Identification of Apolipoprotein B100 Polymorphisms That Affect Low-Density Lipoprotein Metabolism: Description of a New Approach Involving Monoclonal Antibodies and Dynamic Light Scattering†

Jon E. Chatterton,‡,§ Peter Schlapfer,|| Esther Bütler,|| Monica M. Gutierrez,‡ Donald L. Puppione,‡ Clive R. Pullinger,⊥ John P. Kane,⊥ Linda K. Curtiss,▽ and Verne N. Schumaker*,‡

Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, California 90024, Central Laboratory of the Swiss Red Cross Blood Transfusion Service, CH-3012 Bern, Switzerland, Cardiovascular Research Institute and Department of Medicine, University of California, San Francisco, California 94143, and Research Institute of Scripps Clinic, La Jolla, California 92037

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ABSTRACT: Rare mutations in apolipoprotein B (apoB) can cause defective binding of low-density lipoproteins (LDLs) to the LDL receptor, leading to elevated plasma cholesterol levels and premature atherosclerosis. This communication describes a novel approach to study the effects of apoB mutations on LDL metabolism. Monoclonal antibody MB19 identifies a common polymorphism in apoB, an Ile/Thr substitution at residue 71, by binding with a 60-fold higher affinity to apoB(Ile^{71})-containing LDL. Because each LDL contains a single apoB, a maximum of two LDLs may be bound by the bivalent monoclonal antibody. Thus, at the appropriate concentration, an equivalent amount of MB19 will promote substantial dimer formation of LDL containing the strongly binding apoB(Ile^{71}), but little dimer formation of LDL containing the weakly binding apoB(Thr^{71}). For LDL isolated from heterozygous individuals, the amount of dimer formed, determined by dynamic light scattering, yields an estimate of the allelic ratio of the two forms of LDL. For such individuals, not only the effect of the polymorphism recognized by MB19 but also the effects of other polymorphisms on the LDL allelic ratio can be determined. Examination of six normolipemic MB19 heterozygotes gave percent allelic ratios between 48:52 and 51:49 tight:weak-binding LDL, not significantly different from a 50:50 ratio. These individuals were also heterozygous for six common apoB polymorphisms, allowing calculation of the odds that each of these polymorphisms caused significant alterations in lipid levels. In contrast, the rare mutation at residue 3500 causing defective binding to the LDL receptor and familial defective apoB100 (FDB) resulted in substantial changes (26:74 and 13:87) in LDL allelic ratio in both of two FDB individuals examined.

Elevated plasma levels of low-density lipoproteins (LDLs) are an important risk factor for atherosclerosis. Approximately 20 nm in diameter, LDLs contain a core of cholesteryl ester surrounded by a phospholipid, cholesterol monolayer in which is embedded a single molecule of apolipoprotein B (apoB), a large glycopeptide of 4536 residues (Schumaker et al., 1994). ApoB is necessary for the assembly and secretion of very low density lipoproteins (VLDLs) (Young et al., 1990), it remains associated with the lipoprotein throughout its conversion to LDL (Eisenberg et al., 1973), and it is necessary for the endocytosis of LDL via the LDL receptor (Weisgraber et al., 1978; Young et al., 1986a).

Rare mutations (frequency < 1%) in apoB have been identified which affect plasma LDL cholesterol levels. In familial hypobetalipoproteinemia heterozygotes, mutations causing premature truncations of the apoB polypeptide decrease the concentration of the LDL bearing the truncated apoB (Young et al., 1990; Linton et al., 1994). In contrast, in familial defective apoB100 (FDB) heterozygotes, amino acid substitutions in apoB which disrupt binding to the LDL receptor increase the concentration of the LDL bearing the altered apoB relative to the normal LDL. Thus far, two such amino acid substitutions have been identified: an Arg → Gln substitution at residue 3500 (Soria et al., 1989; Innerarity et al., 1990; Arnold et al., 1994) and an Arg → Cys substitution at residue 3531 (Pullinger et al., 1995). Even though these polymorphisms are classified as rare, with an incidence of about 1 in 700, heterozygous FDB causes elevated cholesterol levels and premature atherosclerosis in an estimated 0.5 million persons in the United States, Canada, and parts of Europe, and possibly many more in populations where its frequency remains to be determined.

In addition to these rare polymorphisms, many common apoB polymorphisms (frequency > 1%) are known. Some of these are listed in Table 1 together with appropriate references. Extensive studies using human alloantisera from multiply transfused patients have identified 10 epitopes resulting from six single amino acid substitutions scattered along the length of the apoB polypeptide. These epitopes constitute the "Ag" polymorphisms (Butler, 1990). Other

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* To whom correspondence should be addressed.

‡ University of California, Los Angeles.

§ Current address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

|| Central Laboratory of the Swiss Red Cross Blood Transfusion Service.

⊥ University of California, San Francisco.

▽ Research Institute of Scripps Clinic.

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Table 1: Common Polymorphisms in Apolipoprotein B Analyzed in Nine Normolipemic Subjects

common name	location on mature ApoB100	substitution	frequency	associated RFLP	ref[a]	ref to association with abnormal lipid levels[a,b]
Ins/Del	−14 to −16	3 residues	50:50		1	8
Ag(c/g)	71	Ile/Thr	30:70	Apa LI	2	9
Ag(a_1/d)	591	Val/Ala	50:50	Alu I	3	
XbaI	2488	(Thr/Thr)	50:50	Xba I	4	10−17
Ag(x/y)	2712[c]	Leu/Pro	25:75	Mae I	5	10, 18
Ag(h/i)	3611	Gln/Arg	06:94	Msp I	6	
Ag(t/z)	4154	Glu/Lys	80:20	Eco RI	7	12, 17, 19

[a] References: (1) Boerwinkle and Chan, 1989. (2) Ma et al., 1989. Yound and Hubl 1989. (3) Wang et al., 1988. (4) Ludwig et al., 1987. (5) Wu et al., 1991. (6) Xu, et al., 1989. (7) Ma et al., 1987. (8) Xu et al., 1990. (9) Tikkanen et al., 1988. (10) Law et al., 1986. (11) Berg, 1986. (12) Hegele et al., 1986. (13) Talmud et al., 1987. (14) Monsalve et al., 1988. (15) Dunning et al., 1988. (16) Aalto-Setala et al., 1988. (17) Paulweber et al., 1990. (18) Berg et al., 1976. (19) Rajput-Williams et al., 1988. [b] Other studies have found no significant differences in lipid levels between groups of individuals distinguished by polymorphisms at these loci (Young et al., 1987; Aburanti et al., 1987, 1988; Talmud et al., 1987; Xu et al., 1989; Genest et al., 1990). [c] Two different polymorphisms are tightly associated with Ag(x/y), a Leu/Pro substitution at 2712 and a Ser/Asn substitution at 4311 (Dunning et al., 1992). Only the former affects a restriction endonuclease cutting site.

common polymorphisms have been identified through RFLP analysis and DNA sequencing, including a three amino acid insertion/deletion in the signal sequence and a silent mutation at codon 2488 that creates an *Xba*I restriction site.

Significant associations have been reported between the common apoB polymorphisms and plasma apoB, cholesterol, and triglyceride concentrations and the incidence of coronary heart disease. These common polymorphisms include the Ins/Del and Ag(c/g) loci in the 5′ region of the gene, the *Xba*I and Ag(x/y) loci in the central region, and the Ag(t/z) locus (also called the *Eco*RI polymorphism) in the 3′ region (See Table 1 for references). However, the lack of significant association between these same polymorphisms and plasma lipid concentrations or coronary artery disease has also been reported (see Table 1 for references). The conflicting results may be due to large variations in plasma lipid levels in the general population, caused by non-apoB genetic and environmental or behavioral factors, which mask the effect of apoB genetic factors.

Here we report an approach which determines specifically whether apoB polymorphisms are responsible for differences in lipoprotein metabolism. Since each LDL contains a single copy of apoB, individuals heterozygous for one or more apoB polymorphisms have two types of LDL which can be identified by the polymorphic form of apoB on their surface. Furthermore, all metabolic factors should act equally on the two allelic forms of apoB, except those recognizing the polymorphic nature of the message or protein (Gavish et al., 1989). Thus, if a particular polymorphism in apoB affects a rate-limiting synthetic or catabolic step anywhere along the metabolic chain from transcription of the mRNA for apoB to the removal of apoB-containing lipoproteins from the plasma, the concentration of the end product, i.e., the plasma LDL bearing that particular apoB polymorphism, will be affected. Accordingly, in apoB heterozygotes, the concentration ratio of the LDL bearing different polymorphic forms of apoB will be shifted from 50:50. Indeed, this has already been observed in individuals with heterozygous FDB (Arnold et al., 1994). Especially powerful is the negative hypothesis: If the concentrations of the two LDLs bearing a particular polymorphism are not significantly altered from a 50:50 allelic ratio in the heterozygote, that polymorphism does not significantly affect lipoprotein metabolism.

In the present study, the LDL ratio in apoB heterozygotes was determined using monoclonal antibody MB19, which recognizes the Ile/Thr polymorphism at amino acid 71 of apoB. MB19 binds more tightly to apoB(Ile^{71})LDL than to apoB(Thr^{71})LDL (Young & Hubl, 1989). At an appropriate concentration, apoB(Ile^{71})LDL will bind to MB19, but apoB(Thr^{71})LDL will not. Thus, in an apoB(Ile^{71}/Thr^{71}) heterozygote, an increase in the amount of apoB(Ile^{71})LDL will result in an increase in the amount of LDL binding to MB19. Binding of LDL to MB19 was quantitated by measuring the increase in Stokes radius caused by formation of LDL−MB19−LDL complexes using dynamic light scattering.

MATERIALS AND METHODS

LDL Isolation. Total LDL (density 1.019−1.055 g/mL) was obtained from human plasma by a modification of the sequential flotation procedure described by Chatterton et al. (1991). Briefly, for most subjects, sufficient LDL was obtained from 10 mL of blood. In addition to EDTA, sodium azide, and gentamicin sulfate, a constant background concentration of 40 μM ascorbic acid (Sigma) was maintained throughout the isolation to protect LDL from oxidation (Jialal et al., 1990). After the first centrifugation at a density of 1.063 g/mL (44 000 rpm for 24 h at 20 °C in a Beckman Ti-70.1 rotor and a Beckman L5-65 ultracentrifuge) and a second centrifugation at a density of 1.019 g/mL and 20 °C, the bottom layer (LDL) was adjusted to a density of 1.055 g/mL (20 °C), the centrifugation was repeated a third time, and total LDL was isolated from the top of the centrifuge bottle. The isopycnic gradient ultracentrifugation step previously used to obtain subfractionated LDL (Chatterton et al., 1991) was omitted.

Total LDL was dialyzed at 4 °C against buffer A [0.195 M NaCl, 0.04% (w/v) EDTA, 0.05% (w/v) sodium azide, 0.005% (w/v) gentamicin sulfate, and 40 μM ascorbic acid, pH 7.6]. Large particulates (e.g., dust, aggregated LDL, etc.) were removed by centrifugation in an Eppendorf centrifuge for 60 min at 4 °C. The protein concentration in the supernatant was determined by a modified Lowry assay (Markwell et al., 1978) using bovine serum albumin (RIA grade, Sigma) as a standard, corrected for apoB hyperchromicity (Fisher & Schumaker, 1986). Molar LDL concentration, which is numerically equal to the molar apoB concentration, was determined by dividing the protein concentration by the molecular mass of the apoB protein (513 000 g/mol) (Knott et al., 1986).

Monoclonal Antibodies. Monoclonal antibodies MB19 and MB47 were partially purified from ascites fluid by ion-

exchange chromatography. The production and characterization of these antibodies have been previously described (Curtiss & Edgington, 1982; Young et al., 1986a,b; Young & Hubl, 1989). Following centrifugation to remove large particulates (see LDL Isolation above), protein concentration was estimated spectrophotometrically at 280 nm, using an extinction coefficient of 1.36 mL/mg cm.

Dynamic Light Scattering. LDL and LDL−antibody complex diameters (twice the Stokes radius) were determined by dynamic light scattering using a NICOMP Model 270 particle sizer (Particle Sizing Systems, Santa Barbara, CA) equipped with a Stabilite 2016 argon ion (wavelength = 514.5 nm) laser (Spectra Physics). This instrument utilizes the autocorrelation function to calculate the diffusion coefficient of the macromolecule from fluctuations in the scattering intensity. The Stokes radius is then calculated from the Stokes−Einstein relationship, $D = kT/(6\pi\eta R)$, where D is the diffusion coefficient, k is the Boltzmann constant, T is the temperature, η is the solvent viscosity, and R is the Stokes radius.

LDLs with or without monoclonal antibody were diluted to the desired concentration (total volume = 1.0 mL) with buffer A, which had been filtered through a 0.45-μm Millex HA filter unit (Millipore) to remove dust or other particulates. The diluted sample was allowed to equilibrate at 23 °C for 1 h and then transferred to a standard 1.0-cm quartz fluorescence cuvette for light-scattering measurements. Measurements were performed at 23 °C. At this temperature, buffer A has a viscosity of 0.951 cP and a refractive index of 1.334. All measurements on LDL from a particular subject (LDL alone, LDL + MB19, LDL + MB47) were performed in the same cuvette to avoid variations due to minor differences between cuvettes. However, cuvettes were always washed, rinsed thoroughly with filtered (0.45-μm) water, and dried with filtered air between samples.

Genotype Determination for Common ApoB Polymorphisms. DNA was isolated from leukocytes as previously described (Fodor & Doty, 1977). Genotype determination for the Ins/Del polymorphism was determined by direct size analysis of PCR products on 7% NuSieve GTG agarose gels (FMC BioProducts, Rockland, ME) stained with ethidium bromide as described by Boerwinkle and Chan (1989). Genotypes for the remaining six apoB polymorphisms were determined by PCR−RFLP analysis. Primers and PCR conditions previously described for analyses of the *Apa*LI (Young & Hubl, 1989), *Alu*I (Ludwig & McCarthy, 1990), *Xba*I (Soria et al., 1989), *Mae*I, *Msp*I (Wu et al., 1991), and *Eco*RI (Dunning et al., 1992) RFLPs were used without modification with one exception: the base substitutions in the 5′ ends of the oligonucleotides described by Young and Hubl (1989) for the *Apa*LI RFLP were eliminated.

Oligonucleotides were synthesized on a Gene Assembler Plus from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). The amplification reactions for the RFLP analyses were performed in final volumes of 100 μL containing 50−100 ng of genomic DNA, 2 units of Taq polymerase (Promega, Madison, WI) in the reaction buffer provided by the manufacturer, 50 pmol of each primer, 20 pmol of each dNTP (Pharmacia), and 4 μg of gelatin. To prevent evaporation, 50 μL of mineral oil was layered on top. The first denaturation step was performed at 94 °C for 5 min, followed by annealing at 55 °C for 0.7 min and extension at 72 °C for 2 min for the first cycle. In subsequent cycles, the duration of the denaturation step was reduced to 0.7 min. For the 35th and final cycle, the extension time was increased to 10 min. For the Ins/Del polymorphism, 50 cycles were employed, and the time of denaturation was increased to 1 min after the first cycle; the time and temperature of annealing and extension were 1.5 min at 65 °C until the final cycle, when the time was increased to 10 min. The programmable thermal controller was purchased from MJ Research, Inc.

For restriction enzyme digestion, 5 μL of PCR product (14 μL for *Apa*LI) was added to 2 μL of the appropriate digestion buffer concentrate (10×) and 1−3 μL (5−40 units) of restriction enzyme in a total volume of 20 μL and digested overnight at 37 °C (45 °C for *Mae*I). *Alu*I and *Eco*RI were obtained from Life Technologies Inc. (Gibco BRL, Gaithersburg, MD); *Apa*LI, *Xba*I, and *Msp*I were obtained from New England BioLabs (Beverly, MA); and *Mae*I was obtained from Boehringer Mannheim (Indianapolis, IN). The digests were separated on 1.5% (*Eco*RI) or 2% (*Mae*I and *Msp*I) agarose gels (Eastman Kodak Company, Rochester, NY) or 4% (*Xba*I and *Alu*I) or 6% (*Apa*LI) NuSieve GTG agarose gels (FMC BioProducts, Rockland, ME). Gels were stained with ethidium bromide, visualized by UV light, and photographed. DNA ladders (1 kb or 100 bp) were used as markers (Life Technologies, Gibco BRL).

Genotypes for the Ag polymorphisms were confirmed by a passive hemagglutination assay as previously described (Butler, 1990).

Enzymatic Lipid Assays. Plasma was obtained from blood drawn following an overnight fast. Blood drawn for lipid analyses was not treated with the protease inhibitors employed in the isolation of LDL because these agents interfered with the lipid assays. Plasma concentrations of cholesterol and triglyceride were measured at a wavelength of 515 nm using a Spectronic 21 spectrophotometer (Milton Roy). The enzymatic cholesterol reagent was prepared as described by Puppione and Charagundula (1994). The enzymatic triacylglycerol reagent used to measure glycerol was prepared from a solid blend of enzymes (International Bioproducts, Streamwood, IL) and was reconstituted in 1.0 g/L of Triton X-100, 1.5 mM sodium 2-hydroxy-3,5-dichlorobenzenesulfonic acid, and 5 mM $MgCl_2$. Plasma triglyceride concentrations were measured with this reagent following the addition of a bacterial lipase at a concentration of 40 μg/mL. All measurements were corrected for free plasma glycerol using the reconstituted reagent without the lipase.

Plasma HDL cholesterol (α-lipoprotein cholesterol) was measured following precipitation of apoB-containing lipoproteins with heparin (Liquaemin Sodium, Organon, W. Orange, NJ) and $MnCl_2$ using a modification of the procedure of Burstein et al. (1970). Forty microliters of 5000 units/mL heparin and 50 μL of 1.25 M $MnCl_2$ were added separately to 1 mL of plasma. Following centrifugation at 4 °C, supernatant cholesterol was measured using the reagent described above.

Plasma LDL cholesterol was calculated using the formula of Friedewald et al. (1972): [LDL-C] = [TC] − ([TG]/5 + [HDL-C]), where [TC], [LDL-C], and [HDL-C] are the plasma concentrations (mg/mL) of total cholesterol, LDL cholesterol, and HDL cholesterol, respectively, and [TG] is the plasma concentration of triglyceride.

Table 2: Serum Triglyceride and Cholesterol Levels in Nine Normolipemic Subjects

subject	triglyceride (mg/dL)	total cholesterol (mg/dL)	HDL cholesterol (mg/dL)	LDL cholester[a] (mg/dL)
94-001	45	166	66	91
94-002	85	154	63	74
94-003	82	171	54	101
94-004	90	139	51	70
94-005	87	187	38	132
94-006	34	200	94	99
94-007	86	134	49	68
94-009	38	165	62	95
94-010	62	170	52	106

[a] These values were calculated using the formula described in Materials and Methods.

RESULTS

LDL Isolation and Characterization. LDLs (d = 1.019−1.055 g/mL, 20 °C) were isolated from nine unrelated individuals whose plasma triglyceride, total cholesterol, and LDL and HDL cholesterol levels were within normal limits (Table 2). The nine subjects also were typed for common apoB polymorphisms by both PCR−RFLP and passive hemagglutination inhibition for the Ag polymorphisms, including the Ag(c/g) or MB19 polymorphism at amino acid 71, by PCR−RFLP analysis for the *Xba*I polymorphism and by direct size analysis of the PCR products for Ins/Del polymorphisms (Table 3). Mean hydrated LDL diameter was determined for each sample by dynamic light scattering (Table 4). Values ranged from 23.4 to 24.0 nm; however, these were hydrated diameters. Actual LDL diameter was determined by dividing by the frictional ratio, f/f_0, which for LDL is equal to 1.11 (Fisher et al., 1971). Once corrected for hydration, mean LDL diameter ranged from 21.1 to 21.6 nm, which was within the normal range for LDL (Schumaker et al., 1994). However, hydrated diameters were used for all subsequent data analysis since independently measured values for f/f_0 for LDL−antibody complexes were not available.

Determination of Dissociation Constants for the Binding of MB19 to apoB(Ile^{71})- and apoB(Thr^{71})-Containing LDL. A preliminary experiment was performed to select a concentration which would result in a maximum MB19 binding difference between apoB(Ile^{71}) LDL and apoB(Thr^{71}) LDL. LDL was isolated from two subjects, an apoB(Ile^{71}/Ile^{71}) homozygote and an apoB(Thr^{71}/Thr^{71}) homozygote, and analyzed by dynamic light scattering. In the absence of antibody, LDLs from these two subjects had diameters of 23.1 and 22.9 nm, respectively. LDL from each subject was combined with an approximately equivalent amount of MB19

Table 3: apoB Haplotypes of Nine Normolipemic Individuals

	individual haplotype:[a] residue number on mature apoB and substitution						
subject	−14 to −16[b]	71	591	2488[c]	2712	3611	4514
homozygotes							
94-001	ND	Ile/Ile	ND	ND	ND	ND	ND
94-004	ND	Thr/Thr	ND	ND	ND	ND	ND
94-005	ND	Thr/Thr	ND	ND	ND	ND	ND
heterozygotes							
94-002	Ins/Del	Ins/Thr	Val/Ala	ACT/ACC	Pro/Pro	Arg/Arg	Glu/Lys
94-003	Ins/Del	Ile/Thr	Ala/Ala	ACT/ACC	Pro/Pro	Arg/Arg	Glu/Glu
94-006	Ins/Del	Ile/Thr	Val/Ala	ACC/ACC	Pro/Pro	Gln/Arg	Glu/Glu
94-007	Ins/Del	Ile/Thr	Val/Ala	ACT/ACC	Pro/Pro	Arg/Arg	Glu/Lys
94-009	Ins/Del	Ile/Thr	Val/Ala	ACT/ACC	Pro/Pro	Arg/Arg	Glu/Glu
94-010	Ins/Del	Ile/Thr	Ala/Ala	ACT/ACC	Pro/Pro	Arg/Arg	Glu/Glu

[a] Heterozygotic polymorphisms are underlined. Only heterozygotes for Ile/Thr at residue 71 may be studied by this technique, since MB19 recognizes this site. [b] A three amino acid insertion/deletion in the signal sequence of apoB which is strongly linked to the Ile/Thr polymorphism at residue 71. [c] This nucleotide substitution does not result in an amino acid change.

Table 4: Diameters, Percentage Increases in Diameters, and Allelic Ratios for Normolipemic and FDB LDL

		diameter (nm)			increase in diameter			
donor	type	LDL	LDL + MB47	LDL + MB19	with MB47[a] (%)	with MB19[a] (%)	MB19/MB47[b] (%)	percent apoB (Ile^{71}) LDL[c]
				Normolipemic Homozygotes				
94-001	Ile/Ile	23.80	31.75	31.20	33.4	31.1	93.1	(100)
94-004	Thr/Thr	23.70	31.40	26.10	32.5	10.1	31.2	(0)
94-005	Thr/Thr	23.50	31.40	25.85	33.6	10.0	29.7	(0)
				Normolipemic Heterozygotes				
94-002	Ile/Thr	23.35	31.00	27.60	32.8	18.2	55.6	51.1
94-003	Ile/Thr	23.60	31.50	27.90	33.5	18.2	54.4	49.4
94-006	Ile/Thr	24.00	31.70	28.30	32.1	17.9	55.8	51.4
94-007	Ile/Thr	23.60	31.60	27.90	33.9	18.2	53.7	48.2
94-009	Ile/Thr	23.70	31.80	28.10	34.2	18.6	54.3	49.1
94-010	Ile/Thr	23.60	31.90	28.10	35.2	19.1	54.2	48.9
				FDB				
94-011	Ile/Thr	22.70	30.50	25.90	34.4	14.1	41.0	26.2
94-021	Ile/Thr	23.60	31.10	26.20	31.8	11.0	34.7	12.9

[a] Percent diameter increase (columns 6 and 7) = 100 × (diameter of LDL with antibody − diameter of LDL)/(diameter of LDL). [b] Relative percent diameter increase (column 8) = 100 × (column 7)/(column 6). [c] Values in column 8 were converted to % apoB(Ile^{71}) LDL using the calibration curve of Figure 5.

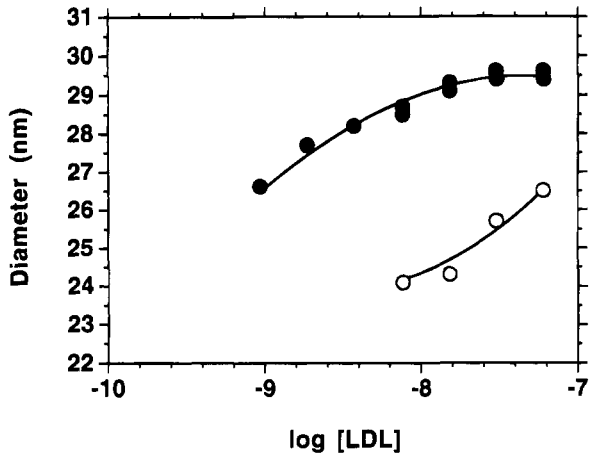

FIGURE 1: Estimation of the MB19 binding constants through dilution. Mixtures of MB19 antibody and strongly binding or weakly binding LDL were prepared at different molar concentrations of LDL, as indicated on the horizontal axis, and the Stokes radii were determined by dynamic light scattering. This experiment was carried out at an LDL:MB19 ratio of 1.38:1, before the equivalence ratio was well established. Binding constants were estimated by a computer program which simulated the titration and determined the average radius, which is plotted as the smooth curve through the data points: (●) strongly binding, apoB(Ile^{71}) LDL; (○) weakly binding, apoB(Thr^{71}) LDL.

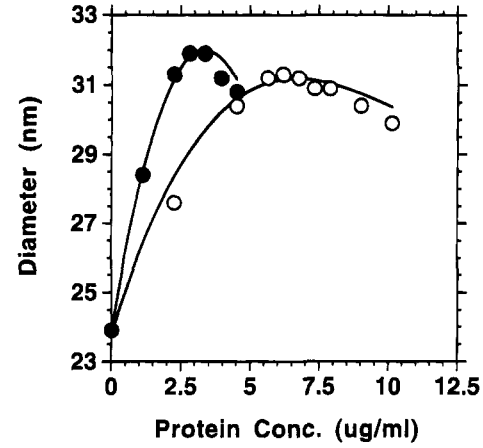

FIGURE 2: Titration of apoB(Ile^{71}/Ile^{71}) LDL with monoclonal antibodies MB47 and MB19. LDL from subject 94-001, an apoB-(Ile^{71}/Ile^{71}) homozygote, was mixed with various amounts of monoclonal antibodies MB19 (○) or MB47 (●) and diluted to a final LDL concentration of 15 nM with buffer A. Mean LDL−antibody complex diameter, which increased due to formation of LDL−Ab−LDL complexes, was determined by dynamic light scattering. The equivalence point was taken as the antibody concentration which resulted in the maximum LDL−antibody complex diameter. The specific activity of the antibody was unknown; therefore, antibody concentration was expressed as micrograms of protein per milliliter (horizontal axis) as determined by absorbance at 280 nm.

at a total LDL concentration of 60 nM, and the resulting mixtures were used to make a series of 1:2 dilutions. For each dilution series, the size of the LDL−antibody complex was studied as a function of total LDL concentration (Figure 1).

At an LDL concentration of 60 nM, addition of MB19 to LDL from the apoB(Ile^{71}/Ile^{71}) subject increased the diameter from 23.1 nm to approximately 29.5 nm due to the formation of LDL−MB19−LDL complexes. Addition of MB19 to LDL from the apoB(Thr^{71}/Thr^{71}) subject at the same concentration resulted in a less substantial increase in the diameter, from 22.9 to 26.5 nm, consistent with the weaker binding of MB19 to apoB(Thr^{71}) LDL. As the total LDL concentration decreased, the mean diameter of the LDL−antibody complex also decreased due to dissociation of the LDL−MB19−LDL structures. The difference in MB19 binding to apoB(Ile^{71}) LDL compared to apoB(Thr^{71}) LDL was easily measured at an LDL concentration of 15 nM (Figure 1). Therefore, all subsequent experiments were performed at 15 nM LDL.

These data were also used to estimate dissociation constants for the binding of MB19 to apoB(Ile^{71})- and apoB-(Thr^{71})-containing LDL. A computer program was developed to simulate the reaction between LDL and MB19 and to calculate the average diameter of the resulting LDL−antibody complex as a function of LDL concentration for a given value of the dissociation constant (see Discussion). The experimental data and the computer-generated theoretical binding curves were in closest agreement when dissociation constants of 0.4 and 24 nM were used for the binding of MB19 to the tightly binding apoB(Ile^{71}) LDL and the weakly binding apoB(Thr^{71}) LDL, respectively.

Titration of LDL with Monoclonal Antibodies MB19 and MB47. The specific activities of partially purified monoclonal antibodies MB19 and MB47 were determined by titration. One of the normolipemic subjects (94-001) was found to be homozygous for Ile at amino acid 71 of apoB, which binds tightly to MB19 (Table 3). At a constant LDL concentration of 15 nM, various amounts of monoclonal antibody (MB19 or MB47) were added to LDL isolated from this subject and allowed to equilibrate. The mean diameter of the resulting LDL−antibody complex was determined by dynamic light scattering (Figure 2). As the concentration of antibody increased, the mean diameter of the mixture increased, due to the formation of LDL−Ab−LDL complexes, until it reached a peak value of 31.3 nm for MB19 and 31.9 nm for MB47 at 6.19 and 3.09 μg/mL, respectively. The peak was taken to be the equivalence point, where the maximum amount of LDL−Ab−LDL complex was formed, resulting in the largest mean diameter. Above this antibody concentration, excess antibody resulted in a decrease in the amount of LDL−Ab−LDL complex and a corresponding decrease in the mean complex diameter.

Binding of MB19 and MB47 to LDL from Nine Normal Subjects. LDLs from the nine normolipemic subjects listed in Table 2 were analyzed for their abilities to bind to monoclonal antibody MB19. LDL was mixed with MB19 at equivalence, as determined by the titration of apoB(Ile^{71}) LDL in Figure 2, and diluted to a final LDL concentration of 15 nM. The mean diameter of the resulting LDL−antibody complex was determined by dynamic light scattering (Table 4 and Figure 3). To correct for the variation in LDL size, the results are expressed as percent diameter increase relative to the mean diameter of the LDL measured in the absence of antibody. The nine subjects could be divided into three classes on the basis of binding to MB19: the LDL from one subject, 94-001, bound tightly to MB19 (31% diameter increase); two, 94-004 and 94-005, bound weakly (10% diameter increase); and the remaining six displayed an intermediate level of binding (18% diameter increase).

As a control for variation in antibody binding due to nongenetic factors (e.g., oxidation, aggregation, etc.), LDL

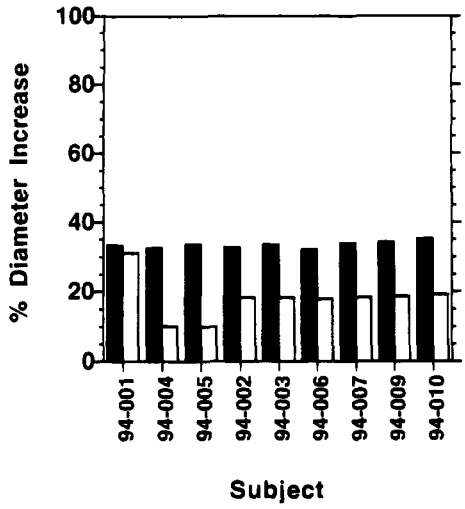

FIGURE 3: Binding of LDL from nine normal subjects to MB19 and MB47. LDL (15 nM) was mixed with an equivalent amount of MB19 (open bars) or MB47 (solid bars), and the average sizes of the resulting LDL−Ab complexes were determined by dynamic light scattering. Results are expressed as % Diameter Increase = 100(diameter of LDL−Ab complex − diameter of LDL alone)/(diameter of LDL alone), as listed in Table 4.

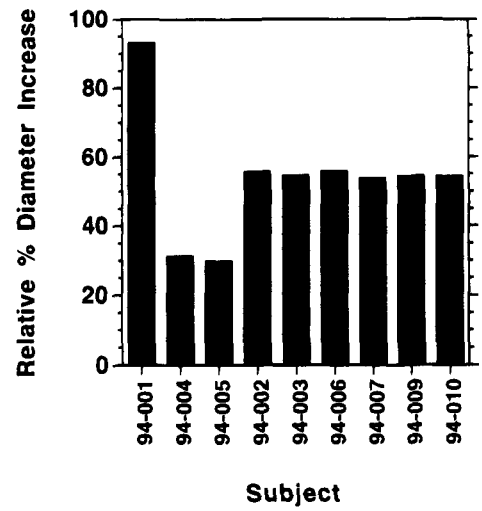

FIGURE 4: Relative percentage increase in diameter upon addition of MB19 to LDL for nine normolipemic subjects. The binding of monoclonal antibody MB19 to LDL relative to the binding of monoclonal antibody MB47 was calculated from the data of Table 3. Relative % Diameter Increase = 100(diameter increase with MB19/(% diameter increase with MB47). The increase in diameter upon addition of MB19 is expressed as the percentage of the increase which occurs upon addition of MB47, which serves as a reference to correct for different particle sizes and for nongenetic variation in antibody binding. The first subject, 94-001, is homozygotic for strongly binding apoB(Ile^{71}) LDL, and the next two subjects, 94-004 and 94-005, are homozygotic for weakly binding apoB(Thr^{71}) LDL. The remaining six subjects are heterozygotic, possessing both strong-binding and weak-binding MB19 epitopes.

from these same nine subjects was also tested for binding to monoclonal antibody MB47. Binding of LDL to MB47 is not affected by the (Ile^{71}/Thr^{71}) polymorphism of apoB (Young et al., 1986b). Furthermore, the LDL concentration in all experiments was 15 nM, which is 60 times greater than the MB47 dissociation constant (Young et al., 1986a); therefore, MB47 was also a control for maximum binding. LDL was mixed with MB47 at equivalence (determined by the titration of LDL in Figure 2), and the mean diameter of the LDL−antibody complex was determined as for MB19 above (Table 4). Binding to MB47 was relatively constant for all nine LDL samples (Figure 3).

In Figure 4, the binding of the nine LDL samples to MB19 was expressed as a function of binding to MB47 by dividing the percent diameter increase with MB19 by the percent diameter increase with MB47. Under these conditions, the apoB(Ile^{71}/Ile^{71}) homozygote LDL bound 93% as well to MB19 as to MB47; the two apoB(Thr^{71}/Thr^{71}) homozygote LDLs, 30% as well; and the six apoB(Ile^{71}/Thr^{71}) heterozygote LDLs, 55% as well. Thus, the tight-binding phenotype corresponded to homozygosity for Ile at amino acid 71; the weak-binding phenotype, to homozygosity for Thr at amino acid 71; and the intermediate-binding, to heterozygosity at this locus, as had been previously observed in solid-phase radioimmunoassays (Young & Hubl, 1989; Robinson et al., 1986).

Determination of the LDL Allelic Ratios for Six Subjects Heterozygous for Ile/Thr at Amino Acid 71 of ApoB. To estimate the allelic ratios of strongly to weakly binding LDL in apoB(Ile^{71}/Thr^{71}) heterozygotes, a calibration curve was constructed. LDLs from apoB(Ile^{71}/Ile^{71}) and apoB(Thr^{71}/Thr^{71}) homozygotes were mixed at known ratios. At LDL concentrations of 15 nM, the mean diameter was determined by dynamic light scattering for these mixtures in the absence of antibody, in the presence of MB19 at equivalence, and in the presence of MB47 at equivalence. The results were plotted as a function of the percentage of the strongly binding apoB(Ile^{71}) LDL and fitted to a second-order polynomial with $r^2 = 0.998$ (Figure 5).

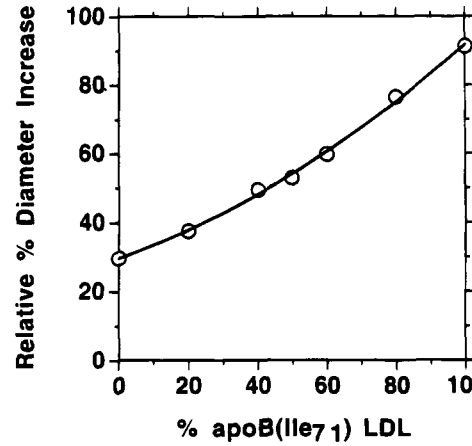

FIGURE 5: Calibration curve to convert relative percent diameter increase to percent strongly binding apoB(Ile^{71})LDL. Artificial mixtures of homozygous apoB(Ile^{71}) LDL and homozygous apoB(Thr^{71}) LDL were created in known apoB(Ile^{71}):apoB(Thr^{71}) ratios. Samples were diluted to final LDL concentrations of 15 nM in the absence of antibody, in the presence of MB19 at equivalence, and in the presence of MB47 at equivalence. Mean diameters were determined by dynamic light scattering for each sample and were used to calculate values of the Relative % Diameter Increase, as explained in the caption to Figure 4. The data were plotted as a function of the percentage of apoB(Ile^{71}) LDL and were fitted to a second-order polynomial ($r^2 = 0.998$).

The resulting calibration curve was then used to convert values of relative percent diameter increase from Figure 4 to percent apoB(Ile^{71}) LDL for the six apoB(Ile^{71}/Thr^{71}) heterozygotes (Figure 6). All six of the heterozygotes studied had allelic ratios [apoB(Ile^{71}):apoB(Thr^{71})] near 50:50, ranging from 48:52 to 51:49 (Table 3). To further determine whether any differences from a 50:50 ratio were significant,

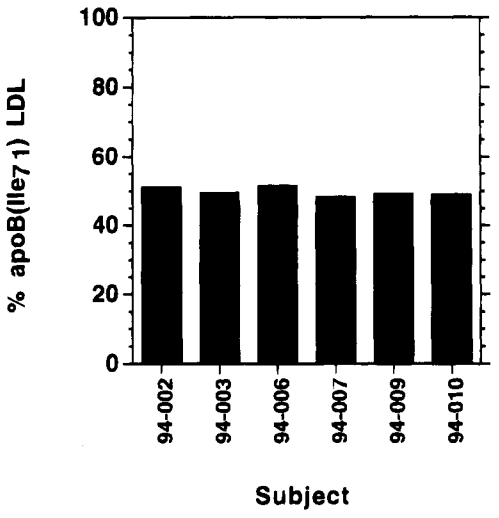

FIGURE 6: Allele-specific LDL ratios for six normolipemic heterozygotes. Values of Relative % Diameter Increase for the six heterozygotes in Figure 4 were converted to % apoB(Ile^{71}) LDL using the calibration curve in Figure 5. The average value and 1 SD are 49.7 ± 1.3 for the data shown. The range was between 48.2 and 51.4.

Table 5: Reproducibility of the Determination of Allelic Ratio as Assessed by Repeated Measurements on Two Subjects

	% apoB(Ile^{71}) LDL	
	subject 93-103	subject 93-104
	48.1	50.3
	49.8	50.3
	48.1	53.8
	49.8	48.5
	46.3	48.5
mean	48.4	50.3
SD	1.5	2.2

the allelic ratio was determined in five independent measurements on samples from two apoB(Ile^{71}/Thr^{71}) heterozygotes (Table 5). The mean percent apoB(Ile^{71}) LDL values for these samples were 48 and 50, with standard deviations of ±2. Therefore, variations from a 50:50 allelic ratio of less than 2 (48:52 or 52:48) are within the inherent error in the measurement and are not significant at the $p < 0.05$ level. The minimum difference in ratio from 50:50, which can reliably be measured using this technique, is 47:53 or 53:47. Thus, no significant differences in the metabolism of the two allelic forms of apoB were found for the six apoB heterozygotes shown in Figure 5, as reflected by the ratio of the LDL concentrations in plasma.

To determine whether the allelic ratio was constant with time, it was measured on six separate occasions from the same subject over a 14-month period. The values ranged from 48:52 to 52:48 (data not shown). Therefore, within the limits of resolution of this technique (±2), the allelic ratio measured for this subject did not change with time.

Determination of the LDL Allelic Ratios for Two Familial Defective Apolipoprotein B Heterozygotes. Individuals heterozygous for the Arg3500 → Gln substitution in apoB, which causes familial defective apoB (FDB), have apoB(Arg3500):apoB(Gln3500) ratios in their LDL of approximately 25:75 (Arnold et al., 1994). LDL was isolated from two apoB(Arg3500/Gln3500) heterozygotes who were apoB(Ile^{71}/Thr^{71}) heterozygotes as well. The allelic ratio [apoB(Ile^{71}):apoB(Thr^{71})] was determined as above. This ratio was

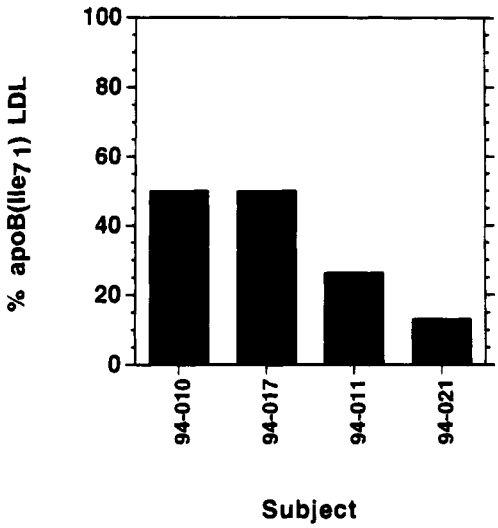

FIGURE 7: Allelic ratios for two FDB subjects and two normolipemic controls. A clear distinction between the allelic ratios, plotted as percent of the apoB(Ile^{71}) LDL, for two FDB subjects, 94-011 and 94-021, and for two normolipemic controls, 94-010 and 94-017, is illustrated. Values for the FDB subjects were 26.2 and 12.9, respectively, whereas the values for the normal controls, measured in the same set of experiments, were both 49.8.

also determined on two normal apoB(Ile^{71}/Thr^{71}) heterozygotes. The results are displayed in Figure 7. In one of the FDB heterozygotes, the allelic ratio was 26:74; however, the other FDB heterozygote had a more extreme ratio, 13:87. This was consistent with the significant variation in the ratio between FDB subjects reported by Arnold et al. (1994). Both of the normal control apoB(Ile^{71}/Thr^{71}) heterozygotes had allelic ratios of 50:50.

DISCUSSION

Some Advantages and Disadvantages of the Monoclonal Antibody/Dynamic Light Scattering Technique. In their pioneering study of heritable allele-specific differences in amounts of apoB and LDL, Gavish et al. (1989) employed monoclonal antibody MB19 in a competitive displacement ELISA assay to detect individuals with an "intermediate immunoaffinity phenotype". This phenotype was demonstrated to result from an unequal allelic ratio of the two LDLs. ELISA assays distinguished the 3:1 variation in allelic ratio of the two LDLs which these authors reported. The approach reported here, dynamic scattering from LDL−antibody complexes, may be more precise, because the degree of precision required to distinguish between allelic ratios of 53:47 and 50:50, about 0.2 nm, seems accessible by dynamic light scattering.

To measure diameters reproducibly within 0.2 nm, contamination with dust or aggregated material, which causes the apparent diameters to increase, must be minimized by filtering solvents, centrifuging lipoproteins, careful cleansing of the light-scattering cell between experiments, and employing relatively fresh, unoxidized lipoproteins. An important parameter was lipoprotein diameter measured before antibody addition, which ranged between 23.0 and 23.5 nm and was not larger than 24 nm in this study. Diameters smaller than 23.0 nm were occassionally found.

Among the advantages of the technique were the low concentrations employed that allowed for multiple measurements. Study of the dimerization of LDL by a monoclonal

antibody permitted a theoretical analysis of the results from thermodynamic and hydrodynamic theory.

Calculation of Expected Values for Monomeric and Dimeric Lipoproteins. Low-density lipoproteins contain about 20% apoB as virtually their only protein [reviewed by Schumaker et al. (1994)], and because the molecular weight of apoB is known, the LDL molecular weight is readily approximated as 513 000 g $mol^{-1}/0.20 = 2.6\ 10^6$ g mol^{-1}. With a density of 1.032 g/mL, the radius, assuming an unhydrated sphere, is given by $(3 \times 2.6 \times 10^6$ g $mol^{-1}/(4 \times 3.14 \times 1.032$ g $cm^{-3} \times 6.022 \times 10^{23}$ $mol^{-1}))^{1/3} =$ 10.0 nm.

Experimentally determined radii, often called Stokes radii, differ from unhydrated sphere radii by the frictional ratio. This is 1.11 for LDL (Fisher et al., 1971). When this correction is applied to the estimated unhydrated radius of the LDL spere, the calculated Stokes radius becomes 11.1 nm, and the Stokes diameter becomes 22.2 nm. Because LDLs are heterogeneous in size, however, this value represents an average diameter, in this case, the number average diameter. Dynamic light scattering using the "intensity mode" of the particular algorithm employed with the software supplied by the manufacturer (the proprietary NICOMP distribution analysis) yields an intensity average Stokes diameter (equal to the sum of the products of molar concentration times molecular weight squared times diameter divided by the sum of the products of molar concentration times molecular weight). For a heterogeneous distribution of particle sizes, the intensity average diameter is always larger than the number average diameter, and values of 23–24 nm generally found for LDL diameters by this technique seem reasonable.

It may be calculated from eqs 10–33 of Cantor and Schimmel (1980) that two touching, identical spheres will have a Stokes radius that is a factor of 4/3 greater than the Stokes radius of one of the spheres. For the 9 normolipemic individuals listed in Table 4, the average increase in Stokes radius of the LDL preparations upon the addition of MB47 was 33.5 ± 0.9% (Table 4), close to the theoretical estimate, suggesting that at equivalence and 15 nM essentially all of the LDL forms dimer with this antibody. A smaller percentage increase in radius of 31.1% (Table 4) was observed when monoclonal antibody MB19 bound to LDL homozygous for the tight-binding site. Complete binding was not achieved at 15 nM, accounting for the smaller percentage increase.

Prediction of the Shapes of the Titration, Binding, and Calibration Curves. The curves drawn through the experimental points of Figures 1 and 2 were generated by a computer program written in BASIC that calculated the concentrations of LDL–Ab and LDL–Ab–LDL complexes given the initial concentrations of antibody and LDL and the dissociation constants. The program then calculated the intensity average particle diameter for the mixture as a function of LDL or antibody concentration (solid lines through the experimental points in Figures 1 and 2). The input data included LDL, LDL–Ab, and LDL–Ab–LDL diameters and K_d values for the interactions of LDL with MB19. The best fit of the theoretical curves to the experimental data was obtained using 23.5 nm for LDL diameter, 25.0 and 33.0 nm for the diameters of LDL–Ab and LDL–Ab–LDL complexes, respectively, and K_d values of 0.4 and 24 nM for the binding of MB19 to strongly and weakly binding LDL.

If Ile^{71}/Thr^{71} Individuals Were Selected at Random, What Fraction Would Be Expected To Show Deviation from a 50:50 Allelic Ratio of Their LDL? The six normal heterozygotes listed in Table 3 were members of an ethnically diverse population of graduate students. These six individuals showed LDL allelic ratios falling within the 48–52% limits of tightly binding LDL, considered not significantly different from 50%. If the probability of selecting an individual whose ratio lay outside these limits was P, then the probability that none of six individuals would be found outside these limits would be $(1 - P)^6$. Thus, if $P = 0.5$, that is, if half of the members of this population actually fell outside these limits, the chance of selecting at random 6 individuals who did not would be only 1 in 64. Therefore, this study suggests, with a significant degree of confidence (<0.02), that fewer than half of the heterozygotes belonging to this population of graduate students have LDL containing less than 48% or more than 52% of the tightly binding apoB phenotype. If $P = 0.25$, the probability of selecting six individuals who did not fall outside the limits would rise to 1 in 5.6; therefore, it is not possible to state with any significant degree of confidence that fewer than one-quarter of individuals will not fall outside these limits. The present preliminary study was not large enough to adequately test this limit of $P = 0.25$. Since 1 in perhaps 500 individuals in the American population is believed to be an FDB heterozygote, the lower limit for P might be about 0.002. Clearly, a larger study to establish the value of P within this rather wide range would be valuable.

What Is the Probability That Common ApoB Polymorphisms Significantly Affect LDL Allelic Ratios? For the allelic ratio to be measured by this technique, subjects had to be heterozygotic at the locus recognized by monoclonal antibody MB19. Thus, it was important to know whether having Ile or Thr at position 71 affected the ratio of the two LDLs. From Table 4 it may be calculated that the percentage of apoB(Ile^{71}) LDL for these six heterozygotic, normolipidemic individuals was 49.7 ± 1.3% (mean ± 1 S D, s). Use of the Student $t_{0.95}$ with $N - 1 = 5$ degrees of freedom ($t_{0.95} = 2.57$) yielded 95% confidence limits (mean ± $t_{0.95}s/(N)^{0.5}$) of 48.3–51.0% for the mean percentage of apoB(Ile^{71}) LDL in the plasma of normolipidemic graduate students heterozygotic at position 71. Thus, it was concluded that the Ile/Thr substitution at residue 71 was not the cause of a significant change in LDL metabolism reflected by an altered apoB(Ile^{71}):apoB(Thr^{71}) LDL ratio. As used here, "a significant change" was defined as an allelic ratio lying outside the limits between 48:52 and 52:48; thus, the precision of the technique allowed the negative hypothesis to be asserted within these 95% confidence limits by an examination of six normolipidemic individuals.

In calculating confidence limits for the remaining polymorphisms, an additional complication arose because it was not known how the different polymorphisms were associated with the two genes coding for apoB. For example, all of the normolipidemic (Ile^{71}/Thr^{71}) heterozygotes in Table 3 were also heterozygotic for the Ins/Del substitution. However, it was not known for any individual whether the insertion was present on the gene coding for the strongly binding apoB(Ile^{71})LDL or whether it was associated with the gene coding for the weakly binding apoB(Thr^{71})LDL.

This lack of knowledge would seriously affect the calculation of confidence limits, because the allelic ratio would be reversed for any particular individual if the association were reversed; for example, for individual 94-002, if the same apoB gene carried both the Ins and the Thr^{71} polymorphisms, then the Ins would be associated with having 48.9% strongly binding LDL, while if the converse were true, the Ins would be associated with having 51.1% strongly binding LDL. Without additional information it was not known which of the two possibilities was correct for any of the six heterozygotes in this study. The additional information could be obtained through family studies, but it was not available here.

In order to circumvent this ambiguity, a computer program was written to compute the confidence limits for each member of the set of all permutations of the two possible allelic ratios for each of the six heterozygotes, and to select the largest confidence limits from among the results. Since the actual permutation must be a member of this set, the actual confidence limits must be equal to or less than those selected. In this manner, maximum 95% confidence limits of 48.2−51.8 were calculated for the Ins/Del polymorphism, which may be compared with the actual 95% confidence limits of 48.3−51.0 for the Ile/Thr polymorphism at residue 71. The maximum limits calculated by this algorithm will always be wider than the actual limits, which could be calculated if the linkage were known, and they will be symmetrical about a mean of 50.

For the five heterozygotes who were polymorphic for nucleotide substitution ACT/ACC in codon 2488, the analogous calculation with $N - 1 = 4$ degrees of freedom gave 95% confidence limits of 48.0−52.0.

For the remaining polymorphisms which appeared four times or fewer (Table 3), the analogous calculation gave 95% confidence limits which increased rapidly as sample size decreased, because the values of Student's t increased markedly due to uncertainty in the estimation of the variances from the limited sample sizes. If the "true" variance, σ^2, were determined from a very large number of measurements, then 95% confidence limits would be equal to the mean $\pm 1.96\ \sigma/(N)^{0.5}$ (Dixon & Massey, 1951), where ±1.96 SD includes 95% of the area beneath a Gaussian curve. If the variance is estimated from the heterozygote data in Tables 3 and 4, then its square root $\sigma = 1.7$. The maximum 95% confidence limits which were calculated employing this expression were a little larger than those calculated from $t_{0.955}/(N)^{0.5}$ as described above for the Ile/Thr, Ins/Del, and ACT/ACC polymorphisms. But this approach enabled reasonable confidence limits to be assigned to the other polymorphisms. Thus, maximal 95% confidence limits for the Ala/Val polymorphism at residue 591 were found to be 47.9−52.1; maximal 95% limits for the Glu/Lys polymorphism at 4514 were 47.3−52.7; and maximal 95% limits for the single instance of Gln/Arg polymorphism at 3611 were 46.9−53.1.

The preliminary data presented here suggest that the Ins/Del, ACT/ACC, Ile/Thr, Ala/Val, Glu/Lys, and Glu/Arg apoB polymorphisms do not significantly affect the allelic ratio and therefore do not affect any rate-limiting step in LDL metabolism.

LDL Ratio Determined for Individuals Heterozygotic for the Rare Mutation Arg3500 → Gln. The allelic ratios for the two FDB subjects (Figure 6) showed an abundance of the apoB(Thr^{71}) LDL, the percentages of apoB(Ile^{71}) LDL being 26% and 13% for FDB subjects 94-011 and 94-021, respectively. This is consistent with the reported association between the $Arg_{3500} \rightarrow$ Gln substitution, responsible for the defective interaction with the LDL receptor, and a single apoB haplotype bearing the Thr^{71} polymorphism (Ludwig & McCarthy, 1990). Thus, LDL bearing apoB(Thr^{71},Gln^{3500}) would bind to the LDL receptor with reduced affinity, leading to an increase in their concentration relative to LDL bearing apoB(Ile^{71},Arg^{3500}). However, a definitive demonstration that Thr^{71} and Gln^{3500} resided on the same apoB allele required family studies, and unfortunately family data were not available for the two FDB individuals studied in this communication. Subsequently, two other FDB individuals heterozygous for both apoB(Arg^{3500}/Gln^{3500}) and apoB(Thr^{71}/Ile^{71}) were identified from a lipid clinic population. These FDB individuals (94-036 and 94-037) were brothers who were receiving Lovastatin at the time of study by dynamic light scattering. Their allelic ratios were 16:84 and 14:86 Ile^{71}:Thr^{71}, respectively. A third sibling also has been haplotyped and found to be heterozygous for apoB(Arg^{3500}/Gln^{3500}) and homozygous for apoB(Thr^{71}/Thr^{71}), demonstrating that the mutant allele was apoB(Thr^{71}, Gln^{3500}). This result was consistent with four reports in which the Gln^{3500} allele has been associated with Thr^{71} in every FDB individual examined thus far (Ludwig et al., 1990; Bersot et al., 1993; Rauh et al., 1993; Arnold et al., 1994). Interestingly, in the study by Bersot et al. (1993), a Chinese man with FDB had a different overall apoB haplotype; however, Thr^{71} remained associated with the mutant Gln3500 allele. A German family with yet another FDB apoB haplotype was not examined at the Ile^{71}/Thr^{71} locus (Rauh et al., 1993).

Since the FDB LDL should have had a longer plasma residence due to a lower fractional catabolic rate, a smaller particle might have resulted due to additional processing by transfer proteins and lipases. The average size of the LDL for the 8 normal subjects was 23.7 nm, and the range was 23.35−24.0 nm. Smaller particles were seen for one of the two FDB subjects, 94-011, which at 22.6 nm were the smallest LDL observed in this study. An average size of 23.6 nm was observed for the other FDB LDL.

Monoclonal antibody MB47 binds very tightly to human LDL, and it was assumed that MB47 caused essentially 100% dimerization of the LDL when an equivalent concentration was added at 15 nM. The Stokes radius obtained with MB47 provided the upper calibration point in calculating the percentage increase in radius upon addition of MB19. It was reported (Weisgraber et al., 1988) that MB47 binds more tightly to the mutant LDL from FDB subjects. Therefore, it was assumed that MB47 would provide a good calibration for complete dimerization when added to FDB LDL as well. The data in Table 4 support this assumption. Upon the addition of MB47, the six normal LDLs showed an average increase in diameter of 33.6%, with a range from 32.1% to 35.2%. Dimerization of FDB LDL by MB47 was similar to that of normal LDL, yielding increases of 34.4% and 31.8%.

The new technique described here should lead to a number of interesting studies. For example, one of the two FDB subjects, 94-021, was taking an HMG-CoA reductase inhibitor (Lovastatin) to lower plasma cholesterol. This individual also had the lowest percentage of apoB(Ile^{71}) LDL, consistent with the hypothesis that the induction of additional receptor

in response to this medication would result in a further lowering of the concentration of the normal LDL. But this critical point requires a tightly controlled study. Finally, in a collaborative study with Pullinger et al. (1995), the technique has been employed to investigate the consequences of a new apoB mutation, $Arg_{3531} \rightarrow Cys$, showing that this mutation also results in defective binding to the LDL receptor.

REFERENCES

Aalto-Setala, K., Tikkanen, M. J., Taskinen, M.-R., Nieminen, M., Holmberg, P., & Kontula, K. (1988) *Atherosclerosis 74*, 47−54.

Aburatani, H., Murase, T., Takaku, F., Itoh, H., Matsumoto, A., & Itakura, H. (1987) *N. Engl. J. Med. 317*, 52.

Aburatani, H., Matsumoto, A., Itoh, H., Yamada, N., Murase, T., Takaku, F., & Itakura, H. (1988) *Atherosclerosis 72*, 71−76.

Arnold, K. S., Balestra, M. E., Krauss, R. M., Curtiss, L. K., Young, S. G., & Innerarity, T. L. (1994) *J. Lipid Res. 35*, 1469−1476.

Berg, K. (1986) *Clin. Genet. 30*, 515−520.

Berg, K., Hames, C., Dahlen, G., Frick, M. H., & Krishan, I. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 937−940.

Bersot, T. P., Russell, S. J., Thatcher, S. R., Pomernacki, N. D., Mohley, R. W., Weisgraber, K. H., Innerarity, T. L., & Gox, C. S. (1986) *J. Lipid Res. 25*, 1149−1154.

Boerwinkle, E., & Chan, L. (1989) *Nucleic Acids Res. 17*, 4003.

Burstein, M., Scholnick, H. R., & Morfin, R. (1970) *J. Lipid Res. 11*, 583−595.

Butler, R. (1990) in *DNA and Other Polymorphisms in Forensic Science* (Lee, H. C., Gaenssler, R. W., Eds.) pp 135−139, Mosby Yearbook, Chicago.

Cantor, C. R., & Schimmel, P. R. (1980) in *Biophysical Chemistry, Part II*, p 568, W. H. Freeman and Company, San Francisco.

Chatterton, J. E., Phillips, M. L., Curtiss, L. K., Milne, R. W., Marcel, Y. L., & Schumaker, V. N. (1991) *J. Biol. Chem. 266*, 5955−5962.

Curtiss, L. K., & Edgington, T. S. (1982) *J. Biol. Chem. 257*, 15213−15221.

Dixon, W. J., & Massey, F. J., Jr. (1951) *Introduction to Statistical Analysis*, Chapter 9, McGraw-Hill Book Company, New York.

Dunning, A. M., Duriez, P., Vu Dac, N., Fruchart, J. C., & Humphries, S. E. (1988) *Clin. Genet. 33*, 181−188.

Dunning, A. M., Renges, H.-H., Xu, C.-F., Peacock, R., Brasseur, R., Laxer, G., Tikkanen, M. J., Butler, R., Saha, N., Hamsten, A., Rosseneu, M., Talmud, P., & Humphries, S. E. (1992) *Am. J. Hum. Genet. 50*, 208−221.

Eisenberg, S., Bilheimer, D. W., Levy, R. I., & Lindgren, F. T. (1973) *Biochim. Biophys. Acta 326*, 361−377.

Fisher, W. R., & Schumaker, V. N. (1986) *Methods Enzymol. 128*, 247−262.

Fisher, W. R., Granade, M. E., & Mauldin, J. L. (1971) *Biochemistry 10*, 1622−1629.

Fodor, E. J. B., & Doty, P. (1977) *Biochem. Biophys. Res. Commun. 77*, 1478−1485.

Friedewald, W. T., Levy, R. I., & Fredrickson, D. S. (1972) *Clin. Chem. 18*, 499−502.

Gavish, D., Brinton, E. A., & Breslow, J. L. (1989) *Science 244*, 72−76.

Genest, J. J., Jr., Ordovas, J. M., McNamara, J. R., Robbins, A. M., Meade, T., Cohn, S. D., Salem, D. N., Wilson, P. W. F., Masharani, U., Frossard, P. M., & Schaefer, E. J. (1990) *Atherosclerosis 82*, 7−17.

Hegele, R. A., Huang, L.-S., Herbert, P. N., Blum, C. B., Buring, J. E., Hennekens, C. H., & Breslow, J. L. (1986) *N. Engl. J. Med. 315*, 1509−1515.

Innerarity, T. L., Mahley, R. W., Weisgraber, K. H., Bersot, T. P., Krauss, R. M., Vega, G. L., Grundy, S. M., Friedl, W., Davignon, J., & McCarthy, B. J. (1990) *J. Lipid Res. 31*, 1337−1349.

Jialal, I., Vega, G. L., & Grundy, S. M. (1990) *Arteriosclerosis 82*, 185−191.

Knott, T. J., Pease, R. J., Powell, L. M., Wallis, S. C., Rall, S. C., Jr., Innerarity, T. L., Blackhart, B., Taylor, W. H., Marcel, Y., Milne, R., Johnson, D., Fuller, M., Lusis, A. J., McCarthy, B. J., Mahley, R. W., Levy-Wilson, B., & Scott, J. (1986) *Nature 7503*, 734−738.

Law, A., Powell, L. M., Brunt, H., Knott, T. J., Altman, D. G., Rajput, J., Wallis, S. C., Pease, R. J., Priestley, L. M., Scott, J., Miller, G. J., & Miller, N. E. (1986) *Lancet*. 1301−1302.

Linton, M. F., Farese, R. V., Jr., & Young, S. G. (1994) *J. Lipid Res. 34*, 521−541.

Ludwig, E. H., & McCarthy, B. J. (1990) *Am. J. Hum. Genet. 47*, 712−720.

Ludwig, E. H., Blackhart, B. D., Pierotti, V. R., Caiati, L., Fortier, C., Knott, T., Scott, J., Mahley, R. W., Levy-Wilson, B., & McCarthy, B. J. (1987) *DNA 6*, 363−372.

Ma, Y., Schumaker, V. N., Butler, R., & Sparkes, R. S. (1987) *Arteriosclerosis 7*, 301−305.

Ma, Y., Wang, X., Butler, R., & Schumaker, V. N. (1989) *Arteriosclerosis 9*, 242−246.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206−210.

Monsalve, M. V., Young, R., Jobsis, J., Wiseman, S. A., Dhamu, S., Powell, J. T., Greenhalgh, R. M., & Humphries, S. E. (1988) *Atherosclerosis 70*, 123−129.

Paulweber, B., Friedl, W., Krempler, F., Humphries, S. E., & Sandhofer, F. (1990) *Arteriosclerosis 10*, 17−24.

Pullinger, C. R., Hennessy, L. K., Chatterton, J. E., Liu, W., Love, J. A., Mendel, C. M., Frost, P. H., Malloy, M. J., Schumaker, V. N., & Kane, J. P. (1995) *J. Clin. Invest.* (in press).

Puppione, D. L., & Charugundla, S. (1994) *Lipids 29*, 595−597.

Rajput-Williams, J., Knott, T. J., Wallis, S. C., Sweetnam, P., Yarnell, J., Cox, N., Bell, G. I., Miller, N. E., & Scott, J. (1988) *Lancet 2* (Dec 24−31), 1442−1446.

Rauh, G., Schuster, H., Schewe, C. K., Stratmann, G., Keller, C., Wolfram, G., & Zollner, N. (1993) *J. Lipid Res. 34*, 799−805.

Robinson, M. T., Schumaker, V. N., Butler, R., Berg, K., & Curtiss, L. K. (1986) *Arteriosclerosis 6*, 341−344.

Schumaker, V. N., Phillips, M. L., & Chatterton, J. E. (1994) *Adv. Protein Chem. 45*, 205−248.

Soria, L. F., Ludwig, E. H., Clarke, H. R. G., Vega, G. L., Grundy, S. M., & McCarthy, B. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 587−591.

Talmud, P. J., Barni, N., Kessling, A. M., Carlsson, P., Darnfors, C., Bjursell, G., Galton, D., Wynn, V., Kirk, H., Hayden, M. R., & Humphries, S. E. (1987) *Atherosclerosis 67*, 81−89.

Tikkanen, M. J., Viikari, J., Akerblom, H. K., & Pesonen, E. (1988) *Br. Med. J. 296*, 169−170.

Wang, X., Schlapfer, P., Ma, Y., Butler, R., Elovson, J., & Schumaker, V. N. (1988) *Arteriosclerosis 8*, 429−435.

Weisgraber, K. H., Innerarity, T. L., & Mahley, R. W. (1978) *J. Biol. Chem. 253*, 9053−9062.

Weisgraber, K. H., Innerarity, T. L., Newhouse, Y. M., Young, S. G., Arnold, K. S., Krauss, R. M., Vega, G. L., Grundy, S. M., & Mahley, R. W. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9758−9762.

Wu, M.-J., Butler, E., Butler, R., & Schumaker, V. N. (19910 *Arterioscler. Thromb. 11*, 379−384.

Xu, C., Nanjee, N., Tikkanen, M. J., Huttunen, J. K., Pietinen, P., Butler, R., Algelico, F., Del Bon, M., Mazzarella, B., Antonio, R., Miller, N. G., Humpries, S., & Talmud, P. J. (1989) *Hum. Genet. 82*, 322−326.

Xu, C.-F., Tikkanen, M. J., Huttunen, J. K., Pietinen, P., Butler, R., Humphries, S., & Talmud, P. (1990) *J. Lipid Res. 31*, 1255−1261.

Young, S. G., & Hubl, S. T. (1989) *J. Lipid Res. 30*, 443−449.

Young, S. G., Witztum, J. L., Casal, D. C., Curtiss, L. K., & Bernstein, S. (1986a) *Arteriosclerosis 6*, 178−188.

Young, S. G., Bertics, S. J., Curtiss, L. K., Casal, D. C., & Witztum, J. L. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1101−1105.

Young, S. G., Bertics, S. J., Scott, T. M., Dubois, B. W., Beltz, W. F., Curtiss, L. K., & Witztum, J. L. (1987) *Arteriosclerosis 7*, 61−65.

Young, S. G., Hubl, S. T., Smith, R. S., Snyder, S. M., & Terdiman, J. F. (1990) *J. Clin. Invest. 85*, 933−942.

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