Monoclonal Antibodies to Human Plasma Low-Density Lipoproteins. II.
Evaluation for Use in Radioimmunoassay for Apolipoprotein B in Patients
with Coronary Artery Disease

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We describe the importance of the low-density lipoproteins (LDL) used in preparing radioimmunoassay standard curves and the clinical application of monoclonal antibodies to LDL. LDL isolated from five normal men produced five parallel displacement curves with conventional mouse antiserum but there was a significant difference of immunoreactivity among the LDL. None of our four monoclonal antibodies could eliminate the heterogeneous immunoreactivity of different LDL. Thus, the determination of plasma apolipoprotein (apo) B will vary depending on the selection of LDL standards, and the comparison of absolute apo B values between laboratories will be of questionable value unless they use the same LDL standard. Nonetheless, in a radioimmunoassay our monoclonal antibody, LP-22, detected a more significant \((p < 0.0001)\) increase of plasma apo B in patients with angiographically documented coronary artery disease than did conventional antiserum \((p < 0.001)\). In addition, the overlap of apo B concentrations for patients with and without disease was less when monoclonal antibody LP-22 was used. We conclude that patients with coronary artery disease have a significant increase in the form of plasma apo B that is specifically recognized by LP-22 monoclonal antibody. Perhaps monoclonal antibodies will be able to sort out the various components of apo B, delineate their possible atherogenic roles, and offer us a predictive value for diagnosing such patients.

Additional Keyphrases: biochemical individuality · cholesterol

Lipoprotein particles defined as low-density lipoproteins (LDL; \(d 1.019-1.063\)) differ in size, hydrated density, chemical composition, metabolic rate, and physicochemical properties (1-10). Apolipoprotein (apo) B, which constitutes 95% of the LDL mass (3), is also heterogeneous in circulating plasma (4-10). We have developed several monoclonal antibodies against human LDL and support the view that apo B is immunochemically heterogeneous (11), as shown by the unique binding of \(^{125}\text{I}-\text{LDL} \left(125\text{-LDL}\right)\) to each monoclonal antibody, maximal binding ranging from 20 to 95% of total \(^{125}\text{I}-\text{LDL}\) (12). Unlike conventional serum antibodies, the binding between LDL and monoclonal antibodies is thermal dependent, with optimal binding at 4 °C (13).

High concentrations of apo B-containing lipoproteins—chylomicrons, very-low-density lipoproteins (VLDL), and LDL—and low concentrations of high-density lipoproteins (HDL) are associated with the development of atherosclerosis (14-16). Because patients with coronary artery disease (CAD) are known to have increased concentrations of plasma apo B (14, 16, 17), a sensitive technique to quantify apo B in plasma remains a high priority. Immunoassays have been widely used for this purpose (16-22), but the reported normal ranges of apo B in plasma vary from laboratory to laboratory. In the present study we have sought to define the immunochemical properties of LDL used in standard solutions and investigate the usefulness of monoclonal antibodies to LDL in radioimmunoassays. We have compared assays involving monoclonal and conventional antibodies to assess concentrations of apo B in patients with and without CAD.

Materials and Methods

Production and characterization of monoclonal antibodies, isolation and iodination of LDL, preparation of conventional serum antibodies, and radioimmunoassays are identical to those described in the preceding paper (12).

To study standard displacement curves, we isolated LDL from five healthy men whose plasma triglycerides, cholesterol, HDL cholesterol, and phospholipids are normal for their age and sex and are described in the preceding paper (12).

Patients with CAD. Age-matched male patients \((n = 131)\) undergoing diagnostic coronary angiography for chest pain or suspected CAD were evaluated at the Mayo Clinic. Coronary angiography was performed by Judkin’s or Sone’s technique, and multiple views of the right and left coronary arteries were recorded (23) over a 10-month period. Patients with obstruction of less than 30% in all segments were defined as normal or insignificant CAD. Upon collection, we diluted without delay fresh plasma samples of these patients 100-fold in a standard radioimmunoassay buffer containing per liter, 0.1 mol of sodium borate, 0.01 mol of Tris·HCl, 1 mmol of Na₂EDTA, and 0.1 g of NaN₃ (pH 8.5), and stored them at 4 °C until HDL were determined enzymically with a cholesterol kit (cholesterol ES-reagent; Beckman Instruments, Inc., Carlsbad, CA 92008) (24), or were isolated for cholesterol analysis by polyethylene glycol 6000 precipitation (25). To validate the latter method, we determined HDL cholesterol by the MnCl₂-heparin precipitation method (24) in 25 plasma samples selected without conscious bias from the total samples. The HDL-cholesterol values were virtually identical by either method.

Radioimmunoassay. The intra-assay variation (CV) for apo B determination was less than 4%; the interassay CV was less than 9.5%. We saw no significant change (less than 10%) in apo B determinations for plasma stored (at 4 °C) as long as 12 months.
Results

Analytical Considerations

Stability of \(^{125}\)I-LDL during storage. To study the immunoreactive stability of \(^{125}\)I-LDL, we stored it at 4 °C. Antibodies were diluted to bind 40–70% of the total labeled LDL added at 4 °C, except the L1C6F2 monoclonal antibody, which bound no more than 30% of the total LDL (13). As Figure 1 shows, there was no significant loss of immunoreactivity of labeled LDL for up to seven weeks, although there were slight losses when conventional and LP-47 monoclonal antibodies were used to assess stability. Nevertheless, the labeled LDL used in the studies reported in this paper were stored for no more than three weeks.

Radioimmunoassay standard curves based on isolated LDL from five individuals. The specificity of mouse serum antibodies against LDL has been described previously (12, 26). They do not cross react with HDL, apoA-I, apoA-II, albumin, or the nonlipoprotein fraction of plasma (d > 1.21). The sensitive range of the radioimmunoassay for LDL was between 80 and 1000 ng of LDL added (Figure 2A). LDL isolated from five normal individuals' plasma resulted in five parallel displacement curves when conventional mouse antiserum (diluted 4000-fold) was used, with a significant difference in immunoreactivity among each isolated LDL. Such differences were not due to the error of the procedure of Lowry et al. (27) for protein determination, because the protein concentration of isolated LDL agreed closely with that determined by amino acid analysis (data not shown), indicating that the immunoreactivity of isolated LDL from each individual is not identical. In the preceding paper (12), we showed that each individual's binding pattern of \(^{125}\)I-LDL to conventional and (or) monoclonal antibodies was similar; we now show that standard displacement curves with their individual LDL are not identical. Thus, a question is raised concerning the most appropriate LDL standard to use for plasma apo B radioimmunoassays: the concentrations of apo B detected will vary depending on the immunoreactivity of the isolated LDL.

Radioimmunoassay standard curves based on monoclonal antibodies to LDL. Conventional serum antibodies to LDL are heterogeneous in terms of binding affinities to a given LDL antigen, owing to multiple antigenic sites (13). We have used monoclonal antibodies to attempt to eliminate the different immunoreactivities of LDL between each individual as described above. Our monoclonal antibodies, however, did not eliminate heterogeneous immunoreactivities among different LDLs, as shown by typical standard curves prepared with monoclonal antibody LP-22 (Figure 2B).

Evaluation of monoclonal antibodies to LDL for determination of plasma apo B. Standard displacement curves for measuring the reactivity of conventional and monoclonal antibodies to plasma and apo B-containing lipoproteins (LDL and VLDL) are shown in Figure 3. Typical parallelism between plasma, VLDL, and LDL (from subject 2) was achieved except with monoclonal antibodies LP-47 (Figure 3) and L1C6F2 (not shown), indicating that neither of these monoclonal antibodies will be suitable for radioimmunoassay. In addition, monoclonal antibody LP-47 reacted poorly with plasma, indicating that the antigenic structure of isolated LDL differs from that of native LDL in plasma. Thus, actual concentrations of apo B in plasma will not be ascertained simply by using immunoassays developed from monoclonal antibodies against LDL.

Analytical recovery. To further evaluate the feasibility of using any of our monoclonal antibodies in radioimmunoassays, we added known amounts of isolated plasma LDL standards (28–112 ng) to aliquots of normal plasma from which the LDL concentration had previously been determined and then assayed for LDL. The LDL added and the LDL used for standards in this study were isolated from the same individual to avoid differences in immunoreactivities of apo B. As shown in Table 1, with conventional mouse antiserum analytical recovery of added LDL was 95–108%.
Fig. 3. Standard displacement curves of $^{125}$I-LDL by LDL (•), VLDL (○), and plasma (△).

Monoclonal antibodies LP-22 (A), LP-34 (B), and LP-47 (C), and mouse antiserum (D) were diluted 640-, 80-, 640-, and 8000-fold, respectively. Reactions were allowed to equilibrate for 16-24 h at 4°C. Total volume in each assay was 300 μL.

Table 1. Analytical Recovery of Isolated LDL in Plasma by Radioimmunoassay

<table>
<thead>
<tr>
<th>LDL added, ng</th>
<th>Conventional</th>
<th>LP-22</th>
<th>LP-34</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh</td>
<td>Freeze-thawed</td>
<td>Fresh</td>
</tr>
<tr>
<td>28</td>
<td>27.4 ± 1.8 (98)</td>
<td>28.7 ± 7.8 (103)</td>
<td>29.9 ± 7.4 (107)</td>
</tr>
<tr>
<td>56</td>
<td>54.7 ± 8.1 (98)</td>
<td>55.8 ± 6.9 (100)</td>
<td>50.2 ± 1.4 (90)</td>
</tr>
<tr>
<td>112</td>
<td>116.5 ± 4.8 (104)</td>
<td>120.5 ± 7.0 (108)</td>
<td>109.6 ± 11.0 (98)</td>
</tr>
</tbody>
</table>

*Assays were run on fresh plasma, which was then stored at -20°C and thawed at 24°C before re-assay. Each value represents the mean ± SD of four determinations.

and was unaffected by subsequent freezing (-20°C for one week) and thawing (23°C) of plasma; the same was true with monoclonal antibody LP-22. Use of LP-34 gave good recovery at low doses but overestimation of added LDL at higher doses. Of these antisera, apparently only LP-22 is appropriate for setting up a radioimmunoassay.

We also performed the same recovery study with isolated VLDL, which may be increased in patients with hypertriglyceridemia. With both conventional and LP-22 antibodies, analytical recovery of added VLDL (103-2060 ng) ranged from 88 to 100% (data not shown).

Apo B Concentrations in Normal Subjects and in Patients with Hypertriglyceridemia

We used both our conventional and monoclonal (LP-22) antibodies to measure apo B in normal (n = 10) and hypertriglyceridemic subjects (n = 10). Previous studies have shown increased concentrations of apo B in these patients (18, 20). The LDL standard for this study was from subject 2 (Figure 2). Both conventional and monoclonal antibodies detected significant increases in concentrations of apo B in plasma of these patients (Table 2), but the monoclonal antibodies detected more apo B than did conventional antibody.

Comparison between Conventional and Monoclonal Antibodies in Radioimmunoassay of Patients with CAD

In Table 3 we show the mean (± SD) immunoreactivity of plasma apo B in 41 angiographically documented normal subjects. The apo B concentrations determined by the monoclonal antibody LP-22 method (y) were slightly, but not significantly (p > 0.3), greater than the concentrations determined with the conventional antibody method (x). The
Table 2. Apo B Concentrations (mean ± SD) in 10 Normolipemic and 10 Hypertriglyceridemic Subjects

<table>
<thead>
<tr>
<th>Conc, g/L</th>
<th>Normal lipemia</th>
<th>Hypertriglyceridemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides</td>
<td>1.25 ± 0.28</td>
<td>4.11 ± 0.81</td>
</tr>
<tr>
<td>(0.77–2.15)</td>
<td>(3.18–5.51)</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.84 ± 0.30</td>
<td>2.38 ± 0.67</td>
</tr>
<tr>
<td>Apo B, conventional</td>
<td>1.00 ± 0.27</td>
<td>1.54 ± 0.59</td>
</tr>
<tr>
<td>Apo B, LP-22</td>
<td>1.04 ± 0.24</td>
<td>1.95 ± 0.44</td>
</tr>
</tbody>
</table>

*Mean age 54 (SD 13) years; *Mean age 55 (SD 9) years. * p < 0.001.

Table 3. Plasma Lipid and Apo B Composition (mean ± SD) of Subjects Undergoing Coronary Angiography

<table>
<thead>
<tr>
<th>Conc, g/L</th>
<th>No CAD*</th>
<th>Normal HDL*</th>
<th>Low HDL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL-cholesterol</td>
<td>0.37 ± 0.09</td>
<td>0.42 ± 0.05</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.89 ± 0.32</td>
<td>2.22 ± 0.35</td>
<td>2.08 ± 0.49</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.51 ± 0.68</td>
<td>1.35 ± 0.65</td>
<td>2.44 ± 1.01</td>
</tr>
<tr>
<td>Apo B, conventional</td>
<td>1.044 ± 0.18</td>
<td>1.261 ± 0.32</td>
<td>1.268 ± 0.38</td>
</tr>
<tr>
<td>Apo B, monoclonal</td>
<td>1.081 ± 0.20</td>
<td>1.672 ± 0.41</td>
<td>1.877 ± 0.30</td>
</tr>
</tbody>
</table>

* Mean age 52 (SD 12) years; n = 41. * Mean age 59 (SD 8) years; n = 42. * Mean age 57 (SD 8) years; n = 48. * p < 0.001 compared with NO CAD. * p < 0.0001 compared with NO CAD. * p < 0.001 compared with conventional method. * p < 0.01 compared with CAD subjects with normal HDL-cholesterol.

Two methods correlated significantly (r = 0.91, p < 0.001): y = 0.21 + 0.87x.

Previous studies (14, 16, 17) have also shown increased concentrations of plasma apo B in patients with CAD. Thus, we decided to evaluate our assays on two groups of patients with documented coronary angiography: one (n = 42) with normal concentrations of HDL cholesterol (0.42 ± 0.05 g/L) and the other (n = 48) with significantly lower concentrations of HDL cholesterol (0.18 ± 0.04 g/L). We observed a striking difference between the groups when we used our two antibodies to measure apo B concentrations (Table 3). With the monoclonal antibodies, both groups of CAD patients showed significant increases of apo B. As compared with normals, the increase was more obvious in CAD patients with low concentrations of HDL cholesterol (1.877 vs 1.081 g/L, p < 0.0001) than in patients with normal HDL cholesterol (1.672 vs 1.081 g/L, p < 0.0001). When using conventional antibody, however (Table 3), although both CAD groups again showed significant increases of apo B (p < 0.001), these changes were not distinguishable between the two groups (p > 0.3). Thus, monoclonal antibody LP-22 appears to be a better reagent in discriminating concentrations of apo B in patients with CAD than was the conventional antibody used in this study. Again, the LP-22 monoclonal antibody method (y) and the conventional antibody method (x) correlated in CAD patients with low concentrations of HDL cholesterol (r = 0.80; p < 0.001; y = 1.069 + 0.637x) and but less so in CAD patients with normal values for HDL cholesterol (r = 0.60; p < 0.001; y = 0.480 + 0.462x).

Using conventional antiserum, we also found significant correlations between plasma cholesterol and apo B in all subjects (r = 0.758; p < 0.001), those with CAD (r = 0.738; p < 0.001), and those without CAD (r = 0.761; p < 0.001). Using monoclonal antibody LP-22, we found significant but lower correlations between plasma cholesterol and apo B in all subjects (r = 0.540; p < 0.001), those with CAD (r = 0.567; p < 0.001), and those without CAD (r = 0.572; p = 0.003). There were no significant correlations between apo B and plasma triglycerides in either patient group, with either antibody.

The advantage of using monoclonal antibody LP-22 is illustrated graphically in Figure 4. The overlap between subjects with and without CAD is pronounced with conventional antibodies, while there is better differentiation with the monoclonal antibody.

**Discussion**

The finding that five individuals had different standard displacement curves is of obvious importance to laboratories performing apo B assays. It suggests that each individual's LDL are immunochemically different, so that comparison of absolute values between laboratories will be of questionable value unless the same LDL standard is used. Within the same laboratory, assay data should be more consistent if the same LDL source is used for all standards. These discrepancies could not be eliminated simply by using monoclonal antibodies (Figure 2), which is further evidence that different apo B values established in laboratories could be related to the choice of LDL used.

The different apo B immunoreactivities for each LDL may...
be explained by the chemical and structural heterogeneity of apo B components in isolated LDL (4–10). Most likely, use of a more homogeneous LDL preparation as a standard for radioimmunoassays will be beneficial.

That monoclonal antibody GP-47 reacted with isolated LDL but not with LDL in plasma indicates that the isolation procedure may alter the structure of LDL used for immunization. If the GP-47 monoclonal antibody is used in an assay, there will be apparent underestimation of the apo B concentrations in plasma. This poor interaction with LDL in plasma is not related to the low binding affinity of the antibody to $^{125}$I-LDL, as shown in the preceding paper (12).

Recent studies support the view that apo B (14, 16, 17), in addition to HDL cholesterol (16, 28), is a potential discriminator for patients with and without CAD. Increased concentrations of apo B in these patients has been established (14, 16, 17), although some patients still apparently have apo B concentrations in the normal range. For this reason, we have tried to determine if our monoclonal antibodies can act as a sensitive reagent in discriminating concentrations of apo B in patients with and without disease. Because some of our CAD patients had apo B values within the normal range, as determined with monoclonal antibody GP-22, we cannot predict the presence of CAD solely by measuring apo B. Nonetheless, our data do suggest that use of monoclonal antibody GP-22 (Figure 4B) discriminates disease from nondisease better than conventional antibodies (Figure 4A); eventually, monoclonal antibodies may provide the needed discriminatory sensitivity.

No doubt, conventional serum antibodies from other laboratories may have a sensitivity equal to that of our monoclonal antibodies, but the specificity of antibodies toward the antigenic site of apo B is also important. In the preceding paper (12), we showed that the GP-22 monoclonal antibody bound ~60% of $^{125}$I-LDL in contrast to the L$_2$C$_3$F$_3$ monoclonal antibody, which binds ~25% (13). If this 60% binding was not an artifact (i.e., degradation of LDL, or iodination blocking the antigenic site recognized by the monoclonal antibody), it would mean that GP-22 binds a specific apo B component in plasma and suggests that patients with CAD have a significant increase in the apo B component specifically recognized by GP-22 monoclonal antibody. Further biochemical and immunochromical characterization of monoclonal antibodies and of LDL from patients with and without CAD will be required to elucidate the exact mechanism. In addition, the patients presented in this study represent a highly selected group undergoing coronary angiography for the evaluation of CAD and do not represent the population at large. Continued long-term evaluation is required to determine the efficacy of using monoclonal antibodies as a screening reagent for predicting CAD.

The concentrations of apo B in our normal subjects, as determined by our radioimmunoassay, are in close agreement within the range of values reported by others (16–22). These values should vary, however, depending on the LDL standard used, as discussed above. The increases of apo B, as determined by conventional antibodies, in hypertriglyceridemic patients are in good agreement with previous reports (18, 20). We noted much greater increases, however, when using monoclonal antibodies; this has not been shown previously.

In summary, we believe that the preparation of LDL and the different antibodies used will be essential in the future standardization of apo B radioimmunoassays. Owing to the heterogeneity of apo B in plasma, each subspecies may play different atherogenic roles (9, 29–31); conceivably, monoclonal antibodies to LDL may provide an important reagent for studying CAD.

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References


