Enzyme Immunoassay for Human Apolipoprotein B, the Major Protein Moiety in Low-Density- and Very-Low-Density Lipoproteins

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We used enzyme immunoassay to measure apolipoprotein B concentration in human plasma. Pure lipoprotein B was isolated from serum samples of fasting normolipidemic subjects by sequential preparative ultracentrifugation and coated to a polystyrene tube surface by adsorption. Human serum samples and rabbit antiserum to human apolipoprotein B were incubated with the solid-phase lipoprotein B. Soluble antigen competed with solid-phase antigen for binding to antibodies. After washing, peroxidase-labeled sheep antibodies against rabbit immunoglobulins were added, and after further washing the bound label was assayed. This provided a direct measurement of the soluble antigen. The best technical conditions for the assay were determined. The minimum detectable concentration was 1 µg per assay. The enzyme immunoassay yielded values that compare favorably with those obtained by radial immunodiffusion (r = 0.84) and by rocket immunoelectrophoresis (r = 0.80). The assay offers several advantages over existing techniques: sensitivity, specificity, simplicity, and non-use of radioisotopes.

Additional Keyphrases: intermethod comparison • solid-phase antigen • screening for hyperlipoproteinemia • diagnostic aids

The lipoproteins of human plasma contain several distinct polypeptides that are specifically associated with lipids to form complexes, which can be separated by sequential ultracentrifugation (1), gel filtration (2), precipitation (3), or preparative electrophoresis (4). To account for the protein heterogeneity of lipoprotein preparations isolated by these techniques, Alauopvic et al. (5) proposed a classification system based on apolipoproteins as the only distinct components for the differentiation of lipoprotein families. Five such lipoprotein families are now recognized and are designated LP-A, LP-B, LP-C, LP-D, and LP-E.3 To establish the relative or absolute concentration ranges of these families in normal and hyperlipoproteinemic subjects, it is important to develop methods of measuring apolipoproteins. Interest in Apo-B, which is the major protein moiety of LDL and VLDL, has led to the development of immunoassays such as immunocrit (6), automated immunoprecipitation by the techniques of nephelometry (7), gel immunodiffusion (8), rocket immunoelectrophoresis (9), and radioimmunoassay (10, 11). For the measurement of low concentrations of Apo-B (in VLDL or in experimental situations where small concentrations need to be measured), radioimmunoassay is very sensitive and usually relatively simple to perform. However, it requires sophisticated equipment for determining radioactivity, and compounds labeled with gamma-emitting isotopes have a relatively short shelf-life. Enzyme immunoassay has been recently developed as a good technique for determining antigens and antibodies in body fluids (12, 13). Its main advantages are the widely available equipment, the long shelf-life of the reagents, and the fact that no separation step is required. We have developed an original inhibition enzyme immunoassay to measure human serum Apo-B with use of simpler equipment. Our results show that the method is sensitive, accurate, precise, simple to perform, and rapid.

Materials and Methods

Blood Samples

After an overnight fast, blood was samples from apparently normal subjects and some patients with hyperlipoproteinemia attending the Clinique Médicale Générale, Cité Hospitalière, Lille, France; the blood was collected into ethylenediaminetetraacetate-containing tubes and plasma was separated immediately at 4 °C. Thimerosal, 200 mg/liter, was added before storage. Plasma samples were assayed within two weeks if stored at 4 °C, within three months if stored at -20 °C.

Methods

Preparation of lipoprotein B (LP-B). Low-density lipoprotein of narrow density range (d = 1.040–1.053 kg/liter) was purified from pooled plasma of healthy

human donors by centrifugation at 100,000 × g. This LDL subfraction consists of apo-B uncontaminated by other apolipoproteins (14). The density of the plasma was adjusted to 1.040 kg/liter with sodium chloride/potassium bromide solution, and after centrifugation for 24 h the supernatant fluid was discarded and the centrifugation repeated at the same density for the same time. The density of the infranatant fluid was then adjusted to 1.053 kg/liter and the supernate obtained after a further 24-h centrifugation was resuspended in solution of similar density and the process repeated. The supernatant fluid thus obtained was dialyzed against 0.15 mol/liter NaCl.

Production of LP-B antibody. New Zealand white rabbits were injected subcutaneously with 2.0 mg of LP-B prepared as above and emulsified with an equal volume of Freund's complete adjuvant, according to Vaitukaitis et al. (15). Antibodies to human Apo-B were detected 10 weeks later. Rabbit blood was collected monthly, and the serum was stored at −20 °C after adding NaN₃ (1 g/liter) as preservative.

Enzyme-conjugated immunoglobulins. Sheep anti-rabbit immunoglobulins conjugated to horseradish peroxidase (EC 1.11.1.7) were obtained commercially from the Institut Pasteur, Paris.

Inhibition enzyme immunoassay of Apo-B. The series of reactions that occur is shown diagrammatically in Figure 1. Disposable polyethylene tubes (13 × 75 mm; Biomat, Hazebruck, France) were coated with LP-B, as follows: 1 ml of the antigen solution in phosphate-buffered isotonic saline (phosphate, 10 mmol/liter, pH 7.2) with 200 mg of NaN₃ per liter was added to each tube. The tubes were incubated in a water bath at 37 °C for 3 h; the fluid was then aspirated, and the tubes were stored at 4 °C for no longer than a week. Immediately before assay, the tubes were washed three times with phosphate-buffered saline containing Tween 20 (0.5 ml/liter) and the fluid was aspirated. Sera or antigen solution and rabbit antiserum to human Apo-B, in 0.5-ml volumes, at appropriate dilutions in phosphate-buffered saline containing Tween 20, were allowed to stand for 4 h at room temperature and then were washed three times as above. After washing, peroxidase-labeled sheep antibody against rabbit immunoglobulins (conjugate) was added in suitable dilution and the mixture was allowed to stand overnight at 4 °C. Excess conjugate was then thoroughly washed away and the amount of peroxidase fixed to the tubes was determined by using H₂O₂ as substrate and o-dianisidine as the hydrogen donor (16). After 1 h at room temperature, the reaction was stopped by adding a drop of HCl (5 mol/liter) and the yellow color was measured at 405 nm.

Apo-B evaluation by radial immunodiffusion and by rocket immunoelectrophoresis. Radial immunodiffusion was performed by the method of Mancini et al. (17). Antiserum to Apo-B was added to a 10 g/liter solution of agarose at 55 °C. This mixture was poured into a mold and allowed to congeal. Antigen wells of 2-mm diameter were punched out and 5 μl of standard or serum was placed into each well. After this arrangement had been standing for 120 h in a humid chamber at 30 °C the diameters of the immunoprecipitation rings were measured.

Rocket immunoelectrophoresis was performed by the method of Laurell (18) with use of agarose (10 g/liter, containing 5 ml of antiserum to human Apo-B per liter). Samples (3 μl) of serum or standard were applied in duplicate and electrophoresed at 2 V/cm for 20 h. Standard and serum were used undiluted and in dilutions as great as 10-fold. The assay plate was placed in a bath containing NaCl (9 g/liter) for 1–2 h, blotted with Whatman no. 1 filter paper, and dried in a current of hot air. Immunoprecipitates were stained with a mixture.
containing a 1.2 g/liter solution of Coomassie Brilliant Blue R (Sigma Chemical Co., St Louis, Mo. 63178), water, acetic acid, and 95% ethanol (2/2/2/1, by vol) for 10 min, and destained to a clear background in a solution of acetic acid/95% ethanol/water (1/3/5 by vol). Results were read from a standard curve for each plate, relating peak heights to concentration of standard.

Other methods. Plasma cholesterol and triglycerides were enzymatically measured by continuous-flow analysis (19, 20). Protein concentrations in lipoprotein fractions were determined by the method of Lowry et al. (21), with human serum albumin (Sigma Chemical Co.) as a standard because of the similarity of its aromatic amino acid content to that of Apo-B (14).

Results

Conditions of Assays

Our experiments showed that LP-B adhered satisfactorily to polystyrene tubes. Polystyrene tubes were coated with different concentrations of LP-B antigen and incubated with antiserum to Apo-B, diluted 1000-fold. After colorimetric quantitation of peroxidase fixed to the tubes, the absorbances were plotted vs. concentration of LP-B used in coating (Figure 2). The optimal coating concentration (1 mg/liter) was selected for further use.

For determination of the optimal dilution of anti-LP-B serum to use, serial dilutions of the antibody were incubated with a fixed concentration of LP-B (1 mg/liter) coated on polystyrene tubes and the samples were processed as described in Methods. A dilution curve is shown in Figure 3. At serum dilutions of less than 250-fold, there was high absorbance. At dilutions exceeding 20 000-fold, or without serum, nonspecific binding was reduced to less than 5%. Sensitivity was optimized by using the greater dilution yielding 90% binding. This dilution can vary from one batch of anti-

Fig. 2. Determination of optimal coating LP-B concentration for determination of Apo-B by enzyme immunoassay

Binding of anti-LP-B to polystyrene tubes coated with different concentrations of LP-B antigen. Each point is the mean of eight analyses. Bars indicate ± SD. Arrow indicates the optimal coating concentration used for further studies.

serum to another, and was 1000-fold in all subsequent analyses.

The optimal dilution of the conjugate was determined by testing different conjugate concentrations with or without LP-B; the optimal concentration (the highest concentration that gives a high range of extinction values) was 500 µg/liter.

Different concentrations of LP-B were used to obtain a reference curve. Figure 4 shows a representative standard curve for Apo-B. As the amount of added soluble LP-B was increased, the intensity of the color reaction decreased progressively. The working range of the assay was 1 to 15 µg.

Sensitivity and Precision of the Assay

The minimum detectable concentration (1 µg per assay) exceeds that required to measure the Apo-B in plasma, and a 200-fold dilution of plasma was made for routine study.

Within-batch precision was calculated from the difference between duplicate measurements of 56 sera. The coefficient of variation was 4.5% between 0.14 and 3.94 g/liter.

For a rapid assessment of the day-to-day imprecision, we analyzed two specimens, one with low value for Apo-B (0.62 g/liter) and one with a high value for Apo-B (1.67 g/liter), once per day for 20 consecutive working days. The coefficients of variation were, respectively, 10.8 and 8.9%.

Accuracy of the Assay

Studies of analytical recovery. When pure LP-B solution was added to several sera, the mean percentage recovery was 98.3.

Specificity. The antiserum selected for use in the assays gave a single peak on crossed immunoelctro-
phoresis against whole human serum, showing that it was directed against a single antigen.

Interference studies. Bilirubin, hemoglobin, and commonly used drugs (nicotinic acid, clofibrate) did not interfere with the determination.

Comparison with other methods. Enzyme immunoassay was more sensitive than rocket immunoelectrophoresis (14) and radial immunodiffusion (8) and required a 200- or 400-fold sample dilution with phosphate-buffered saline. Results obtained by the three methods correlated well (Figures 5 and 6). The mean concentrations found by enzyme immunoassay were somewhat higher than those obtained by rocket immunoelectrophoresis or radial immunodiffusion, but it is well known that these last two methods may be adversely affected if migration of large particles (VLDL) through agarose is impeded.

Discussion

Epidemiological studies have established that above-normal plasma cholesterol and triglyceride concentrations are associated with premature coronary heart disease (22, 23). The risk appears to be particularly associated with increased plasma LDL and VLDL, whereas increased HDL is thought to prevent premature atheroma (24).

Apolipoprotein B is a major protein of LDL and VLDL but not of HDL, which gives great interest to its determination. In the past, the lipoproteins have been more intensively studied in terms of their lipid rather than of their protein moiety, because the methodology to measure apolipoproteins was cumbersome, time consuming, and applicable only to the processing of a small number of samples. Recently a number of assay procedures based upon immunological techniques have been devised for measuring Apo-B in plasma. The earlier methods such as rocket immunoelectrophoresis and radial immunodiffusion suffer from the disadvantage that larger lipoprotein molecules, such as chylomicrons and VLDL, diffuse poorly in solid agarose, give an underestimate of total Apo-B, and the techniques cannot be automated. More recently, radioimmunoassays have become available (10, 11) for apolipoprotein B estimation in whole plasma or in isolated lipoproteins, but not only must the competing substance (LP-B) be radio-labeled but expensive and sophisticated counting equipment, radioisotope licensing, and special protective measures for safe handling of the radioactive reagents are required.

Enzyme immunoassay provides a specific and highly sensitive alternative method for measuring Apo-B in small samples.

The precision of our enzyme immunoassay equalled that reported for radioimmunoassays or rocket immunoelectrophoresis (14), and there was good correlation between Apo-B values obtained by the three techniques.
The great sensitivity of the method should allow estimation of Apo-B in lipoprotein fractions for genetic studies of the hyperlipidemias (11). Enzyme immunoassay is also especially suited for screening studies. Its cost is relatively low and the method could be automated. Because Apo-B concentrations in the plasma of fasting and non-fasting normal subjects were not significantly different, enzyme immunoassay of Apo-B may provide a diagnostic test for dyslipoproteinemias that will be superior to the qualitative techniques now used in clinical practice for population screening.

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References