Improved Method for Quantification of B Apoprotein in Plasma Lipoproteins by Electroimmunoassay

Michael F. Reardon, Mary E. Poapst, Kristine D. Uffelman, and G. Steiner

The B apoprotein occurs in a wide range of plasma lipoproteins, which are heterogeneous both with respect to size and to composition. Immunochemical recognition of the apoprotein is influenced by the nature of the particle in which the apoprotein is found, presumably due to the masking of the antigenic sites by lipoprotein lipid. Consequently, it is difficult to provide suitable standards for use in routine electroimmunoassay procedures of the B apoprotein, particularly for triacylglycerol-rich lipoproteins. We have devised a procedure whereby lipoprotein samples, independent of their initial size and composition, are reduced, by use of a bacterial lipase, to a common size and composition, which is almost identical to that of the standard. This permits an assay that may be easily used routinely. It provides a far more reliable estimate of the absolute B apoprotein mass in plasma lipoproteins of the very-low- and low-density lipoprotein spectrum than has been previously available.

Additional Keyphrases: lipid masking • low- and very-low-density lipoproteins • better immunological match between standard and sample • production of "LDL-like" particles by treatment and lipase

Apoproteins serve as structural elements of lipoproteins and as modulators of their metabolism. Hence, the quantitation of apoprotein mass and concentration has assumed increasing importance in understanding the regulation of lipoprotein metabolism, both in health and disease. Although several techniques have been developed for determination of apoprotein mass, immunological techniques in general have proven the most useful for routine application. These techniques include radioimmunoassay, immunodiffusion, and electroimmunoassay. In practice, their implementation is made difficult because of the heterogeneous nature of the plasma lipoproteins. For example, B apoprotein is the fundamental apoprotein of lipoproteins in the chylomicron—low-density lipoprotein (LDL) spectrum. These particles may vary in diameter from 20 to 600 nm, and also vary markedly in lipid and apoprotein composition. Schonfeld et al. (1) showed that the immunoreactivity of the B apoprotein of the very-low-density lipoprotein (VLDL) changes as VLDL particles are converted to remnants. Likewise, the immunoreactivity of chylomicrons alters as a function of triacylglycerol (triglyceride) hydrolysis (2). Evidently immunological recognition is influenced by the nature of the particle in which the apoprotein is found.

Immunochromatographic quantitation procedures demand that, in the assay system being used, the behavior of the antigen in the test samples be identical to that in the standard samples (3). Marked differences in lipoprotein particle composition (and so immunochemical recognition) may exist, even within particles of the same lipoprotein class, e.g., VLDL (4). Because of such heterogeneity, the provision of suitable standards for the quantitation of lipoprotein apoproteins is inherently difficult.

We have devised a technique for the routine quantitation of the absolute mass of B apoprotein in lipoproteins of the VLDL—LDL spectrum in which all samples may be directly related to the standard. In both test and standard samples, the apoprotein is assayed under almost identical conditions of particle size, charge, and composition. This is achieved by assaying after first treating all samples with a lipase, to convert the lipoprotein particles to "LDL-like" particles.

Experimental Procedures and Results

Preparation of Antibody and Standard

Human LDL (rel. density 1.030–1.040 kg/L) was isolated and washed by ultracentrifugation. It was ascertained to be free of all plasma proteins and apoproteins, other than the B apoprotein, by Ouchterlony immunodiffusion (5). This "narrow-band" LDL was used to raise anti-human B apoprotein antibodies in rabbits by conventional techniques (6). It was also used as the standard in the assay procedure. Protein concentration was determined by the method Lowry et al. (7), with bovine serum albumin as standard (Sigma Chemical Co., St. Louis, MO 63178). The suitability of bovine serum albumin as a standard for B apoprotein when this colorimetric procedure is used has been described elsewhere (6, 9).

Final Assay Protocol

Criteria for selecting these assay conditions will be discussed below.

For routine assay, both standard and test samples were incubated for 60 min at 37 °C, with "Lipase-TG" (commercially available bacterial lipase (EC 3.1.1.3) obtained from Calbiochem Corp., La Jolla, CA 92037). The incubation mixture consisted of 50 μL of assay sample (containing B apoprotein concentration within the range of 20–800 mg/L) and 50 μL of enzyme solution ("Lipase-TG," 2000 kU/L in 25 mmol/L Tris·HCl, pH 8.6).

After incubation at 37 °C for 60 min, 8-μL aliquots of the incubation mixture were taken for electroimmunoassay. We prepared gels (1.0 mm in thickness) of 10 g agarose per liter of 25 mmol/L Tricine buffer (Bio-Rad Laboratories, Richmond, CA 94804), pH 8.6, and containing 6 mL of rabbit anti-human B apolipoprotein serum per liter according to Laurell (3). Samples were electrophoresed for 16 h at a field strength of 8 V/cm. Temperature in the electrophoresis chamber was maintained at 22 °C with a water-recirculating cooling system. The gels were then stained (Coomassie Blue Brilliant R) as previously described (3). Rocket height and/or rocket area (height × width at half height) were ascertained. The within-assay CV was 2%, the between-assay CV 6%. The

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2 "Tricine" is N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Chem. Abstr. registry no. 5704-04-1).
Fig. 1. Immunoprecipitin rockets obtained upon immunoelectrophoresis of (a) VLDL S₁, 60–400, (b) S₁, 12–60, and (c) LDL S₁, 0–12
In each group the first rocket is of particles electrophoresed after lipase treatment, the second rocket is of the same sample electrophoresed without lipase treatment.

B apoprotein mass of the test samples was determined from the standard curve, constructed relating peak height to apoprotein B concentration of the standards (four standards were included in each run). In practice, we found that rocket height alone was a satisfactory index of electrophoretic migration, because rocket width at half height was consistent from one rocket to another.

Effect of Lipase Incubation

To demonstrate the effect of lipase treatment, samples of VLDL, intermediate-density lipoproteins, and LDL, prepared as previously described (10) were electrophoresed with and without lipase treatment. The above-described protocol was followed except for samples without lipase treatment, where the enzyme solution was replaced with the Tris buffer alone.

As shown in Figure 1, the height of the rocket was greater for the lipase-treated samples, an effect particularly marked with triglyceride-rich samples, i.e., VLDL and intermediate-density lipoproteins. Smaller differences were seen with LDL. These observations were consistent with significant lipid masking of immunoreactive B apoprotein in triglyceride-rich lipoprotein, as has been previously demonstrated (1, 2). Evidently these sites must be unmasked if estimates of B apoprotein mass are to be accurate by immunoelectrophoresis with LDL as standard. Similar observations have been found with four antisera, prepared as described with use of separate preparations of LDL for immunization of different rabbits.

Criteria for Selection of Assay Conditions

The lipase incubation procedure was designed to allow for maximum hydrolysis of lipoprotein triacylglycerol in both test samples and standards, the purpose being to produce lipoprotein particles of similar characteristics with respect to size and composition. In so doing, the exposure of immunoreactive B apoprotein in both test and standards should have been identical.

Lipase. “Lipase-TG” was selected for use in this procedure primarily because its activity does not depend on the presence

Fig. 2. Effect of enzyme concentration on rocket height and triacylglycerol hydrolysis
VLDL was incubated with increasing concentrations of lipase for 60 min at 37 °C. Triacylglycerol hydrolysis was determined by release of glycerol into the incubation medium. Glycerol was measured enzymatically (Boehringer Mannheim kit). Samples were immunoelectrophoresed using conditions described in Figure 1.

Fig. 3. Effect of duration of incubation on rocket height and triacylglycerol hydrolysis
VLDL was incubated with lipase (2 MU/L) for increasing times. Triacylglycerol hydrolysis was evaluated from glycerol released into the incubation medium.

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Table 1. Effect of Lipase Treatment on Lipoprotein Particle Characteristics

<table>
<thead>
<tr>
<th>Particle composition</th>
<th>Soluble apoprotein a</th>
<th>Particle diameter b</th>
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<tr>
<td></td>
<td>TG/β c</td>
<td>PL/CE d</td>
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<tr>
<td>VLDL (S, 60–400)</td>
<td></td>
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<tr>
<td>Untreated</td>
<td>10.6</td>
<td>8.3</td>
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<tr>
<td>Treated</td>
<td>0</td>
<td>0.1</td>
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<tr>
<td>Intermediate-density lipoproteins (S, 12–60)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>4.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Treated</td>
<td>0</td>
<td>0.1</td>
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<tr>
<td>LDL (S, 0–12)</td>
<td></td>
<td></td>
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<tr>
<td>Untreated</td>
<td>0.4</td>
<td>0.6</td>
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<tr>
<td>Treated</td>
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<td>LDL standard c</td>
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<tr>
<td>Untreated</td>
<td>0.3</td>
<td>0.7</td>
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<tr>
<td>Treated</td>
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<td>0.1</td>
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* Soluble apoproteins detected by examination of isoelectric focused gels. * Lipoprotein particle diameter was estimated from electron micrographs of osmium-fixed carbon platinum shadowed particles. At least 100 particles of each sample were measured. Grids were examined under a Philips 300 microscope at an instrument magnification of 9640. * Mass ratio (g/g) of particle triacylglycerol to protein. * Mass ratio (g/g) of particle phospholipid to cholesterol ester. Lipid analyses were performed by automated gas–liquid chromatography. Undetectable quantities of triacylglycerol remained in lipase-treated particles. * LDL standard rel. density 1.030–1.040 kg/L.

to the amount of lipoprotein triacylglycerol hydrolyzed, thereby confirming that the changes in rocket height were a consequence of lipoprotein triacylglycerol hydrolysis. The addition of albumin to the incubation mixture, to complex released free fatty acids, was found to have no influence on the rate and extent of lipoprotein triacylglycerol hydrolysis. Because albumin preparations are often contaminated with apoproteins (13), and albumin addition was not necessary for maximal activity of the lipase being used, our incubation mixture did not include any albumin addition.

Suitability of standard. We compared size and apoprotein and chemical composition of the standard and test samples before and after lipase treatment. To do so, we isolated posttreatment particles from their incubation mixtures by ultracentrifugation (at rel. dens. 1.963 kg/L). Lipid profiles were determined by automated gas chromatography as described elsewhere (14). The presence of apoproteins in treated or untreated particles was determined by examination of separations obtained on isoelectric focused gels (15). Table 1 shows the close similarity with respect to all the above-mentioned characteristics over the entire spectrum of lipase-treated particles: all samples were reduced to a similar size and composition and showed “LDL-like” properties. A much closer similarity in these respects existed between test and standard samples after, as compared with before, lipase treatment.

Validation of assay. To validate estimates of B apoprotein mass with this modified procedure, we compared concentration values obtained for the same samples by our procedure with those obtained by the 1,1,3,3-tetramethylurea isolation procedure of Kane et al. (9). As shown in Figure 4, values obtained for untreated samples reflected, but underestimated by about half, values for B apoprotein concentration obtained with the isolation procedure of Kane et al. In contrast, values obtained for lipase-incubated samples closely approximated those obtained with the isolation procedure.

Discussion

Physiologically, VLDL particles are catalyzed first to intermediate-density lipoproteins and then to LDL (10). This cascade process is essentially a delipidation process mediated by lipases (8, 16). The only component to remain in toto with the lipoprotein particle during this catalytic cascade to LDL is the B apoprotein (16). All other components leave the particle entirely (E apoprotein to HDL) (17) or in part (lipids and C apoprotein) (16), so that the particle approaches the size and composition of LDL.

Here, we have used a bacterial lipase to convert all standard and test lipoprotein particles to LDL-like particles. This ensured that the standard and test samples were immunologically identical. We used no organic solvents, detergents, or dissociating agents to unmask antigenic sites, because the B apoprotein is known to undergo conformation changes that affect its solubility on chemical delipidation. Such agents have been used in electroimmunoassay procedures for other apoproteins (18, 19).

In designing the present method, we kept in mind the specific requirements for absolute quantitation outlined by Laurell (3): “The validity of the method requires that the antigen occur with identical molecular size and charge in sample and standards.” The B apoprotein occurs in lipoprotein particles having a wide range of composition and size. Hence, there will be considerable heterogeneity in the test samples. For any routine procedure, it is not feasible to be able to
to provide standards with similar heterogeneity as the test samples. We have therefore designed a procedure that, just before assay, will convert all standard and test particles, independent of their initial size and composition, to a homogeneous and comparable form.

Numerous reports have described problems in the radioimmunoassay of B apoprotein, particularly in triglyceride-rich lipoproteins (1, 2, 20, 21), owing to the lipid masking of antigenic sites on the B apoprotein. Our observations clearly indicate that the presence of triglyceride in the particle influences the electroimmunoassay procedure for B apoprotein mass. Using the modification described, we found relatively small differences, after lipase treatment, in the estimation of B apoprotein mass in LDL, presumably because LDL particles are relatively poor in triacylglycerol. Similarly, small differences were found in normal plasma, where about 90% of the B apoprotein is in the LDL fraction. Because marked lipid masking of immunochemical sites occurs only in the triacylglycerol-rich lipoproteins, plasma (or serum) B apoprotein will be significantly underestimated only in preparations where a substantial proportion of the plasma B apoprotein resides in the triacylglycerol-rich lipoproteins. Others have reported agreement between results by gravimetric and electroimmunoassay procedures for the quantitation of B apoprotein (20, 22). Our observations (Figure 4) demonstrated a correlation between chemical and electroimmunoassay procedures. However, absolute B apoprotein mass was considerably underestimated when untreated triacylglycerol-rich particles were being assayed. Antisera, prepared as described, are a mixture of antibodies directed at various antigenic sites of the B apoprotein molecule. Thus, the effect of any unmasking procedure will depend on the concentrations of antibodies directed against "surface" antigenic sites relative to those directed against "hidden" or "buried" sites. The degree to which B apoprotein mass is underestimated when all antigenic sites are not exposed equally in the standard and test samples will vary from one antibody preparation to another. It has recently been shown that B apoprotein of hepatic and intestinal origin are not identical (23, 24). It remains unknown, however, whether or not antibodies raised against LDL B apoprotein (which is of hepatic origin) are appropriate for use in measuring B apoprotein secreted by the intestine. For this reason, our method has not been adapted for the assay of B apoprotein of chylomicrons. It is also possible that we may not be estimating accurately the relatively minor contribution of B apoprotein in the circulation in intestinally-produced VLDL particles. The present method eliminates errors introduced by lipid masking of antigenic sites, independent of the antibody preparation being used, by making the standard and test samples comparable, by treatment with a lipase, before electroimmunoassay.

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References