Mass Concentration of Plasma Phospholipid Transfer Protein in Normolipidemic, Type IIa Hyperlipidemic, Type IIb Hyperlipidemic, and Non–Insulin-Dependent Diabetic Subjects as Measured by a Specific ELISA

Catherine Desrumaux, Anne Athias, Ginette Bessède, Bruno Vergès, Michel Farnier, Laurence Perségon, Philippe Gambert, Laurent Lagrost

Abstract—Mean plasma phospholipid transfer protein (PLTP) concentrations were measured for the first time by using a competitive enzyme-linked immunosorbent assay. PLTP mass levels and phospholipid transfer activity values, which were significantly correlated among normolipidemic plasma samples (r=0.787, P<0.0001), did not differ between normolipidemic subjects (3.95±1.04 mg/L and 575±81 nmol · mL⁻¹ · h⁻¹, respectively; n=30), type IIa hyperlipidemic patients (4.06±0.84 mg/L and 571±43 nmol · mL⁻¹ · h⁻¹, respectively; n=36), and type IIb hyperlipidemic patients (3.90±0.79 mg/L and 575±48 nmol · mL⁻¹ · h⁻¹, respectively; n=33). No significant correlations with plasma lipid parameters were observed among the various study groups. In contrast, plasma concentrations of the related cholesteryl ester transfer protein (CETP) were higher in type IIa and type IIb patients than in normolipidemic controls, and significant, positive correlations with total and low density lipoprotein cholesterol levels were noted. Interestingly, plasma PLTP mass concentration and plasma phospholipid transfer activity were significantly higher in patients with non–insulin-dependent diabetes mellitus (n=50) than in normolipidemic controls (6.76±1.93 versus 3.95±1.04 mg/L, P<0.0001; and 685±75 versus 575±81 nmol · mL⁻¹ · h⁻¹, P<0.0001, respectively). In contrast, CETP levels did not differ significantly between the 2 groups. Among non–insulin-dependent diabetes mellitus patients, PLTP levels were positively correlated with fasting glycemia and glycohemoglobin levels (r=0.341, P=0.0220; and r=0.382, P=0.0097, respectively) but not with plasma lipid parameters. It is proposed that plasma PLTP mass levels are related to glucose metabolism rather than to lipid metabolism. (Arterioscler Thromb Vasc Biol. 1999;19:266-275.)

Key Words: cholesteryl ester transfer protein ■ lipid transfer ■ ELISA ■ glucose ■ non–insulin-dependent diabetes mellitus

In vivo, plasma lipoproteins do not constitute stable entities but are continuously remodeled through the action of several enzymes and lipid transfer proteins. In particular, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP), 2 related proteins belonging to the lipid transfer/lipopolysaccharide binding protein (LT/LBP) family,1 can promote the exchange of lipid species between plasma lipoprotein fractions. In fact, studies over the past few years have demonstrated that both CETP and PLTP produce multiple effects on lipoprotein structure and composition. Thus, CETP promotes the exchange of neutral lipids, ie, CEs and triglycerides, between plasma lipoprotein fractions, leading to alterations in both the neutral lipid content and the size distribution of lipoproteins.2,3 PLTP can facilitate the transfer of phospholipids between lipoprotein particles,4 and it was lately shown to transfer lipopolysaccharides,3 unesterified cholesterol,6 and α-tocopherol7 as well. In addition, PLTP constitutes an important determinant of the size distribution of HDL.8-12 Taken together, recent advances have raised considerable interest in elucidating the precise function of lipid transfer proteins in lipoprotein metabolism, and a new challenge of in vivo studies is to relate pathophysiological alterations of the plasma levels of CETP and PLTP to atherosclerosis susceptibility.

Mainly, 2 distinct approaches can be applied to the quantification of lipid transfer protein levels, consisting of either evaluation of lipid transfer activities by isotopic or net mass-transfer assays or determination of the mass concentration of lipid transfer proteins by specific immunoassays. Although the determination of lipid transfer rates in plasma has proved helpful and informative, it does not necessarily provide a reliable and specific estimate of the lipid transfer protein mass per se, due in part to the presence of putative modulators in total plasma. The result is that only specific...
immunoassays are suitable for accurate determination of lipid transfer protein mass in plasma samples. In 1990, the first radioimmunoassay of human CETP, proposed by Marcel and coworkers, allowed the determination of mean CETP levels in normolipidemic plasmas, and subsequent clinical investigations with specific immunoassays led to a significant improvement in our knowledge of the metabolism of CETP and its pathophysiological variations. Unlike CETP, PLTP has been quantified only through its ability to exchange phospholipids, and to date no specific immunoassay has been proposed to assay PLTP mass levels in biological samples. In fact, it is noteworthy that phospholipid exchange activity is a property that is shared by several plasma proteins, including CETP, LBP, and soluble CD14, in addition to PLTP. The latter point suggests that only a specific immunoassay would accurately reflect the level of PLTP in plasma, and today the lack of an adapted quantitative tool may account, at least in part, for the paucity of information concerning the pathophysiological relevance of PLTP.

The present report describes the first immunoassay of human PLTP. A competitive ELISA of PLTP was devised by using polyclonal immunoglobulins raised against purified PLTP. This new method was then applied to the determination of PLTP in normolipidemic plasmas, as well as in plasmas from type IIa hyperlipidemic, type IIb hyperlipidemic, and non–insulin-dependent diabetes mellitus (NIDDM) patients.

Methods

Study Subjects
One hundred forty-nine subjects were selected for the study, including 30 normolipidemic subjects (15 males and 15 females; total cholesterol <2.50 g/L and triglycerides <1.30 g/L), 36 patients with type IIa dyslipidemia (27 males and 9 females; total cholesterol >2.50 g/L and triglycerides <1.30 g/L), 33 patients with type IIb dyslipidemia (30 males and 3 females; total cholesterol >2.50 g/L and triglycerides >1.30 g/L), and 50 patients with NIDDM (23 males and 27 females; total cholesterol 1.02 to 3.20 g/L and triglycerides 0.45 to 4.35 g/L). Control normolipidemic subjects were selected from the hospital staff as healthy subjects with normal thyroid, renal, and hepatic functions and without a history of hyperlipidemia, coronary artery disease, or diabetes. Type IIa and type IIb patients did not present secondary causes of dyslipidemia, and patients with diabetes mellitus or those who were overweight...
Determination of Phospholipid and Cholesterol Transfer Activity of PLTP

**Figure 4.** Effect of anti-PLTP polyclonal immunoglobulins on phospholipid transfer and CE transfer activities in human plasma. For phospholipid transfer activity measurements, various amounts of control (○) or anti-PLTP (●) rabbit IgGs were added to 100 μL of human plasma. The mixtures were preincubated for 16 hours at 4°C in a final volume of 200 μL and centrifuged for 30 minutes at 10,000 rpm. The resulting supernatants were then incubated at 37°C for 90 minutes in the presence of [3H]DPPC liposomes (110 nmol phosphatidylcholine), isolated HDL (250 μg protein), and iodoacetate (1.5 mmol/L) in a final volume of 400 μL. For CE transfer activity determinations, 5 μL of plasma was preincubated for 16 hours at 4°C with the indicated amounts of nonimmune (□) or immune (■) rabbit IgG. The mixtures were then centrifuged, and the supernatants were incubated at 37°C for 18 hours with [14C]CE HDL (0.8 μg cholesterol), isolated LDL (150 μg protein), and iodoacetate (1.5 mmol/L) in a final volume of 150 μL. At the end of the incubations, donor and acceptor lipoprotein fractions were separated, and transfer activity values were calculated as the percentage of radioactivity transferred from the donor to the acceptor fraction after deduction of blank values. Data are expressed as percentages of remaining lipid transfer activity compared with controls incubated without immunoglobulin supplementation (phospholipid transfer activity, 692 nmol · mL⁻¹· h⁻¹). Each point represents the mean of duplicate determinations.

(blood mass index >30 kg/m²) were excluded from the type IIa and type IIb groups. Diabetic patients suffered from NIDDM and were treated by either diet alone (n=8) or in combination with oral hypoglycemic drugs (n=42). Among the entire population studied, neither normolipidemic subjects nor dyslipidemic patients received drugs known to affect lipoprotein metabolism. The study was approved by the ethics committee of the Bocage Hospital (Dijon, France), and informed consent was obtained.

**Blood Samples**

Fasting blood samples were collected into EDTA-containing glass tubes, which were placed immediately on ice. Plasma was separated by a 5-minute centrifugation at 3000g, and aliquots were kept at −80°C until analysis.

**Purification of Human Plasma PLTP**

PLTP was purified from 1200 mL of citrated human plasma that was made lipoprotein deficient by the dextran sulfate–MnCl₂ precipitation procedure of Burstein et al.²² PLTP was purified by sequential chromatography on hydrophobic, affinity, and anion-exchange columns as previously described.²³ Only Mono-Q fractions with high specific phospholipid transfer activity and containing virtually only pure PLTP were selected for the study, with the exception of the partially purified Mono-Q fractions used for plate coating in the ELISA. For rabbit immunization and ELISA calibration, Mono-Q fractions containing pure PLTP were further passed through an anti-albumin immunoaffinity column to ensure removal of any traces of human plasma albumin. Finally, before rabbit immunization, the purified PLTP fractions were subjected to an ultimate preparative electrophoresis step to ensure a maximal degree of purity. In brief, purified PLTP fractions were applied to an 8% polyacrylamide gel containing 1% SDS, and electrophoresis was conducted in a 50 mmol/L Tris, 380 mmol/L glycine, and 0.1% SDS, pH 8.3, buffer for 6 hours at 50 mA. After electrophoresis, the portion of gel containing PLTP was cut off and the protein was eluted as previously described.²⁴ The purity of PLTP preparations was assessed by SDS electrophoresis in 80 to 250 g/L polyacrylamide gradient gels (Phastsystem, Pharmacia) and by SDS gel capillary electrophoresis, as indicated. The purified protein was concentrated and used for immunization of a New Zealand White rabbit within 24 hours. For plate coating, a partially purified PLTP fraction was obtained from lipoprotein-deficient fresh plasma (d>1.21 g/mL) by a combination of phenyl-Sepharose, heparin-Ultragel, and anion-exchange chromatography.²³ In brief, after anion-exchange chromatography of heparin-bound proteins, all of the eluted fractions containing detectable phospholipid transfer activity were pooled, and the resulting material used for plate coating corresponded to an ~500-fold increase in specific phospholipid transfer activity compared with the starting plasma.

**Anti-PLTP Polyclonal Antibodies**

Antiserum to purified human PLTP was prepared by immunization of a 3-kg New Zealand White rabbit with 1 initial injection of 250 μg
PLTP emulsified in complete Freund's adjuvant followed by three 150-μg injections of PLTP emulsified in incomplete Freund's adjuvant at 2-week intervals. The rabbit was bled 8 days after the last injection, serum was recovered by low-speed centrifugation, and the serum IgG fraction was prepared by using a protein A column (protein A–Sepharose 4 Fast Flow, Pharmacia) according to the procedure described by the manufacturer. This experiment was performed under the framework of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 81-23, revised 1985).

**Anti-PLTP Immunoblotting**

The specificity of anti-PLTP immunoglobulins was assessed by Western blotting. To this end, plasma and purified PLTP samples were subjected to electrophoresis in 80 to 250 g/L polyacrylamide Phastgels under reducing conditions, and proteins were subsequently transferred to a nitrocellulose membrane by using a Phast semidry electrophoretic transfer system as recommended by the manufacturer (Pharmacia). The resulting blots were blocked overnight at 4°C in 10% low-fat milk before being incubated for 1 hour at 37°C in the presence of anti-PLTP antibodies. After being washed, nitrocellulose membranes were incubated for 1 hour at 37°C with horseradish peroxidase–conjugated secondary antibodies (Bio-Rad). Finally, development was achieved by using the ECL-Western blotting detection reagent kit from Amersham.

**Plate Coating**

A competitive ELISA of PLTP was devised according to the general procedure previously used in our laboratory to quantify human apoA-IV, apoB, CETP, and CETP. All steps of the immunoassays (pipetting, diluting, dispensing, washing, and photometry) were carried out with a Biomek 2000 Biorobotics System (Beckman Instruments).

**Sample Treatment**

PLTP-containing samples were diluted in the albumin-phosphate buffer and mixed with an equal volume of polyclonal anti-PLTP antibodies diluted in albumin-phosphate buffer containing 1% Triton X-100 (Fierce Chemical Co). Total plasma samples were diluted from 1:2 to 1:16 in the albumin-phosphate buffer. The mixtures were incubated overnight at 4°C in 96 Deep-well titer plates (Beckman). Aliquots (100 μL) were then pipetted into the immunoplate micro-wells and incubated for 3 hours at 37°C. At the end of the incubation, the plates were washed 4 times with the Tween-20 solution.

**Detection of Bound Anti-PLTP Antibodies**

One hundred microliters of peroxidase-conjugated anti-rabbit antibodies (Bio-Rad) diluted in the albumin-phosphate buffer was pipetted into each well and incubated for 1 hour at 37°C. After completion of the incubation, the plates were washed 4 times as before, and 100 μL of a freshly prepared 0.4 g/L o-phenylenediamine–0.68 g/L H₂O₂ solution in a 6.6 mmol/L sodium phosphate–3.4 mmol/L citrate buffer (pH 5.2) was pipetted into each well. After 15 minutes at room temperature in the dark, the reaction was stopped by addition of 50 μL of 2.5 mol/L NaH₂PO₄. The absorbances were read at 490 nm with a Photometry tool on the Biomek 2000 Biorobotics station, and data were saved on a PC computer for further treatment.

**Calibration**

Pure PLTP (specific activity, ~10 μmol·mg⁻¹·h⁻¹) was used to standardize the assay. The amount of PLTP in purified fractions was determined by SDS gel capillary electrophoresis with carbonic anhydrase as an internal standard. Capillary electrophoresis was performed with uncoated, fused-silica capillaries (27 cm×100-μm ID) attached to a P/ACE 2100 system that was controlled by Gold software (Beckman Instruments). The P/ACE system 2100 was used in reversed-polarity mode. The electrolyte buffer was a non–cross-linked gel matrix (eCAP SDS 14-200, Beckman). In brief, PLTP-containing samples were diluted in Tris buffer (pH 6.6) containing 1% SDS, and they were supplemented with orange G as a tracking dye and run through the capillary under conditions previously used in our laboratory, quantifying human apoA-IV, apoB, and CETP.

**Table 1. Correlation of CETP and PLTP Mass Concentration With Plasma Parameters in Normolipidemic Subjects (n=30)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PLTP</th>
<th>CETP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>0.148</td>
<td>0.396*</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.139</td>
<td>0.176</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.140</td>
<td>0.362*</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.014</td>
<td>0.120</td>
</tr>
<tr>
<td>Glycemia</td>
<td>0.041</td>
<td>0.066</td>
</tr>
</tbody>
</table>

*P<0.05.
dye and carbonic anhydrase (0.2 g/L) as an internal standard. Electrophoresis was conducted at 20°C at 8.10 kV, and detection was performed at 214 nm. PLTP mass concentration was determined by comparing the area of the PLTP peak to the area of the peak obtained with a known amount of carbonic anhydrase. Finally, an ELISA primary standard curve constructed from a set of dilutions of purified PLTP was used to determine PLTP levels in a pool of frozen, normolipidemic human plasmas that constituted a secondary standard. Routinely, 8 dilutions (PLTP concentrations from 0.0275 to 3.52 mg/L) were used to construct a secondary calibration curve for sample PLTP mass concentration calculation. Four dilutions of each sample were assayed, and the CETP concentration was calculated by averaging the 4 results.

**Isolation of Lipoproteins**

HDLs were isolated as the 1.07 < d < 1.21 g/mL fraction of normolipidemic, fresh, and citrated human plasma at a speed of 55 000 rpm (223 000g) in a 70-Ti rotor on an L7 ultracentrifuge (Beckman) by a 2-hour spin at the density of 1.21 g/mL fraction of normolipidemic human plasma was increased from 0 to 20 minutes in the absence of plasma. Phospholipid transfer after a 2-hour period (Figure 1B). Throughout the study, phospholipid transfer activity measurements among various plasma samples were assayed. Phospholipid transfer activity was calculated as the capacity of a plasma sample to induce the transfer of radiolabeled dipalmitoyl phosphatidylcholine ([14C]DPPC) from [14C]DPPC liposomes to apoB-containing lipoproteins that were subsequently precipitated, and radioactivity was assayed in supernatants. Phospholipid transfer activity was calculated as the amount of total radiolabeled phospholipids transferred from liposomes to HDL after 2-hour period (Figure 1A).

**TABLE 2. Characteristics of the Study Subjects**

<table>
<thead>
<tr>
<th></th>
<th>Normolipidemic</th>
<th>Type Ila</th>
<th>Type Iib</th>
<th>NIDDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>38±11</td>
<td>42±16</td>
<td>49±11*</td>
<td>60±12†</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>15/15</td>
<td>27/9</td>
<td>30/3</td>
<td>23/27</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.2±2.9</td>
<td>23.3±3.1</td>
<td>25.8±2.3</td>
<td>31.3±6.8†</td>
</tr>
<tr>
<td>Total cholesterol, g/L</td>
<td>1.79±0.34</td>
<td>2.96±0.64†</td>
<td>3.13±0.49†</td>
<td>2.13±0.42‡</td>
</tr>
<tr>
<td>Triglycerides, g/L</td>
<td>0.77±0.17</td>
<td>0.89±0.25</td>
<td>1.91±0.55</td>
<td>1.65±0.90†</td>
</tr>
<tr>
<td>HDL cholesterol, g/L</td>
<td>0.58±0.14</td>
<td>0.55±0.18</td>
<td>0.44±0.11*</td>
<td>0.46±0.11*</td>
</tr>
<tr>
<td>LDL cholesterol, g/L</td>
<td>1.05±0.31</td>
<td>2.27±0.72†</td>
<td>2.31±0.50†</td>
<td>1.69±0.41†</td>
</tr>
<tr>
<td>Glycemia, g/L</td>
<td>0.86±0.07</td>
<td>0.88±0.08</td>
<td>0.93±0.12</td>
<td>1.88±0.71†</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>8.72±2.22</td>
</tr>
<tr>
<td>Insulinemia, mU/L</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>9.34±8.19</td>
</tr>
<tr>
<td>C-peptide, µg/L</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3.39±2.70</td>
</tr>
<tr>
<td>PLTP mass, mg/L</td>
<td>3.95±1.04</td>
<td>4.06±0.84</td>
<td>3.90±0.79</td>
<td>6.76±1.93†</td>
</tr>
<tr>
<td>Phospholipid transfer activity, nmol · mL⁻¹ · h⁻¹</td>
<td>575±81</td>
<td>571±43</td>
<td>575±48</td>
<td>685±75†</td>
</tr>
<tr>
<td>CETP mass, mg/L</td>
<td>2.67±0.55</td>
<td>2.99±0.63</td>
<td>3.23±0.73‡</td>
<td>2.34±0.67</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; ND, not determined.

*P<0.001, †P<0.0001, ‡P<0.05 vs normolipidimic subjects.

**CETP ELISA**

CETP concentration in total plasma samples was measured by a competitive ELISA on a Biomek 2000 laboratory automatic workstation (Beckman) as previously described.18 CETP mass concentration values were determined from a calibration curve obtained with a frozen plasma standard, and they were calculated by using data analysis software (Immunofit EIA/RIA data analysis software, Beckman). Four dilutions of each sample were assayed, and the CETP concentration was calculated by averaging the 4 results.

**Phospholipid Transfer Activity Assay**

Plasma phospholipid transfer activity was determined as the capacity of a plasma sample to induce the transfer of radiolabeled dipalmitoyl phosphatidylcholine ([14C]DPPC) from [14C]DPPC liposomes to apoB-containing lipoproteins that were subsequently precipitated, and radioactivity was assayed in supernatants. Phospholipid transfer activity was calculated as the amount of total radiolabeled phospholipids transferred from liposomes to HDL after 2-hour period (Figure 1A). When 10 µL of plasma was added, the phospholipid transfer assay was linear over a 2-hour period (Figure 1B). Throughout the study, phospholipid transfer activity measurements among various plasma samples were conducted by using 10-µL plasma aliquots that were

**TABLE 3. Correlation of CETP and PLTP Mass Concentration With Plasma Parameters in Type Ila Patients (n=36)**

<table>
<thead>
<tr>
<th></th>
<th>PLTP</th>
<th>CETP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLTP</td>
<td>0.292</td>
<td>-</td>
</tr>
<tr>
<td>CETP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>-0.274</td>
<td>0.423*</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.009</td>
<td>0.160</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.316</td>
<td>0.397*</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.253</td>
<td>-0.077</td>
</tr>
<tr>
<td>Glycemia</td>
<td>0.006</td>
<td>-0.115</td>
</tr>
</tbody>
</table>

*P<0.02.
incubated for 90 minutes at 37°C. The assay was proved to be independent of the phospholipid exchange activity catalyzed by CETP.4-23,26-28

CETP Activity Assay
Plasma CETP activity was determined as the capacity of a plasma sample to promote the transfer of radiolabeled CEs ([3H]CE) from [3H]CE HDL₃ to an excess of isolated LDL. Radiolabeled HDL₃ (0.8 μg cholesterol) was incubated for 18 hours at 37°C with isolated LDL (150 μg protein) in the presence of 5 μL of total plasma and iodoacetate (1.5 mmol/L) in a final volume of 150 μL. Donor and acceptor particles were subsequently separated by ultracentrifugation, and CETP activity was calculated as the percentage of total radiolabeled CEs transferred from HDL₃ to LDL after deduction of nonincubated control values.

Other Analyses
All chemical assays were performed on a Cobas-Fara centrifugal analyzer (Roche). Total cholesterol concentration was measured by an enzymatic method using a Boehringer Mannheim reagent. HDL cholesterol was measured after selective precipitation of apoB-containing lipoproteins with Boehringer phosphotungstic acid/MgCl₂ reagent, as recommended by the manufacturer. LDL cholesterol concentration was calculated using the Friedewald formula.29 Triglycerides were assayed by an enzymatic method using Roche reagent, as recommended by the manufacturer. LDL cholesterol concentration was calculated using the Friedewald formula.29 Triglycerides were assayed by an enzymatic method using Roche reagent, as recommended by the manufacturer. HDL cholesterol concentration was calculated using the Friedewald formula.29 Triglycerides were assayed by an enzymatic method using Roche reagent, as recommended by the manufacturer. HDL cholesterol concentration was calculated using the Friedewald formula.29 Triglycerides were assayed by an enzymatic method using Roche reagent, as recommended by the manufacturer. HDL cholesterol concentration was calculated using the Friedewald formula.29 Triglycerides were assayed by an enzymatic method using Roche reagent, as recommended by the manufacturer.

Statistical Analysis
ELISA curves were constructed by polynomial regression analysis. Sigmoidal competitive curves were linearized by logit-log transformation. Coefficients of correlation were calculated by linear regression analysis. Multiple regression analysis was used to determine the contribution of age and diabetic state to the rise in PLTP mass concentration in the diabetic subpopulation. Data means were compared by using a 1-way ANOVA.

Results
Purification and Characterization of Human PLTP
PLTP was purified from fresh human plasma by using a sequential procedure involving dextran sulfate/MnCl₂ precipitation followed by chromatographic and electrophoretic steps (see Methods). As shown in Figure 2, purified PLTP appeared as a single homogeneous band after electrophoresis in denaturing polyacrylamide gradient gels, with an apparent molecular weight of 56 kDa. In good agreement with previous studies,⁷ the apparent molecular weight of pure PLTP rose to 70 kDa when preincubated with β-mercaptoethanol before denaturing polyacrylamide gradient gel electrophoresis (Figure 2). Further analysis of PLTP preparations by the high-resolution capillary electrophoresis technique confirmed the presence of a single protein peak (Figure 3). Complementary experiments revealed that the mean isoelectric point of purified PLTP (pl 5.0) was identical to the pl value previously reported by Tollefsen and coworkers.⁸ Purified PLTP preparations presented elevated specific phospholipid transfer activity, determined as the rate of transfer of radiolabeled phosphatidylcholine from [14C]DPPC-liposome donors to isolated HDL acceptors.

Production of a Specific Anti-PLTP Antiserum and Development of an ELISA
Specific anti-PLTP IgGs were prepared from the serum of the rabbit successively injected with pure PLTP over a 2-month period (see Methods). As shown in Figure 4, anti-PLTP immunoglobulins (concentration range, 0 to 100 μg/μL) were found to inhibit plasma phospholipid transfer activity in a concentration-dependent manner. When 5 distinct plasma samples were supplemented with high concentrations of anti-PLTP immunoglobulins (150 μg/μL), the maximal inhibition of phospholipid transfer activity ranged between 70% and 87%. Under the same experimental conditions, the CE transfer activity of the related CETP remained unchanged (Figure 4). As shown in Figure 5, a single 70-kDa band was detected by Western blotting of either plasma or purified PLTP samples under reducing conditions. Anti-PLTP immunoglobulins were used to establish a competitive ELISA (see Methods). As shown in Figure 6, a typical ELISA displacement curve was obtained with purified PLTP with a 0.1 to 10 μg/mL working concentration range. In contrast, no displacement curves were observed when purified CETP or albumin solutions were used (Figure 6). When displacement curves were obtained by using various PLTP-containing fractions with distinct degrees of purity, the logit-log lines were parallel, indicating that the affinity of polyclonal anti-PLTP

<p>| TABLE 5. Correlation of CETP and PLTP Mass Concentration With Plasma Parameters in NIDDM Patients (n=50) |
|-------------------------|-------------------------|-------------------------|</p>
<table>
<thead>
<tr>
<th>PLTP</th>
<th>CETP</th>
<th>CETP Mass Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLTP</td>
<td>0.199</td>
<td>0.99</td>
</tr>
<tr>
<td>CETP</td>
<td>0.199</td>
<td>0.99</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.116</td>
<td>0.159</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.007</td>
<td>0.093</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.123</td>
<td>0.201</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.044</td>
<td>0.223</td>
</tr>
<tr>
<td>Glycemia</td>
<td>0.341*</td>
<td>-0.022</td>
</tr>
<tr>
<td>HbA1c</td>
<td>0.382†</td>
<td>-0.183</td>
</tr>
<tr>
<td>C peptide</td>
<td>0.036</td>
<td>0.162</td>
</tr>
<tr>
<td>Insulinemia</td>
<td>-0.074</td>
<td>0.135</td>
</tr>
</tbody>
</table>

*P<0.05, †P<0.01.
IgG was unaffected by the presence of other protein components in the mixture to be assayed (Figure 7). In addition, parallel logit-log lines were obtained with serial dilutions of normolipidemic or hyperlipidemic plasmas, indicating that under the experimental conditions used, plasma lipid levels did not alter the immunofinity of immunoglobulins for PLTP (Figure 8).

Intra-assay and interassay coefficients of variation were evaluated by analyzing the same plasma sample 5 times in the same microwell plate on the same day and on 5 consecutive days, respectively. The values of the intra-assay and interassay coefficients of variation were 5.7% and 7.8%, respectively.

**Determination of PLTP and CETP Mass Concentrations in Plasma From Normolipidemic Subjects**

PLTP mass concentration was assayed among plasmas from 30 normolipidemic subjects (15 males, 15 females). The mean plasma concentration of PLTP was 3.95±1.04 mg/L (range, 1.98 to 5.71), with identical levels in males and females. The mean plasma phospholipid transfer activity concentration among the normolipidemic population was 575±81 nmol · mL⁻¹ · h⁻¹. As shown in Figure 9, plasma PLTP mass levels were correlated positively and significantly with phospholipid transfer activity values measured as the transfer of radiolabeled phosphatidylcholine from [14C]DPPC liposomes toward exogenous HDL (r=0.787, P<0.0001). Among the same normolipidemic subpopulation, the mean plasma concentration of CETP was 2.67±0.55 mg/L, with slightly higher levels in females than in males (2.73±0.67 mg/L and 2.61±0.41 mg/L, respectively). Plasma CETP concentration was positively correlated with total and LDL cholesterol levels (r=0.40, P=0.0301; and r=0.36, P=0.0495, respectively). In contrast, plasma PLTP levels were not significantly correlated with any of the plasma lipid parameters determined among the normolipidemic subpopulation (Table 1).

**Determination of PLTP and CETP Mass Concentrations in Plasma From Patients With Type IIa Hyperlipidemia**

As shown in Table 2, a marked and significant rise in LDL cholesterol levels constituted the main abnormality of type IIa hyperlipidemic patients (n=36), accounting for the significantly higher total cholesterol levels compared with normolipidemic controls (P<0.0001), whereas triglyceride and HDL cholesterol levels in type IIa and normolipidemic populations were similar. No significant differences in PLTP mass and phospholipid transfer activity levels were observed between normolipidemic and type IIa groups (Table 2). As observed with the normolipidemic subpopulation, plasma PLTP levels were not significantly correlated with any of the plasma lipid parameters among type IIa patients (Table 3). In contrast, regression analysis revealed a positive correlation of CETP mass levels with both total and LDL cholesterol levels among type IIa patients (r=0.423, P=0.0102; and r=0.397, P=0.0166, respectively).

**Determination of PLTP and CETP Mass Concentrations in Plasma From Patients With Type IIb Hyperlipidemia**

Type IIb hyperlipidemic patients presented significantly higher levels of total cholesterol, LDL cholesterol, and triglycerides (P<0.0001 in all cases) compared with normolipidemic controls (Table 2). In contrast, HDL cholesterol levels were significantly lower in type IIb patients than in normolipidemic subjects (P=0.0004; Table 2). CETP mass concentration was significantly higher in type IIb patients than in normolipidemic controls. No significant differences in mean PLTP mass and phospholipid transfer activity levels were observed between normolipidemic and type IIb groups (Table 2). Neither CETP nor PLTP mass levels were significantly correlated with any of the plasma lipid parameters among the type IIb hyperlipidemic subpopulation (Table 4). Nevertheless, in agreement with data observed in the normolipidemic and type IIa subpopulations, CETP mass levels tended to be positively correlated with plasma LDL cholesterol levels in the type IIb population (r=0.306, P=0.0836).

**Determination of PLTP and CETP Mass Concentrations in Plasma From Patients With NIDDM**

NIDDM patients (n=50) presented significantly higher levels of total cholesterol, LDL cholesterol, and triglycerides (P=0.0324, P<0.0001, and P<0.0001, respectively) and significantly lower levels of HDL cholesterol (P=0.0005) than in normolipidemic subjects (Table 2). In addition, fasting glycemia was markedly and significantly higher in diabetics than in nondiabetics (Table 2). Plasma PLTP mass and phospholipid transfer activity levels were significantly higher in diabetics than in controls, whereas CETP mass levels did not vary significantly between the 2 groups (Table 2). Phospholipid transfer activity levels, but not PLTP mass levels, were lower in diabetics treated with a combination of hypoglycemic drugs and diet than in diabetics treated with diet alone (phospholipid transfer activity, 676±76 versus 750±55 nmol · mL⁻¹ · h⁻¹, respectively, P=0.02; PLTP concentration, 6.75±1.80 versus 7.03±2.15 mg/L, respectively, NS). Whereas no significant relationships between lipid transfer protein levels and plasma lipid parameters were noted among the diabetic subpopulation, PLTP but not CETP was correlated positively and significantly with both fasting glycemia (r=0.341, P=0.0220) and HbA1c (r=0.382, P=0.0097) levels (Table 5 and Figure 10). Because diabetic patients tended to be older than normolipidemic controls, multiple regression analysis was used to determine the contribution of age and the diabetic state to the prediction of PLTP mass and phospholipid transfer activity levels. From this analysis it was found that diabetic/nondiabetic state, but not age, contributed significantly to the rise in both PLTP mass levels (P<0.0001) and phospholipid transfer activity levels (P=0.0009) in the diabetic subpopulation.

**Discussion**

The importance of the roles of CETP and PLTP in lipoprotein metabolism has been suggested by a number of in vitro studies (for a review see Reference 2), and recent observations in animal models indicated that human PLTP expression can markedly influence HDL metabolism.30–32 These observations gave rise to a great interest in evaluating the pathophysiological variations of CETP and PLTP levels in vivo. To this end, a basic requirement is to devise specific immunoassays allowing the quantification of CETP and PLTP mass levels in biological samples from various human populations.
The present report describes the first immunoassay of human plasma PLTP that was applied to the determination of PLTP levels in plasma from 30 normolipidemic subjects, 36 type IIa hyperlipidemic patients, 33 type IIb hyperlipidemic patients, and 50 NIDDM patients. The parallel determination of CETP levels among the same subpopulations allowed a comparative analysis of the variability of lipid transfer protein levels in humans. A specific polyclonal antiserum was raised in rabbit against pure PLTP that was isolated from normolipidemic human plasma, and pure PLTP fractions exhibited the same characteristics as those previously described by others. Hence, PLTP preparations were able to transfer phosphatidylcholine from liposomes toward isolated HDL. It features that is not shared by human CETP.4,25,26,28 In addition, the present work confirmed that the mean apparent molecular weight of pure PLTP, as determined by denaturing polyacrylamide gradient gel electrophoresis, is 70 kDa under reducing conditions, but 56 kDa in the absence of a reducing agent.7 Moreover, anti-PLTP polyclonal antibodies were able to remove most of the phospholipid transfer activity in total human plasma while CE transfer activity remained unaffected. Polyclonal anti-PLTP antibodies were used to establish a competitive ELISA that proved to be a specific, sensitive, and accurate assay for PLTP concentration in plasma samples from normolipidemic and dyslipidemic subjects. No cross-reactivity against other plasma proteins, including the related CETP, was noted, and plasma PLTP mass concentrations were correlated significantly with phospholipid transfer rates as measured by an endogenous lipoprotein-independent assay.

When PLTP was assayed in total plasma from normolipidemic subjects, the mean concentration was 3.95 ± 0.14 mg/L, with no difference between men and women. The mean plasma PLTP level was in the same range as that of other members of the LTP/LBP family,13,33,34 and in the same normolipidemic group, the mean plasma CETP concentration was 2.67 ± 0.55 mg/L. Whereas CETP mass levels were correlated positively and significantly with total and LDL cholesterol levels, PLTP mass levels were not correlated significantly with any of the plasma lipid parameters measured. In support of recent in vivo studies,35 the present observations suggest that plasma LDL cholesterol levels might constitute a key determinant of plasma CETP levels, possibly through upregulation of CETP gene expression.36–38 In contrast, parameters other than LDL cholesterol might constitute the major determinants of PLTP expression. No significant relationship between plasma CETP and PLTP mass levels were noted, and overall observations in normolipidemic subjects indicate that plasma CETP and PLTP expression would be differentially regulated. The latter view is supported by several recent observations: (1) Plasma PLTP activity but not plasma CETP activity is affected by the saturated versus trans-unsaturated fatty acid content of the diet.39 (2) Opposite tendencies in diet-induced variations in CETP and PLTP activities have been reported among various inbred rabbit strains.40 (3) Alcohol withdrawal in alcoholic patients produces different effects on plasma CETP and PLTP activities.41

Another point of the present study was the first determination of PLTP levels in plasmas from type IIa and type IIb dyslipidemic patients. In fact, the PLTP concentration was remarkably similar in normolipidemic, type IIa, and type IIb subpopulations despite marked abnormalities in the plasma lipid levels of the dyslipidemic groups. Again, these observations sustain the hypothesis for the lack of a direct link between PLTP and plasma lipid levels. In good agreement with previous observations,42–43 CETP mass levels were significantly higher in type IIb patients than in normolipidemic controls, and a similar tendency was observed for type IIa patients.

Finally, a specific ELISA was applied to the determination of plasma PLTP levels in another pathological state associated with dyslipidemia, ie, NIDDM. This part of the study was hastened by recent reports addressing alterations in plasma phospholipid transfer activity in diabetic patients. However, the data are controversial, with either no alteration44 or a significant decrease45 in plasma phospholipid transfer activity being reported in NIDDM, as assessed by distinct isotopic activity assays. In addition, circumstantial evidence in favor of increased PLTP-mediated conversion of HDL₃ to HDL₂ in plasma from hypertriglyceridemic NIDDM patients compared with normolipidemic controls has recently been reported.46 In the present study, we found a marked and significant increase in PLTP mass levels in plasmas from NIDDM patients compared with normolipidemic controls. Again, as described above in normolipidemic subjects as well as in type IIa and type IIb patients, no significant correlation of PLTP levels with lipid parameters was observed in NIDDM patients. Because homologies between plasma lipid abnormalities were noted in NIDDM, type IIa, and type IIb patients, it is unlikely that the significant increase in plasma PLTP concentrations in NIDDM is related to the dyslipidemic state per se. In fact, analysis of additional plasma parameters revealed a significant, positive correlation between fasting glycemia and PLTP levels among the diabetic subpopulation, whereas no significant relationship between PLTP mass and insulin levels was found. Together with the positive correlation between HbA1c levels and PLTP levels, the results indicate that plasma glucose might be a putative determinant of plasma PLTP levels, and the significant increase in plasma glucose in NIDDM could account for the concomitant increase in PLTP mass. Interestingly, 1 recent study reported that isotopic transfer of phospholipids in obese men is positively related to both body mass index and fasting blood glucose concentration.47 Because we did not observe a significant relationship between body mass index and PLTP mass levels among diabetics, we postulate that plasma glucose rather than body mass index would determine PLTP levels in NIDDM patients. In fact, increased PLTP levels in the diabetic subpopulation of the present study might actually be related to the insulin resistance that is associated with long-lasting, elevated levels of plasma glucose rather than a rapid response to transiently elevated plasma glucose levels. Indeed, decreased plasma phospholipid transfer activity was recently observed in healthy men under acute hyperglycemia-induced hyperinsulinemia, and a significant negative correlation between plasma phospholipid transfer rates and insulin sensitivity was reported.48 We propose that the latter point might also apply to the NIDDM population and that the increased PLTP levels in these patients would be part of the insulin resistance syndrome. In contrast to PLTP, CETP mass concentrations in normolipidemic and NIDDM groups did...
not differ significantly. The latter point was in good agreement with previous studies that reported normal CETP mass levels in NIDDM patients despite elevated plasma CE transfer rates.49,50

In conclusion, the PLTP ELISA described in the present report constitutes the first tool for the measurement of PLTP mass concentration in plasma from normolipidemic as well as dyslipidemic subjects. Whereas PLTP mass levels, unlike CETP mass levels, did not vary significantly in type IIa and type IIb dyslipidemic groups compared with normolipidemic subjects, a highly significant rise was observed in NIDDM patients. Whether PLTP mass concentration is linked to glucose metabolism rather than to lipid metabolism deserves further attention.

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References


Mass Concentration of Plasma Phospholipid Transfer Protein in Normolipidemic, Type IIa Hyperlipidemic, Type IIb Hyperlipidemic, and Non–Insulin-Dependent Diabetic Subjects as Measured by a Specific ELISA

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