Evaluation of a monoclonal antibody-based enzyme-linked immunosorbent assay as a candidate reference method for the measurement of apolipoprotein B-100

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Abstract A monoclonal antibody-based direct binding enzyme-linked immunosorbent assay (ELISA) for apoprotein (apo) B-100 has been developed for use as a reference method. The assay uses the two well-characterized monoclonal antibodies, MB24 and MB47. MB47, which recognizes an epitope at the low density lipoprotein (LDL) receptor-binding domain of apoB and is specific for apoB-100, is bound to the microtiter plate as the capture antibody. MB24, which binds an epitope in the amino terminal half of the apoB-100 and identifies both apoB-100 and apoB-48, is conjugated to horseradish peroxidase and is utilized as the indicating antibody. The assay was calibrated with LDL (d 1.030–1.050 g/ml) and the LDL protein was determined by a sodium dodecyl sulfate (SDS) Lowry procedure. The working range of the assay is 0.25–1.25 μg/ml. Optimal dilution of whole plasma was found to be 1:2000. In the assay, MB47 bound ~97% of the apoB in all low density lipoprotein, and >90% of the apoB in the majority of very low density lipoprotein preparations. Small dense LDL from subjects with familial combined hyperlipidemia (FCHL) and large bouyant LDL from subjects with familial hypercholesterolemia (FH) exhibited binding properties similar to LDL from healthy normolipidemic subjects when tested in the reference ELISA. The intra- and interassay coefficients of variation averaged 2.5% and 6.0%, respectively. Plasma B-100 levels were not influenced by freezing and thawing or storage at 4°C for up to 3 weeks or storage at −70°C for up to 11 months. Excellent agreement was obtained between the reference ELISA and a polyclonal RIA which measures total apoB ($r = 0.93$, n = 105, mean ELISA B-100 value = 100 mg/dl, mean RIA value = 101 mg/dl, Sy = 9.6). Reference ELISA B-100 values of samples pretreated with bacterial lipase were not significantly increased in most samples with plasma triglyceride levels below 600 mg/dl. To help reduce the large among-laboratories variability of apoB measurements, we recommend that this candidate reference direct binding ELISA be used to assign apoB target values to apoB reference pools. —**Albers, J. J., M. S. Lodge, and L. K. Curtiss.** Evaluation of a monoclonal antibody-based enzyme-linked immunosorbent assay as a candidate reference method for the measurement of apolipoprotein B-100. *J. Lipid Res.* 1989. **30:** 1445–1458.

Supplementary key words apoB-48 • FPLC • lipase • LDL • VLDL

As a result of the implementation of the National Cholesterol Education Program, there is now a new national thrust to identify and treat hypercholesterolemia. The measurement of apolipoprotein (apo) B is of growing clinical interest because of its use as a predictor of the risk of developing coronary heart disease (CHD), as an aid in the diagnosis of dyslipoproteinemic states, and for monitoring the progress of dietary and drug intervention (1). However, apoB measurements have not reached their full potential in the clinical laboratory because of inadequate standardization, lack of suitable reference material, and lack of a reference method for plasma apoB protein.

Human apoB is heterogeneous but exists primarily in two forms: apoB-100 and apoB-48 (2). ApoB-100 is a large apolipoprotein containing 4536 amino acid residues and has a calculated molecular mass of approximately 513 kDa (3–6), based on the complete protein sequence. It is synthesized primarily by the liver and is the major protein moiety of low density lipoprotein (LDL), very low density lipoprotein (VLDL), and VLDL remnants. ApoB-48 is synthesized in the adult intestine as a protein containing

Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; CDC, Centers for Disease Control; cELISA, competitive enzyme-linked immunosorbent assay; CHD, coronary heart disease; DGUC, density gradient ultracentrifugation; EIA, electroimmunoassay; ELISA, enzyme-linked immunosorbent assay; FCHL, familial combined hyperlipidemia; FH, familial hypercholesterolemia; FPLC, fast, high performance liquid chromatography; gPAGE, gradient polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; INA, immunonephelometric assay; LDL, low density lipoprotein; LPDP, lipoprotein-deficient plasma; NWLRC, Northwest Lipid Research Center; PBS, phosphate-buffered saline; RIA, radioimmunoassay; RID, radial immunodiffusion; TCA, trichloroacetic acid; VLDL, very low density lipoprotein; SDS, sodium dodecyl sulfate.

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2152 amino acid residues that are homologous with the first 2152 residues of apoB-100 (7–9). Based on amino acid sequence data, apoB-48 has a molecular mass of about 241 kDa. It is a constituent of chylomicrons and chylomicron remnants.

A reference method for the measurement of apoB protein should not only be accurate and precise but also must be readily standardized and transferred to reference laboratories. Monoclonal antibodies are attractive for use as reagents for a reference assay, because they are highly specific, chemically uniform, and can be readily produced and purified in large amounts. Among the many methodological approaches used to measure plasma apoB protein, a monoclonal antibody-based ELISA has the potential to serve as a reference assay for plasma apoB protein. ELISA procedures are generally reproducible and relatively simple to perform. Furthermore, they use uniform reagents, do not require radioisotopes, and involve convenient incubation times. Recently, several apoB ELISA procedures have been reported (10–14). One of the reports used the well-characterized monoclonal antibodies MB24 and MB47 (12). These two antibodies bind to distinct apoB epitopes expressed by all LDL particles, and both can detect apoB in VLDL. MB24 binds an epitope in the amino terminal portion of apoB-100 within the T4 fragment and therefore identifies both apoB-100 and apoB-48 (12, 15, 16). MB47 binds at or near the LDL receptor recognition site (17), within residues 3350 to 3505 (3) of the carboxyl-terminal one-third of the protein. Thus, MB47 identifies only apoB-100.

Here we report the modification of one of the direct binding ELISA procedures (12) and the evaluation of this modified procedure as a candidate reference method for the measurement of apoB-100 in human plasma using MB47 as the capture antibody and MB24 conjugated to horseradish peroxidase as the detecting antibody.

MATERIALS AND METHODS

Collection of specimens

Blood was collected from hyperlipidemic and normolipidemic individuals by venipuncture into Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) in both the presence and absence of EDTA, following a 12–14 h fast. Upon separation at 4°C by low-speed centrifugation, the serum or plasma was either prepared for the immediate isolation of lipoprotein fractions, the analysis of apoproteins and lipids, or frozen at −70°C for eventual analysis. Plasma from subjects with clinically diagnosed familial combined hyperlipidemia (FCHL) or familial hypercholesterolemia (FH) was kindly provided by Drs. John D. Brunzell and Robert H. Knopp.

Isolation and characterization of lipoproteins

VLDL (d < 1.006 g/ml) was isolated from the plasma of fasting individuals by ultracentrifugation (18). Briefly, using a Beckman L5-50 ultracentrifuge and a Beckman 40.3 Ti rotor, 5 ml of sample was overlaid with 0.15 M NaCl, 1 mM EDTA in a 6.5 ml Beckman polyallomer bell-top Quick-Seal™ tube and centrifuged at 40,000 rpm for 18 h at 10°C. The top fraction of approximately 2.3 ml was collected and recentrifuged at d 1.006 g/ml for 18 h at 40,000 rpm to remove excess albumin. Harvested VLDL fractions were stored at 4°C. For some experiments VLDL was diluted to its plasma equivalent.

LDL (d 1.030–1.050 g/ml) was isolated from both pooled plasma and plasma of individuals by sequential ultracentrifugation as previously described (19) with KBr being substituted for NaCl. After recentrifugation at d 1.050 g/ml, the LDL fraction was dialyzed overnight at 4°C against two changes of 0.02 M boric acid, 0.15 M NaCl, 1 mM EDTA, pH 8.0. LDL was also isolated from the plasma of normolipidemic, FCHL, FH, and hypercholesterolemic subjects by single-spin density gradient ultracentrifugation (20). LDL size and heterogeneity were established using the method of Krauss and Burke (21). Purity was assessed by 0.1% sodium dodecyl sulfate, 3–20% gradient polyacrylamide gel electrophoresis (SDS-gPAGE) (22) and by radioimmunoassay for apolipoproteins B, A-I, A-II, and E (19, 23).

Determination of total LDL protein

Total LDL protein concentration was determined by the method of Lowry et al. (24) as modified by Markwell et al. (25). Sample and reagent volumes were adjusted to minimize the amount of sample required for analysis. A standard curve was prepared by diluting the United States Institute of Standards and Technology (formerly the National Bureau of Standards) bovine serum albumin standard reference material (SRM 927, Washington, DC) to 0.2, 0.15, 0.1, 0.05, and 0.025 mg/ml in 0.15 M NaCl. Samples were diluted with 0.15 M NaCl to three different concentrations within the working range. Two hundred μl of diluted sample, standard, or saline as a nonspecific control was aliquoted in triplicate into 12 × 75 mm borosilicate test tubes. One ml of freshly prepared Lowry reagent C (100 parts 2.0% Na_2CO_3, 0.4% NaOH, 0.16% Na-tartrate, 1% SDS (Kodak) to 1 part 4% $CuSO_4 \cdot 5 H_2O$) was added to all tubes. Samples were vortexed thoroughly and incubated for 10 min at room temperature. This was followed with the addition of 100 μl of Folin-Ciocalteu phenol reagent (Sigma Chemical Co., St. Louis, MO) diluted to half its original concentration with deionized water. Each tube was vortexed immediately upon addition of the reagent. The assay was incubated in the dark at room temperature for 45 min, after which the optical absorbance of each sample was read at 660 nm

using a Gilford 2600 spectrophotometer (CIBA-Corning Diagnostics Corporation, Oberlin, OH). A standard curve generated by least-squares linear regression analysis was used to calculate the concentrations of the unknowns.

Total protein mass in purified LDL was also determined, in some samples, by amino acid analysis. Amino acid analysis on six different LDL preparations was performed in triplicate by AAA Laboratory, Mercer Island, Washington, following the manufacturer's instructions for the Dionex D-500 amino acid analyzer single column system. The mass of LDL apoB was calculated from the mole percents of leucine and phenylalanine in LDL hydrolysates and the amino acid sequence obtained from the apoB-100 cDNA sequence (4, 6).

Preparation of antibodies

The previously characterized monoclonal antibodies MB47 and MB24 used in the candidate reference ELISA procedure have been described (15–17). MB47 is an apoB-100-specific antibody that binds to the highly conserved LDL receptor domain in the carboxyl-terminal one-third of the protein (3, 17). MB24, previously referred to as B24, binds an epitope on the amino-terminal half of apoB-100 that is common to apoB-48 (12). The monoclonal antibody, B1B6, which binds at or near the LDL receptor-binding domain within the T2 fragment and is specific for apoB-100 (26), was a generous gift from Gustav Schonfeld and Elaine Krul.

The monoclonal antibodies were purified from ascites fluid by fast, high-performance liquid chromatography (FPLC) on a Pharmacia (Uppsala, Sweden) Mono-Q HR 5/5 (50 mm × 5 mm I.D.) anion-exchange column (12, 27). IgG was eluted with a 0–1.0 M NaCl gradient in 10 mM Tris buffer, pH 8.2. For comparative binding studies monoclonal antibodies were also purified by protein A affinity chromatography with the use of the Bio-Rad Affi-Gel Protein A MAPSII system (Richmond, CA).

Rabbit anti-LDL polyclonal antibody (RB74), produced as previously described (19), was purified by immunoaffinity column chromatography. Briefly, LDL (d 1.030–1.050 g/ml) was passed three times each over regenerated immunoaffinity columns of goat anti-apoE, rabbit anti-apoA-I, and goat anti-HSA. Purity of the LDL was then reassessed by 3–20% SDS-gPAGE and Western immunoblot (28) with antibodies MB47, goat anti-apoE, rabbit anti-apoA-I, and goat anti-HSA. This LDL was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to manufacturer's instructions. RB74 antisera was passed over this LDL/Sepharose column and the antibody fraction which eluted with 0.5 M HAc, 0.5 M NaCl, 1 mM EDTA, pH 2.6, was used in the subsequent studies.

Direct binding of ^{125}I-labeled ligands

Sodium [^{125}]iodide (Amersham Corp., Arlington Heights, IL) labeling of lipoprotein fractions and IgG was done by the iodine monochloride method of McFarlane (29) as modified by Bilheimer, Eisenberg, and Levy (30). The specific activity of ^{125}I-labeled MB47 was used to calculate the maximum binding capacity of the microtiter plate for the antibody by subtracting the counts removed from plate after incubation from the total number of counts applied. ^{125}I-Labeled LDL and ^{125}I-labeled VLDL were used to validate the maximum binding of these ligands by MB47 and other antibodies bound to microtiter plates. The percent ^{125}I-labeled ligand bound was calculated by subtracting the unbound 12% TCA-precipitable counts removed after incubation with the antibody from the total 12% TCA-precipitable counts applied and dividing this difference by the total 12% TCA-precipitable counts.

Conjugation of antibody to horseradish peroxidase

MB24 was conjugated to horseradish peroxidase (HRP) by the method of Nakane and Kawoi (31) with the citrate reduction and storage considerations of Henning and Nielsen (32). Commercially prepared goat anti-rabbit IgG and rabbit anti-mouse IgG antibody-enzyme conjugates were purchased from Sigma (St. Louis, MO).

Procedure for the candidate ELISA method

All reagents were brought to room temperature (23°C) before use. Nunc Immuno-Plate I (Nunc, Denmark) flat-bottom, polystyrene microtiter plates were coated with 100 μl of FPLC-purified MB47 (5 μg/ml) in 0.1 M sodium bicarbonate buffer, pH 9.6. Plates were sealed with an acetate plate sealer (Flow Laboratories, McLean, VA) and incubated overnight at room temperature with gentle shaking (100 rpm) on an Orbit Shaker (Lab-Line Instruments, Melrose Park, IL). All subsequent incubations were done with sealed plates that were shaken at 100 rpm. Unbound antibody was removed by washing four times with PBS-TB (phosphate-buffered saline, 0.05% Tween-20 (Sigma No. P-1379), 0.1% bovine serum albumin (Sigma No. A-7030), pH 7.4) on a Titertek S8/12 microtiter-plate washer (Flow Laboratories). All subsequent wash steps followed this format. Blocking of remaining binding sites on the plate was achieved by incubating the plate with 3% BSA (150 μl/well) in PBS for 1 h at room temperature. At this point plates were washed and either used immediately or thoroughly dried, sealed, and stored at 4°C in a desiccated chamber for up to 1 month.

Plasma samples and controls were diluted 1:2000 in PBS-TB. Dilution was done in two steps, a 1:50 dilution followed by a 1:40 dilution using a Micromedic Digiflex

automatic pipettor (Horsham, PA). A standard curve was prepared by diluting a primary LDL standard to 1.25, 1.0, 0.75, 0.5, 0.375, and 0.25 μg/ml, respectively. Standard, quality controls, and samples were plated in triplicate (100 μl/well) and incubated overnight at room temperature.

After washing, 100 μl of MB24 conjugated to horseradish peroxidase (MB24-HRP) diluted to a concentration 4.12 μg/ml in PBS-TB was added to the plate using a Flow Multisteppor pipet (Flow Laboratories). The plate was incubated for 1 h at room temperature after which excess second antibody was removed by washing and 100 μl of substrate solution was added (Sigma *o*-phenylamine diamine, 0.67 mg/ml, in distilled water containing 0.0125% hydrogen peroxide). After 30 min, the reaction was stopped by the addition of 50 μl 2 N H_2SO_4. The plate was read for absorbance at 492 nm on an ELISA Processor II (Behring Diagnostics, La Jolla, CA) interfaced with an IBM-AT personal computer. ApoB-100 values were calculated from absorbance readings by log-linear regression analysis of the standard curve using customized software.

Competitive ELISA (cELISA) procedure

All reagents were brought to room temperature (23°C) before use. Nunc Immuno-Plate I (Nunc) flat-bottom, polystyrene microtiter plates were coated with 100 μl of LDL (3.5 μg/ml) in 0.1 M sodium bicarbonate buffer, pH 9.6. Plates were sealed with an acetate plate sealer (Flow Laboratories) and incubated overnight at room temperature with gentle shaking (100 rpm) on an Orbit Shaker (Lab-Line Instruments.) All subsequent incubations were done with sealed plates, that were shaken at 100 rpm. Unbound LDL was removed by washing four times with PBS-TB, and blocking of remaining binding sites on the plate was achieved as described above. At this point plates were washed and either used immediately or thoroughly dried, sealed, and stored at 4°C in a desiccated chamber for up to 1 month.

Plasma samples and controls were diluted 1:500 in PBS-TB. Dilution was done in two steps, a 1:50 dilution followed by a 1:10 dilution using a Micromedic Digiflex automatic pipettor. A standard curve was prepared by diluting a primary LDL standard to 5.0, 4.0, 3.0, 2.0, and 1.0 μg/ml, respectively. Fifty μl of standards, quality controls, and samples were pipetted in triplicate into the wells. After the final addition, 50 μl of either MB47, MB24, RB74, or B1B6 was added to all wells and incubated overnight at room temperature.

After washing, 100 μl of a 1:500 dilution in PBS-TB of commercially prepared rabbit anti-mouse IgG or goat anti-rabbit IgG conjugated to horseradish peroxidase was added to the plate. The plate was incubated for 2 h at room temperature and excess antibody was removed by washing. One hundred μl of *o*-phenylamine diamine substrate solution was added as described above. After 30 min, the reaction was stopped by the addition of 50 μl of 2 N H_2SO_4. Data were collected and analyzed as described above.

Pretreatment of samples with lipase

To 20 μl of plasma, 880 μl of free fatty acid acceptor buffer (0.2 M Tris, 0.15 M NaCl, 5% Sigma A-7030 BSA, pH 8.2, sterile-filtered) was added. This was followed with 50 μl of anti-proteolytic cocktail containing 2 mM *p*-amidinophenyl-methylsulfonyl fluoride hydrochloride, 2 mM D-phenylalanyl-L-prolyl-L-arginine chloromethylketone, 2 mM D-phenylalanyl-L-phenylalanyl-L-arginine chloromethylketone (Calbiochem-Behring, San Diego, CA), and 1 mM aprotinin (Sigma, St. Louis, MO). After vortexing, 25 μl of anti-proteolytic cocktail containing 4 U of lipase (*Chromobacterium viscosum*, Calbiochem-Behring, lot #607446) was added. Following incubation at 37°C for 30 min in a shaking water bath, 25 μl of 6 mM 5,5'-dithio-*bis*(2-nitrobenzoic acid) was added to stop the lipase reaction. Complete hydrolysis of triglyceride was confirmed by lipid analyses performed as described (33). For the apoB-100 ELISA, the sample was diluted 1:40 in PBS-TB containing 100 μM anti-proteolytic cocktail. For the cELISA procedure, treated samples were diluted 1:10 in PBS-TB containing 100 μM anti-proteolytic cocktail.

RESULTS

Antibody preparation and coating concentration

Nunc Immuno-plate I microtiter plates were chosen for their superior reproducibility (between-well variance < 2%) in binding IgG. Other manufacturers' plates tested in our laboratory did not perform as well. These included Falcon (Falcon, Inc.), Immulon II (Dynatech, Vienna, VA), Corning (Corning Glass Works), Linbro Titertek, and EIA II Plus (Flow Laboratories).

MB47 purified by protein A affinity chromatography and FPLC anion exchange chromatography were compared with MB47 murine ascites fluid and a rabbit polyclonal anti-LDL (Rb74), affinity-purified against LDL, for maximum binding of ^{125}I-labeled LDL in Nunc Immuno-plate I microtiter plates. After 18 h incubation of ^{125}I-labeled LDL under conditions of antibody excess, only the FPLC-purified MB47 approached the binding capacity of the polyclonal antibody based on TCA-precipitable counts (95% vs. 97%). MB47 murine ascites bound a maximum of 72% and protein A affinity-purified MB47 bound a maximum of 62%, on average (**Fig. 1**). Three separate LDL isolations and labelings compared with three different antibody preparations by the various methods gave essentially identical results. Thus, purifica-

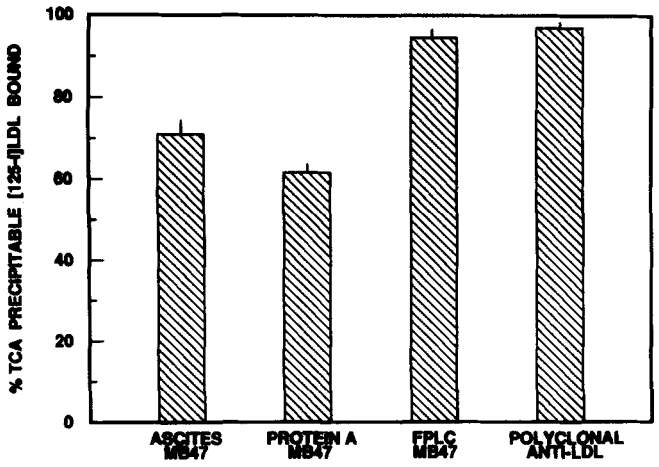

Fig. 1. Maximum binding of ^{125}I-labeled LDL by different antibody preparations. ^{125}I-labeled LDL (0.5 μg/ml) was incubated overnight in microtiter plate wells previously coated with either MB47 ascites, MB47 ascites purified by protein A affinity chromatography, MB47 ascites purified by anion-exchange FPLC, or rabbit anti-LDL affinity-purified against LDL. After incubation the ^{125}I-labeled LDL was removed from the plate and precipitated with 12% TCA. The percent ^{125}I-labeled LDL bound was calculated by subtracting the TCA-precipitable counts in the ^{125}I-labeled LDL removed after incubation from the total counts applied to the plate and dividing this difference by the total TCA-precipitable counts. The bars indicate the results of three ^{125}I-labeled LDL isolations/labelings ± SD, each incubated in sextuplicate. The results of MB47 purified by protein A and FPLC are representative of three different purifications by each method.

tion of MB47 by FPLC anion-exchange chromatography was found to be the optimal antibody preparation as demonstrated by the maximum binding capacity of ^{125}I-labeled LDL per surface area coated.

MB47 is bound to the plate by noncovalent adsorption by incubation at alkaline pH. An optimal antibody coating concentration is one where there is a single monolayer of high avidity antibody. Extreme excess of first antibody is not only inefficient, but also promotes protein-protein binding that is unstable and increases assay variability. Thorough washing of the plate will remove most low avidity, as well as protein-protein bound, IgG thus minimizing the effects of antibody excess. The optimal coating concentration for FPLC-purified MB47 IgG, where MB47 is in slight excess, was found to be between 0.5 and 1.0 μg/100 μl per well (**Fig. 2**).

Assay incubation conditions

In the direct binding ELISA format, antibody is first bound to the plate. The antibody-antigen binding process begins as soon as the antigen is pipetted into the previously coated microtiter plate well. In our experience, 10–15 min was the average time required to load all 96 wells of the microtiter plate. This time requirement resulted in a variance in the binding between the first and last wells loaded. For the assay to be valid, a sample must yield the same apoB value independent of its location on the microtiter plate. The effect of incubation time on this between-well variance for a single sample loaded into all 96 wells within 15 min is shown in **Fig. 3**. An acceptable level of variance, <2%, was achieved only after overnight incubation. Furthermore, when more than 25 min was taken to load the entire plate variance was always >8%.

Assay incubation time and shaking versus not shaking was evaluated for binding of ^{125}I-labeled LDL. Maximum binding of ^{125}I-labeled LDL as determined by TCA-precipitable counts was significantly greater with shaking, 97 ± 2% (mean ± SD), than without, 82 ± 3% (**Fig. 4**). Gentle shaking did not increase the nonspecific binding of the ^{125}I-labeled LDL and it did not alter the incubation time necessary to achieve maximum antigen binding. Thus, maximum binding of ^{125}I-labeled LDL by FPLC-purified MB47 was achieved with gentle shaking of the microtiter plate during overnight incubation.

Blocking of unoccupied microtiter plate binding sites was accomplished by incubating the MB47-coated plates with PBS containing 3% BSA, 0.025% Tween-20 for 1 h with shaking at room temperature. Other blocking agents tested included gelatin (Sigma) and lipoprotein-deficient plasma (LPDP, d>1.21 g/ml). Gelatin resulted in a higher within-assay variance and appeared to inhibit binding of LDL. LPDP was similar to BSA in stability and effectiveness. BSA was chosen for its availability and uniformity. The addition of 0.025% Tween-20 was found necessary to minimize nonspecific binding. However, Tween-20 concentrations above 0.025% in the presence of 0.1% BSA resulted in a decrease in the slope of the LDL standard.

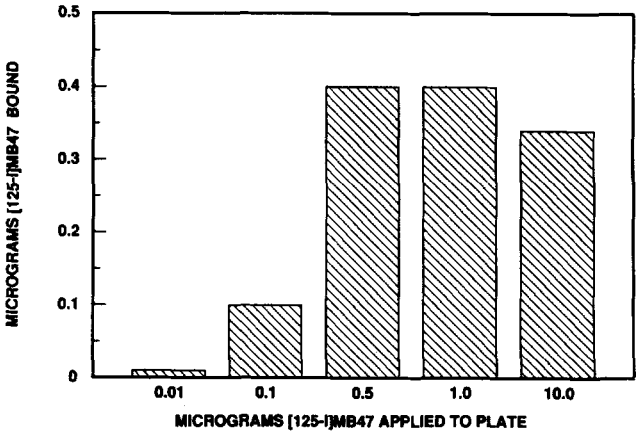

Fig. 2. Maximum binding capacity of the microtiter plate for ^{125}I-labeled MB47. Nunc Immuno-plate I microtiter plates were incubated overnight at room temperature with increasing concentrations of ^{125}I-labeled MB47 (100 μl/well). After incubation, the ^{125}I-labeled MB47 was removed and based on the specific activity, micrograms of ^{125}I-labeled MB47 bound were calculated by subtracting the counts unbound after incubation from the total number of counts applied to the plate. SD were <3%.

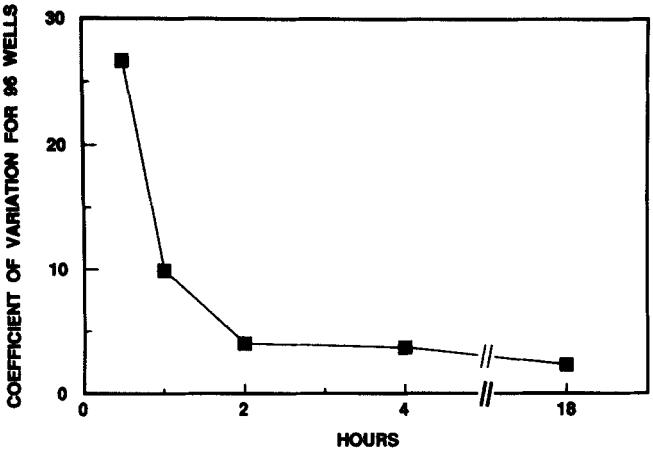

Fig. 3. Variation of apoB-100 binding by MB47 across the microtiter plate as a function of time. Whole plasma, diluted 1:2000, was added to all 96 wells of five microtiter plates coated with MB47. Plating time was 15 min per plate. After the final addition, one plate was incubated for each time point as indicated. Each plate was then washed and the assay was developed as described for the direct binding reference ELISA procedure (see Methods).

MB24-HRP antibody-enzyme conjugate is incubated in the assay under conditions of antibody excess. The binding of MB24-HRP in the assay was found to be both time- and dose-dependent (**Fig. 5**). At higher concentrations of MB24-HRP, less time was required to achieve maximum binding. Maximum binding was achieved after 1 h at a 1:4000 dilution or after 3 h at 1:12,000 (Fig. 5). Because a shorter incubation time is desirable, 1 h at a 1:4000 (4.12 μg/ml) dilution of MB24-HRP was chosen. The Behring ELISA Processor II plate reader is linear to 2.2 absorbance units between 405 nm and 650 nm. Therefore, color development of the highest concentration LDL standard was limited to 1.5 absorbance units at 492 nm by the addition of 4 N H_2SO_4. Depending on the efficiency of the MB24-HRP conjugation, this was achieved between 15 and 30 min after addition of substrate to the microtiter plate.

Assay calibration

The SDS-Lowry protein value for a purified LDL (d 1.030–1.050 g/ml) containing less than 1% nonapoB protein by Western immunoblot analysis was used to calibrate the assay. SDS-Lowry protein values of eight LDL preparations were compared to the protein values derived from amino acid analyses performed in duplicate (see Methods). The average of the eight preparations was 93 ± 3% of the SDS-Lowry value. From the LDL curve the range of linear binding for the assay was determined to be 0.25–1.25 μg/ml and the optimal dilution for whole plasma 1:2000. LDL prepared from four unrelated donors by sequential ultracentrifugation (d 1.030–1.050 g/ml) was diluted based on the SDS-Lowry value and compared for linearity of binding in the B-100 direct binding ELISA. All LDL preparations tested were essentially identical (**Fig. 6A**). Dilution curves of fresh plasma were identical to LDL (not shown).

LDL from FCHL, FH, hypercholesterolemic (without a definitive genetic diagnosis), and normolipidemic subjects isolated by DGUC was tested for the potential effects of LDL size heterogeneity on the accuracy of the assay. Particle size, as determined by nondenaturing gel electrophoresis, ranged from 23.2 nm for the smallest FCHL-LDL to 27.3 for the largest FH-LDL. LDL from two subjects with hypercholesterolemia with distinctly heterogeneous LDL was also tested. Both subjects demonstrated two sizes of particles of 25.4 nm and 26.4 nm. The mean size for three normolipidemic subjects was 25.1 ± 0.2 nm. No significant differences in binding related to size or heterogeneity were observed when these LDLs were tested in the ELISA (Fig. 6B-C).

VLDL ($d < 1.006$ g/ml) prepared from different donors also were compared for linearity of binding. Most of the VLDL preparations tested (12 of 15) were parallel. **Fig. 7** shows five VLDL preparations (a thru e) that bound parallel to the primary LDL standard and two (f and g) that did not. The VLDL cholesterol and triglyceride content ranged from 4 mg/dl and 18 mg/dl to 192 mg/dl and 613 mg/dl, respectively. Lipid values for VLDL-f were 166 mg/dl cholesterol and 702 mg/dl triglyceride. Lipid values for VLDL-g were 245 mg/dl cholesterol and 1479 mg/dl triglyceride. The data suggest that samples with triglyceride levels < 700 mg/dl are accurately measured. Binding curves of VLDL-f and VLDL-g were only slightly affected by pretreatment with lipase (experiment not shown).

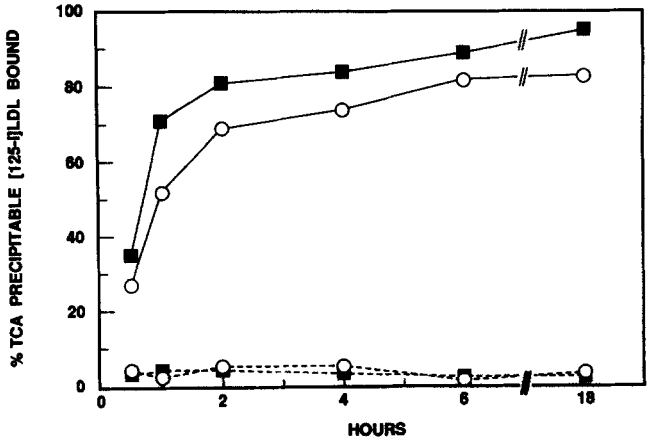

Fig. 4. Effect of time and shaking on ^{125}I-labeled LDL binding by MB47. Microtiter plates coated with MB47 were incubated at the times indicated with ^{125}I-labeled LDL, with (■) and without (○) shaking at 100 rpm. The corresponding nonspecific binding (---) controls were obtained by incubating ^{125}I-labeled LDL in wells coated with mouse IgG.

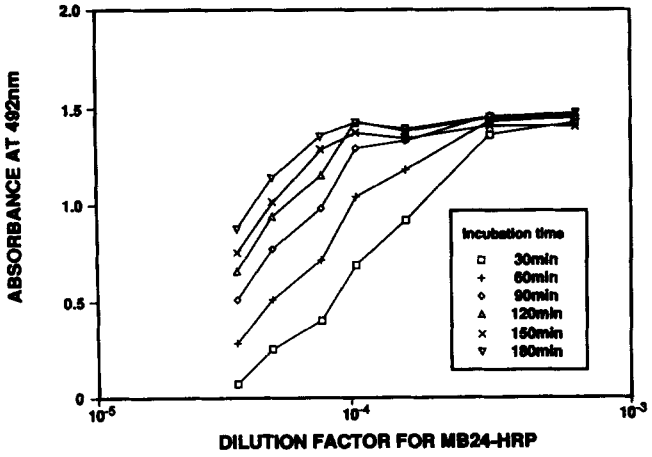

Fig. 5. Titer of MB24-HRP. MB24 conjugated to HRP was diluted between 1:2000 (8.24 μg/ml) and 1:32,000 (0.52 μg/ml) and incubated with plates coated with MB47 and incubated with 150 ng of LDL/well. At the indicated times, conjugate was removed, the plates were washed, and substrate was added. Color development was stopped after 15 min by the addition of 4 N H_2SO_4.

Incubation of ^{125}I-labeled VLDL on MB47-coated microtiter plates, under conditions of antibody excess, resulted in 70% to 94% of the total TCA-precipitable counts being bound for five different preparations (**Fig. 8**).

Intra-assay variance was evaluated by analyzing three different fresh-frozen plasma pools and a lyophilized pool prepared by the NWLRC 12 times each in a single assay (**Table 1**). Results ranged from 1.7% to 3.8%. Interassay variance was established by analyzing freshly thawed plasma, or freshly prepared lyophilized material, from the same pools in 105 assays performed on 41 different days over an 11-month period. The interassay coefficient of variation ranged from 5.1% to 6.9% (Table 1).

Effect of storage on apoB-100 measurements

Plasma could be stored for up to 2 weeks at 4°C without a significant change in measurable apoB-100 (**Fig. 9**). A decrease was observed in one sample after 2 weeks and in all samples after 4 months. Plasma quality control pools stored up to 11 months at −70°C did not exhibit any significant changes in apoB values (Table 1). Freezing plasma or serum for 24 h at either −20°C or −70°C was tested against samples held at 4°C and found not to significantly change apoB-100 measurements (**Table 2**). ApoB-100 values from plasma were slightly lower (4%) than values obtained from serum.

Comparison of apoB-100-specific methods with total B methods

We analyzed 16 normolipidemic samples by six different methods, three that measure only apoB-100 (MB47-MB24 direct ELISA, MB47 cELISA, B1B6 cELISA) and three that measure total apoB (MB24 cELISA, RB74 cELISA, and RB74 NWLRC RIA). Excellent agreement was obtained between all six methods

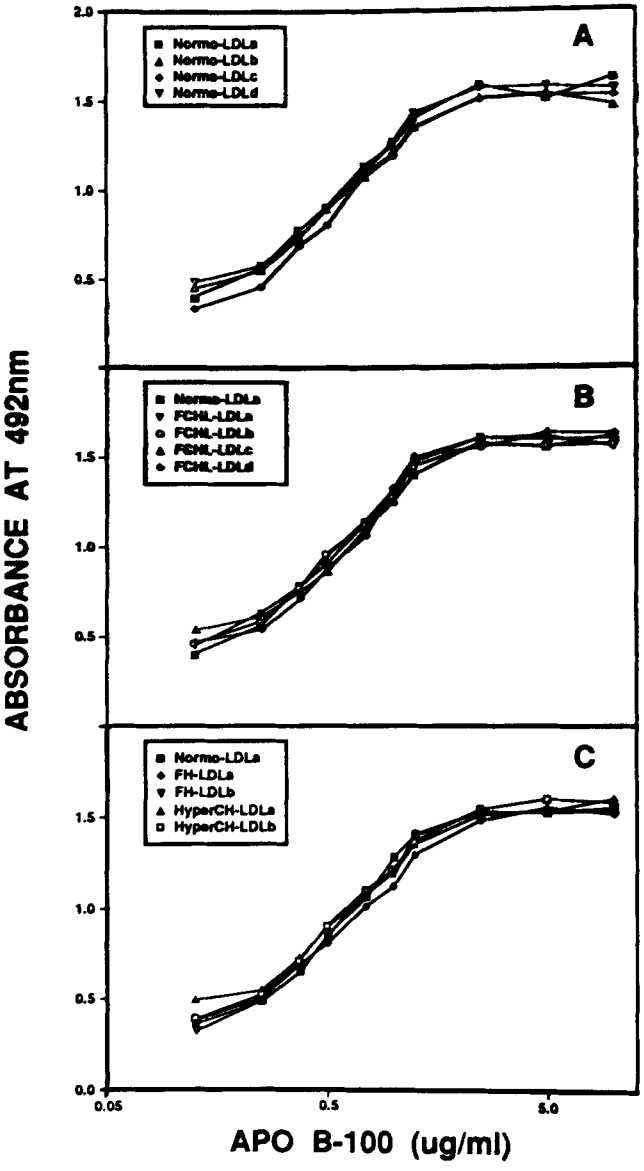

Fig. 6. Binding curves of LDL preparations from twelve unrelated subjects. Dilutions based on the SDS-Lowry protein concentration were incubated in the B-100 reference ELISA and the assays were developed as described (see Methods). A: LDL (d 1.030–1.050 g/ml) isolated from four healthy normolipidemic subjects (Normo-LDL) by sequential spin ultracentrifugation. B: LDL isolated by DGUC from one normolipidemic subject (Normo-LDL) and four subjects with familial combined hyperlipidemia (FCHL-LDL). C: LDL isolated by density gradient ultracentrifugation (DGUC) from one normolipidemic subject (Normo-LDL), two subjects with familial hypercholesterolemia (FH-LDL), and two hypercholesterolemic subjects (HyperCH-LDL) with distinct heterogeneous LDL. Particle size (nm), as determined by nondenaturing gel-electrophoresis was as follows; Normo-LDLa, 24.9; Normo-LDLb, 25.2; Normo-LDLc, 25.3; Normo-LDLd, 24.9; FCHL-LDLa, 23.6; FCHL-LDLb, 23.5; FCHL-LDLc, 23.8; FCHL-LDLd, 23.2; FH-LDLa, 27.3; FH-LDLb, 26.3; HyperCH-LDLa, 25.5 and 26.5; HyperCH-LDLb, 25.3 and 26.5.

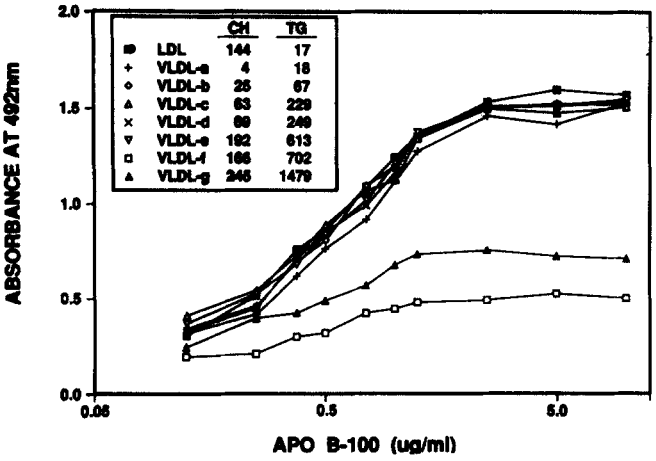

Fig. 7. Binding curves of seven unrelated VLDL (d < 1.006 g/ml) preparations. Dilutions of VLDL were incubated in the B-100 reference ELISA as described (see Methods). The concentration of apoB-100 in the VLDL was determined by the candidate reference apoB-100 ELISA. CH (cholesterol) and TG (triglyceride) are expressed in mg/dl.

on all 16 plasma samples from normolipidemic subjects with between-method coefficients of variation ranging from 2.4% to 4.9% (**Table 3**). The measurement of apoB was highly correlated with both the measurement of total cholesterol (r = 0.66 to 0.77, $P < 0.001$) and LDL cholesterol (r = 0.70 to 0.80, $P < 0.001$) in these samples. Comparison of the six methods on six hypertriglyceridemic samples with an average triglyceride of 765 mg/dl, ranging from 308 mg/dl to 1619 mg/dl, showed that the

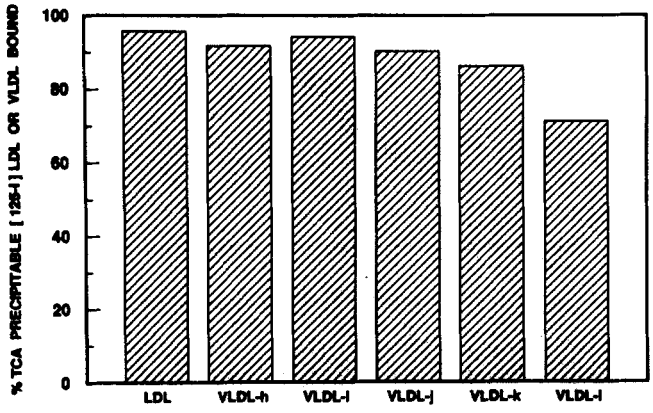

Fig. 8. Binding of ^{125}I-labeled VLDL. ^{125}I-labeled VLDL (0.5 μg/ml ELISA apoB-100) from five unrelated subjects was incubated overnight in microtiter plate wells previously coated with MB47 ascites purified by anion-exchange FPLC. After incubation the ^{125}I-labeled VLDL was removed from the plate and precipitated with 12% TCA. The percent ^{125}I-labeled LDL bound was calculated by subtracting the TCA-precipitable counts in the ^{125}I-labeled VLDL removed after incubation from the total TCA-precipitable counts applied to the plate and dividing this difference by the total TCA-precipitable counts. The results are representative of three different labelings of each subject's VLDL. ^{125}I-labeled LDL was included as a control. Cholesterol triglyceride values (mg/dl) were as follows: VLDL-h, 13, 10; VLDL-i, 10, 53; VLDL-j, 16, 120; VLDL-k, 39, 213; VLDL-l, 247, 1212.

TABLE 1. Intra- and interassay variance of the apoB-100 ELISA

	QC-1[a]	QC-2[a]	QC-3[a]	QC-4[b]
Intra-assay (n = 12)				
Mean (mg/dl)	106	94	146	95
SD	3.0	2.0	3.3	1.6
CV (%)	3.8	2.2	2.2	1.7
Interassay (n = 105)				
Mean (mg/dl)	107	96	144	94
SD	7.2	6.6	7.9	4.8
CV (%)	6.7	6.9	5.5	5.1

[a]Fresh-frozen plasma quality control pool, stored at −70°C, freshly thawed.
[b]Lyophilized serum pool, stored at −20°C, freshly reconstituted.

total apoB values were 15% higher, on average, than the apoB-100 values (Table 3). A comparison of apoB-100 measurements obtained with the MB47-MB24 direct ELISA with total B measurements obtained with the RB74 NWLRC RIA from 105 normotriglyceridemic samples yielded a correlation coefficient of 0.93, mean ELISA B-100 value = 100 mg/dl, mean RIA value = 101 mg/dl, and Sy = 9.6 (**Fig. 10**).

Effect of triglyceride hydrolysis on plasma apoB measurements

To determine to what extent the MB47 epitope on apoB-100-containing particles is expressed in lipemic samples, plasma was incubated with bacterial triglyceride lipase in the presence of proteolytic inhibitors (see Methods) under conditions of complete triglyceride hydrolysis. For most samples with triglyceride levels < 610 mg/dl there was little difference in apoB-100 values before and after lipase treatment (**Table 4**). For plasma samples with triglyceride levels > 610 mg/dl the B-100 values ranged from 10% lower to 30% higher after lipase treatment.

DISCUSSION

Most clinical studies suggest that either total plasma apoB or LDL apoB is a better predictor of CHD than that of cholesterol (1). Nearly all (10 of 11) CHD case-control studies have demonstrated that subjects with coronary artery disease have significantly higher apoB levels than controls (1). Familial hypercholesterolemia and the two closely related disorders familial combined hyperlipidemia (FCHL) and hyperapobetalipoproteinemia are associated with premature CHD (34, 35). Subjects with FH usually have an elevation of apoB and LDL cholesterol. Subjects with FCHL usually have an elevation of apoB and often have borderline high LDL cholesterol (34). Subjects with hyperapobetalipoproteinemia have high levels of LDL apoB in the absence of an elevated

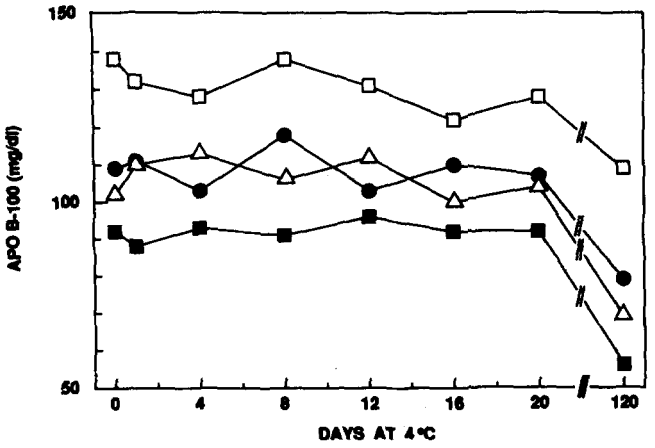

Fig. 9. Effect of storage of plasma at 4°C on the measurement of apoB-100. Four plasma pools were aliquoted, stored the indicated times at 4°C, and then frozen at −70°C. At 120 days, all samples were thawed and assayed for apoB-100 in the direct binding reference ELISA as described (see Methods).

TABLE 2. Effect of sample handling on reference apoB-100 ELISA measurements

	Serum			Plasma		
Sample[a]	4°C	−20°C	−70°C	4°C	−20°C	−70°C
		mg/dl			*mg/dl*	
1	83	88	83	86	80	81
2	90	87	95	88	84	92
3	94	101	101	90	93	98
4	105	100	100	98	92	100
5	107	100	111	94	100	102
6	107	106	112	108	105	107
7	121	131	128	122	128	124
8	131	123	132	123	124	127
9	135	142	130	128	135	122
10	147	155	156	146	144	152
Mean	112	113	115	108	108	110
SD	20	22	21	19	21	20

[a]Aliquots were stored for 24 h at the indicated temperatures.

LDL cholesterol (35). Among male subjects less than 60 years of age with coronary heart disease, approximately one-third have an elevation of apoB above the 95th percentile, and more than half of those with an elevation of apoB have normal LDL cholesterol levels (1). A recent prospective study in male U. S. physicians indicates that apoB levels are predictive of premature CHD (Albers, J. J., M. J. Stampfer, C. H. Hennekens, unpublished observations). Thus, the measurement of apoB is of clinical importance.

However, the measurement of apoB has not reached its full potential in the clinical laboratory because of problems in methodology, inadequate standardization, the lack of a suitable common reference material, and lack of a reference method. To assess a person's risk for coronary disease accurate age-, race-, and sex-specific population-

TABLE 3. Comparison of apolipoprotein B methods

CH	LDL-CH	TG	Direct ELISA MB47, 24 B-100	cELISA MB47 B-100	cELISA B1B6 B-100	cELISA MB24 B-100, -48	cELISA RB74 B-100, -48	RIA RB74 B-100
				mg/dl				
Normolipidemic subjects (n = 16)								
177	125	48	98	92	92	94	86	92
201	136	40	126	129	125	130	134	132
157	82	40	67	73	69	69	69	74
187	125	74	108	105	112	115	108	118
193	120	54	123	119	122	122	125	130
168	114	46	86	86	82	92	90	91
183	97	48	89	86	84	90	92	87
220	139	56	104	99	107	110	107	101
140	81	64	79	69	79	77	77	74
196	131	69	93	99	89	93	88	95
165	103	84	78	71	77	75	73	75
206	112	80	87	89	81	87	82	84
173	105	42	76	73	79	82	85	78
205	134	105	95	91	96	99	98	105
163	89	126	83	83	85	92	89	90
226	155	156	129	125	121	117	117	124
Mean 185	116	71	95	93	94	97	95	96
SD 23	21	32	18	18	17	17	18	20
Hypertriglyceridemic subjects (n = 6)								
Mean 326	225	914	143	146	146	165	166	169
SD 85	64	417	58	60	58	60	61	58

Abbreviations: CH, total cholesterol; LDL-CH, LDL cholesterol; TG, plasma triglyceride.

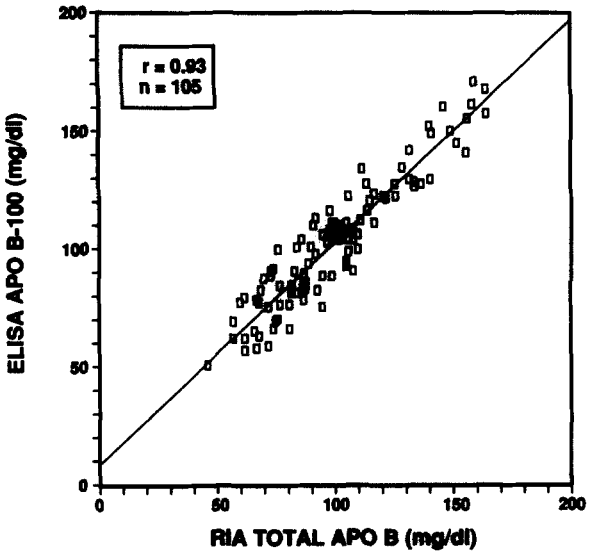

Fig. 10. Comparison of apoB-100 measurements obtained with the MB47/MB24-HRP ELISA with total apoB measurements obtained with the NWLRC RB74 RIA. Samples from 105 normotriglyceridemic subjects were measured. The line of regression is described by the equation ELISA apoB-100 = [0.92 RIA apoB] + 8.7. Sy = 9.6.

based reference measurements are needed for a uniform interpretation of apoB values. With proper standardization, all laboratories can adopt uniform apolipoprotein cutpoints for identifying subjects at high risk for coronary disease. This requires that all laboratories use appropriate methods and calibration procedures and common reference materials to minimize method-specific biases. This proper standardization also requires careful standardization of the method used for the measurement of the apoB protein standard. Historically, investigators have applied the protein procedure of Lowry et al. (24) or some modification such as organic solvent extraction or addition of detergent (25). Some investigators, including ourselves (19), have applied a chromogenicity factor (0.82) to convert Lowry protein to absolute protein mass. The chromogenicity factor used has ranged from 0.77 (36, 37) to 0.9 (38, 39). Our present study suggests that the Lowry protein value is slightly higher than the absolute protein mass determined by amino acid analysis. In view of the lack of a universally accepted chromogenicity factor and the impracticality and expense of amino acid analysis for the determination of protein mass, we recommend that apoB measurements be expressed in terms of a Lowry-based protein procedure as described here in detail, including the addition of SDS to minimize the potential interference of both lipoprotein lipids and lipoprotein aggregation. However, the bias between the Lowry-based protein procedure and amino acid analysis should ultimately be taken into account with future standardization efforts. Because the Lowry protein value can vary with the source of the albumin standard, we recommend that bovine serum albumin obtained from the United States Institute of Standards and Technology be used in all Lowry determinations of LDL protein. Also, because of the microheterogeneity of ultracentrifugally isolated LDL (40), we recommend that narrow cut LDL (d 1.030–1.050 g/ml) obtained from normolipidemic donors be used to ensure a minimal degree of contamination of nonapoB-100 protein.

The candidate reference procedure uses the monoclonal antibody MB47 as the capture antibody and MB24 conjugated to horseradish peroxidase as the detecting antibody. These antibodies bind to distinct apoB epitopes expressed by all LDL particles and detect apoB in VLDL as well. MB47 binds an epitope in the carboxyl-terminal portion of apoB-100 and does not bind apoB-48. Therefore, the assay is specific for apoB-100. An assay for apoB-100 was chosen because the clinical significance of apoB-48, a constituent of intestinal-derived chylomicrons and chylomicron remnants, remains to be established. Secondly, the large amount of lipid in chylomicrons is likely to mask or block some of the epitopes, making it difficult to accurately measure apoB-48 by immunoassay.

TABLE 4. Effect of lipase treatment on apoB measurements

			B-100 ELISA	
Analyte	CH	TG	Pre-Lipase	Post-Lipase
LDL			180	183
Plasma (n = 28)	146	27	91	94
	260	151	112	111
	192	232	96	101
	113	244	91	93
	159	319	71	82
	290	338	139	142
	232	341	127	135
	259	366	140	147
	301	396	125	144
	190	402	95	92
	223	407	129	133
	344	411	146	210
	189	513	98	102
	179	518	77	77
	212	572	111	110
	230	609	133	129
	314	618	139	157
	322	686	125	162
	219	738	87	99
	255	742	83	96
	461	746	113	150
	246	910	105	105
	249	1177	77	86
	305	1262	90	105
	329	1571	103	119
	305	2127	54	64
	595	4437	112	102
	517	7877	49	63

See Methods for incubation conditions. CH, plasma cholesterol; TG, plasma triglyceride.

Thirdly, accurate calculation of total apoB mass in serum is not possible because LDL is not an appropriate primary standard for the measurement of apoB-48-containing particles. The direct binding double antibody "sandwich" format was chosen rather than the competitive single antibody format because the apoB-100 in LDL displays a high degree of heterogeneity in terms of antibody affinity and the direct binding format is less sensitive to this property. Also, binding of LDL to the microtiter plate could alter epitope expression resulting in higher variability than observed with binding of the chemically uniform monoclonal antibody to the plastic.

Young et al. (12) previously described a direct binding ELISA using MB47 and MB24 for the measurement of apoB-100 and compared it to a competitive ELISA assay using MB24 for the measurement of total apoB. In this report we have modified those assay conditions and specifically evaluated the direct binding assay as a candidate reference method for the measurement of apoB-100. The key methodologic modifications include incubation of the antigen with the MB47 capture antibody for 18 h versus 30 min to ensure maximal binding of the antigen, and the sequential addition of antigen and MB24-HRP detecting antibody rather than a simultaneous incubation of antigen and detecting antibody to ensure uniform binding across the plate. Additional modifications include coating the plate with an optimal 100 μl of 0.5 μg MB47/ml instead of 150 μl of 1.0 μg/ml, the inclusion of 0.1% BSA and 0.025% Tween-20 to the diluent instead of 1.0% BSA and 0.5% Tween-20, and a validated working range of 0.25–1.25 μg apoB-100/ml. The use of FPLC Mono-Q-purified MB47 in both assays was evaluated in these studies and found to be essential for achieving maximal binding. The reason why the specific LDL binding capacity of protein A-purified MB47 is less than the FPLC Mono-Q-purified antibody remains unclear.

We have also demonstrated that the candidate reference method is not sensitive to the size heterogeneity of LDL. LDL isolated from a variety of sources, including large bouyant LDL from subjects with FH and small dense LDL from subjects with FCHL, exhibited binding properties similar to LDL from healthy normolipidemic subjects. Furthermore, we have shown that MB47, when used according to the assay format, not only bound nearly all LDL, but it bound nearly all VLDL from the majority of VLDL preparations as well. The fact that some VLDL is not bound by MB47 is not surprising, in view of the work of Schonfeld et al. (41), Tikkanen et al. (42), and Gianturco and Bradley (43) who demonstrated that the hydrolysis of VLDL triglyceride results in increased VLDL apoB epitope expression and increased binding by the LDL receptor. Furthermore, MB47 will not bind the B-48-containing particles found in the $d < 1.006$ g/ml fraction of human plasma.

Although it would be ideal to use a monoclonal antibody that identifies an epitope that is expressed on all VLDL and LDL particles, such an epitope is yet to be described. The use of MB47 is still desirable because it binds to the highly conserved LDL receptor-binding domain of apoB-100 and the expression of this epitope presumably reflects the number of apoB-100-containing particles that are functionally capable of binding to the LDL receptor. Furthermore, we have also shown that similar apoB values are obtained with competitive ELISAs using either MB47 or B1B6. The monoclonal B1B6 recognizes an epitope between amino acid residues 3665 and 3780 that is very close to the MB47 epitope (44), which is between residues 3350 and 3505. The decreased slope of the MB47 binding curve of some triglyceride-rich particles presumably reflects altered apoB conformation which results in an altered form of the MB47 epitope. Additionally, Krul et al. (44) and Marcel et al. (45) have shown that the expression of epitopes near the putative LDL receptor-binding region of apoB-100 increases as the size of the triglyceride-rich particles decreases, with LDL having the highest expression. Therefore, masking of the MB47 epitope would be expected to increase as size of the triglyceride-rich particle increases. The treatment of hypertriglyceridemic samples with triglyceride lipase under conditions that minimize proteolysis resulted in little change in MB47-measurable apoB for most samples with triglyceride levels < 610 mg/dl (Table 4). These results suggest that the vast majority of the MB47 epitopes on plasma apoB-100-containing particles are expressed. Thus, plasma apoB-100-containing particles are not significantly underestimated in the assay, even for most hypertriglyceridemic samples. Indeed, experiments shown here on the absolute binding of ^{125}I-labeled LDL and ^{125}I-labeled VLDL suggest that for the majority of samples, MB47, as used in our assay format, is an accurate means for detecting all available apoB-100-containing particles.

Measurement of total apoB in normotriglyceridemic samples by competitive ELISA procedure using either MB24 or polyclonal anti-LDL and by RIA using polyclonal anti-LDL gave results nearly identical to the three procedures for the measurement of apoB-100 (Table 3). These findings indicate that apoB-48 is usually negligible in normotriglyceridemic plasma. In contrast, in some hypertriglyceridemic plasma, significant differences were obtained between the measure of apoB-100 and total apoB due to the presence of higher levels of apoB-48. Assuming only one MB24 epitope per apoB-48 particle, the difference in the total apoB and the apoB-100 assays should approximate twice the mass of apoB-48. Our studies do not, however, determine whether the MB24 epitope is expressed in all apoB-48-containing particles. It is likely that the expression of the MB24 epitope can be masked in some hypertriglyceridemic samples.

Reference materials with appropriate assigned target values and population base reference values are needed for uniform apolipoprotein cutpoints for identifying subjects at high risk for CHD. Sex- and age-specific apoB values in whites obtained from four independent studies (13, 19, 46, 47) have been shown to be quite similar. For example, for males age 40–49, the apoB mean values from each study ranged from 96 to 113 mg/dl, with an overall mean of 105 mg/dl. The mean value recently obtained from a study of males age 40–49 using our RIA was 108 mg/dl (Unpublished results. Little, R. E., E. Wisjman, J. J. Albers, A. G. Motulsky, J. D. Brunzell. Host and environmental effects on apolipoprotein B.). Mean values obtained with the candidate reference apoB-100 ELISA method agree very well with our RIA (r = 0.93, n = 105, mean ELISA value = 100 mg/dl, mean RIA value = 101 mg/dl) and with the consensus population-based apoB values obtained from the literature (1).

The most commonly used reference material in the United States is the International Union of Immunological Scientists (IUIS) Matrix apoA-I and apoB Reference Material (Pool 1883) prepared by the Centers for Disease control (48). However, this and other lyophilized materials are not suitable for use as reference materials or calibrators for apoB because these pools exhibit matrix interactions when used in certain methods. We recently have evaluated five different lyophilized materials, including the IUIS reference pool, for matrix interactions. ApoB was measured in the lyophilized pools and in four frozen serum pools by the candidate reference ELISA, RIA (19), two immunonephelometric (INA) methods, two immunoturbidometric methods (ITA), and two radial immunodiffusion (RID) methods. Similar apoB values were observed among the different methods for the frozen serum pools, while the lyophilized materials exhibited a large range of variation for the apoB values obtained by the different methods (Adolphson, J. A., and J. J. Albers, unpublished observation). Thus, use of lyophilized materials for apoB standardization or calibration can result in substantial biases with some methods. For this reason, fresh-frozen serum reference pools that reflect actual patient specimens have been recommended for apoB (49).

We recommend that the direct binding candidate reference ELISA, using the SDS-Lowry protein value for purified LDL as a primary standard, be used to assign apoB values to frozen serum apoB reference materials, secondary standards, and other reference materials that have been shown not to exhibit matrix effects. Adoption of the candidate apoB-100 reference method and its transfer to reference laboratories should help reduce the large among-laboratories variability seen with apoB measurements and permit determination of reference ranges for clinical use.

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