Double Pre-Beta Lipoprotein
Isolation and Characterization of the Two Populations of Very Low Density Lipoproteins by Zonal Ultracentrifugation

Enzo Manzato, Antonio Pagnan, Lorena Ziron, Adriana Gasparotto, and Monica Braggion

Two electrophoretic populations of very low density lipoprotein are frequently observed in both normal and hyperlipidemic human sera. This gives the appearance of two pre-beta bands, called the "double pre-beta lipoprotein phenomenon." The slow and fast bands forming the double pre-beta lipoprotein were isolated by ultracentrifugation in the zonal rotor under rate flotation conditions. On the basis of the effluent position, the slow band showed a lower flotation coefficient than the fast one. The lipid and apoprotein composition of the two very low density lipoprotein populations isolated by zonal ultracentrifugation was in close agreement with those obtained by preparative agarose gel electrophoresis. The slow and fast pre-beta fractions had the same triglyceride fatty acid composition. Both fractions contained only higher molecular weight apo B-100, while the slow fraction was relatively enriched in apo E and apo C-III. Since the slow pre-beta fraction showed the typical properties of the remnant particle, studying this fraction could clarify the possible relationship between remnant lipoproteins and atherogenesis. Zonal rotor ultracentrifugation may be useful to characterize remnant particles in normal and hyperlipidemic subjects.

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Two separate populations of very low density lipoprotein (VLDL) that showed fast and slow pre-beta mobility upon agarose gel electrophoresis were isolated by preparative agarose gel electrophoresis and characterized in our laboratory in 1977. The lipid and protein composition of the slow moving pre-beta component is similar to that of remnant particles because the slow moving component is poor (contains a low amount) in triglycerides and apo C and it is rich (contains a high amount) in cholesteryl esters and arginine-rich proteins.

However, it should be stressed that these two VLDL particles are clearly distinguishable from the beta VLDL of dysbetalipoproteinemia (Type III) because of two main characteristics: 1) the electrophoretic mobility of the VLDL particles is strictly pre-beta rather than beta, and 2) the isoelectric focusing (IEF) pattern of the apoprotein E in the VLDL particles is normal.

The separation and characterization of remnant particles similar to those presented by double pre-beta lipoproteinemia (DPBL), sometimes called late pre-beta lipoproteinemia, is important because of the possible relationships between remnant particle accumulation and the development of atherosclerosis. Clinical studies show a very high prevalence of DPBL in uremia and hypothyroidism, conditions that are frequently associated with atherosclerotic complications.

The present study was undertaken to find a faster and simpler method than the one we had previously used (preparative agarose gel electrophoresis) to isolate these two electrophoretic VLDL populations. We found that zonal rotor ultracentrifugation under flotation conditions enabled us to indirectly calculate the flotation coefficients of the two VLDL components and to measure their serum concentrations.
We report here on this method of separation and on the lipid and protein characterization of the slow and fast pre-beta components isolated from four subjects.

**Methods**

The procedures we followed were in accordance with the ethical standards of our medical institution. Each subject gave informed consent to the study.

Venous blood was obtained after a 12-hour fast from four members of a large family. These subjects were the propositus, two brothers, and one male cousin and they repeatedly showed double pre-beta bands upon agarose gel electrophoresis of their serum and their VLDL fractions (Table 1, Figure 1). The propositus suffered from myocardial infarction, but his relatives were healthy. None of the four subjects had kidney or thyroid disease.

The VLDL fraction was separated by preparative ultracentrifugation according to the method of Havel. To separate the two VLDL components, variable amounts of serum, ranging from 3 to 20 ml according to serum triglyceride concentrations, were placed in the zonal ultracentrifuge, as already described. Briefly, a Ti-14 Beckman zonal rotor was filled with a NaBr linear density gradient (d = 1.00 to 1.15 g/ml). The sample density was adjusted to 1.15 g/ml with solid NaBr and then injected into the rotor. Lipoprotein separation was performed at 98,000 g for 45 minutes in a L 5-65 Beckman ultracentrifuge. The temperature of the gradient, sample, and rotor was 18° C and no differences in the separation of DPBL were observed when the centrifugation temperature was maintained at 8° C. The rotor content was pumped out and the effluent was continuously monitored for absorbance at 280 nm before the 25 ml fractions were collected.

Suitable fractions of the zonal effluent were exhaustively dialyzed against 100 mM NaCl containing 1 mM disodium EDTA and 1 mM NaN₃ (pH 7.6) at 4° C and were concentrated in a Diaflo cell (Model 32) by using an XM 100 membrane (Amicon Corporation, Lexington, Massachusetts).

Zonal ultracentrifugation, dialysis, and concentration were performed on the day the blood was collected. Serum and VLDL fractions were analyzed by analytical agarose gel electrophoresis, as previously described.

Total and free cholesterol, triglycerides, phospholipids, and proteins were determined according to the methods of Röschlau et al., Whalefeld, Bartlett, and Lowry et al., respectively. The turbidity caused by the high concentration of lipids in the protein estimation samples was extracted before making the reading by diethyl ether at room temperature. The IEF of the VLDL apoproteins was performed in urea gel according to the method of Pagnan et al.

**Table 1. Serum Lipid, Cholesterol Levels, and Cholesterol/Triglyceride Ratio in the VLDL Fraction of Subjects with Double Pre-Beta Lipoproteinemia and of Control**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Serum Cholesterol (mg/dl)</th>
<th>VLDL Cholesterol (mg/dl)</th>
<th>LDL Cholesterol (mg/dl)</th>
<th>HDL Cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>Cholesterol/Triglyceride VLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>DT G</td>
<td>378</td>
<td>112</td>
<td>232</td>
<td>27</td>
<td>600</td>
<td>0.19</td>
</tr>
<tr>
<td>DT B</td>
<td>295</td>
<td>28</td>
<td>209</td>
<td>47</td>
<td>151</td>
<td>0.24</td>
</tr>
<tr>
<td>DT F</td>
<td>317</td>
<td>119</td>
<td>160</td>
<td>29</td>
<td>404</td>
<td>0.32</td>
</tr>
<tr>
<td>DT L</td>
<td>334</td>
<td>111</td>
<td>171</td>
<td>29</td>
<td>430</td>
<td>0.28</td>
</tr>
<tr>
<td>Mean ± so</td>
<td>331 ± 35</td>
<td>92 ± 43</td>
<td>193 ± 33</td>
<td>33 ± 9</td>
<td>396 ± 165</td>
<td>0.26 ± 0.06</td>
</tr>
<tr>
<td>Control (n = 1)</td>
<td>203</td>
<td>24</td>
<td>130</td>
<td>46</td>
<td>132</td>
<td>0.38</td>
</tr>
</tbody>
</table>
The apoprotein composition of the unfractionated VLDL was analyzed by electrophoresis in 3.0\% polyacrylamide gel with SDS.\(^{13}\) Cholesterol was measured directly in the effluent from the zonal rotor as previously described.\(^ {14}\) High density lipoprotein (HDL) cholesterol was measured by low density lipoprotein (LDL) precipitation in the d > 1.006 g/ml fraction obtained by preparative ultracentrifugation,\(^ {15}\) and LDL cholesterol was calculated by the difference.

Lipoprotein lipids were extracted with chloroform/methanol (2:1, vol/vol) and washed according to the method of Folch, et al.\(^ {16}\) Triglycerides were separated by thin-layer chromatography with hexane/diethyl ether/acetic acid (85:15:1, vol/vol/vol) and were extracted with 10 ml of chloroform. After transesterification with boron trifluoride methanol, the fatty acid composition was determined using a Carlo Erba gas chromatograph equipped with a hydrogen flame ionization detector and an electronic integrator.\(^ {17}\)

The same procedures used in the four subjects with double pre-beta lipoproteinemia were applied to a normolipidemic control subject. In this subject, the agarose gel electrophoresis of the VLDL fraction showed a single pre-beta band.

Statistical analysis were performed with standard procedures.\(^ {18}\)

**Results**

Table 1 shows serum lipid, VLDL, LDL, and HDL cholesterol levels and the VLDL total cholesterol/triglycerides ratio in the four subjects with the double pre-beta lipoprotein phenomenon and in the normal subject with a single VLDL component.

After zonal ultracentrifugation VLDL were recovered in the first 350 ml of rotor effluent. In the four subjects, the zonal effluent absorbance profiles at 280 nm were similar and resembled those observed in the control subject with a single VLDL component. An example of this profile is shown in Figure 2.

The fast and slow pre-beta VLDL bands were clearly separated, with no significant contamination, in the effluent from 20 to 100 ml and from 250 to 350 ml, respectively. The agarose gel electrophoresis of the unfractionated VLDL and of the slow and fast VLDL components that were recovered from zonal ultracentrifugation showed a very good separation (Figure 3). The two VLDL populations that were again ultracentrifuged in the zonal rotor maintained the same effluent position.

In the control subject with a single VLDL band, two VLDL fractions were collected, one in the 20 to 100 ml effluent and another in the 250 to 350 ml effluent. However, the electrophoretic mobility of the VLDL fraction from the 250 to 350 ml effluent was distinctly fast, not slow moving, pre-beta. This was not the case in subjects with double pre-beta lipoproteinemia.

The 0 to 25 ml zonal effluent contained lipoproteins which do not migrate on agarose gel electro-
Table 2. Protein-Lipid Composition and Cholesterol/Triglyceride Ratio of Fast and Slow Fractions in Subjects with Double Pre-Beta Lipoproteinemia

<table>
<thead>
<tr>
<th>Subject</th>
<th>DTG</th>
<th>DTB</th>
<th>DTF</th>
<th>DTL</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast band</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol* Free</td>
<td>3.7 ± 2</td>
<td>5.7 ± 6</td>
<td>7.0 ± 6</td>
<td>6.6 ± 1</td>
<td></td>
</tr>
<tr>
<td>Cholesterol* Esterifiedf</td>
<td>2.5 ± 1</td>
<td>3.4 ± 4</td>
<td>4.4 ± 4</td>
<td>11.1 ± 2</td>
<td></td>
</tr>
<tr>
<td>Triglycerides*</td>
<td>71 ± 7</td>
<td>65 ± 6</td>
<td>70 ± 6</td>
<td>68 ± 15</td>
<td></td>
</tr>
<tr>
<td>Phospholipids*</td>
<td>17 ± 2</td>
<td>19 ± 1</td>
<td>16 ± 4</td>
<td>15 ± 7</td>
<td></td>
</tr>
<tr>
<td>Protein*</td>
<td>7 ± 1</td>
<td>4 ± 1</td>
<td>4 ± 1</td>
<td>7 ± 1</td>
<td></td>
</tr>
<tr>
<td>Cholesterol/Triglyceride</td>
<td>0.07</td>
<td>0.18</td>
<td>0.14</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Slow band</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol* Free</td>
<td>5.7 ± 1</td>
<td>7.7 ± 1</td>
<td>6.6 ± 1</td>
<td>6.6 ± 1</td>
<td></td>
</tr>
<tr>
<td>Cholesterol* Esterifiedf</td>
<td>10 ± 1</td>
<td>13 ± 1</td>
<td>11 ± 1</td>
<td>9 ± 1</td>
<td></td>
</tr>
<tr>
<td>Triglycerides*</td>
<td>47 ± 3</td>
<td>46 ± 3</td>
<td>48 ± 3</td>
<td>53 ± 3</td>
<td></td>
</tr>
<tr>
<td>Phospholipids*</td>
<td>24 ± 2</td>
<td>23 ± 1</td>
<td>22 ± 1</td>
<td>21 ± 1</td>
<td></td>
</tr>
<tr>
<td>Protein*</td>
<td>14 ± 1</td>
<td>11 ± 1</td>
<td>12 ± 1</td>
<td>11 ± 1</td>
<td></td>
</tr>
<tr>
<td>Cholesterol/Triglyceride</td>
<td>0.32</td>
<td>0.43</td>
<td>0.38</td>
<td>0.28</td>
<td></td>
</tr>
</tbody>
</table>

Data were obtained by zonal ultracentrifugation.
*Values are in percentage by weight.
†The data for esterified cholesterol are calculated as the mass of cholesterol.

Figure 4. IEF pattern of the urea-soluble very low density lipoprotein apoproteins in unfractionated (A) slow (B), and fast (C) fractions of a subject with double pre-beta lipoproteinemia.

Table 3. Apoprotein Composition of Urea-Soluble Peptides of the Fast and Slow Fraction of VLDL from Subjects with Double Pre-Beta Lipoproteinemia and of VLDL from Controls

<table>
<thead>
<tr>
<th>Fraction</th>
<th>E-I</th>
<th>E-IV</th>
<th>C-III</th>
<th>Apo E</th>
<th>Apo C</th>
<th>Apo C-III</th>
<th>Apo E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast*</td>
<td></td>
<td></td>
<td></td>
<td>5 ± 3</td>
<td>5 ± 3</td>
<td>11 ± 1</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Slow*</td>
<td></td>
<td></td>
<td></td>
<td>9 ± 3</td>
<td>9 ± 3</td>
<td>13 ± 1</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>VLDL control†</td>
<td></td>
<td></td>
<td></td>
<td>8 ± 1</td>
<td>8 ± 1</td>
<td>14 ± 1</td>
<td>14 ± 1</td>
</tr>
</tbody>
</table>

*Isolated with zonal ultracentrifugation from four subjects.
†Isolated with preparative ultracentrifugation from 19 male control subjects.
‡ = p < 0.05.
Table 4. Cholesterol Levels in VLDL of Sf 100-230 and Sf 20-100 of Double Pre-Beta Lipoproteinemia Subjects and Corresponding VLDL Fraction of Control

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sf 100-230</th>
<th>Sf 20-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>DT G</td>
<td>16</td>
<td>90</td>
</tr>
<tr>
<td>DT B</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>DT F</td>
<td>30</td>
<td>84</td>
</tr>
<tr>
<td>DT L</td>
<td>14</td>
<td>85</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>16 ± 11</td>
<td>71 ± 32</td>
</tr>
<tr>
<td>Control (n = 1)</td>
<td>8</td>
<td>17</td>
</tr>
</tbody>
</table>

Values are mg/dl. Sf 100-230 includes the fast fraction; Sf 20-100 includes the slow fraction.

(Table 2) we calculated that the mean lipoprotein mass in the slow band in these subjects was 418 mg/dl and in the fast band it was 177 mg/dl.

Polyacrylamide gel electrophoresis in SDS demonstrated that there was apo B 100, but not apo B 48, in the unfractionated VLDL after a 12-hour fast.

The fatty acid composition of triglycerides from the slow and fast VLDL components was analyzed in three subjects (DTG, DTF, DTL). The difference in the percent composition of fatty acids was always less than 1% confirming that the composition of the two components was similar.

Discussion

The results of our study show that zonal ultracentrifugation represents a simple, rapid and reproducible technique for isolating the different electrophoretic populations of VLDL. In fact, the lipid and protein composition percent by weight of the slow and fast pre-beta VLDL components turned out to be remarkably similar to that obtained after preparative agarose gel electrophoresis, as previously reported by Pagnan et al.1

In particular, the slow VLDL component had less triglyceride and more esterified cholesterol, apo E, and apo C-III than the fast VLDL component, suggesting that the slow component is made up of remnant particles. Moreover, the findings that the triglyceride fatty acid compositions of both the slow and fast components were similar and that there was apo B-100 only in the unfractionated VLDL indicate that both the fast and slow VLDL components have a common hepatic origin, although we still lack definitive metabolic evidence for this hypothesis.

This method of separating remnant particles by zonal ultracentrifugation is important because such pathological conditions as hypothyroidism, uremia, and dysbetalipoproteinemia are characterized by remnant particle accumulation and are also highly atherogenic.3-5 It should also be stressed that the calibration of the zonal rotor under the technical conditions we described allows us to calculate the flotation characteristics of the slow and fast pre-beta bands on the basis of their zonal effluent position. In fact, because the particle diameters and the flotation coefficients are directly correlated, we confirmed that the slow moving VLDL population (made up by smaller particles) shows a lower flotation coefficient than the fast band. Thus, the slow moving VLDL particles have an Sf from 20 to 50, while the fast one has an Sf from 125 to 230. These flotation properties agree with the data on particle size (mean diameter) of the two different VLDL populations as previously calculated.

As already demonstrated, in the control subject (with the single pre-beta band) we isolated a VLDL fraction in the zonal effluent volume corresponding to the volume of the slow moving VLDL pre-beta band of the DPBL subjects. However, the electrophoretic mobility of this fraction typically is fast pre-beta. This finding confirms that normal VLDL (i.e., the single, fast-moving pre-beta band) represents a continuous spectrum of particles of varying size, from the largest (Sf 400) to the smallest (Sf 20), and that the slow-moving VLDL component (the DPBL phenomenon) represents a clearly separable electrophoretic species, rather than a part of the continuous VLDL spectrum. This behavior probably reflects a disturbance in VLDL catabolism, leading to the appearance of remnant particles (the slow-moving pre-beta band) that are not completely degraded and therefore accumulate in plasma. Further metabolic studies during prolonged fasting and in the postprandial phase would be useful to definitely clarify the origin and the physiological meaning of this remnant.

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References


Index Terms: very low density lipoprotein • double pre-beta lipoprotein • remnant • zonal ultracentrifugation • lipoprotein metabolism • atherogenesis
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