Isolation and Characterization of an Abnormal Alpha Slow-Moving High-Density Lipoprotein Subfraction in Serum from Children with Long-Standing Cholestasis

Jacqueline Peynet, Madeleine Fénéant-Thibault, Alain Legrand, Denis Marot, François Rousselet, and Alain Lemonnier

An abnormal high-density lipoprotein (HDL) subfraction, detected during periods of mild jaundice in the serum of seven children with chronic cholestasis from birth, was isolated and characterized. This fraction, identified by its slow alpha electrophoretic migration, is present in addition to normal HDL and differs from the abnormal HDL previously described in cholestatic syndromes. It is devoid of apolipoprotein B but is precipitated with phosphotungstate-MgCl₂. These properties allowed its isolation by double selective precipitation. This subfraction is undetectable with this procedure in the serum of healthy subjects, is rich in cholesterol, and contains a large amount of apolipoprotein E, which may explain its precipitation by phosphotungstate-MgCl₂. These apo E-containing HDL may play a major role in the lipid metabolism of patients with long-standing cholestasis during periods of mild jaundice.

Additional Keyphrases: chronic cholestasis • apolipoprotein E-containing HDL • apolipoprotein (E-Al) complex • icterus

Cholestasis frequently is associated with changes in plasma lipoproteins and sometimes with the occurrence of abnormal lipoproteins, the best known of which is lipoprotein X (LPX) (1). Abnormalities have also been reported in plasma high-density lipoproteins (HDL). Danielsson et al. (2) and Ameszj et al. (3), who analysed the plasma of patients with severe biliary obstruction, isolated HDL rich in phospholipids, free cholesterol, and apolipoprotein E (apo-E), which closely resembled nascent HDL. Watanebe (4, 5) detected HDL "S," so called because of their slow electrophoretic migration, in the serum of patients with mild cholestasis. These HDL S were rich in triglycerides, contained apolipoprotein A₁ (apo-A₁) and traces of apo-E but no apo-

lipoprotein B (apo-B), and were precipitated with dextran sulfate-MgCl₂.

Here we report an abnormal HDL subfraction, identified because of its slow electrophoretic migration, in serum from seven children presenting with chronic cholestasis from birth. We describe the isolation of this subfraction by double selective precipitation and report its composition.

Materials and Methods

Serum Specimens

Serum was sampled from seven children from the Department of Pediatric Hepatology (Prof. Alagille, Hôpital Bicêtre). They all had cholestasis from birth and had been regularly examined. In three cases, two samples taken six months apart were investigated. Table 1 gives the relevant data concerning the patients (6) and the results of liver-function tests, including values for serum bilirubin, alkaline phosphatase, gamma-glutamyltransferase (γGT), and alanine aminotransferase. Patients 2 and 6 underwent hepatporto-enterostomy, which only partly restored bile flow.

Control sera were obtained from apparently healthy donors.

Blood samples were collected from patients and control subjects after an overnight fast. The sera were separated by centrifugation and analyzed within 24 h.

Lipid and Lipoprotein Assay

The concentrations of lipid constituents in serum were determined enzymatically (7–9) in a centrifugal analyzer (Rotochem II; Kontron S.A., 78194 Trappes, France). Reagents for total and free cholesterol were from Biotrol, 96380 Louvres, France; for triglycerides from Boehringer Mannheim, 82440 Meylan, France; and for phospholipids from Wako (via Biolyon, 69570 Dardilly, France). HDL cholesterol was measured in the supernate resulting from VLDL and LDL precipitation with phosphotungstate–MgCl₂ (NaPT-MgCl₂) as described by Grove (10). Serum apolipoproteins A and B were determined by radial immunodiffusion (Behring-Hoechst, 92504 Rueil Malmaison, France). Lipoproteinograms were made on agarose gel (Corning, 78110 Le Vesinet, France) and on acrylamide–agarose plates with a discontinuous acrylamide gradient, 20 and 30 g/L (Lipofilm; Sebia, 92130 Issy les Moulineaux, France).

Isolation of HDL by Ultracentrifugation

HDL were isolated by sequential ultracentrifugation (density range: 1.063 to 1.21) performed at 40 000 rpm and 4 °C for 24 h in a "50 TY" rotor in a Beckman–Spinco L₂ ultracentrifuge. HDL control for study of apoprotein distribution were obtained from pooled normal serum. They were refloated at density 1.21, then diazylzed.
Table 1. Diagnosis and Results of Liver-Function Tests of Patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Date of birth</th>
<th>Diagnosis</th>
<th>Date of study</th>
<th>Total bilirubin</th>
<th>Conjugated bilirubin</th>
<th>Alkaline phosphatase</th>
<th>γGT</th>
<th>Alanine aminotransferase</th>
<th>Biliary salts, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (A)</td>
<td>02/23/76</td>
<td>Paucity of interlobular bile ducts (syndromatic)</td>
<td>12/82</td>
<td>39</td>
<td>32</td>
<td>1368</td>
<td>35</td>
<td>40</td>
<td>110</td>
</tr>
<tr>
<td>2</td>
<td>08/30/76</td>
<td>Extrahepatic biliary atresia</td>
<td>03/83</td>
<td>66</td>
<td>35</td>
<td>462</td>
<td>110</td>
<td>20</td>
<td>105</td>
</tr>
<tr>
<td>3 (A)</td>
<td>03/03/71</td>
<td>Paucity of interlobular bile ducts (syndromatic)</td>
<td>06/83</td>
<td>32</td>
<td>20</td>
<td>788</td>
<td>110</td>
<td>20</td>
<td>45</td>
</tr>
<tr>
<td>3 (B)</td>
<td>04/28/80</td>
<td>Paucity of interlobular bile ducts (syndromatic)</td>
<td>12/83</td>
<td>30</td>
<td>21</td>
<td>110</td>
<td>110</td>
<td>20</td>
<td>90</td>
</tr>
<tr>
<td>4 (B)</td>
<td>02/26/88</td>
<td>Paucity of interlobular bile ducts (non-syndromatic)</td>
<td>06/83</td>
<td>86</td>
<td>78</td>
<td>1770</td>
<td>110</td>
<td>20</td>
<td>190</td>
</tr>
<tr>
<td>5</td>
<td>01/06/81</td>
<td>Extrahepatic biliary atresia</td>
<td>01/84</td>
<td>76</td>
<td>64</td>
<td>828</td>
<td>110</td>
<td>20</td>
<td>39</td>
</tr>
<tr>
<td>7</td>
<td>11/03/82</td>
<td>Paucity of interlobular bile ducts (syndromatic)</td>
<td>11/84</td>
<td>23</td>
<td>18</td>
<td>793</td>
<td>110</td>
<td>20</td>
<td>102</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>49.4</td>
<td>39.3</td>
<td>1025</td>
<td>110</td>
<td>20</td>
<td>103</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td></td>
<td>21</td>
<td>20</td>
<td>477</td>
<td>110</td>
<td>20</td>
<td>65</td>
</tr>
</tbody>
</table>

*Usual values in children. *ND, not determined.

Isolation of α Slow-Moving Lipoproteins from the Patients’ Sera

LDL and VLDL were precipitated by adding 200 μL of antisera to apolipoprotein B (T = 16 g/L; Behring–Hoechst) to 500 μL of serum. After a 20-min incubation at room temperature, the mixture was centrifuged (4000 rpm, 5 min, 4 °C) and the supernatant fluid was removed.

The α slow-moving HDL were then separated from other HDL by selective precipitation, as follows. Add 50 μL of a reagent containing, per liter, 40 g of phosphotungstic acid and 0.5 mol of magnesium chloride, pH 6.15 (10), to 500 μL of supernate. After 10 min at room temperature, centrifuge the mixture at 4000 rpm for 10 min. Collect the pellet and wash it twice with 1-mL portions of precipitation reagent diluted 11-fold in a 0.15 mol/L sodium chloride solution. Then dissolve the pellet in 500 μL of a 2 mol/L sodium chloride solution.

We checked the purity of the different lipoprotein fractions resulting from this isolation procedure by electrophoresis on agarose and polyacrylamide gels, and their immunoreactivities with apolipoprotein A and apolipoprotein B antisera were investigated by Ouchterlony immunodiffusion.

The α slow-moving lipoproteins from patient 1 (sample 1A) were also precipitated by NaPT-MgCl₂ reagent from HDL prepared by sequential ultracentrifugation. The pellet was then collected, washed, and redissolved as described above.

Analysis of α Slow-Moving Lipoproteins

Total and unesterified cholesterol, phospholipids, and triglycerides in the redissolved pellet were measured with the same enzymatic reagents as those we used to determine the lipid components in the serum. The weight of cholesteryl esters was estimated as the amount of esterified cholesterol × 1.7. The lipid composition was expressed as the percentages of total lipid weight. The α slow-moving lipoprotein concentrations in serum samples were expressed in grams of lipids per liter: concentration in the redissolved fraction × 1.4.

Total proteins were measured by the method of Lowry et al. (11), with bovine serum albumin as standard. Apoprotein distribution was studied by electrophoresis on sodium dodecyl sulfate/polyacrylamide gel (SDS-PAGE) after lyophilization of dialyzed lipoproteins, delipidation with ethanol–ether (12), and redissolution of apoproteins in a pH 7.4 buffer containing, per liter, 2 mmol of sodium phosphate, 5 mol of urea, and 0.2 g of sodium azide. Apoproteins were incubated for 5 min at 95 °C in the presence of 17 g of SDS per liter. SDS-PAGE was done as described by Weber and Osborn (13), 50-μg aliquots of protein being applied to 110 g/L gels. The molecular-mass calibration kit was from Bio-Rad Labs., Richmond, CA 94804.

Analysis of HDL Not Precipitated by NaPT-MgCl₂

Lipid components of the HDL in serum of patients and controls, remaining after addition of NaPT-MgCl₂ to the
Results

Serum Lipids

The lipid components found in the serum of patients (Table 2) showed that in all cases (except for number 2) total cholesterol and phospholipid concentrations were above normal (up to 16.2 and 9.8 mmol/L, respectively). On the other hand, triglyceride concentrations were consistently normal (<1.35 mmol/L). HDL-cholesterol values determined after VLDL and LDL precipitation were in the upper range of the normal reference interval. Serum apolipoprotein A concentrations were normal or augmented, whereas apo-B concentrations were <1.20 g/L in all cases except for number 7 (1.95 g/L). The α slow-moving lipoproteins were detected in both agarose and polyacrylamide gel electrophoresis (Figure 1).

Reliability of Double Selective Precipitation for Isolating the α Slow-Moving Lipoproteins from Patients’ Serum

When the α slow-moving lipoproteins were detected on electrophoresis, they were isolated by the double selective precipitation described. When this procedure was applied to serum from normal subjects, no precipitate was detected during the second step.

To check the selectivity of the method used, we verified each step. Electrophoresis (Figure 2) showed that the first supernatant contained the α slow band and normally migrating HDL, but neither VLDL nor LDL; the electrophoretic mobility of the redissolved pellet lipoproteins was similar to that of the α slow band in serum. The lipoproteins remaining in the supernatant fluid migrated like normal HDL, and their lipid composition (Table 3) was close to that found in the supernate of serum from the normal control subjects after double selective precipitation. We therefore designated these lipoproteins as “normal” patient HDL.

Lipid Composition of the α Slow-Moving Lipoproteins

As shown in Table 3, the percent lipid composition were similar for all the α slow-moving lipoproteins analysed, including those of patient number 1 (sample 1A), isolated from total HDL prepared by sequential ultracentrifugation. Note that the α slow-moving lipoproteins were undetectable in the LDL-VLDL fractions (d<1.063) of this sample. The abnormal fractions contained more cholesterol, especially its unesterified form, than did the “normal” HDL of patients or the HDL of normal subjects (Table 3).

The concentrations of α slow-moving lipoproteins (expressed in terms of lipids) in serum varied from one patient to another and in different samples from the same patient.

Apolipoprotein Distribution in the α Slow-Moving Lipoproteins

The α slow-moving lipoproteins reacted with an apolipoprotein A antiserum by Ouchterlony immunodiffusion. SDS-

| Table 2. Concentrations of Lipid Components and of Apolipoproteins A and B in Patients’ Sera |
|-----------------------------------------------|-----|------------|-----------|-----------|-----------|
| Patient no.                                    | Total cholesterol | Triglycerides | Phospholipids | HDL-cholesterol | Apolipoprotein A | Apolipoprotein B |
| No.                                      | mmol/L       | <1.50 | 2.1-3.2   | 1.00-1.9   | g/L        | g/L            |
| 1 (A)                                      | 13.8         | 0.65  | 7.6       | 1.71       | 2.20       | 0.50           |
| 1 (B)                                      | 12.8         | 0.80  | 6.0       | 1.80       | 2.30       | 0.53           |
| 2                                          | 4.3          | 1.05  | 3.3       | 1.81       | 2          | 0.50           |
| 3 (A)                                      | 9.8          | 0.75  | 6.2       | 1.80       | 3.1        | 0.92           |
| 3 (B)                                      | 9.2          | 0.70  | 7.0       | 1.85       | 3.4        | 1              |
| 4 (A)                                      | 16           | 1.05  | 8.1       | 1.71       | 3.24       | 1.20           |
| 4 (B)                                      | 13.8         | 1.20  | 9.8       | ND*        | 2.9        | 0.90           |
| 5                                          | 9.2          | 1.0   | 5.2       | 1.78       | 3.95       | 1.08           |
| 6                                          | 12.45        | 0.60  | 7.1       | 1.55       | 2.70       | 1              |
| 7                                          | 16.2         | 1.35  | 9.2       | ND*        | 3.45       | 1.95           |

*ND, not determined.
Fig. 2. Electrophoretic verification of each step in the double selective precipitation procedure on (A) agarose and (B) polyacrylamide gels. (a) patient's serum; (b) first supernate after anti-apo B serum addition ("normal" and α slow-moving HDL); (c) second supernate after NaPT-MgCl₂ reagent addition (normally migrating HDL); (d) redissolved second precipitate (α slow-moving LDL). Spots observed at the deposits and at the limit of the two gels on PAGE slabs (lane 3) correspond to soluble HDL–phosphatidylglycerate complexes.

PAGE of delipidated fractions revealed an apolipoprotein composition quite different from that of the control HDL (Figure 3). The apolipoprotein distribution was similar in all the α slow-moving HDL fractions we studied, but there were some quantitative differences. Compared with control HDL, a sharp drop in apolipoproteins Apo-A₁ (Mr 28 000) and Apo-A₂ (Mr 17 500) was always observed. Other bands of variable intensity, not detected in control HDL, appeared. The most intense of these bands had an apparent relative molecular mass of 37 000, suggesting that it was apo-E. Another band (Mr 45 000–47 000) was also observed and might have been apo-B.

Table 3. Concentrations and Lipid Compositions of the Patient HDL Subfractions

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Concentration expressed as g of lipid per liter</th>
<th>α slow-moving HDL *</th>
<th>Normal HDL *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(expressed as % of total lipids wt/wt)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cholesteryl esters</td>
<td>Phospholipids</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>Control (n = 20)</td>
<td>5.90</td>
<td>17.2</td>
<td>31.5</td>
</tr>
<tr>
<td>1 (B)</td>
<td>4.20</td>
<td>15.1</td>
<td>35.3</td>
</tr>
<tr>
<td>2</td>
<td>0.50</td>
<td>3.7</td>
<td>31.2</td>
</tr>
<tr>
<td>3 (A)</td>
<td>3.30</td>
<td>20</td>
<td>35.1</td>
</tr>
<tr>
<td>3 (B)</td>
<td>5.50</td>
<td>15.9</td>
<td>34.7</td>
</tr>
<tr>
<td>4 (A)</td>
<td>6.60</td>
<td>13.4</td>
<td>39.7</td>
</tr>
<tr>
<td>4 (B)</td>
<td>5.20</td>
<td>16</td>
<td>36.1</td>
</tr>
<tr>
<td>5</td>
<td>3.60</td>
<td>20.2</td>
<td>28.8</td>
</tr>
<tr>
<td>6</td>
<td>6.80</td>
<td>20.2</td>
<td>35.9</td>
</tr>
<tr>
<td>7</td>
<td>1.30</td>
<td>10.6</td>
<td>33</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Isolated by NaPT-MgCl₂ precipitation after immunoprecipitation of lipoproteins containing apo-B; "HDL not precipitated by the NaPT-MgCl₂ reagent after immunoprecipitation of lipoproteins containing apo-B; "expressed as g of lipids carried by HDL subfractions in 1 L of serum (sum of the different lipid components); "cholesterol esters, estimated as the concentration of esterified cholesterol x 1.7; "HDL obtained from normal subjects after immunoprecipitation of lipoproteins containing apo-B and addition of the NaPT-MgCl₂ reagent; "fraction of sample 1A precipitated by NaPT-MgCl₂ from HDL obtained by sequential ultracentrifugation (density range: 1.063 to 1.21). ND: Not determined.
the apo (E–monomeric A1) complex described by Weis-
graber and Malheyy (14). The electrophoretic patterns also
revealed the presence of non-negligible amounts of albumin,
probably the result of insufficient purification of the isolated
fractions.

Discussion

We have demonstrated the presence, in serum from seven
young patients with long-standing cholestasis, of an abnor-
mal HDL subfraction characterized by its α slow electropho-
retic migration and its richness in apo-E.

Hitherto, α slow-moving HDL have seldom been de-
scribed. The circumstances in which they appeared in our
patients deserve some comment. They were detected during
periods of moderate icterus, i.e., under the conditions also
reported by Watanabe (5) for HDL S. Others (15, 16) found α
slow-migrating lipoproteins in the sera of subjects in the
early stage of primary biliary cirrhosis. The cases described
by Danielsen et al. (2) and Arnesjö et al. (3) were quite
different, because the cholestatic HDL they studied occurred
in serum of patients with pronounced obstructive jaundice.

As regards serum lipids, our patients exhibited normal
triglycerides but high values for total cholesterol and phos-
pholipids. Electrophoretic patterns and determinations of
serum apo B indicated that LDL and VLDL were not
increased, except for patient number 7. The “normal” HDL
always remained at the usual values, in contrast with the
sharp diminution of HDL described by Arnesjö et al. (3).
Taken together, these data suggest that the high values for
serum total cholesterol and phospholipids were due to the
presence of the α slow-moving lipoproteins.

Several characteristic features of these lipoproteins are
worth noting. By definition, they belong to the HDL class,
because of their presence after ultracentrifugation in the d
1.063–1.21 fraction. Further, although they are precipitated
by NaPT–MgCl2, they lack apo-B. These original properties
enabled us to separate them from “normal” patient HDL.
Each step of separation procedure was checked, and the
separation was satisfactory. The composition of the α slow-
moving fraction precipitated from HDL was similar, wheth-
er total HDL were obtained by ultracentrifugation or by
immunoprecipitation of lipoproteins containing apo-B. Con-
sequently, because our patients are sometimes young chil-
dren, we chose the double selective precipitation procedure
because it allowed us to work on 500–μl volumes of serum
sample. However, we did observe some discrepancies in
the protein values, which were probably overestimated on ac-
count of subfraction contamination by albumin.

The α slow-moving HDL subfraction is richer in cholester-
ol, especially its unesterified form, than is “normal” HDL.
The most striking modifications affected the apoprotein
distribution, including the presence of apo(E–A11) complex
and of a substantial quantity of apo-E. This composition is
quite different from that of LPX, which is also precipitable
with NaPT–MgCl2 but not by antiapo-B sera (17). There are
many other differences between the HDL subfraction stud-
ied here and the HDL previously described in cholestatic
syndromes. The particular composition of HDLα, (3), rich in
free cholesterol and apo-E, was partly attributed to an
LCAT deficiency. In our study, the LCAT activities mea-
sured for patients 1, 2, and 4 were normal. With respect to
HDL S, they contained far more triglycerides but only traces
of apo-E (4).

The presence of apo-E in the α slow-moving HDL subfrac-
tion of our patients seems of great importance, for analytical
and physiopathological reasons. It probably accounts for the
precipitation of this fraction by the NaPT–MgCl2 reagent.
Thus, Gibson et al. (18) reported that apo-E-containing
lipoproteins devoid of apo-B might be precipitable by the
reagents commonly used to determine serum HDL-chole-
sterol. Malheyy et al. observed that HDLα, which are chole-
sterol-enriched and apo-E-containing HDL present in the
serum of cholesterol-fed animals (19), were variously precip-
itated by heparin–manganese and that the most readily
precipitable HDLα were those richer in apo-E (20).

Weisgraber and Malheyy (14) reported that apo E and
apo(E–A11) complex are present in HDLα, which constitute a
minor HDL subclass in normal subjects. It may therefore be
assumed that the abnormal HDL subfraction detected in our
patients results from the accumulation of HDLα or from the
appearance of lipoproteins analogous to HDLα themselves
formed from HDLα and HDLβ by the acquisition of chole-
sterol and apo-E. However, our procedure did not allow
detection of precipitable HDL in the serum of normal
subjects, and in this connection the work of Schmitz and
Assmann (21) points out that HDLα are not precipitable by
NaPT–MgCl2. Consequently, the abnormal HDL subfrac-
tion of our patients rather resembles HDLα of cholesterol-fed
animals, which also displayed an α slow electrophoretic
migration.

The presence of HDL containing apo-E in these cholesta-
tic children certainly has some metabolic significance. Apo-
E was reported to play a role in the transport and exchange
of esterified cholesterol among lipoproteins (22) and to
mediate receptor binding (23). HDLα, especially those of
their subpopulations in which apo-E is the major apoprotein
constituent, competed very effectively with LDL for both
binding to B,E receptors and degradation by human fibro-
blasts (20). Funke et al. (24) demonstrated that the hepatic
parenchymal cells were chiefly responsible for apo-E–HDLα
uptake, which was mediated by apo-E receptors. Therefore
it has been suggested that HDLα rich in apo-E might be
formed in response to the deposition of cholesterol in periph-
eral tissues and the need to transport the cholesterol from
these tissues to the liver (24, 25). The pathogenesis of the
appearance in the serum of our patients of a slow-moving
HDL containing apo-E remains unknown. However, accord-
ing to the preceding hypothesis, this subfraction might
mainly contribute to eliminate cholesterol excess during
moderate jaundice in these children with long-standing
chronic cholestasis.

We are greatly indebted to Prof. Olivier Bernard, Department
of Pediatric Hepatology (Prof. Alagille, Director), Bicêtre Hospital,
94270 Le Kremlin-Bicêtre, France, for supplying us with the
patients’ serum samples and for his interest throughout this inves-
tigation.

References

obstructive jaundice. I. Method for quantitative separation
and identification of lipoprotein in jaundice subjects. J Clin Invest
2. Danielsen B, Ekman R, Peterson BG. An abnormal high
density lipoprotein in cholestatic plasma isolated by sonal ultracen-
3. Arnesjö B, Danielsen B, Ekman R, Johansson BG, Peterson
B. Characterization of high density lipoproteins in human chole-
4. Watanabe M. Lipoprotein abnormalities in cholestasis. I. Elec-
trophoretic and ultracentrifugal analyses. Acta Med Okayama


