Low-Density-Lipoprotein Apoprotein B in Plasma as Measured by Radial Immunodiffusion and Rocket Immunoelectrophoresis

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Radial immunodiffusion (RID) and rocket immunoelectrophoresis (RIE) are compared with respect to determination of LDL-bound apo B in plasma. Isolated VLDL could not enter a 15 g/L agarose gel when either technique was used. However, in the presence of plasma proteins, migration of VLDL into agarose was enhanced. Only when plasma samples were kept frozen before the assay was plasma VLDL unable to enter the agarose gel when RID was used. With RIE the contribution of plasma VLDL to the apo B determination under these conditions was not always negligible. Besides enhancing the entry of VLDL into the agarose, the presence of proteins also influences apo B immunoreactivity of LDL and VLDL. For measuring LDL-bound apo B directly in unfraccionated plasma we recommend: (a) RID in 15 g/L agarose gel; (b) freezing the plasma samples before assay; (c) diluting the plasma samples in saline supplemented with protein in the same concentration as is present in plasma (70 g/L); and (d) using plasma as the assay standard.

The main risk factors for vascular diseases seem to be the increased concentration of cholesterol transported as low-density lipoprotein (LDL) and the decreased concentration of cholesterol present in the high-density lipoprotein (HDL) fraction (1, 2).

LDL is usually quantitated by measuring its cholesterol component (3). According to some recent studies the apolipoproteins are equally good or even better discriminators for cardiovascular risk than the lipid components of the lipoproteins. Apo B is almost the only protein in LDL, and it accounts for about 40–50% of the protein moiety of VLDL (4). According to Brown and Goldstein (5), apo B of LDL regulates lipoprotein metabolism and would therefore play a key role in the atherogenic process. There are many methods for quantifying apo B in plasma—for example RID, RIA, RIE (see for example ref. 6), AIP (7), and INA (8)—but many of them are unsuitable for the direct determination of LDL-bound apo B in unfraccionated plasma. With most assay methods the apo B measured directly in plasma is derived not only from LDL but also in part from VLDL. For specifically measuring LDL-bound apo B by immunological techniques, chylomicrons and VLDL had to be separated from plasma by ultracentrifugation before the apo B determination.

Sniderman et al. (9) presented an RID technique by which LDL-apo B only could be directly determined in plasma. Their method is based on the use of agarose (15 g/L) as supporting medium.

In this paper, we compare RID and RIE with respect to the contribution of the VLDL-apo B protein moiety to the apo B determination under different conditions. We describe conditions under which specifically LDL-bound apo B can be directly determined in unfractionated plasma.

Materials and Methods

Samples

Blood was collected into EDTA-containing tubes (1 mg/mL final concentration) and blood cells and platelets were separated by low-speed centrifugation.

Isolation of Lipoproteins

VLDL and LDL with density ranges of d < 1.006 kg/L and 1.030 < d < 1.050 kg/L, respectively, were isolated by sequential ultracentrifugation (10). Both lipoprotein preparations were recentrifuged through 1.006 kg/L and 1.050 kg/L, respectively, to remove any residual plasma proteins. Double-immunodiffusion techniques showed the isolated VLDL and LDL samples to be free of albumin. In addition, only apo B could be detected in the LDL preparations. The apo B protein content from both lipoprotein preparations was determined after precipitation and washing twice with 4.2 mol/L tetramethyl urea (11), followed by washing once with chloroform/methanol (2:1 by vol). Protein was determined in the precipitate according to Lowry et al. (12), with human serum albumin (Cohn Fraction V) as standard, and corrected for chromogenicity by multiplying by the factor 0.90 (6). Unless otherwise indicated, the LDL preparations were stabilized by adding BSA to give a concentration of 20 g/L immediately after the isolation. The BSA (Cohn Fraction V) we used was obtained from Sigma Chemical Co., St. Louis, MO 63178, and contained no human apo B-immunoreactivity as tested by double-immunodiffusion with use of antiserum to apo B in serial dilutions.

Chemical LDL-apo B Determination in Plasma

We centrifuged 3.6 mL of EDTA plasma by density-gradient ultracentrifugation according to Redgrave et al. (13). The LDL band in the tube was sliced off between the densities of about 1.02 and 1.06 kg/L. From this LDL preparation, the apo B protein moiety was determined as described above. This plasma with a known amount of LDL-bound apo B was then used as assay standard for the immunological determination of apo B in plasma samples.

Immunological Apo B Determination in LDL, VLDL, and Plasma Samples

For the immunological apo B determination we used radial immunodiffusion (RID) and rocket immunoelectrophoresis (RIE). For both methods we used as the supporting medium agarose (10 or 15 g/L, in 30 mmol/L barbiturate buffer, pH 8.6) supplemented with monospecific rabbit antiserum to apo B (10 mL/L).

We applied 5 μL of diluted LDL, VLDL, or plasma samples to the antigen well. The dilutions were made in PBS unless otherwise indicated. A five- to 10-fold dilution of normal plasma proved to be appropriate. RID plates were incubated in a humid chamber at 37 °C for 16 to 20 h; RIE plates were
electrophoresed at 15 V/cm at 12 °C for 4.5 h. After drying, washing, and staining the plates with Coomassie Brilliant Blue and destaining, we measured the diameters of the ring-shaped (RID) or the heights of peak-shaped (RIE) immunoprecipitates in tenths of a millimeter, using a micrometer lens. The within- and between-assay coefficients of variation were 3% and 6%, respectively, for RID and 5% and 7% for RIE.

Results

Figure 1 shows the results of RID (Figure 1A–C) and RIE (Figure 1D–F) of LDL and VLDL preparations. When RID with 10 g/L agarose plates was used, the slopes of the curves were less steep for VLDL-apo B than for LDL-apo B (Figure 1A). When we instead used 15 g/L agarose plates, the VLDL did not enter the agarose at all (Figure 1B). After storage for two months at 4 °C VLDL could not migrate even into 10 g/L agarose (Figure 1C). The same is true for frozen VLDL preparations (results not shown). Apparently, when based on the concentration of apo B, the RID technique was more sensitive for LDL than for VLDL, especially when 15 g/L agarose plates were used.

The RIE technique also appeared to be more sensitive for LDL than for VLDL when 10 g/L agarose plates and freshly isolated lipoprotein samples were used (Figure 1D). Also, when RIE was used, isolated VLDL could not migrate into 15 g/L agarose (Figure 1E).

In contrast to the RID technique, with the RIE technique, stored VLDL preparations could still enter 10 g/L agarose, although VLDL penetrated less far than LDL (Figure 1F).

Because LDL-apo B usually must be measured in unfractionated plasma samples, we determined the effects of added LDL and VLDL on the apparent apo B concentrations in plasma. Figure 2 shows that, with the RID technique, addition of some LDL-apo B to plasma results in a greater increase in the apparent apo B concentration than when the same amount of VLDL-apo B was added (compare Figure 2A with 2B). This was the case for either 10 or 15 g/L agarose plates. Apparently, in contrast with pure VLDL preparations, plasma-VLDL can still diffuse into 15 g/L agarose plates (compare Figure 2B with 1B). However, when the VLDL-enriched plasma samples had
been stored at \(-20\text{}^\circ\text{C}\) for one week, the plasma-VLDL did not further contribute to the apparent plasma apo B (Figure 2D).

The same LDL- and VLDL-enriched plasma samples as used in Figure 2 were assessed for apparent apo B by the RIE technique (Figure 3). It is remarkable that with 10 g/L agarose plates the addition of VLDL-apo B increased the apparent plasma apo B much more pronouncedly than when the same amount of LDL-apo B was added to the plasma (Figures 3A and B). This was also observed when the samples were stored at \(-20\text{}^\circ\text{C}\) (Figures 3C and D). Also, the use of 15 g/L agarose plates could not eliminate the effect of plasma-VLDL on the apparent plasma apo B concentration when RIE was used (Figure 3D).

Figure 4A shows that an increase in the BSA concentration in the LDL preparation resulted in an increase in the apo B-immunoreactivity when RID was used, an effect that was much more pronounced when RIE was used with low concentrations of LDL (Figure 4B).

Figure 5 shows the effect of BSA on the apo B-immunoreactivity of VLDL. The control experiment without BSA could not be done, because under these conditions VLDL could not diffuse into 15 g/L agarose at all (see Figures 1B and E). With RID, the apo B-immunoreactivity of VLDL increased only slightly with increasing concentrations of BSA (Figure 5A). With RIE, however, the apo B-immunoreactivity of VLDL decreased with increasing BSA, especially when we used low concentrations of VLDL (Figure 5B).

From the results presented in Table 1 it appears that, in normal plasma samples, apo B concentrations as measured by RIE correlate highly with those measured by RID. Freezing the plasma samples before apo B quantitation did not affect the value calculated for Spearman's Rank Correlation Coefficient. The coefficient of correlation between the concentrations of apo B and LDL-cholesterol [calculated according to Friedewald et al. (3)] was about 0.7, irrespective of whether

RID or RIE was used and irrespective of whether or not these normal plasma samples had been stored at \(-20\text{}^\circ\text{C}\). From the results presented in Table 1 it can be concluded that the difference between the apo B concentrations in fresh normal plasma samples as measured by RIE and RID, respectively, correlates slightly with the plasma triglyceride concentration (p < 0.1). After the samples have been frozen this difference between both apo B measurements does not depend on the triglyceride concentration (p > 0.1). In frozen and thawed hypertriglyceridemic plasma samples, RIE and RID values correlated with each other, corresponding with the correlation found with normal fresh or frozen plasma samples. The difference between results for apo B by the two assay methods, for these frozen hypertriglyceridemic plasma samples, is not correlated significantly with the triglyceride concentration (p > 0.1). With fresh normal plasma samples the mean apo B concentration as measured by RIE significantly exceeds that measured by RID (Table 2). After storage of normal plasma samples at \(-20\text{}^\circ\text{C}\), no significant difference between the two means for apo B could be observed, but frozen hypertriglyceridemic samples still showed a significant difference.

**Discussion**

From the results presented in Figure 1 it appears that LDL and VLDL behave differently in quantitation of apo B with

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\text{RID or RIE was used and irrespective of whether or not these normal plasma samples had been stored at } -20\text{}^\circ\text{C}}.
\]

**Fig. 4.** Effect of BSA on the apo B-immunoreactivity of LDL immediately after isolation the LDL preparation was dialyzed vs PBS for 3 h. The LDL samples were then centrifuged to remove any precipitate and diluted in PBS containing BSA to the final concentrations indicated. No visible precipitate formed in the LDL samples during the whole procedure. The concentration (mg of apo B per ml) of the LDL samples was as indicated. 15 g/L agarose plates were used. The immunoreactivity at 0% BSA was taken as 100%.

**Fig. 5.** Effect of BSA on the apo B-immunoreactivity of VLDL immediately after isolation, VLDL was diluted in PBS containing BSA in the final concentrations indicated. Measurement of apo B-immunoreactivity in VLDL without added BSA (0%) was omitted because VLDL could not enter 15 g/L agarose in the absence of BSA. Immunoreactivity at 10 g of BSA per liter (1%) was taken as 100%. Further details in legend to Figure 4.
Table 1. RID Compared with RIE for Plasma Apo B Determination

<table>
<thead>
<tr>
<th>Samples derived from healthy subjects</th>
<th>Fresh samples</th>
<th>Frozen samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>y = ax + b</td>
<td>r_s</td>
</tr>
<tr>
<td>RID</td>
<td>y = 1.13x - 4.9</td>
<td>0.88</td>
</tr>
<tr>
<td>34 RID</td>
<td>y = 1.28x + 24.1</td>
<td>0.69</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>y = 1.02x + 41.0</td>
<td>0.73</td>
</tr>
<tr>
<td>RID</td>
<td>y = 2.76x +129</td>
<td>0.23</td>
</tr>
<tr>
<td>RID-RID</td>
<td>y = 0.78x + 19.9</td>
<td>0.82</td>
</tr>
<tr>
<td>49 RID</td>
<td>y = 1.12x + 46.9</td>
<td>0.72</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>y = 1.21x + 39.8</td>
<td>0.72</td>
</tr>
<tr>
<td>RID-RID</td>
<td>y = 2.28x + 150</td>
<td>0.10</td>
</tr>
<tr>
<td>Samples derived from hypertriglyceridemic subjects (&gt;3.0 g/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>49 RID</td>
<td>y = 0.92x + 12.6</td>
<td>0.92</td>
</tr>
<tr>
<td>RID-RID</td>
<td>y = 14.5x + 698</td>
<td>0.15</td>
</tr>
</tbody>
</table>

15 g/L agarose plates were used. Concentrations in mg/dL. Samples diluted sixfold in saline supplemented with BSA (70 g/L). Plasma with chemically determined LDL-apo B concentration (see Materials and Methods) was used as assay standard.

Abbreviations (see also footnote 1): TG: triglyceride; n: number of samples tested; r_s: Spearman’s Rank Correlation Coefficient; LDL-cholesterol: cholesterol in LDL calculated according to Friedewald et al. (9) (for the abnormal samples this calculation is not valid).

either RID or RIE. This difference in behavior may be ascribed firstly to differences in the disposition of apo B upon the surface of VLDL and LDL particles (14). However, from the results presented in Figure 1 we also conclude that a reduced diffusion or migration rate of VLDL into the agarose gel is at least partly responsible for the different immunological response of isolated LDL and VLDL, respectively. The marked size difference between VLDL and LDL particles would affect their migration into the gel. The results shown in Figure 1 suggest that the use of 15 g/L agarose as the supporting medium provides the opportunity to measure LDL-bound apo B by either RID or RIE without any contribution of VLDL-apo B.

Because LDL-apo B generally has to be measured in unfractionated plasma containing LDL and VLDL, we wondered whether the use of 15 g/L agarose plates would also provide the opportunity to measure LDL-bound apo B specifically in unfractionated plasma samples. Comparison of the results shown in Figures 2 and 3 with those of Figure 1 shows that the presence of plasma proteins affects the migration rate of VLDL, at least, into the agarose gel and therefore the determination of LDL-bound apo B. We suggest that when only LDL-apo B must be measured directly in unfractionated plasma samples, RID with 15 g/L agarose is preferable to RIE with 15 g/L agarose. Furthermore, freezing the plasma samples before the determination appears to be necessary.

The addition of BSA to LDL or VLDL preparations influences apo B immunoreactivity (Figures 4 and 5). This suggests that in addition to an effect on the migration rate of VLDL into the agarose (compare Figures 2 and 3 with Figure 1), plasma proteins also affect apo B immunoreactivity. Although we found no precipitate of LDL during the course of the experiment it is still possible that in the absence of proteins, LDL loses apo B immunoreactivity, whereas in the presence of proteins the immunoreactivity is preserved. Or protein may increase the apo B immunoreactivity by affecting the exposure of antigenic determinants. At present we can not distinguish between these two possibilities.

The effect on apo B immunoreactivity of the presence of protein in LDL and VLDL samples depends on the concentration of apo B. For standardization of the assay it is therefore necessary to keep the total protein concentration the same in each sample that is applied. Accordingly, for accurate measurement of apo B we propose the use of plasma with known amounts of LDL-apo B as assay standard and that each sample be appropriately diluted in phosphate-buffered saline supplemented with protein (BSA) in the same concentration that is present in plasma (70 g/L).

Sniderman et al. (9) found that addition of isolated VLDL to isolated LDL samples did not increase the apparent apo B concentration. They concluded that under their conditions (RID, 15 g/L agarose) VLDL did not contribute to the apparent apo B in plasma samples. In addition, they showed a close correspondence between apo B measured in the plasma and in the LDL fraction of plasma of nine patients with type IV hyperlipidemia. This suggested that their method for specifically measuring LDL-bound apo B directly in plasma remained valid in patients with high concentrations of VLDL and low concentrations of LDL. However, our present paper shows that the presence of plasma proteins does enhance the ability of VLDL to diffuse freely (RID) or migrate (RIE) into 15 g/L agarose gel, and that in normal plasma samples VLDL contributes to the apparent apo B except when plasma samples have been frozen before the RID assay (Table 2). When RID was used, the contribution of VLDL to apparent apo B was even more severe. Freezing normal plasma samples before assay eliminates the difference between mean apo B as measured by RID and RIE. In frozen hypertriglyceridemic plasma samples, however, there is still a slight but significant difference in mean apo B values as measured by the two assay methods (p = 0.018, Table 2). This significance is due to a few relatively extreme values for which Student’s t-test is sensitive. The hypertriglyceridemic plasma samples tested indicate different types of hypertriglyceridemia. Differences in particle size or sensitivity to freezing of VLDL of different types of hypertriglyceridemia possibly interfere with the difference between the mean apo B as measured by RID and RIE.

In summary, for measuring LDL-bound apo B in unfractionated plasma samples, RID with 15 g/L agarose is prefer-

Table 2. RID and RIE Compared for Apo B Determination with Respect to Mean Apo B Values

<table>
<thead>
<tr>
<th>Apo B, mg/dL, mean (and SD)</th>
<th>Fresh samples</th>
<th>Frozen samples</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>RID</td>
<td>RID-RID</td>
<td>RID-RID</td>
<td></td>
</tr>
<tr>
<td>Normal subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>97 (22)</td>
<td>105 (28)</td>
<td>0.001</td>
</tr>
<tr>
<td>34</td>
<td>91 (25)</td>
<td>89 (25)</td>
<td>0.782</td>
</tr>
<tr>
<td>34</td>
<td>91 (25)</td>
<td>89 (25)</td>
<td>0.003</td>
</tr>
<tr>
<td>34</td>
<td>105 (28)</td>
<td>89 (25)</td>
<td>&lt;10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hypertriglyceridemic subjects</td>
<td>86 (38)</td>
<td>92 (39)</td>
<td>0.018</td>
</tr>
</tbody>
</table>

These data are obtained from the same apo B measurements as used for the data presented in Table 1. p is the level of significance as determined by Student’s t-test for paired samples.
able over RIE, and freezing the plasma samples before assay is necessary. In addition, to avoid the effect of proteins on the apo B-immunoreactivity we recommend dilution of plasma samples in saline supplemented with BSA (7 g/L final concentration).

References