Radioimmunoassay of Apolipoprotein B with Use of Monoclonal Antibodies

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We describe a double-antibody radioimmunoassay for apolipoprotein B with use of monoclonal antibodies. Values for 32 patients with various lipid disorders, obtained with use of one of the monoclonal antibodies (464B1B6), were similar to those obtained with use of a polyclonal antisera. When we used another monoclonal antibody (465C3D1) the values were significantly lower. Monoclonal antibody 464B1B6 recognized apolipoprotein B determinants in the very-low-density-lipoprotein and low-density-lipoprotein fractions of plasma in a similar fashion as in unfractioned plasma, but monoclonal 465C3D1 gave lower values with the fractionated plasma. For precipitation of the antibody–apolipoprotein complex to be complete, use of polyethylene glycol was necessary with one of the monoclonal antibodies but not with the other. The inter assay CV was 12.3% and 11.6% for the assays with the monoclonal antibodies. Analytical recoveries ranged from 85 to 94%. Owing to variations in the B50 from one preparation of standard to another, we found it necessary to normalize the results to those of a serum pool. We conclude that certain monoclonal antibodies can be used effectively in radioimmunoassays for apolipoprotein B in plasma and may help in the standardization of these assays.

A correlation between increased LDL-cholesterol and increased risk of atherosclerotic heart disease has been well established (1–3). More recently, some investigators have found that the concentration of apolipoprotein B, the major protein component of LDL, may have better predictive value than the more widely measured LDL-cholesterol concentration (4–8). Many techniques have been used to measure apolipoprotein B (9 and its references): radioimmunoassay, enzyme immunoassay, electroimmunoassay, rocket electrophoresis, radioimmunodiffusion, and immunonephelometry. These methods have differing but adequate sensitivity and precision and appear to be specific for measuring apolipoprotein B. One of the difficulties in applying these techniques has been that they all depend on polyclonal antisera, which may not be reproducible from one laboratory to another and may recognize different epitopes of the apolipoprotein B molecule.

The production of monoclonal antibodies to apolipoprotein B has provided precise probes for defining and measuring epitopes of apolipoprotein B. Monoclonal antibodies developed in our laboratories (10–13) and in others (14–21) have been used to study isolated LDL and apolipoprotein B molecules, with emphasis on elucidating the structure of the molecule. We were interested in assessing the use of monoclonal antibodies in radioimmunoassays to measure apolipoprotein B in the plasma of hyperlipidemic patients. A double-antibody RIA involving polyclonal antisera against LDL has been in use in one of our laboratories (G.S.) for many years (22). A comparison of the apolipoprotein B values obtained with monoclonal antibodies with those from this polyclonal-antiserum assay demonstrated that certain monoclonal antibodies may indeed be useful for measuring total apolipoprotein B, but that other monoclonal antibodies may measure only part of that species in plasma.

During our preparation of this manuscript, Patton et al. (23) published results of a RIA in which a monoclonal antibody was used to measure apolipoprotein B in patients with coronary heart disease.

Materials and Methods

Reagents

Production and affinity purification of two anti-LDL monoclonal antibodies (464B1B6 and 465C3D1) have been described previously (10). A rabbit anti-LDL polyclonal antibody (R198-2) and purified LDL (d 1.025–1.050) were prepared as previously described (22) and stored at 4 °C. The apolipoprotein B content of the purified LDL preparations was evaluated by measuring the protein by the classical method of Lowry et al. A serum pool was prepared from excess serum submitted for routine chemical analysis at Barnes Hospital and stored in aliquots at −70 °C. A commercial product (Omega™ Lipid Fraction; Hyland Diagnostics, Deerfield, IL 60015), used as a quality-control material, was stored lyophilized at 4 °C. Plasma samples from patients seen at the Lipid Research Clinic were collected in 10-mL Vacutainer Tubes containing tri-potassium EDTA and centrifuged at room temperature. The plasma was stored at 4 °C and assayed within a month.

Na125I was purchased from Amersham, Arlington Heights, IL 60005; Iodo-gen™ from Pierce Chemical Co., Rockford, IL 61105; bovine serum albumin (Cohn Fraction V) from U.S. Biochemicals, Cleveland, OH 44122; and non-immune sera and second antibodies from Pel-Freeze, Rogers, AR 72756. All other chemicals, including polyethylene glycol (M, 8000), were from Sigma Chemical Co., St. Louis, MO 63178.

Iodination of LDL

Purified LDL was iodinated by a modification of the method involving 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (iodo-gen) (24). We mixed 15–25 μg of LDL in 200 μL of 0.25 mol/L Tris HCl, pH 7.6, at room temperature with 0.5 mCi of Na125I and transferred the mixture to a polypropylene tube precoated with 100 μg of Iodo-gen. The tubes were each coated with 100 μL of 1 g/L Iodo-gen solution in chloroform, the chloroform then being evaporated under a stream of nitrogen. The reaction was allowed to proceed at
room temperature for 30 min and the iodinated LDL was then desalted on a Sephadex G-50 column and eluted with sodium barbital (0.1 mol/L, pH 8.3), containing 1 mmol of disodium EDTA per liter. The pooled void-volume fractions, identified with a Geiger counter, were stored at 4 °C for as long as a month after addition of bovine serum albumin to give a final concentration of 10 g/L. Before use in the assay, aliquots of 125I-labeled LDL were repurified by chromatography on Sephadex G-200 with the albumin-barbital buffer, to remove any remaining free iodine or LDL breakdown products, which can form during storage. The specific activity ranged from 1.3 to 3.7 Ci/g of LDL if complete recovery from the columns is assumed. The radioactivity was 92% precipitable with trichloroacetic acid, and >90% precipitable by excess polyclonal or monoclonal anti-LDL antibody.

Radioimmunoassay

A double-antibody radioimmunoassay was developed based on competition between 125I-labeled LDL and unlabeled LDL (standard or sample) for a monoclonal or polyclonal antibody. The selected dilution of the primary antibody was based on the amount of antibody necessary to precipitate the maximum radioactivity (i.e., number of counts). Initially we used a dilution of primary antibody that precipitated 50 to 60% of the radioactivity. This assay was more sensitive but less precise than the final assay. Because sensitivity was not a problem, we selected the higher amount of primary antibody. The concentration of the second antibody was then re-titrated, to ensure maximum precipitation. The 125I-labeled LDL was more than 90% precipitated with polyclonal antiserum R198-2 and monoclonal antibody 464B1B6, but only 20–25% of the counts were precipitated with monoclonal antibody 465C3D1. These results were the same whether or not non-immune mouse serum (0.3 mL/L) was present. We then assessed the influence of polyethylene glycol on the precipitability of labeled LDL in the presence and absence of antibody 465C3D1. We selected a concentration of polyethylene glycol that caused precipitation of >90% of the labeled LDL with <2% increase in nonspecific binding. The degree of dilution of the samples was such that the average sample would displace 50% of the available counts (B0). The minimum time required for the primary antibody reaction to reach equilibrium was 16 h at 4 °C or 2 h at 37 °C. For the second-antibody reaction, equilibrium was reached after 16 h at 4 °C or 2 h at 37 °C. The time and temperature for the final assay conditions were chosen to ensure equilibrium and to provide technical flexibility. Under the final assay conditions, dilutions of the serum pool produced displacement curves paralleling those of the LDL standard for all three antibodies. Dilutions of plasma samples also paralleled the LDL standard curves, but we did not run complete displacement curves on every sample.

Table 1 details final assay conditions for the two monoclonal antibodies and one polyclonal antiserum. The assays were performed in polystyrene tubes, and the assay buffer was sodium barbital (0.1 mol/L, pH 8.3) containing 10 g of bovine serum albumin and 1 mmol of Na2EDTA per liter. The assays were performed in duplicate as follows:

1. 125I-labeled LDL, 36 000–40 000 counts/min, in 100 μL of assay buffer was added to each tube.
2a. 50 μL of assay buffer containing no standard or sample was added to establish the maximum amount of counts bound (B0).
2b. 50 μL of assay buffer containing no standard or sample was added to establish the nonspecific binding (NSB).
2c. 50 μL of appropriate dilutions of the LDL standard with assay buffer was added to the standard tubes.

### Table 1. RIA Conditions for Determination of Apolipoprotein B with Monoclonal and Polyclonal Antibodies

<table>
<thead>
<tr>
<th>LDL std and range</th>
<th>Serum pool and sample dilution (fold)</th>
<th>Quality-control dilution (fold)</th>
<th>Secondary incubation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>464B1B6</td>
<td>50–2000 ng</td>
<td>1000</td>
<td>250</td>
</tr>
<tr>
<td>Monoclonal</td>
<td></td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>465C3D1</td>
<td></td>
<td>3000</td>
<td>1000</td>
</tr>
<tr>
<td>Polyclonal</td>
<td></td>
<td>25–1000 ng</td>
<td>1000</td>
</tr>
</tbody>
</table>

*4 °C overnight; **37 °C, 2 h.

2d. A dilution of sample or controls appropriate for each antibody was made with assay buffer, and 50 μL of the diluted sample or control was added. The appropriate dilutions resulted in binding equal to 40–70% of B0.

3. 100 μL of the appropriate dilutions of the primary antibodies in assay buffer was added to each tube except that used to assess NSB. The affinity-purified monoclonal antibodies were diluted 200-fold, resulting in the addition of 1.65 μg of 464B1B6 and 0.55 μg of 465C3D1. The polyclonal antiserum was diluted 10 000-fold.

4. All tubes were incubated overnight at 4 °C.

5. 50 μL of a 10-fold dilution of the second antibody in assay buffer was added. For the assay with use of monoclonal antibody 465C3D1, we also added 50 μL of a 300 g/L solution of polyethylene glycol in assay buffer.

6. The tubes were incubated for 2 h at 37 °C. We then added 1 mL of assay buffer to all tubes and pelleted the precipitated LDL–antibody complex by centrifugation (3000 × g, 25 min). The pellet was washed with 1 mL of assay buffer and the pellet recenterfuged and its radioactivity counted. Data reduction included subtraction of NSB counts from the counts of each tube, averaging of duplicates, and calculation of B/B0 for each dose of standard, control or sample. Typical standard curves are shown in Figure 1. The concentrations of apolipoprotein B in control and sample were determined from a logit transformation of the standard curve (done with software available in the gamma counter).

We occasionally saw considerable variation in values for the serum pool and the Omega quality-control material.

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**Fig. 1. Standard curves for radioimmunoassay of apolipoprotein B (Apo B) with use of polyclonal antibody (R198-2) ▲, or two monoclonal antibodies, 464B1B6 ● and 465C3D1 ■.**
from one assay to another. These fluctuations paralleled fluctuations in the BLD for the assays and so we attributed them to differences in the various LDL preparations, each of which was prepared from a single donor. We therefore normalized all results to those obtained on our pooled human serum. This was done by arbitrarily setting the value of the pooled human serum to 100 normalized units (N.U.) per deciliter. Final results were then calculated by the following formula:

\[ \text{LDL value of unknown} \times (100/\text{LDL value of serum pool}) = \text{N.U./dL} \]

The average value for the serum pool used for normalization was determined in the polyclonal assay to be 173.4 mg/dL, a much higher value than generally reported by others and by one of us previously (22). We believe this is ascribable to the particular LDL preparation from different individuals that we used to prepare the standard curves during this study. A second serum pool prepared and assayed more recently with different LDL standard preparations had an Apo B value of 116 mg/dL when assayed with 464B1B6 and 120 mg/dL when assayed with a polyclonal antibody with use of the same LDL standards. When analyzed in the same assay (antibody 464B1B6), the two serum pools give indistinguishable Apo B values. The commercial quality-control material, Omega Lipid Fraction, was specified by the manufacturer to have a mean Apo B value of 64 mg/dL. The values we obtained after normalization to the initial serum pool were 69.9–71.0 N.U./dL with the three antibodies. More recently we assayed the same quality-control material with 464B1B6 and normalized with the second serum pool, obtaining a value of 74.3 (SD 4.6) N.U./dL, not statistically different from the values with the initial serum pool.

In addition to whole plasma, we fractionated several plasma samples by differential density centrifugation into a VLDL and an LDL + HDL fraction. The apolipoprotein B values for the fractions were determined and compared with the values for whole plasma.

Cholesterol and triglyceride were determined by the standardized methods used in the Lipid Research Clinic protocols.

Analytical-recovery experiments were performed by supplementing the serum pool with known amounts of purified LDL and measuring the difference in measured apolipoprotein B concentration in supplemented and unsupplemented serum. Percentage recovery was calculated as (amount recovered/amount added) × 100. Precision was assessed by measuring the control sera or patients' samples repeatedly in the same assay or in multiple assays done during several weeks. The stability of patient samples was assessed by measuring the sample on the day it was drawn and then periodically re-assaying aliquots stored at 4 or −70 °C for up to 60 days. The change in values for samples assayed in 10-day intervals was assessed by the Wilcoxin signed rank test.

**Results**

**Precision, Recovery, and Stability**

Precision data for the assay are shown in Table 2. The interassay precision (CV) for the various assays as assessed by use of the Omega control was 15–20%; for patients' samples it was 12–21%. The assays in which monoclonal antibodies were used were substantially more precise than when polyclonal antisera were used.

Analytical recovery in assays involving monoclonal antibodies was 85–94%, which compares well with the 89.8% recovery for polyclonal antisera.

The stability of 39 plasma samples was assessed by periodically assessing patients' samples that had been stored at 4 or −70 °C for various intervals up to 60 days. The values obtained with antibodies R198-2 or 465C3D1 showed no statistically significant deterioration with either storage temperature. The samples analyzed with antibody 464B1B6 gave lower results after 30 days at −70 °C but were stable if kept at 4 °C. For this reason we routinely store the plasma samples at 4 °C and analyze them within 30 days.

**Apolipoprotein B Values for Patients**

Plasma samples were obtained from 32 patients with various lipid disorders (12 with Type IIa, 11 with Type IIb, three with Type III, five with Type IV, and one with Type V). In Figure 2 we compare apolipoprotein B values obtained with the three antibodies. The line equations were determined by use of the linear function slope-estimation technique, an alternative to the least-squares regression technique that does not require the assumption that one of the methods is error free (25). The values obtained with the monoclonal antibodies correlated well with each other and with the polyclonal antisera. However, using the Wilcoxin signed rank test, we found that the values of samples obtained with the monoclonal antibodies differed significantly from each other and from the polyclonal assay values. This difference was not observed for the quality-control material (Table 2). The apolipoprotein B values obtained...

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**Table 2. Precision Data for Apo B Values (N.U.) Determined for Control, Plasma Samples, or Serum Pool**

<table>
<thead>
<tr>
<th>Lyophilized quality-control material</th>
<th>Plasma samples</th>
<th>Serum pool, within-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo B value (N.U.)</td>
<td>Within-assay CV, %</td>
<td>Between-assay CV, %</td>
</tr>
<tr>
<td>Monoclonal 464B1B6</td>
<td>69.9</td>
<td>8.9</td>
</tr>
<tr>
<td>71.0</td>
<td>6.9</td>
<td>6.6</td>
</tr>
<tr>
<td>n = 21</td>
<td>n = 12</td>
<td>4.6</td>
</tr>
<tr>
<td>Monoclonal 465C3D1</td>
<td>69.9</td>
<td>8.9</td>
</tr>
<tr>
<td>71.0</td>
<td>6.9</td>
<td>8.8</td>
</tr>
<tr>
<td>n = 21</td>
<td>n = 12</td>
<td>4.6</td>
</tr>
<tr>
<td>Polyclonal R-1B9</td>
<td>69.9</td>
<td>8.9</td>
</tr>
<tr>
<td>71.0</td>
<td>6.9</td>
<td>9.5</td>
</tr>
<tr>
<td>n = 20</td>
<td>n = 20</td>
<td>7.1</td>
</tr>
</tbody>
</table>

*Av from six assays (n = 3–6). †Av of assays of 32 plasma samples (n = 3–6). ‡Av from 10 assays (n = 6–10).
with any of the antibodies correlated significantly with total cholesterol values ranging from 116 to 438 mg/dL \((r = 0.83,\) monoclonal antibody \(464B1B6; r = 0.79,\) monoclonal antibody \(465C3D1;\) and \(r = 0.78,\) antibody R198-2) but not with the total triglyceride values (range of values 48 to 423 mg/dL).

**Apolipoprotein B in Fractionated Plasma**

Plasma from each of eight patients with various lipoprotein disorders was separated by ultracentrifugation into a VLDL fraction \((d < 1.006)\) and a LDL + HDL fraction \((d > 1.006)\). Each of the fractions was analyzed with all three antibodies and compared with the value obtained for whole plasma. Adding the values for the two lipoprotein fractions and dividing by the value of the whole plasma gave median values of 100.7\%, 97.1\%, and 82.5\% for antibodies R198-2, 464B1B6, and 465C3D1, respectively, suggesting that the presence of the lipid-rich VLDL fraction alters the immune reactivity with antibody 465C3D1 but not 464B1B6. Others have also reported monoclonal antibodies with different reactivity to isolated LDL and LDL in plasma \((19, 22)\).

**Discussion**

We have developed reliable double-antibody RIAs for apolipoprotein B in which monoclonal antibodies are used. With one of the antibodies, use of polyethylene glycol was necessary to ensure complete precipitation; with the other, polyethylene glycol was not necessary. Because the monoclonal antibodies bind at one epitope, the antigen–antibody complex is smaller than that obtained with polyclonal antisera unless the epitope is repeated in the molecule. Therefore it is not surprising that polyethylene glycol may be necessary for complete precipitation. Our experience suggests that polyethylene glycol should be tried whenever a low percentage of counts are found in double-antibody RIA with use of monoclonal antibodies. We found that non-immune serum was not needed in our assays.

The monoclonal antibodies we utilized in our assay were from seven that have been developed to five different epitopes of LDL \((10–13)\). The two antibodies chosen have both shown reactivity to the B-100 and B-74 forms \((12)\) of apolipoprotein B found in LDL. In addition, antibody 464B1B6 has reactivity to the B-26 form found in LDL and to the B-48 form found in the VLDL fraction, but not to the chylomicron fraction. This antibody also inhibits binding of LDL to fibroblasts. The binding of both antibodies correlates only weakly, if at all, with the phosopholipid content of the LDL. Because of these reactivities it is attractive to attribute the difference in values between antibodies 465C3D1 and 464B1B6 to the amount of B-26 and B-48 lipoprotein present in the samples, but this has not been established. Alternative explanations include conformational differences of the epitopes in plasma. Both antibodies bind less avidly if the lipid of LDL is extracted and 465C3D1 is more reactive to chemically modified LDL. In addition, antibody 464B1B6 reacts more avidly with apolipoprotein B from LDL than VLDL \((11)\). Both antibodies produced parallel displacement to LDL isolated from 17 individuals with serum cholesterol values ranging from 121 to 454 mg/dL, but the epitopes expressed relative to the standard preparation were more variable with antibody 465C3D1 \((12)\). Interestingly, a lyophilized quality-control material did not show any difference in apolipoprotein B values by the three antibodies \((Table 2)\). This is probably due to the loss of some epitopes during the preparation of the control material and reemphasizes the need for work with fresh human samples when developing immunoassays.

The specificity of these antibodies to various lipoprotein
fractions has been described elsewhere (10–13). Their specificity in plasma appears good, as judged by the correlation of results with a polyclonal antiserum and total cholesterol and lack of correlation with triglyceride.

A major problem with all of the assays was their reproducibility. This variability correlated with changes in the Bapo, suggesting that variations in the LDL standard were responsible. This assumption is further supported by the assay of a second serum pool in two different laboratories by different antibodies (564BI6 and a polyclonal) but the same standard LDL preparation. The values obtained with the different antibodies were 116 and 120 mg/dL, respectively, which is lower than reported for the serum pool used in this work. However, when the serum pools were analyzed in the same assay, the Apo B values were indistinguishable.

We have used a narrow density band of LDL (d 1.025–1.050 kg/L), which is freshly prepared every four weeks as standard and tracer. A mild iodination scheme was used and the tracer repurified before each assay. Nevertheless, we, like others working with apolipoproteins (9, 26, 27), have had to utilize a secondary standard to obtain reproducible results. This problem appears related to the unique biochemical properties of apolipoproteins rather than to the monoclonal antibodies.

In conclusion, we have shown that monoclonal antibodies can be used for the measurement of apolipoprotein B in plasma and that differences in the epitopes measured can lead to different values for the antigen. We are now performing studies to establish the clinical utility of measuring apolipoprotein B with monoclonal antibodies.

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References