Monoclonal Antibodies Can Precipitate Low-Density Lipoprotein. II.
Radioimmunoassays with Single and Combined Monoclonal Antibodies for Determining Apolipoprotein B in Serum of Patients with Coronary Artery Disease

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We have established four lines of monoclonal antibodies against human low-density lipoproteins (LDL) that, mixed in equal proportions, can precipitate LDL in gel and so can be used for apolipoprotein (apo) B determination in plasma. One monoclonal antibody (clone A), with a relatively low affinity to LDL ($K_a = 0.6 \times 10^9$ L/mol) and recognizing only two species of apo B, significantly underestimated the concentration of apo B in 74 patients with and 27 without coronary artery disease (CAD). High-affinity monoclonal antibody C ($K_a = 3.8 \times 10^8$ L/mol), which recognized all four apo B species, gave the same values for apo B as determined with the mixture of monoclonal antibodies. The latter results (by radioimmunoassay, $y$) correlated well with those by radial immunodiffusion ($x$): $y = 0.994x + 0.003 \ (r = 0.987)$. The CAD patients showed an increased concentration of apo B as compared to the angiographically documented CAD-negative patients. Except for the values determined by clone B ($p = 0.07$), the increase was statistically significant ($p = 0.002$–$0.018$) for values determined by use of the other clones or their mixture.

Additional Keyphrases: single vs combined monoclonal antibodies in radioimmunoassay · radial immunodiffusion compared · heart disease detection

Apolipoprotein B (apo B),4 a large and immunochemically heterogeneous protein (1–3) always found on low-density lipoprotein (LDL), plays a significant role in the catabolism of LDL (4). High concentrations of apo B-containing lipopro-

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4 Nonstandard abbreviations: apo B, apolipoprotein B; LDL, low-density lipoprotein; CAD, coronary artery disease; SORB, suspension of killed staphylococci containing "Protein A."

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LDL (standard or sample) for single or combined monoclonal antibodies. Monoclonal antibodies A, B, C, and D—titered so as to bind <50% of the maximal binding—were diluted 300-, 400-, 3000-, and 600-fold, respectively. The mixture of monoclonal antibodies was formed by combining equal volumes of each of these dilutions. The titer of the rabbit anti-mouse antibody was optimized for maximum precipitation.

Table 1 shows assay conditions for use with the single and combined monoclonal antibodies. We used 12 × 75 mm polystyrene disposable tubes (Lancer, American Scientific Products, McGaw Park, IL 60085) for the assays. The assay buffer (RIA buffer) was sodium borate (0.1 mol/L, pH 8.6) containing, per liter, 10 g of bovine serum albumin, 1 mmol of Na₂EDTA, and 0.1 g of Na₃. We performed the assays in duplicate, as follows:

1. To each tube add 125I-LDL (20 000–25 000 cpm, corresponding to about 10 ng of protein) in 100 μL of RIA buffer.
2a. To establish the maximum amount of counts bound (B₀), add to one tube 100 μL of RIA buffer containing no standard or sample.
2b. To establish the non-specific binding, add to one tube 100 μL of normal hybridoma medium.
2c. Add 100 μL of appropriate dilutions of LDL standard to the standard tube.
2d. Add 100 μL of diluted sample to the sample tube.
3. Add 100 μL of appropriately monoclonal antibodies to each tube except that for non-specific binding.
4. Incubate all tubes for 10–12 h at 4 °C.
5. Add 100 μL of appropriately diluted second antibody.
6. Incubate the contents of all tubes for 8–10 h at 4 °C.
7. To separate unbound 125I-LDL, add 100 μL of IgG SORB (see below) to all tubes.
8. After a further 30 min at 4 °C, wash the assay tubes with 2 mL of RIA buffer, and centrifuge to separate the bound 125I-labeled LDL (2000 × g, 30 min, 4 °C).

Calculate the percent bound as (B/B₀) × 100, where B is the total bound 125I-LDL in the presence of unlabeled LDL standard or plasma (minus non-specific binding) and B₀ is the total bound 125I-LDL in the absence of unlabeled LDL or plasma (minus non-specific binding). Determine the concentrations of apo B by use of a logit-log transformed standard curve.

Table 2 shows precision data assessed with use of lyophilized pooled human serum proposed as reference material (10). The intra-assay precision was established by analyzing the serum 10 times in the same assay. Between-assay precision was evaluated by analyzing the serum twice a week for four weeks. In all the tests we used a single batch of labeled LDL, second antibody, and monoclonal antibodies.

Patients

Male patients (n = 101) undergoing diagnostic coronary angiography for chest pain or suspected CAD or both, 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 over a 12-month period. Patients with obstruction of less than 30% in all segments were defined as normal or with insignificant CAD.

Blood was sampled by venipuncture, from patients who had fasted overnight, to tubes containing 0.2 mL of a 5 mmol/L solution of disodium EDTA. Plasma samples were stored at 4 °C. We saw no significant change (<10%) in apo B determinations for plasma stored at 4 °C for as long as 12 months (12).

On the basis of results of coronary angiography we divided the patients into two groups: 74 patients with and 27 without CAD. The mean values for total cholesterol, HDL-cholesterol, and triglycerides in plasma were 2.24, 0.29, and 1.94 g/L, respectively, for the patients with CAD and 2.14, 0.29, and 1.92 g/L, respectively, for those without.

Results

Binding of Monoclonal Antibodies to IgG SORB

Twenty monoclonal antibodies to human LDL were produced, of which four could precipitate LDL and mimic the properties of polyclonal serum antibodies (9). We used these four monoclonal antibodies and mixtures of them for evaluating our LDL radioimmunoassay. In a previous study (11) we developed a direct binding assay involving monoclonal antibodies of the IgG2b class to quantify apo B in plasma. Because these four monoclonal antibodies (all of the IgG1 class), complexed with 125I-LDL, did not bind to Protein A, we developed a double-antibody radioimmunoassay involving rabbit anti-mouse Ig. Nevertheless, because the monoclonal antibodies bind one epitope and the resulting antigen-antibody complex is small, the use of a suspension of killed staphylococci containing Protein A (IgG SORB; The Enzyme Center Inc., Boston, MA 02111) was necessary for complete precipitation. Even though the IgG SORB was not necessary when the mixture of monoclonal antibodies was used, we added it in all the assays.

Radioimmunoassay Standard Curves

Partly purified monoclonal antibodies prepared by precipitation with ammonium sulfate (~2 mg of protein per milliliter) were titered so as to give ~50% of the maximal binding to 125I-LDL for generating the radioimmunoassay standard curves.

Table 1. Reagents for RIA of Apo B with Single and Combined Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Antibodies, and dilution (fold)</th>
<th>Range of apo B standards, ng per tube</th>
<th>Sample dilution (fold)</th>
<th>2nd antibody dilution (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, 300</td>
<td>12.5–3200</td>
<td>200</td>
<td>500</td>
</tr>
<tr>
<td>B, 400</td>
<td>12.5–3200</td>
<td>200</td>
<td>500</td>
</tr>
<tr>
<td>C, 2000</td>
<td>12.5–3200</td>
<td>200</td>
<td>500</td>
</tr>
<tr>
<td>D, 800</td>
<td>12.5–3200</td>
<td>200</td>
<td>500</td>
</tr>
<tr>
<td>All 4</td>
<td>12.5–3200</td>
<td>200</td>
<td>500</td>
</tr>
</tbody>
</table>

*Equivalent mixture of the above dilutions each monoclonal antibody.

Table 2. Precision Data for Apo B As Determined with Use of Lyophilized Pooled Human Sera*

<table>
<thead>
<tr>
<th></th>
<th>Within assay (n = 10)</th>
<th>Between assay (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clones</td>
<td>Mean apo B, g/L</td>
<td>CV, %</td>
</tr>
<tr>
<td>A</td>
<td>0.67</td>
<td>3.75</td>
</tr>
<tr>
<td>B</td>
<td>0.86</td>
<td>2.87</td>
</tr>
<tr>
<td>C</td>
<td>0.76</td>
<td>2.34</td>
</tr>
<tr>
<td>D</td>
<td>0.72</td>
<td>2.68</td>
</tr>
<tr>
<td>All 4</td>
<td>0.77</td>
<td>2.49</td>
</tr>
</tbody>
</table>

*From the Centers for Disease Control, Atlanta, GA (10).
Monoclonal antibodies A, B, C, and D were diluted 300-, 400-, 3000-, and 600-fold, respectively, and mixed in equal volumes of each dilution. Protein content in LDL and apo B content in the standard serum ranged from 12.5 to 3200 ng per tube. Total volume in each assay was 300 µL.

Previous studies (4, 7, 12) have shown that concentrations of plasma apo B are increased in patients with CAD. Thus we decided to evaluate our assays on patients with and without CAD. In Table 3, we show that the 74 CAD patients had an increased concentration of apo B as compared with the 27 patients with angiographically documented negative or insignificant CAD. Except for the values determined with use of clone B (p = 0.07), the increases for the values determined with the other monoclonal antibodies or with their mixture were statistically significant (p = 0.002–0.018).

Thus, in general, each monoclonal antibody, or the mixture of all four of them, could be used to discriminate between patients with and without CAD.

Correlation between Results by Radioimmunoassay and Radial Immunodiffusion

In the previous paper (9) we found a high correlation between the apo B values as determined by use of the monoclonal antibodies mixture and by use of polyclonal antibodies, with the radial-immunodiffusion technique. A high correlation was also found between the same immunoprecipitation method and a method such as enzyme immunoassay that does not require immunoprecipitation.

We determined the correlation between radioimmunoassay (RIA) and radial immunodiffusion (RID), using the same reagent (the mixture of monoclonal antibodies) and the same secondary standard. Figure 3 shows that our results by RIA (γ) are almost identical to those obtained by RID (α): r = 0.987; y = 0.994x + 0.003.

Discussion

We have developed a double-antibody RIA for apolipoprotein B in which single and combined monoclonal antibodies

Table 3. Apo B Values (g/L) Compared in Patients with (n = 74) and without (n = 27) Coronary Artery Disease

<table>
<thead>
<tr>
<th>Clones</th>
<th>CAD +, x ± SD</th>
<th>CAD −, x ± SD</th>
<th>Δx</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.64 ± 0.22</td>
<td>0.48 ± 0.17</td>
<td>0.16</td>
<td>0.002</td>
</tr>
<tr>
<td>B</td>
<td>1.15 ± 0.40</td>
<td>0.99 ± 0.36</td>
<td>0.16</td>
<td>0.006</td>
</tr>
<tr>
<td>C</td>
<td>0.97 ± 0.26</td>
<td>0.82 ± 0.26</td>
<td>0.15</td>
<td>0.018</td>
</tr>
<tr>
<td>D</td>
<td>0.84 ± 0.27</td>
<td>0.67 ± 0.28</td>
<td>0.17</td>
<td>0.006</td>
</tr>
<tr>
<td>All 4</td>
<td>0.99 ± 0.26</td>
<td>0.84 ± 0.25</td>
<td>0.15</td>
<td>0.006</td>
</tr>
</tbody>
</table>

x = mean value; Δx = difference from mean value.
are used to evaluate the feasibility of using monoclonal antibodies for accurate and reproducible determination of apo-B in plasma. Previous studies (12, 13) reported that apo-B radioimmunoassay with use of the same monoclonal antibody would yield different values for apo B depending on the LDL preparations. Our studies (9) showed that, using the same monoclonal antibodies mixture and the same secondary standard in techniques that differ completely in principle and sensitivity, apo B values can be comparable.

The present studies show that, when we used the same secondary standard with the same technique, the means for the apo-B concentrations in plasma from 101 patients also varied with the monoclonal antibody used in the assay. We proposed that this is because each epitope is expressed in a different fashion in interacting with each monoclonal antibody in LDL, and therefore resulted in different values.

The results suggest that not all our monoclonal antibodies are readily practical for immunoassays. However, the concentrations as determined with each monoclonal antibody were significantly correlated (p <0.001).

The values for apo B as determined by use of clone A were significantly lower than those determined with the other clones and those values generally reported in the literature. Moreover, we found a lower concentration for this antibody as compared with the mixture or with the other three antibodies. The reason for this is not clear. Possibly the antigenic sites of the apo B to which the monoclonal antibody A is directed are partly masked by lipids or by conformational changes of the polypeptide chain in plasma. Or it may be due to the low binding affinity of LDL, approximately five times less than the mean $K_d$ for the other clones (9). However, monoclonal antibodies B and D, which possessed almost the same LDL binding affinity, did not give an equal apo B determination and clone D also underestimated apo-B values. Thus, the bias is not solely explained by binding affinity. Because both monoclonal antibodies A and D showed immunoreactivity to the species B-100 and B-74 of apo B but not to B-48 and B-26, it is tempting to explain the lower concentrations of apo-B as determined with these monoclonal antibodies as being a result of the amount of B-48 and B-26 lipoprotein present in the samples. In fact, the other two clones, B, and C, which showed immunoreactivity to all the species of apo-B (B-100, B-74, B-48, and B-26) gave higher apo B values. This hypothesis, which has been also put forward by Maynard et al. (13) to explain the different apo B values obtained by two monoclonal antibodies, has not been experimentally established, but it could be very interesting to attempt to verify it in future studies.

To avoid the problems associated with use of monoclonal antibodies in immunoassays, arising from their extreme specificity, Goding (14) suggests the preparation of antibodies formed by pools of several clonal products. In the previous (9) and present study we showed that the results obtained with the mixture of monoclonal antibodies correlated significantly (p <0.001) with those for polyclonal antibodies, and that the monoclonal antibodies mixture gave comparable apo B values by different techniques. Assuming that the mixture of monoclonal antibodies is a superior reagent to single antibody in terms of the accuracy in detecting apo B values, it is of interest that monoclonal antibody C, which possessed the higher binding affinity, could bind 100% of 125I-LDL and reacted with all the species of apo B (B-100, B-74, B-48, and B-26), giving the same apo B concentrations as assessed by use of the antibodies mixture.

Different studies (6-7, 12) support the view that apo B is a potential discriminator for patients with and without CAD. To test this ability of monoclonal antibodies, we determined apo B values in patients with and without angiographically documented CAD, using single or combined monoclonal antibodies. Except for clone B, each monoclonal antibody (or their mixture) showed a discriminatory ability for patients with and without CAD. The number of patients studied does not allow us to confirm the hypothesis that different subpopulations of apo B may play different atherogenic roles and that it may be possible to identify specific epitopes of the atherogenic peptides by using monoclonal antibodies. Continued long-term evaluation of a large population is therefore required to determine such efficacy of monoclonal antibodies as a screening reagent for predicting or confirming CAD.

In summary, we have developed a precise and reproducible radioimmunoassay in which single or combined monoclonal antibodies are used. Our studies indicate that the pool formed by four different clonal products can provide a controlled and standardized reagent that can be used in different assays for the immunochemical determination of apo B in plasma. We also found that not all the monoclonal antibodies are equally suitable for use in accurate determination of apo B. The use of monoclonal antibodies and a common secondary standard can be essential for the standardization of the determination of this protein. To establish the clinical value of the determination of apolipoprotein B using monoclonal antibodies, accurate studies on large population are required.

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References