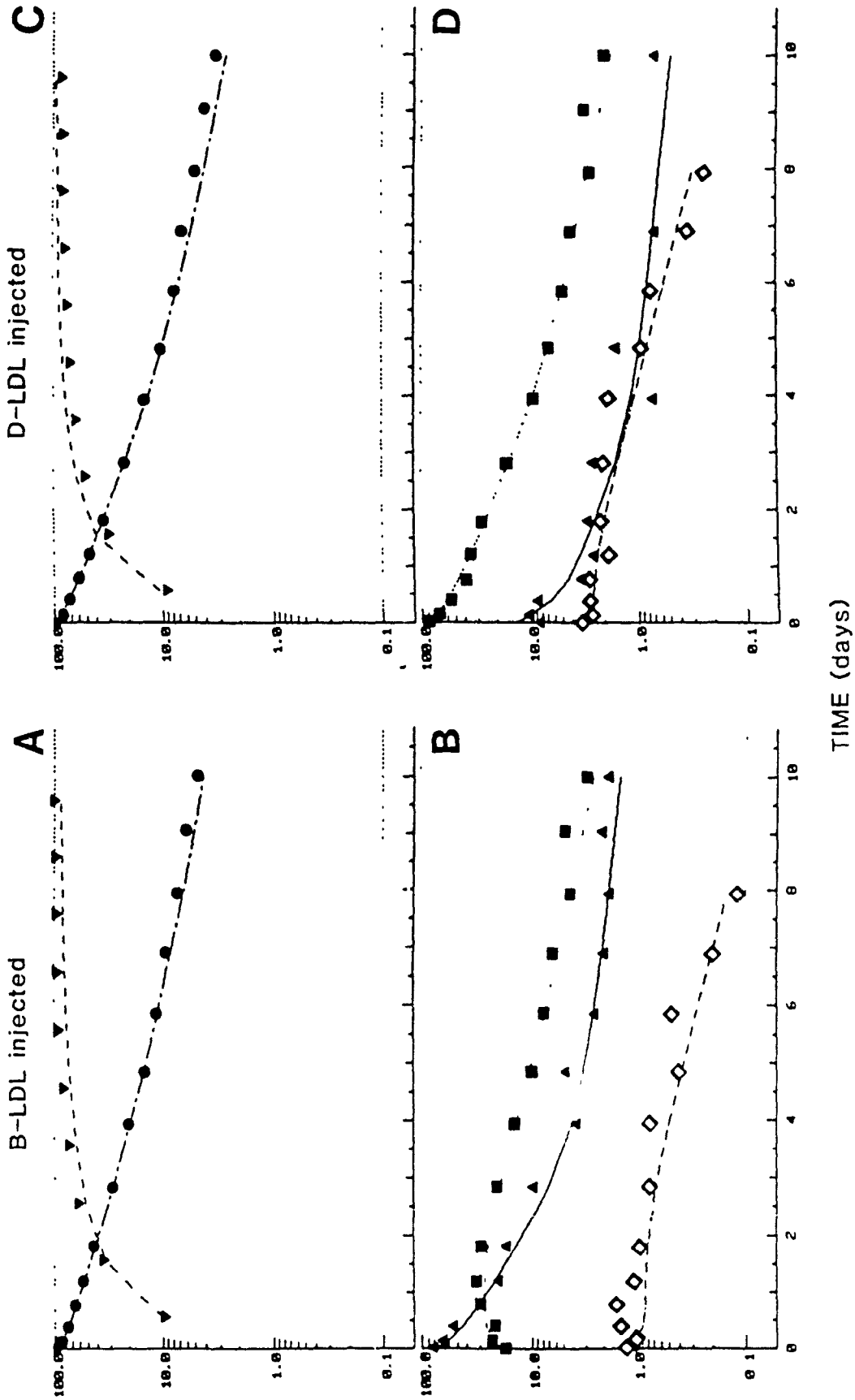
A
B
C
D
B-LDL injected
D-LDL injected
PERCENT OF INJECTED DOSE
TIME (days)

Fig. 8. The simultaneous model generated best fitted line of HyperapoB patient KB. The symbols are the observed values and the lines are predicted by the model.

Figs. 8A and 8B are data after injection of radiolabeled B-LDL whereas Figs. 8C and 8D are data after injection of radiolabeled D-LDL.

The plasma decay curve (●———●) and the accumulated urine curve (▼- - -▼) are shown in Figs. 8A and 8C after B-LDL and D-LDL injection respectively. The LDL subfraction curve, fraction 1 (▲——▲), fraction 2 (■·····■), and fraction 3 (◇·—·◇) are shown in Figs. 8B and 8D after B-LDL and D-LDL injection respectively.

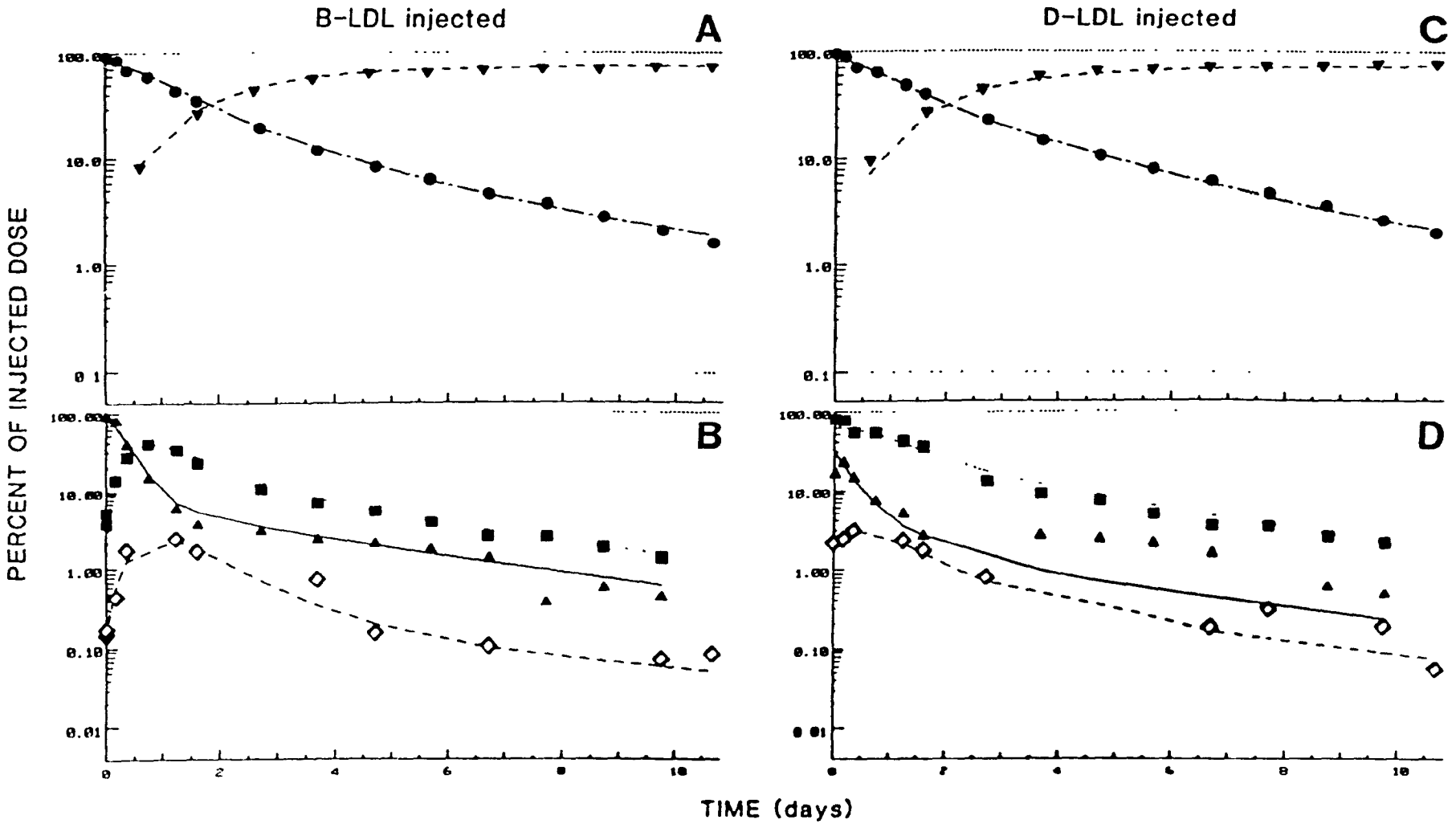
B-LDL injected
A
C
D-LDL injected
B
D
PERCENT OF INJECTED DOSE
TIME (days)

Fig. 9. The simultaneous model generated best fitted line of HyperapoB patient AB. The symbols are the observed values and the lines are predicted by the model.

Figs. 9A and 9B are data after injection of radiolabeled B-LDL whereas Figs. 9C and 9D are data after injection of radiolabeled D-LDL.

The plasma decay curve (●----●) and the accumulated urine curve (▼----▼) are shown in Figs. 9A and 9C after B-LDL and D-LDL injection respectively. The LDL subfractions curves, fraction 1 (▲—▲), fraction 2 (■·····■), and fraction 3 (◇·—·◇) are shown in Figs. 9B and 9D after B-LDL and D-LDL injection respectively.

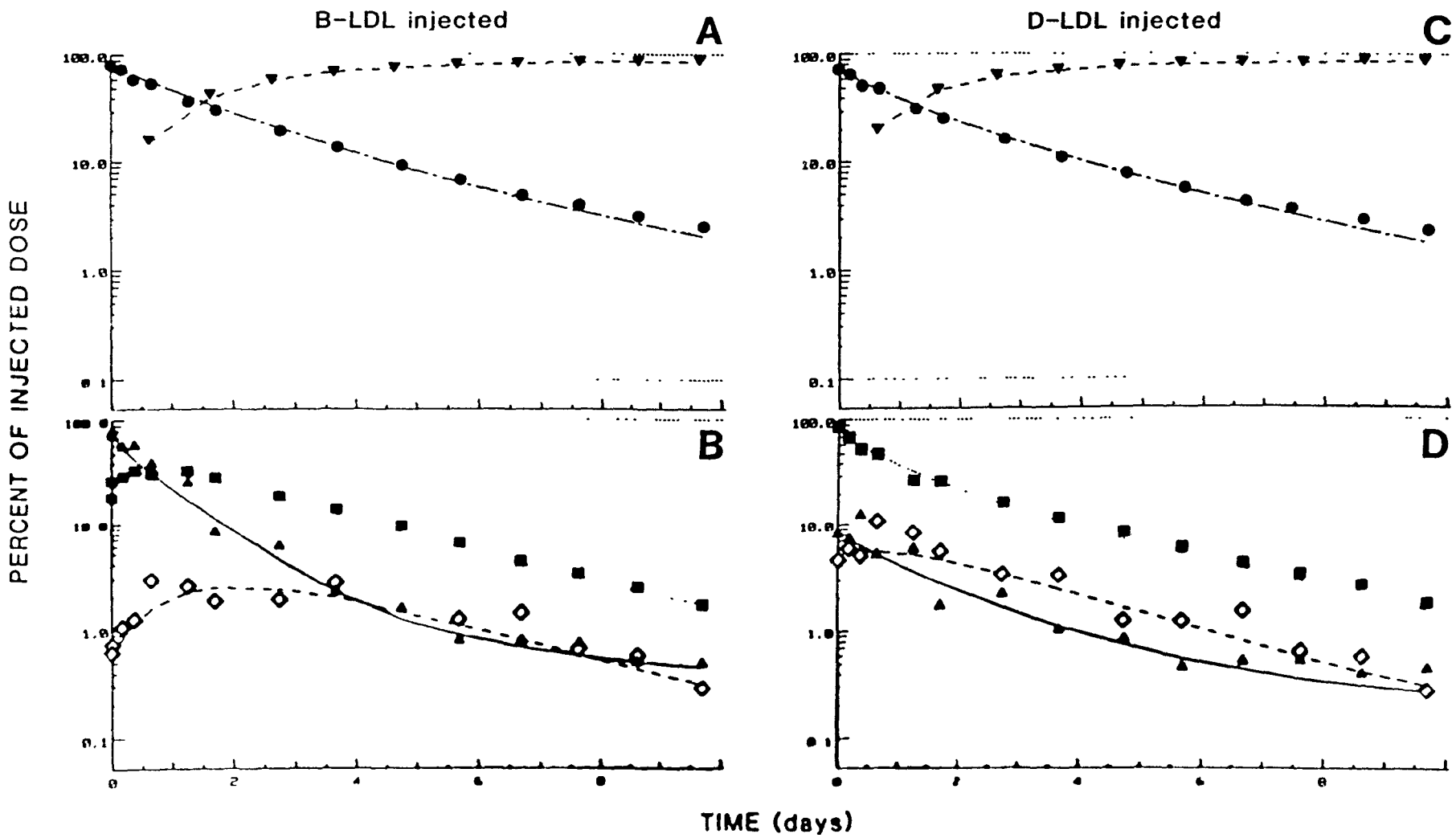
B-LDL injected
A
D-LDL injected
C
B
D
PERCENT OF INJECTED DOSE
TIME (days)

Fig. 10. The simultaneous model generated best fitted line of HyperapoB patient EB. The symbols are the observed values and the lines are predicted by the model.

Figs. 10A and 10B are data after injection of radiolabeled B-LDL whereas Figs. 10C and 10D are data after injection of radiolabeled D-LDL.

The plasma decay curve (●-—-●) and the accumulated urine curve (▼----▼) are shown in Figs. 10A and 10C after B-LDL and D-LDL injection respectively. The LDL subfractions curves, fraction 1 (▲—— ▲), fraction 2 (■·····■), and fraction 3 (◇·—·◇) are shown in Figs. 10B and 10D after B-LDL and D-LDL injection respectively.

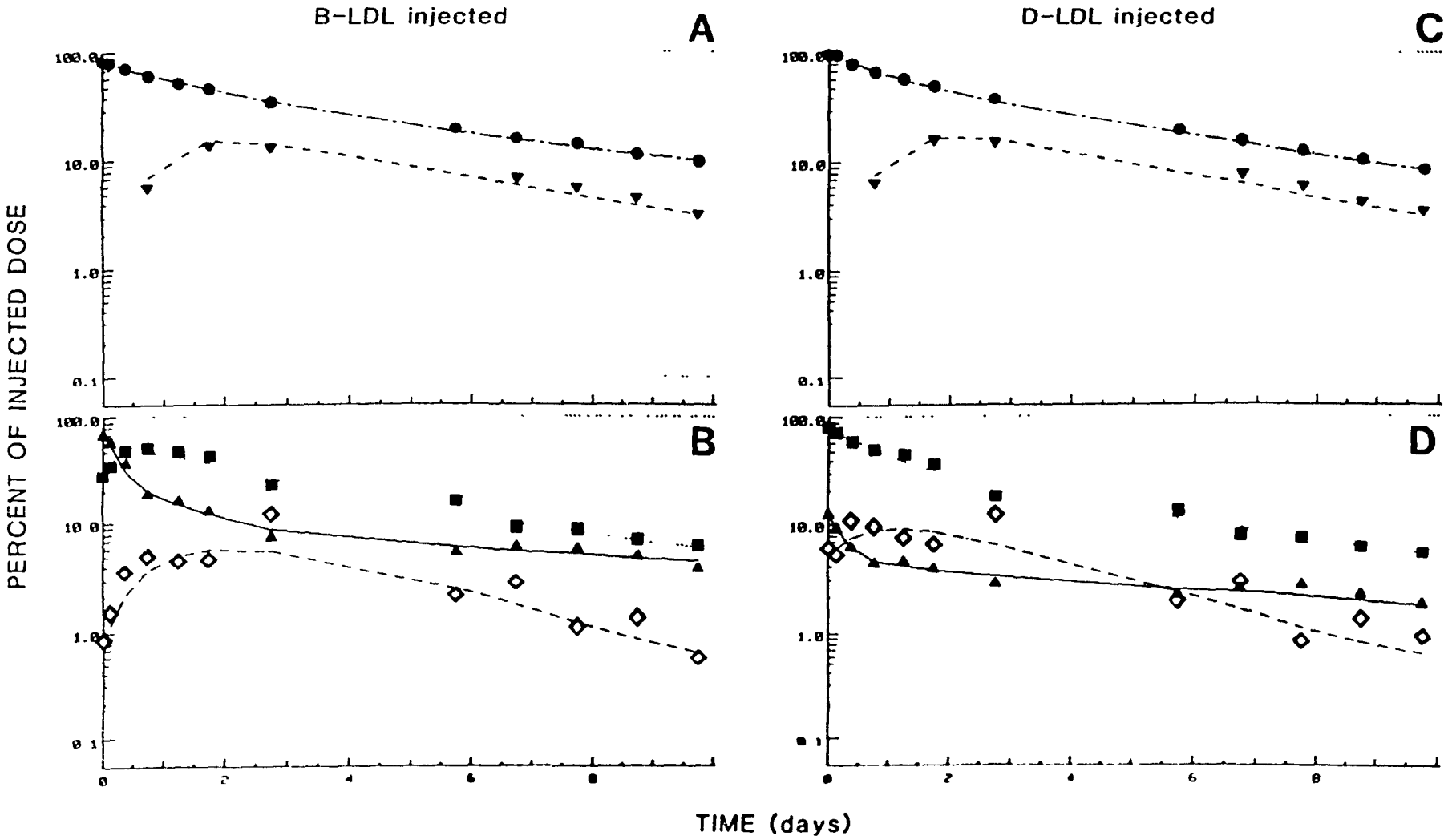
B-LDL injected
A
D-LDL injected
C
B
D
PERCENT OF INJECTED DOSE
TIME (days)

Table 1. Clinical and biochemical data of the subjects

Subject	Age	RBW	TC	TG	VLDL-C	LDL-C	HDL-C	LDL-B	WHO type
		%	mg/dl plasma						
Control									
DF	40	97	160	127	5	105	30	73	N
HyperapoB									
KB	38	94	135	318	24	94	17	121	IV
AB	44	95	209	147	20	166	23	124	N
EB	61	95	250	226	--	186	19	144	IV

TC = total cholesterol; TG = triglyceride;
VLDL-C, LDL-C, and HDL-C = VLDL-, LDL-, and HDL-cholesterol;
LDL-B, LDL apolipoprotein B in d > 1.019 fraction of plasma;
RBW, relative body weight calculated as kg/(cm-100) x 100%

Table 2. Distribution of ApoB and Cholesterol to ApoB Ratios in LDL Subfractions*
(1=buoyant LDL, 2=dense LDL, 3=very dense LDL)

	apoB, % of total apoB			cholesterol: apoB ratio	
Subject	1	2	3	1	2
Control					
DF	27±3.7%	73±3.7%	0	1.58±0.10	1.29±0.11
HyperapoB**					
KB	28±4.8%	66±3.9%	6.4±4.0%	1.13±0.10	0.88±0.06
AB	26±5.0%	65±5.0%	8.8±2.5%	1.73±0.14	1.18±0.10

* All the data are expressed as mean±SD.
** Samples from patient EB were not measured and mean values were used to calculate fractional pool size.

Table 3. Production rate of LDL apoB in Control and Patients with HyperapoB

	Control	HyperapoB		
	DF	KB	AB	EB
De novo synthesis of B-LDL; U(1)*:				
mg/d.	436	2797	1123	1284
mg/kg-d.	6.58	35.1	13.9	18.9
De novo synthesis of D-LDL; U(23)*:				
mg/d.	391	431	995	546
mg/kg-d.	5.91	5.44	12.3	8.02
Production rate of total LDL:				
mg/d.	827	3228	2119	1828
mg/kg-d.	12.5	40.6	26.2	26.9
Production rate of D-LDL:				
mg/d.	576	1093	1708	1445
mg/kg-d.	8.70	13.8	21.1	21.2

U (I)*: De novo synthesis input rate represents the rate of entry of new material into compartment I from outside the system.

Table 4. Fate and transport rate of LDL apoB in the model system

	Control	HyperapoB		
	DF	KB	AB	EB
Transport rate through Comp(1); U(1):				
mg/d.	436	2797	1123	1284
fraction equilibrium with liver:	43%	100%	65%	71%
fraction degraded irreversibly:	57%	0%	35%	29%
Transport rate through liver; Comp (2):				
mg/d.	306	3340	2481	996
1)* fraction from B-LDL,	62%	84%	29%	91%
fraction from recirculating D-LDL,	38%	16%	71%	9%
2)** fraction entering deplipidation chain,	100%	36%	99%	100%
fraction degrading irrevesibly,	0%	64%	1%	0%
Transport rate through deplidation chain:				
mg/d.	306	1208	2465	996
fraction converted to D-LDL,	98.9%	99%	100%	99%
fraction entering slow turnover pool,	1.1%	1%	0%	1%

Comp; compartment
* ; represents the fraction of transport material that comes from
** ; represents the fraction of transport material that leaves to

Table 5. Fate and transport rate of LDL apoB in the subfractions in the model system

	Control	HyperapoB		
	DF	KB	AB	EB
Transport rate through fraction 1:				
mg/d.	553	3337	2875	1374
1)* fraction from U(1),	79%	84%	39%	93%
fraction from recirculating D-LDL,	21%	16%	61%	7%
2)** fraction converted to D-LDL,	55%	36%	86%	72%
fraction degraded irreversibly,	45%	64%	14%	28%
Transport rate through fraction 2:				
mg/d.	693	1630	3460	1532
1)* fraction from U(23),	56%	26%	29%	36%
fraction from conversion of U(1),	27%	41%	21%	58%
fraction from recirculating D-LDL,	17%	33%	51%	6%
2)** fraction recirculating back to liver,	17%	33%	51%	6%
fraction converted to very dense LDL,	11%	34%	5.7%	45%
fraction degraded irreversibly,	72%	33%	44%	49%
Transport rate through fraction 3:				
mg/d.	76	546	199	693
fraction degraded irreversibly,	100%	100%	100%	100%

* ; represents the fraction of transport material that comes from
**; represents the fraction of transport material that leaves to

Table 6. Direct removel of LDL apoB in the model system

		Irreversible removal of LDL									
		Liver; Comp*(2)		Fraction 1				Fraction 2		Fraction 3	
	PR of total LDL	removal in the liver	% of LDL PR	total removal rate	% of LDL PR	removal before equilib-rating in liver	removal from the slow turnover pool	total removal rate	% of LDL PR	total removal rate	% of LDL PR
Subject		mg/d.	%	mg/d.	%	mg/d.	mg/d.	mg/d.	%	mg/d.	%
Control											
DF	827	0	0	251	30%	247	4	501	61%	76	9.2%
HyperapoB											
KB	3228	2128	66%	10	0.	0	10	546	17%	546	17%
AB	2119	16.4	0.8%	394	19%	394	0	1510	71%	199	9.4%
EB	1828	0	0	384	21%	378	6.36	751	41%	693	38%

PR; production rate
* ; compartment

Table 7. Fractional catabolic rate (FCR) of LDL and LDL subfractions in Control and patients with HyperapoB

Subject	FCR (per day)		
	LDL	Fraction 1	Fraction 2
Control			
DF	0.376	0.751	0.367
HyperapoB			
KB	0.791	2.385	0.396
AB	0.468	0.846	0.581
EB	0.359	1.165	0.437

CHAPTER 6

ADIPOSE TISSUE GLYCERIDE SYNTHESIS IN PATIENTS WITH HYPERAPOBETALIPOPROTEINEMIA

ABSTRACT

Adipose tissue was obtained at thoracotomy in 8 patients with valvular heart disease, all of whom were free of coronary disease and were normolipidemic with normal LDL apoB levels, and 10 patients with coronary artery disease, all of whom had hyperapobetalipoproteinemia. In both groups, the rates at which linoleic acid and palmitic acid were incorporated into diglyceride and triglyceride were determined *in vitro*. The data indicate that fatty acid incorporation into adipose tissue glycerides was twice as rapid in controls as in patients with hyperapobetalipoproteinemia. By contrast there was no difference between the groups in the rate of hydrolysis of adipocyte triglyceride. On average the adipocytes in the patients with hyperapobetalipoproteinemia were larger than controls. However, when compared to a subgroup of the controls with similar cell size, the difference in glyceride synthesis between controls and patients with hyperapobetalipoproteinemia was even more pronounced. These observations may explain, at least in part, the overproduction of VLDL in hyperapobetalipoproteinemia and also suggest that the basic defect in the disorder may be impaired fatty acid metabolism in critical peripheral sites such as adipose tissue.

INTRODUCTION

Hyperapobetalipoproteinemia (HyperapoB) is defined as the combination of a normal or near normal low density lipoprotein (LDL) cholesterol with a disproportionately elevated LDL apolipoprotein B (apoB) (1). The disorder is common in patients with premature coronary artery disease (1,2), and although HyperapoB is frequently familial (3), its genetic basis is not well understood with the findings in one large Amish kindred most consistent with a single gene effect (4).

Phenotypically, HyperapoB is characterized by increased numbers of LDL particles in plasma, most of which are smaller and denser than normal (5). Both in vitro (6) and in vivo (7) studies have not identified any significant fault in catabolism with the data pointing instead to overproduction of LDL. This overproduction of LDL apoB is secondary to oversynthesis of very low density lipoprotein (VLDL) (7), and given this, the frequency of hypertriglyceridemia in patients with HyperapoB is not surprising (2). The reason for overproduction of VLDL apoB in patients with HyperapoB though is not clear. It could, for example, be a primary abnormality of hepatocyte metabolism due to impaired regulation of protein synthesis, or alternatively, secondary to increased free fatty acid (FFA) flux to the liver (8-10, 39-41). Studies with normolipidemic subjects (39,41) showed that the output of triglyceride (Tg) in VLDL was dependent upon the uptake of FFA in the splanchnic region. Howard et al. (40) further demonstrated in an obese population with low plasma lipids that VLDL triglyceride synthesis was not significantly related to fasting FFA levels but was significantly correlated with post-

prandial FFA. In the case of hypertriglyceridemia, Boeg et al. (41) explained that some patients had the correlation between FFA uptake with VLDL-Tg output whereas some patients apparently had a higher VLDL-Tg secretion rate which had a precursor other than plasma FFA. Havel et al. (39) instead pointed out that the uptake of FFA in the splanchnic region in the hyperlipidemic groups is greater than the normolipidemic group. In all cases, when the FFA flux to the splanchnic region increases, the VLDL output increases.

Reports have appeared previously pointing to abnormal adipose tissue metabolism in hypertriglyceridemia with either triglyceride synthesis diminished or triglyceride hydrolysis increased (11-15). Both could produce the same consequence -- namely, an increased flux of FFA to the liver which might then lead to increased VLDL synthesis. Therefore the present study was designed to measure adipose tissue triglyceride synthesis in patients with HyperapoB and compare these results to those found in controls.

SUBJECTS AND METHODS

Subjects: The 18 patients studied had been admitted to hospital to undergo either aortocoronary bypass surgery or cardiac valve replacement. Based on coronary angiography, the first group had coronary artery disease, the second did not. Further, all 10 patients with coronary artery disease had HyperapoB while the 8 with valvular disease who served as controls had normal levels of plasma LDL apoB (1).

Methods: After an overnight fast, blood samples were obtained from the patients prior to their operation. VLDL was isolated by ultracentrifugation at d < 1.006 g/ml. Total cholesterol, triglyceride and VLDL cholesterol were measured enzymatically (Cholesterol kit No. 683197, and Triglyceride kit No. 683248, Beckman Instrument Corp., California). High density lipoprotein (HDL) cholesterol was determined after heparin-manganese chloride precipitation of plasma (16). Intermediate density lipoprotein (IDL, d 1.006-1.019 g/ml) was removed by ultracentrifugation and d > 1.019 g/ml LDL apoB was then measured by radial immunodiffusion (17). LDL cholesterol was calculated by subtracting VLDL and HDL cholesterol from total cholesterol (18).

Subcutaneous adipose tissue was obtained after the thorax was opened at surgery. The tissue was first freed from visible blood vessels and connective tissue, and then divided into small segments weighing between 20 and 50 mg. Each segment was pre-incubated for 30 minutes at 37°C in 2 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) with 2% human serum albumin (HSA) fraction V (Sigma, St. Louis) and 0.2% glucose (19). The concentration of FFA to HSA in the incubation medium was 1.56 mEq/l :0.33 mmol/l. The adipose tissue segment was then transferred to 2 ml of the same incubation medium which had been gassed with 95% O_2 and 5% CO_2 for 30 minutes and to which was added either ^{14}C-linoleic acid-HSA or ^{3}H-palmitic acid-HSA (20) (approximately 10^6 cpm/ml; specific activities of ^{14}C-linoleic acid and ^{3}H-palmitic acid were 52.6 uCi/umol and 500 uCi/umol respectively -- New England Nuclear, Boston, Mass.). Each incubation was performed either in duplicate or triplicate with atmosphere as the gas

phase. The incubation time ranged from 0 to 300 minutes. After incubation, aliquots of the medium were used for the determination of glycerol concentration enzymatically (21) and FFA concentration colorimetrially (22).

To extract glycerides, the tissue was first washed with warm saline and then homogenized in 2 ml of isopropanol:heptane solvent (4:1, V/V). One ml of heptane and one ml of 0.05% KOH were added to the homogenate to extract the glycerides into the heptane phase. The mixture was then mixed and left standing for at least ten minutes. After centrifugation, the heptane phase was washed once with 2 ml of isopropranol-heptane-0.05% KOH (4:1:3, by volume). The heptane phase fraction thus obtained was used for thin-layer chromatography on polysilic acid-impregnated fibreglass sheet with a developing solvent of hexane:diethyl ether: acetic acid:methanol (75:25:2:3, by volume) to separate triglycerides, diglycerides, and monoglycerides. The radioactivity in each glyceride fraction was then eluted and counted in scintillation fluid. Insignificant incorporation of labelled fatty acid into adipose tissue cholesteryl esters and phospholipids was observed in both controls and patients with hyperapoB.

Fat cell size and number were determined by the methods of Hirsch and Gallian (23). The adipose tissue was weighed, fixed with 2% osmium tetroxide in collidine buffer for 48 hours, and the mixture then filtered through a 250 um and 25 um mesh nylon filter so that adipocytes were retained. These were then suspended in Isoton II and the suspension counted at various appertures using an electronic Coulter counter. The dry weight of

the total lipid content in the adipose tissue was measured after extraction with chloroform: methanol (2:1, v/v).

To calculate the absolute amount of fatty acid taken up from the medium and incorporated into glycerides, it is necessary to correct for the decrease in specific activity of medium FFA due to the release of FFA from adipose tissue. Consequently, mean FFA specific activity was calculated by using initial and final concentrations of FFA and radioactivity in each incubation time, as suggested by Dole (24) using the differential equation. $A = Ao\ (k/\alpha)$ where A is the mean specific activity, Ao is the initial specific activity and $k = \ln\ (1+\alpha)$ where α is the fractional increase in the fatty acid concentration of the medium at the end of each incubation time.

Statistical analyses were performed using Student's unpaired t-test. Values in the tables and figures are given as the mean ± standard error of the mean.

RESULTS

The clinical and biochemical data of the 18 subjects studied are listed in Table 1. The two groups did not differ significantly in age. All the patients with HyperapoB had significant coronary artery disease documented by coronary angiography while none of the patients in the control group had coronary disease evident angiographically. Total cholesterol, triglyceride and LDL cholesterol were all significantly higher in the HyperapoB group, whereas HDL cholesterol was significantly lower. One of patients with HyperapoB had a plasma triglyceride level above the 95th percentile while three had HDL cholesterol

values below the 5th percentile based on Lipid Research Clinic screening values (25). All control patients, however, were normolipidemic and none had lowered HDL cholesterol. In addition, it should be noted that the HyperapoB group had a significantly higher relative body weight index than did the controls in which two of the HyperapoB patients had a body weight index > 125% (135% and 142%).

The data on adipocyte morphology are presented in Table 2 with the cells characterized by number (i.e. cells/mg tissue), by lipid content, and by weight. The adipocytes from the HyperapoB patients were significantly larger than the controls (Table 2A). Further, the average cell lipid content was significantly greater and the cells were heavier in the HyperapoB group than in the controls. However, the variation in cell size amongst the controls was considerable whereas the results were much more uniform amongst the HyperapoB patients. In addition since adipocyte metabolism may be affected by cell size, the control group was subdivided into those with realtively smaller (SC) and those with relatively larger cells (LC). These data are presented in Table 2B. Note that the larger control cells (LC) were slightly but not significantly smaller than the adipocytes from the HyperapoB patients whereas within the control group the SC cells were significantly smaller than the LC cells. These relations also held for cell lipid content and cell weight: in neither instance was there a significant difference between the LC and HyperapoB groups, whereas in both the differences were significant when SC cells were compared to LC cells.

The incorporation of linoleic acid into adipose tissue glycerides in the control group and the HyperapoB groups are shown in Table 3 with the results expressed as nEq linoleic acid per 10^6 cells incorporated into either diglycerides (Dg) or triglycerides (Tg). Note that at each time point, significantly more linoleic acid was incorporated into the glycerides of control adipocytes compared to HyperapoB adipocytes, with just over twice as much incorporated into diglycerides, the difference being even greater when triglyceride synthesis was compared.

These differences can be seen in greater detail in Fig. 1 where SC an LC control subgroups are shown separately. Note that in both control subgroups as well as the HyperapoB group, the incorporation of linoleic acid into adipose tissue glycerides is most rapid during the first 60 minutes, the rate decreasing thereafter. The rate of diglyceride (Fig. 1A) and triglyceride (Fig. 1B) synthesis did not differ significantly between the controls subgroups. However when HyperapoB cells were compared to the LC control subgroups, both with similar cell sizes, the difference in diglyceride synthesis (Fig. 1A) was significant at all time points ($p < 0.0125$, < 0.01, < 0.05, and < 0.025, respectively). Similarly Fig. 1B demonstrates the incorporation of linoleic acid into adipocyte triglycerides in both control subgroups and the HyperapoB group. Triglyceride synthesis was two to three fold more rapid in the SC group than in the HyperapoB group at all time points ($p < 0.005$, < 0.005, < 0.005, and < 0.0125, respectively). This was also the case when the LC control group was compared to the HyperapoB group with triglyceride synthesis higher at all time points ($p < 0.05$, < 0.0005, < 0.0025, and $<$

0.025, respectively).

Fig. 2 compares the incorporation of palmitic acid into adipose tissue diglycerides and triglycerides in both control subgroups and the HyperapoB group. The results are similar to those obtained with linoleic acid in which the LC subgroup metabolize somewhat more active than the SC subgroup. With regard to diglyceride synthesis, when the SC subgroup and the HyperapoB group were compared, although the control group was higher, the difference was not statistically significant at either 60 or 300 minutes. However, with respect to triglyceride synthesis, the difference was statistically significant at both time points ($p < 0.025$, and < 0.0125, respectively). When the LC subgroup and the HyperapoB were compared with regard to incorporation of palmitic acid, both diglyceride and triglyceride synthesis at 60 and 300 minutes were significantly higher in the LC group than in the HyperapoB group (diglyceride: $p < 0.0025$ and < 0.01; and triglycerides: $p < 0.0025$ and < 0.0005, respectively).

Net incorporation of fatty acids is, of course, a balance between synthesis and hydrolysis of glycerides. The rate of hydrolysis was therefore also examined. The data are shown in Fig. 3 which illustrates the change in medium FFA (Fig. 3A) and glycerol (Fig. 3B) over the course of the experiment. Note that there is no significant difference in either parameter between the control and the HyperapoB groups. Thus while differences in synthesis were apparent, no difference in hydrolysis was evident between the groups.

DISCUSSION

Adipose tissue glyceride synthesis was examined in two groups: one, which served as the control group, was composed of patients with valvular heart disease; all of these were normolipidemic and free of coronary artery disease. The second was made up of patients with coronary atherosclerosis. Most of these were normolipidemic but with elevated LDL apoB level. The present in vitro experiments demonstrate that both linoleic acid and palmitic acid were incorporated much less rapidly into adipocyte glycerides in the HyperapoB group compared to the controls. However, while on the one hand there was evidence of diminished glyceride synthesis in the HyperapoB group, on the other, there was no evidence of any difference in hydrolysis.

Before considering the possible implications of these findings with regard to the pathophysiology of HyperapoB, the limitations of the experimental methods must first be noted. For example, when fatty acids are released from the tissue to the medium throughout the incubation, the specific activity of the medium FFA declines during the experiment. Fortunately, mean FFA specific activity can be calculated as suggested by Dole (24), thus overcoming this difficulty. However, were the intracellular FFA pool to be diluted disproportionately by unlabelled material released from hydrolysis of glyceride, no correction would be possible and the esterification rate consequently would be underestimated. Previous work though (15,24,26) indicates this is unlikely to be the case since there appears to be discrete entry and exit FFA pools within the adipocyte. Still there were separate glyceride pools within the adipocyte with some having

more rapid turnover rates than others (27), it must be recognized that the calculations in this and similar studies (12-15) would be insensitive to this eventuality.

In addition to the above methodologic issues, we must also consider whether the differnces observed experimentally between the two groups might be attributed to the fact that, on average, the adipocytes in the patients with HyperapoB were larger than those in the control patients. In this regard, it should first be appreciated that, though larger, the HyperapoB adipocytes were, in fact, normal in size (28-30). As well, there is considerable evidence both in human and rat adipose tissue to indicate that larger fat cells are metabolically more, not less, active than smaller cells (31-35). In this study the HyperapoB adipocytes though larger were, by contrast, less active in terms of glyceride esterification than the control cells. Beyond this though, when matched for cell size, the differences between control and HyperapoB adipocytes were even sharper. Therefore, the data indicate that the impaired glyceride esterification in adipocytes from these patients with HyperapoB does not appear to be a function of cell size.

Other investigators have previously studied fatty acid incorporation into adipose tissue glycerides in an effort to uncover the pathogenesis of hypertriglyceridemia. Their findings, while similar, have not been entirely consistent: for example, Carlson and Waldius (13) and later Rubba (14) found decreased esterification in adipose tissue in hypertriglyceridemic patients compared to normal controls. However, Larson et al. (10) noted

increased lipolysis in large adipocytes studied in patients with endogenous hypertriglyceridemia while Arner et al. (15) found re-esterification was decreased in adipocytes from obese hypertriglyceridemic patients compared to obese normolipidemic controls. Whether due to decreased incorporation or increased hydrolysis, all advanced the hypothesis that an increased fatty acid flux to the liver might be expected to increase VLDL synthesis and thus plasma triglycerides. In the present study, however, all those studied except one from the HyperapoB group had normotriglyceride levels and so this study represents the first time that decreased esterification has been found in normotriglyceridemic patients. Since hypertriglyceridemia is such a frequent accompaniment of HyperapoB, it will be important in the future to determine whether there is a common basis for the present and previous observations.

We believe, however, that the present in vitro studies may shed important light on the pathophysiology of the increased VLDL synthesis reported in HyperapoB (7) in that this phenomenon might result from at least two different mechanisms: first, were adipose tissue FFA uptake reduced, but hepatic FFA uptake not, there might then be an increased flux of FFA to the liver leading to increased VLDL synthesis so as to maintain hepatic lipid homeostasis. Alternatively, it has now been recognized that apoproteins are acylated within the hepatocytes (36), and it is possible, therefore, that this post-translational step might be important in the regulation of apoB synthesis. As well, the present studies may also help explain the observation of delayed chylomicron clearance after an oral fat load in normotrigly-

ceridemic patients with HyperapoB (37) since impaired FFA uptake into adipose tissue might lead to increased FFA levels locally inhibiting lipoprotein lipase. Finally the present results appear important because they now provide evidence that adipocytes as well as fibroblasts from patients with HyperapoB (38) differ metabolically from normal, raising the possibility that the defect(s) are, in fact, sited within the cell.

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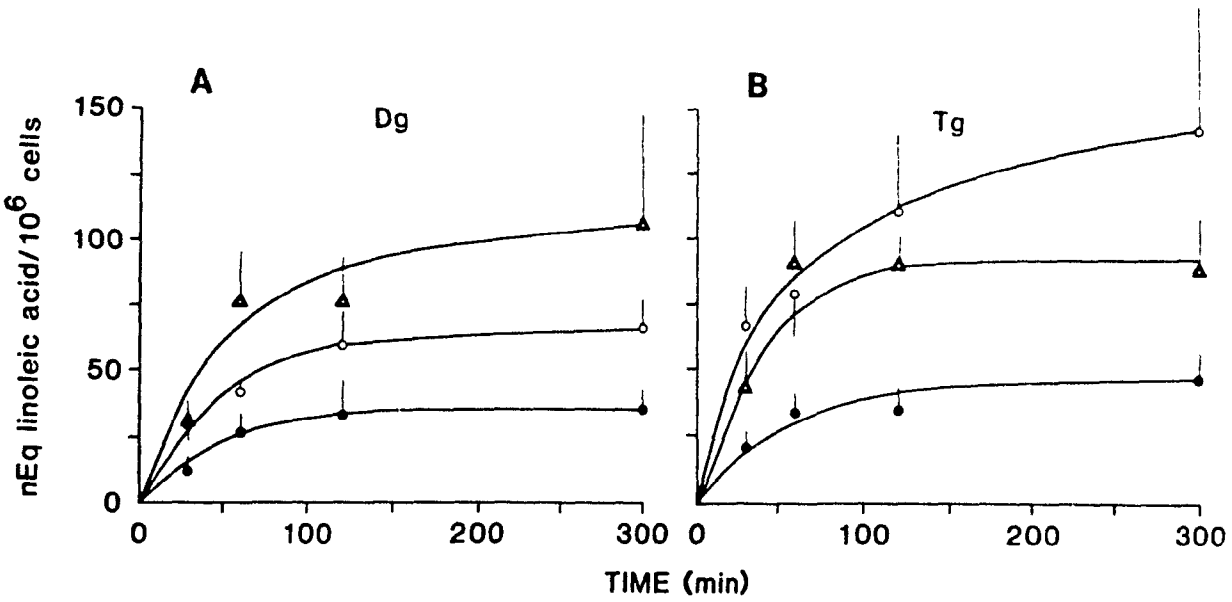

Figure 1. Incorporation of linoleic acid into adipose tissue diglycerides (Dg) and triglycerides (Tg) is expressed as nEq linoleic acid per 10^6 cells. Values are shown as mean ± standard error of the mean with large control cells in triangles, small control cells in open circles, and HyperapoB cells in closed circles.

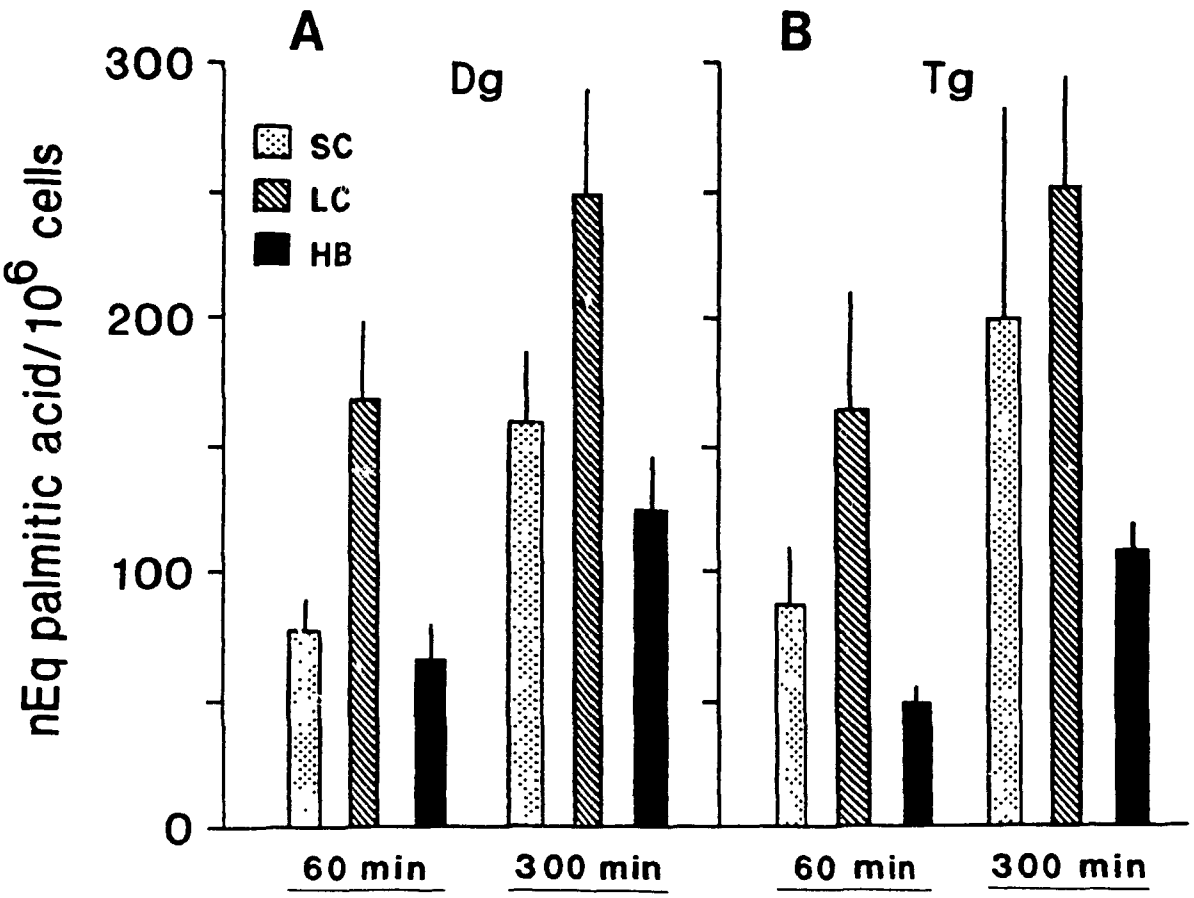

Figure 2. Incorportion of palmitic acid into adipose tissue diglycerides (fig. A) and triglycerides (fig. B) with small control cells (SC) in dotted bar, large control cells (LC) in slash bar, and HyperapoB cells (HB) in black bar. The incubation was either 60 or 300 min and values are shown as mean ± standard error of the mean.

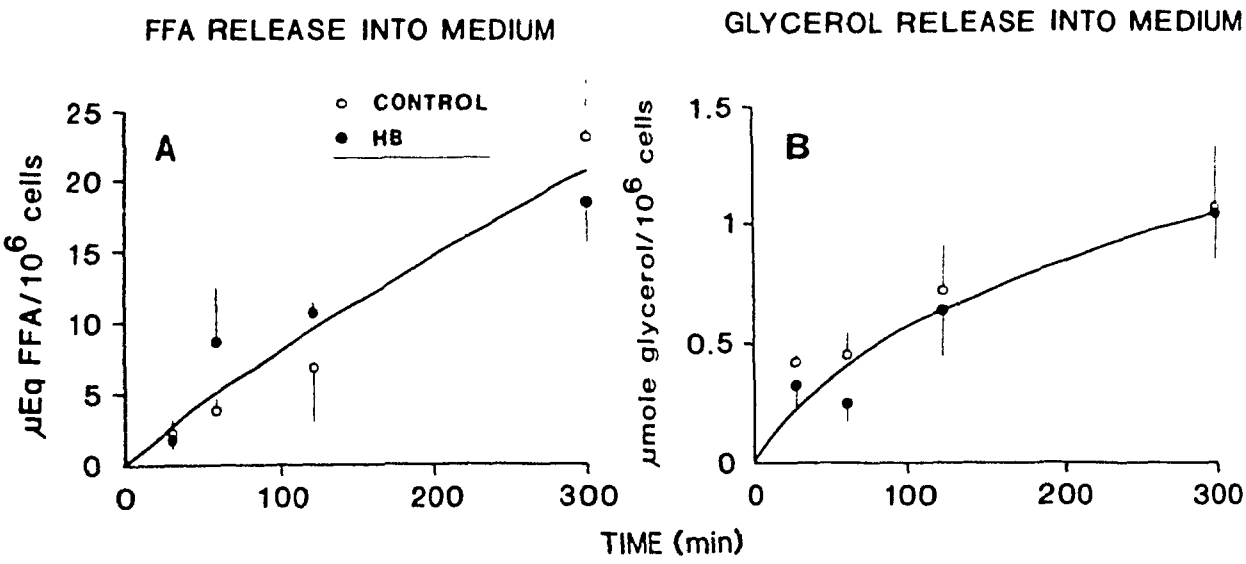

Figure 3A. FFA release into medium during the incubation is shown with control in open circles and HyperapoB (HB) in closed circles. Values are expressed as mean ± standard error of the mean.

Figure 3B. Glycerol release into the medium during the incubation is shown with control in open circles and HyperapoB (HB) in closed circles. Values are expressed as mean ± standard error of the mean.

Table 1. Clinical and biochemical data of controls and coronary artery disease patients with HyperapoB

	Sex	Age	RBW	TC	TG	HDL-C	LDL-C	LDL apoB
	m/f	yr.	%	mg/dl plasma				
Control	6/2	60± 5	87±5	181±8	80±11	48±4	117±7	86±4
HyperapoB	8/2	59±32	114±5	218±8	193±20	38±3	142±8	137±3
p		NS	<.0025	<.0025	<.0005	<.025	<.025	<.0005

Values are shown as mean ± standard error of the mean.
TC; total plasma cholesterol
TG; total plasma triglyceride
RBW; relative body weight calculated as (Kg/cm-100) x 100%
HDL-C, LDL-C; high density lipoprotein- , low density lipoprotein-cholesterol
LDL apoB; LDL apolipoprotein B

Table 2A. Adipocyte morphology of controls and HyperapoB patients

	No.	cell number	cell lipid	cell weight
		cells/mg tissue	ug/cell	ug/cell
Control	8	2976±648	0.34±0.07	0.49±0.10
HyperapoB	10	1343±185	0.74±0.16	0.90±0.16
p		<0.02	<0.05	<0.05

Table 2B. Adipocyte morphology of the subgroups of controls

	No.	cell number	cell lipid	cell weight
		cells/mg tissue	ug/cell	ug/cell
SC	4	4574±425	0.17±0.02	0.51±0.05
LC	4	1377±103	0.23±0.02	0.74±0.06
p		<0.001	<0.0005	<0.0005

Values are shown as mean ± standard error of the mean.
SC; small cell control subgroup
LC; large cell control subgroup

Table 3. nEq Linoleic acid incorporation into adipose tissue glycerides in controls and HyperapoB patients

	Dg				Tg			
	nEq linoleic acid/10^6 cells							
	30	60	120	300	30	60	120	300
	minutes				minutes			
Control (n=8)	30±3.8	59±14	67±13	85±25	55±12	85±12	99±19	115±30
HyperapoB (n=10)	12±4.5	27±7.6	34±14	35±8.4	20±6	34±7.7	35±9.4	47±11
p	<0.0025	<0.005	<0.005	<0.025	<0.005	<0.0005	<0.0005	<0.0125

Values are shown as mean ± standard error of the mean.
Dg; diglyceride
Tg; triglyceride

CHAPTER 7

SUMMARY AND DISCUSSION

SUMMARY

Hyperapobetalipoproteinemia (HyperapoB) was defined as the combination of a normal, or near-normal, LDL cholesterol in the face of an elevated plasma LDL apoP. In this disorder, an increased number of LDL particles in plasma can frequently occur without hypercholesterolemia. This contrasts with familial hypercholesterolemia (FH), where an increased number of LDL particles always produces hypercholesterolemia. Our task was to elucidate the physicochemical mechanisms responsible for HyperapoB.

Using density gradient ultracentrifugation, LDL can be separated into three subfractions: fraction-1, buoyant-LDL (B-LDL), d 1.019-1.043 g/ml; fraction-2, dense-LDL (D-LDL), d 1.043-1.055 g/ml; and fraction-3, very dense LDL, d 1.055-1.063 g/ml. In normal subjects, less than one-third of the apoB is in the B-LDL, just over two-thirds is in the D-LDL, and less than 5% is in the very-dense LDL. A similar distribution was evident in HyperapoB, except that almost 10% of the apoB was recovered in very-dense LDL, whereas in FH, over 40% was in B-LDL, 55% in D-LDL and less than 2% in very-dense LDL. D-LDL particles are smaller and have a lower cholesterol-to-protein ratio than do B-LDL particles. D-LDL particles in HyperapoB are even smaller, denser, more depleted in cholesteryl ester and enriched in protein, compared to normals, with cholesterol-to-protein ratios of 1.16 and 1.33, respectively. This difference is even more marked in hypertriglyceridemic HyperapoB patients with a cholesterol-to-protein ratio in D-LDL of 0.88. By contrast, patients with FH have an increased cholesterol-to-protein ratio in B-LDL (homozygote, 2.14 and heterozygote, 1.81) compared to both normals and patients

with HyperapoB, both of whom have a cholesterol-to-protein ratio in B-LDL of 1.50. In FH, the B-LDL particles are larger, cholesteryl ester-enriched and relatively protein-depleted. Compared with control subjects, patients with FH have an abnormal form of B-LDL, which is cholesteryl ester enriched, whereas HyperapoB patients have an abnormal form of D-LDL which is cholesteryl ester-depleted. Therefore, characteristic differences in LDL composition occur in both FH and HyperapoB, such that HyperapoB patients have a low cholesterol-to-protein ratio in LDL, whereas FH patients have a high cholesterol-to-protein ratio in LDL.

ApoB not only provides structural integrity to the LDL molecule, but also interacts specifically with the apoB/E receptor. In this regard, the immunoreactivities of six monoclonal antibodies against LDL apoB of LDL subfractions, B-LDL, D-LDL, and very dense LDL, were studied. The immunoreactivities of the LDL subfractions with antibodies 2D8, 3F5, and 4G3 were highly correlated with the LDL cholesterol-to-protein ratio. That is, B-LDL in each individual was more immunoreactive than D-LDL, which in turn was more reactive than very-dense LDL. These results suggest that as LDL particle size decreases, and as the cholesterol-to-protein ratio decreases, the structural interaction of apoB with the lipids changes progressively. It is of considerable interest that this determinant recognized two of these antibodies, 3F5 and 4G3, which are located very close to the cell receptor binding domains of the apoB molecules.

In order to understand the mechanism responsible for the heterogeneity of LDL and its metabolic characteristics in HyperapoB, the turnover of apoB in VLDL, IDL, and two major subfractions of LDL (B-LDL and D-LDL) was studied in 7 patients with HyperapoB, 6 normolipidemic subjects, and 5 patients with heterozygotic FH. Most subjects exhibited precursor-product relationships between VLDL and IDL and all did so between IDL and B-LDL. The same relationship between B-LDL and D-LDL was evident in most controls and patients with HyperapoB, but not in FH patients; in addition, the existence of VLDL-independent synthesis of D-LDL in this disorder was confirmed.

Kinetic analysis performed after injection of tracer ^{125}I-VLDL showed that HyperapoB patients had a higher rate of synthesis of VLDL-apoB than controls (40.1 vs. 21 mg/kg-d, $p < 0.05$), but a reduced fractional catabolic rate (FCR) (0.230 vs. 0.366 per hour, $p < 0.01$). After an injection of tracer ^{131}I-LDL, HyperapoB patients had higher rates of LDL apoB synthesis than did controls (23.1 vs. 13.0 mg/kg-d, $p < 0.001$); this was also the case for FH patients (22.7 mg/kg-d). The FCR of LDL was similar in HyperapoB patients and controls (0.386 vs. 0.366 per day), but was markedly decreased in FH patients (0.192 per day). These data show that the increased concentration of LDL apoB in HyperapoB is due to increased LDL synthesis which is secondary to increased VLDL synthesis; in FH, however, there is both an increased synthesis (which is partly VLDL-independent) and reduced catabolism of LDL.

Analysis of the relationship of LDL subfractions in the subjects studied showed that in each instance the turnover of B-

LDL was initially much faster than that of D-LDL. This difference is more obvious in both the control and HyperapoB subjects, but much less marked in FH. In control and HyperapoB subjects, injection of ^{125}I-B-LDL gave rise to D-LDL with a precursor-product relationship similar to that observed between VLDL and IDL. However after injection of ^{131}I-D-LDL, the plasma decay curves of B-LDL and D-LDL are almost identical. This suggests that D-LDL gives rise to B-LDL and this reverse process seems more marked in FH.

The proportion of B-LDL converted to D-LDL was first estimated quantitatively using deconvolution analysis. With this technique and measurement of the sizes of the two intravascular pools of LDL, it is possible to estimate the rates of turnover of each fraction. The results show that B-LDL turnover is faster than D-LDL and B-LDL is largely converted into D-LDL in controls and HyperapoB subjects. By contrast, the FCR of both subfractions are reduced in FH and there is less than 50% conversion of B-LDL into D-LDL. This then results in twice as much expansion of the B-LDL pool in FH as occurs in HyperapoB, whereas the D-LDL pool is increased to a similar extent in both disorders. The data also show that the increased total LDL synthesis in HyperapoB is due to overproduction of both B-LDL and D-LDL. In HyperapoB patients, there is overproduction of VLDL, then overproduction of B-LDL and D-LDL. By contrast, in FH patients, the prolonged plasma residence time of LDL might cause the accumulation of cholesteryl ester in LDL, thus producing larger, cholesteryl ester enriched B-LDL particles.

Since LDL has been shown to be both structurally and kinetically heterogeneous, it was necessary to develope a multicompartmental model to study LDL apoB metabolism. To do so, the turnover of apoB in B-LDL and D-LDL was studied in one normal subject and three patients with HyperapoB and a preliminary multicompartmental model has been developed. The model contains: 1) a B-LDL delipidation cascade with a slowly catabolizing pool derived from this cascade; 2) a pathway by which B-LDL is converted to D-LDL, and D-LDL can return to the liver and re-enter the plasma space; 3) pathways by which B-LDL, D-LDL, and very dense LDL can each be directly catabolized. Using this model, quantitative analysis confirmed that the transport rates of B-LDL and D-LDL are increased in HyperapoB compared to control (6.55 vs. 22.4, 8.70 vs. 19.5 mg/kg-d, respectively). The FCR of B-LDL apoB is three times higher than that of D-LDL apoB (1.325 vs. 0.421 per day) and an average of 54% (36% to 71%) of B-LDL goes through the cascade to be converted to D-LDL. Although the precise physiological processes involved in the model remain to be defined, the model underscores the potential physiological importance of cholesteryl ester-triglyceride exchange, by which the cholesteryl ester in B-LDL exchanges with the triglyceride in VLDL, and subsequently, hepatic or lipoprotein lipase hydrolyzes the triglyceride-enriched B-LDL particles to generate D-LDL which, as a consequence, is a cholesteryl ester-depleted, smaller, denser particle.

In summary, all the data to date indicate that the characteristic abnormalities of HyperapoB are consequences of the overproduction of hepatic apoB. In this regard, a preliminary

study with adipose tissue suggests that this oversynthesis of hepatic apoB might be secondary to a defect in peripheral triglyceride biosynthesis.

DISCUSSION

The principal concept dealt with in this thesis is that low-density lipoproteins (LDL) are heterogeneous in their composition and metabolism. The evidence in support of this recently developed concept is now very diverse and widespread, but the metabolic and clinical consequences of this concept are just beginning to be explored.

LDL is heterogeneous in several different senses. Within any normal individual, LDL is heterogeneous in that some of the LDL particles are larger, with more cholesterol, and some smaller with less, although all contain the same amount of apoB. Most important, there is a predictable relationship between size, buoyancy, and composition, with the larger particles being more buoyant because they contain more lipid but relatively less protein. While these differences are clear, within normals they are not marked, and thus LDL heterogeneity can be seen more clearly in other situations. For example, even in normals, LDL differs in composition at different times in life. LDL isolated from human umbilical-cord blood by density gradient ultracentrifugation differs in composition from adult LDL in that it is a relatively triglyceride-enriched and still cholesteryl ester-depleted particle (1). At birth, plasma FFA and triglyceride levels are low (2), while just after birth both rise sharply (3-

5), as hepatic apoB production commences. As the end product of VLDL metabolism, it is not surprising that much of the composition of LDL might be related to factors which affect its antecedent, as discussed below in more detail.

Dyslipoproteinemia produces characteristic changes in LDL, and these changes are another type of LDL heterogeneity; these changes, however, are differences in degree not in kind from those found in the normal. By that we mean the variations imposed by disease highlight the basic structure of LDL and point to the processes which control its composition. LDL are spherical particles which vary in size, but not in basic structure. In brief, every LDL particle has two major parts: a surface coat of invariate thickness, the width of a phospholipid bilayer, and a core of variable composition and diameter. The coat is made up of free cholesterol and phospholipid, while the core is made up of the most non-polar lipids, cholesteryl ester and triglyceride. The apoprotein, apoB, entwines the surface lipid environment of the particle, dipping from time to time into the core (7). The amount of protein per LDL particle is constant (8), but the size of the LDL particle is variable. Because the thickness of the coat is constant, as LDL particle size changes the major differences in composition occur in the core. Because the core contains principally cholesteryl ester, the ratio of LDL cholesterol to apoB mirrors the LDL size and composition, such that larger particles have a higher LDL-cholesterol-to-apoB ratio than do smaller particles. Since lipids are less dense than protein, and since the protein per LDL particle is constant, the larger

LDL particles are more buoyant, and smaller particles are more dense.

HyperapoB is characterized by an increased LDL particle number, where most of the LDL particles are smaller and denser than normal. The smaller, denser particles contain less cholesteryl ester than normal, and because most LDL particles are so altered, HyperapoB is characterized by an increased LDL apoB with a low LDL cholesterol-to-apoB ratio. In FH, LDL particle number is also increased, but in this case, a major portion of the LDL particles are enriched in cholesterol and so FH is characterized by an increased LDL apoB, with an elevated LDL-cholesterol-to-apoB ratio.

The validity of our observations obviously rests on the validity of the techniques used to measure both the lipid and protein components of LDL. ApoB accounts for almost all the protein in LDL and had been quantitated by chemical methods until Lees introduced immunological techniques, first a radial immunodiffusion method (9) and then a radioimmunoassay (10). Since apoB is present in VLDL and IDL as well as in LDL, though in much smaller amounts, the measurement of LDL apoB required separation by ultracentrifugation of LDL from these other components. We modified the method proposed by Lees so that LDL could be measured directly in plasma (11). This method has been criticized recently: Havekes (12) has suggested that plasma samples should be frozen, particularly in hypertriglyceridemic patients, to hinder the entry of VLDL particles into the gel; Lutalo-Bosa (13) has argued that the modified radial immunodiffusion assay overestimates LDL apoB in plasma from hypertriglyceridemic patients.

However, Teng et al. (14) reinvestigated this and did not confirm their findings. Therefore we remain satisfied that the modified radial immunodiffusion assay does not, to any significant degree, measure VLDL apoB. Furthermore, the immunoreactivity of apoB in VLDL could be masked by lipids, especially with hypertriglyceridemic VLDL particles (15,16). The apoB in Lp(a) and IDL, however, would be included under most circumstances. Except for type III hyperlipoproteinemia, IDL apoB genearlly accounts for less than 10 percent of the plasma LDL apoB. In type III Hyperlipoproteinemia, LDL apoB measured by the modified assay is usually in the normal to low-normal range (17). This occurs either because the IDL is, in fact, abnormal in composition in this disorder and so may not enter the gel rapidly, or the immunoreactivity of the apoB in these particles is masked by lipids. Finally, it should be noted that all our concepts of LDL heterogeneity are based, in the final analysis, on direct measurement of LDL apoB in the 1.019-1.063 g/ml density fraction, as isolated by ultracentrifugation, with precise correspondence between chemical and immunologic measurements.

If the differences in LDL composition that we and others have demonstrated are real, what then are the mechanisms responsible for LDL heterogeneity, and what are the potential physiologic, and even pathologic, implications of these mechanisms? In this regard, the exchange and transfer of the non-polar lipids, cholesteryl ester and triglyceride amongst the plasma lipoproteins is an essential phenomenon underlying LDL heterogeneity. Triglycerides in plasma are principally in chylomicrons

and VLDL, with lesser, but still important, amounts in LDL and HDL. Normally, triglycerides are synthesized in the gut (18) and liver (19) and any triglyceride in LDL or HDL must have first originated from either chylomicrons or VLDL. Cholesteryl esters are components of the core of all the plasma lipoproteins. Except perhaps for chylomicrons, it is agreed that all the cholesteryl esters in plasma are synthesized by LCAT (20), and as such, appear first in HDL (21), and only afterwards, in other lipoproteins. Thus the entry points into plasma of triglyceride are chylomicrons and VLDL, and the entry point of cholesteryl ester, HDL.

It was long thought that while free cholesterol and phospholipid could exchange amongst the plasma lipoproteins, cholesteryl ester and triglyceride could not. It is now clear, however, this is not the case. But cholesteryl ester and triglyceride can exchange or transfer between the lipoproteins, only if a transfer protein is present (22). The requirement for this transfer protein is absolute and clearly distinguishes such transfer from the movement of free cholesterol, which does not utilize any carrier protein. Two different events can occur -- exchange or transfer. By the first is meant movement of either cholesteryl ester or triglyceride from one lipoprotein, with return of the same constituent from another. Thus, for example, a cholesteryl ester may move from HDL to LDL, in return for which, another moves from LDL to HDL. This results in an undisturbed balance of mass between the two. The second process, transfer, does, however, result in net changes. By this we mean the movement of, for example, triglyceride from VLDL to LDL, in return

for which cholesteryl ester moves from LDL to VLDL. In this instance, in contrast to the first, the composition of both lipoproteins has been altered by the process. When the two cholesteryl ester-rich lipoproteins, HDL and LDL, interact, cholesteryl ester exchange is the dominant process. When either interacts with chylomicrons or VLDL, cholesteryl ester-triglyceride transfer predominates.

There is strong evidence that these processes occur in vivo and undoubtedly account for the movement of cholesteryl ester from HDL to the other plasma lipoproteins (22,33,34). There is also evidence from our laboratory that they form the basis for LDL heterogeneity. In brief, we observed, in humans, that the composition of LDL entering the splanchnic bed was different from the composition of LDL leaving it (23). Specifically, hepatic vein LDL had less cholesteryl ester, but more triglyceride than arterial LDL. LDL apoB level was the same in both, and thus there was a difference in LDL composition, but not in LDL particle number on either side of the splanchnic bed. In addition, we observed that VLDL cholesterol content increased proportionally to the decrease in LDL cholesterol. It must also be appreciated that about one-quarter of LDL is extravascular, in a pool which is in rapid equilibrium with plasma; in pigs (24), and very probably in humans as well, the liver is the major site of this pool. That is to say, a large number of LDL particles are at any time either attached to, or in very close proximity to, hepatocytes. LDL particles enter this pool from plasma, and after a time leave it, and return to plasma. It has been demonstrated

that the human hepatic receptor may reversibly bind LDL, such that it disengages within 5 minutes; it has also been suggested that these proteins are distinct from the fibroblast LDL apoB/E receptor (25).

Taken together, these observations suggest the following scheme: since ACAT activity in human liver is very low (18,26), nascent VLDL contains very little core cholesteryl ester. However, nascent VLDL particles interact with LDL near the margin of the hepatocyte, causing cholesteryl ester to leave LDL and triglyceride to enter it from nascent VLDL. This transformed LDL particle then reenters the plasma space, with the exodus of cholesteryl ester from LDL to VLDL accounting for the drop in LDL cholesterol level across the splanchnic bed (23).

How then does this explain LDL heterogeneity? We would suggest that triglyceride which enters the LDL is then hydrolyzed, the effect of which is to now produce an LDL particle with less core lipid, in particular, an LDL particle with less cholesteryl ester. The extent to which this process operates depends in part on the number of LDL particles coming in contact with VLDL particles. This contact may occur in plasma, or more likely, we believe, in the extravascular hepatic space. Thus, there is first cholesteryl ester-triglyceride transfer and then triglyceride hydrolysis, by either hepatic or lipoprotein lipase (27,28). In HyperapoB patients, we have shown VLDL production to be markedly increased and LDL particle number to be elevated as well. The conditions for cholesteryl ester-triglyceride transfer near the hepatocyte are thus maximized, such that the process is accelerated with consequently greater replacement of LDL core

cholesteryl ester by triglyceride. When this triglyceride is hydrolyzed, the product is a smaller, denser, lipid-depleted, and relatively protein-enriched LDL particle.

What are the predictions of such a scheme and what evidence is there that it exists? First, buoyant LDL should be the precursor of dense LDL, and indeed in normals and patients with HyperapoB, that is exactly what we observed (14): VLDL was the precursor of IDL, IDL of B-LDL, and B-LDL the precursor of D-LDL. This concept, of course, was greatly strengthened by the development of the multicompartmental model of LDL, produced as part of the experimental work of this thesis. Analysis of the LDL subfractional turnover in plasma makes it evident that most of the LDL in the extravascular pool is made up of buoyant LDL, as demonstrated in the multicompartmental model -- another necessary prediction therefore verified. Transfer of triglyceride into LDL was, as noted above, directly validated by studies of splanchnic bed metabolism in humans (23). Further, hydrolysis of this triglyceride should be a step-wise process, evidence for this being the necessity to include a sequential cascade in the multicompartmental model. One would also expect the cholesteryl ester-triglyceride exchange to be unequal in the sense that some LDL particles in the extravascular space would have greater substitution of triglyceride than others. If so, some but not all LDL particles should pass all the way down the cascade. A variable degree of processing depends, therefore, on a variable degree of exchange, as predicted by the multicompartmental model.

This is, as it were, the 'down pathway', in which LDL particles are divested of core lipids. But there is also an 'up pathway', by which LDL particles acquire cholesteryl ester. Again, the existence of this pathway has been demonstrated by the multicompartmental analyses. This is the case, both in normals and in HyperapoB, in which the dense LDL recirculates back to the extravascular space. But just as HyperapoB exaggerates the down pathway, FH exaggerates the route up.

In FH, LDL clearance is delayed (35) and, as a consequence, LDL plasma residence time is prolonged and thus the LDL particles have a much greater opportunity to acquire cholesteryl ester. It is not surprising, therefore, that these particles become so enriched in this lipid. It is also possible that LDL particles may be synthesized de novo with excess cholesteryl ester from the liver. Under normal conditions, the liver secretes a triglyceride-rich lipoprotein, VLDL, but substantial amounts of cholesterol are also produced de novo, plus the liver acquires cholesterol from chylomicrons, LDL, and HDL. Given the limited capacity of the liver for cholesterol catabolism, apoB plays an important, but usually unappreciated, role in maintaining hepatic cholesterol balance. In FH, this role may become more exaggerated.

The present studies establish that overproduction of hepatic apoB likely accounts for the characteristic abnormalities of the plasma lipoproteins in HyperapoB. Overproduction of VLDL apoB has also been observed in obesity (29,30) and familial combined hyperlipidemia (31,32). The issue for future research is to understand what underlies this overproduction of apoB: is it

related to a primary fault in the protein's structure, the regulation of apoB gene expression, or is it due to a secondary fault in the regulation of the production of apoB? Is HyperapoB basically a disorder of protein metabolism -- or of lipid metabolism? The study of triglyceride synthesis in adipose tissue suggests that an underlying defect may be in peripheral tissues. At this stage it is, of course, entirely possible that the observation that adipose tissue from patients with Hyperapobetalipoproteinemia synthesizes triglyceride less rapidly than that from controls may be unrelated to the hepatic overproduction of apoB, characteristic of this disorder. Our knowledge, as yet, is much too incomplete to rule for or against this view. What these observations do provide, however, is an experimental route to approach these questions and so discover the cause or causes of the increased hepatic apoB synthesis in HyperapoB.

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. This research provides the first evidence that changes in the composition of subfractions of low density lipoprotein occur in disease: namely, in familial hypercholesterolemia an LDL subfraction is cholesterol-enriched while in HyperapoB, the majority of LDL particles are depleted of cholesteryl ester.

2. The data therefore provide a physical basis to interpret the previous, purely phenomenologic, definition of HyperapoB -- namely an elevated LDL apoB in the face of normal or near-normal LDL cholesterol. Most of the LDL particles in LDL HyperapoB were shown to be smaller than normal because they contain less cholesteryl ester in their core. However, they contain the normal amount of apoB and thus are denser than normal and have a low cholesterol-to-apoB ratio.

3. These studies provide the first data demonstrating a systematicrelation between the immunoreactivity of certain apoB epitopes and LDL composition. These observations thus provide novel evidence of LDL heterogeneity. In addition they suggest new hypotheses to be tested. For example these data suggest the dense LDL particles might be bound by the LDL receptor less avidly than larger, more buoyant LDL particles.

4. The apoB turnover studies are the first such studies in HyperapoB and thus establish the hallmarks of the disorder: overproduction of LDL apoB due to overproduction of VLDL apoB without any evidence of a catabolic defect. These are also the first such studies with data on LDL subfractional turnovers using iodinated lipoproteins.

5. The subfractional turnover studies establish precursor-product relationships between buoyant and dense LDL, thus establishing sequential order of origin amongst the LDL subclasses.

6. A new multicompartmental model of LDL turnover has been developed which incorporates and extends these concepts. This model is faithful to all the physiological observations and allows, therefore, valid quantitative estimates of production and breakdown of individual lipoprotein subclasses. Such a model will be essential for future studies to document the impact of pharmacologic treatment of HyperapoB as well as demonstrate the full differences between LDL metabolism in HyperapoB and the other dyslipoproteinemias.

7. Finally there is a study of adipose tissue triglyceride synthesis in normals and HyperapoB. Until this study, the entire emphasis in HyperapoB was on the plasma lipoproteins. This is the first evidence of abnormal cellular behaviour in HyperapoB other than the increased hepatic apoB production deduced from the turnover studies. The findings that adipose tissue triglyceride synthesis is reduced in HyperapoB may, or may not, be related to the hepatic overproduction of apoB; only further studies will determine this. They do, however, raise clearly the question as to how adipose tissue triglyceride synthesis is controlled and indicate HyperapoB to be a potentially important biologic model which can be used to elucidate these concepts.