Cholesterol efflux from Fu5AH cells to the serum of patients with Alagille syndrome: importance of the HDL-phospholipids/free cholesterol ratio and of the HDL size distribution

A. Davit-Spraul,[1,*,†] V. Atger,[§] M. L. Pourci,[*,†] M. Hadchouel,[**] A. Legrand,[†,††] and N. Moatti[*,§]

Laboratoire de Biochimie,* Faculté des Sciences Pharmaceutiques et Biologiques de l'Université Paris-Sud, 92290 Châtenay Malabry, France; Laboratoire de Biochimie 1,[†] Hôpital Bicêtre, 94275 Le Kremlin Bicêtre, France (AP-HP); Laboratoire de Biochimie,[§] Hôpital Broussais, 75014 Paris, France (AP-HP); INSERM U347,** Hôpital Bicêtre, 94275 Le Kremlin Bicêtre, France; and Laboratoire de Biochimie,[††] Faculté de Pharmacie, 75005 Paris V, France

Abstract **We have previously described the lipoprotein abnormalities in cholestatic children with paucity of interlobular bile ducts (PILBD), and we have shown that two different profiles emerged among these patients, depending on the level of lecithin:cholesterol acyltransferase (LCAT) activity. Reduced LCAT activity was associated with hypo-α-lipoproteinemia (group I) whereas normal LCAT activity was associated with hyper-α-lipoproteinemia (group II). In both groups, high density lipoproteins (HDL) were enriched with phospholipids and LpA-I particles were predominant. Here, we have investigated the ability of serum and of isolated HDL, obtained from PILBD and control subjects, to promote cellular cholesterol efflux, from Fu5AH rat hepatoma cells. The mean fractional efflux to 5% serum in each group was, on average, following the differences in HDL concentrations (control: 30.1 ± 4.2%; group I: 23.7 ± 7.9%, ns; group II: 44.2 ± 6.5%, $P < 0.001$). The variations in efflux values in group II were positively correlated to the variations in HDL-PL concentrations ($P < 0.0001$) and in HDL-PL to serum apo-AI ratio ($P < 0.003$). By contrast, the variation in efflux in group I was only positively related to the large range of HDL-PL to free cholesterol (FC) ratio values ($P < 0.0004$). Fractional efflux to isolated HDL, measured at a constant HDL-PL amount, confirmed this relationship ($P < 0.0001$). Two-dimensional gel electrophoresis of the HDL size and apo A-I distribution in serum, revealed that small size HDL_3 and pre-β HDL were predominant in the serum of patients from group I, especially those exhibiting low HDL-PL to FC ratio, whereas in the serum of patients from group II, both small HDL_3 and large HDL_2 were present. These results suggest that a combination of an imbalance between phospholipids and free cholesterol in the HDL particles and a deficit in large acceptors of cholesterol will be responsible for an impairment of cellular cholesterol efflux in PILBD patients with reduced lecithin:cholesterol acyltransferase activity.—**Davit-Spraul, A., V. Atger, M. L. Pourci, M. Hadchouel, A. Legrand, and N. Moatti. **Cholesterol efflux from Fu5AH cells in the serum of patients with Alagille syndrome: importance of the HDL-phospholipids/free cholesterol ratio and of the HDL size distribution.** *J. Lipid Res.* **1999.** 40: **328–335.**

Supplementary key words paucity of interlobular bile ducts (PILBD) • HDL • pre-β HDL • LCAT

Alagille syndrome is a rare hereditary multisystem disorder, associating five typical features: chronic cholestasis, congenital heart disease, embryotoxon, vertebral abnormalities, and typical facies. The hepatic lesions are characterized by a paucity of interlobular bile ducts (PILBD). Increased levels of plasma cholesterol and phospholipids are common in cholestatic children with Alagille syndrome. This condition is induced in part by biliary regurgitation (1). Despite the lipid abnormalities and the frequent presence of xanthomas, patients with PILBD rarely suffer from ischemic heart disease.

Recently, we have shown that hypercholesterolemia and hyperphospholipidemia in patients with PILBD were associated with either hypo or hyper α-lipoproteinemia depending upon lecithin:cholesterol acyltransferase (LCAT) activity (2). Decreased LCAT activity observed in severely icteric patients led to an accumulation of unesterified cholesterol that associates with phospholipids to form LpX. These patients exhibited low total HDL concentrations although they were characterized by normal LpA-I

Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; LpA-I, lipoprotein particle A-I; LpA-I:A-II, lipoprotein particle A-I:A-II; LCAT, lecithin:cholesterol acyltransferase; PILBD, paucity of interlobular bile ducts; PL, phospholipids; TC, total cholesterol; FC, free cholesterol; EC, esterified cholesterol.

[1] To whom correspondence should be addressed.

concentrations. In contrast, in mild icteric patients, LCAT activity was maintained and excess phospholipids and cholesterol were transported, in large part, by HDL particles that accumulate in plasma as revealed by an increased concentration of all HDL components. Last, because of the importance of the phospholipid regurgitation, the HDL of both mild and severely icteric patients were phospholipid-enriched, compared with normal HDL (2).

HDL is believed to have a crucial role in the transfer of cholesterol from peripheral tissues to the liver, a process called reverse cholesterol transport (3). This function partly explains the inverse association between HDL-cholesterol concentrations and the risk of coronary artery disease (4). A large variety of structural and biochemical features of HDL particles, reflected by the heterogeneity of the HDL pattern, influence their capacity to act as cholesterol acceptors. An enrichment of HDL with phospholipids (5, 6) or with apoE (7) facilitates the ability of HDL to remove cellular free cholesterol. Within HDL, the LpA-I that contain only apoA-I have been shown to be more efficient than the LpA-I:A-II in removing cholesterol from cells and in enhancing reverse cholesterol transport (8, 9).

In addition, LCAT activity is also postulated to modulate this process by esterifying the cell-derived cholesterol, leading to its retention and transport in the plasma compartment (10). The absence of LCAT activity is associated with elevated amounts of unesterified cholesterol and reduced number of HDL particles (11). Moreover, the accumulation of nascent and discoidal pre-β HDL particles in plasma with LCAT deficiency illustrates the importance of LCAT in the HDL maturation process (12). Thus, a decreased LCAT activity leads to a diminished overall number of cholesterol acceptors but also to enhanced pre-β HDL particles considered as the initial acceptors of cellular cholesterol (13).

In the present study, we investigated the ability of PILBD serum to mediate the efflux of cellular cholesterol, which is considered as the first step of reverse cholesterol transport. We examined this process with respect to the peculiar HDL features determined in these patients. To study this, we used Fu5AH hepatoma cells as cholesterol donors and we measured the efflux capacity of both total serum and isolated HDL from both mild and severely icteric PILBD patients, compared to samples from a group of control children. Our results emphasize the importance of both quantitative and qualitative HDL parameters in promoting cholesterol efflux.

MATERIALS AND METHODS

Subjects studied

Twenty-five patients with paucity of interlobular bile duct (PILBD) were studied. Children with PILBD were seen annualy for clinical and biological testing. For this study, patients were assigned to group I or group II according to their bilirubin levels (>100 μmol/l or <100 μmol/l total bilirubin, respectively). Thirteen patients had high bilirubin levels (group I >100 μmol/l) and 12 had lower bilirubin levels (group II <100 μmol/l). Control samples were obtained from 14 age-matched children without hepatic disease undergoing presurgery biological tests. This study has received the approval of the ethical committee of Hospital Bicetre and parental consent was obtained for all patients and control subjects before inclusion.

Serum lipid analysis and apolipoprotein quantitation

Blood was drawn into tubes without anticoagulant (Becton Dickinson, Rutherford, NJ) and centrifuged at 4°C by low speed centrifugation.

Total and free cholesterol, triglyceride, and phospholipid concentrations in serum were determined enzymatically.

Plasma apoA-I and apoB concentrations were determined by immunonephelometric assay (Beckman Array System, Gagny, France). Plasma LpA-I concentration was measured by immunoelectrophoresis (Sebia, Issy les Moulineaux, France) and was expressed as the mass of apoA-I (g) in LpA-I per liter of plasma. The apoA-I concentration in LpA-I:A-II was obtained by subtracting the LpA-I value from the total plasma apoA-I concentration.

HDL composition

High density lipoproteins (HDL, d 1.063–1.210 g/ml) were isolated from PILBD and control sera by density-gradient ultracentrifugation as previously described (14). Then, isolated HDL were dialyzed against phosphate-buffered saline, pH 7.4 (PBS). Total and free cholesterol, triglyceride, and phospholipid levels were determined enzymatically. Protein concentrations were determined by Peterson's modification of the Lowry procedure (15) using bovine serum albumin as a standard.

Measurement of cellular cholesterol efflux

Cellular cholesterol efflux was determined by using rat Fu5AH hepatoma cells following the procedure previously described (16). Briefly, cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 100 μg/ml penicillin-streptomycin, 1% l-glutamine (Gibco, Cergy Pontoise, France) and 5% fetal calf serum (Gibco). Fu5AH cells (2 ml; 20,000/ml) were plated on 22-mm 12-well plates (Falcon, Polylabo Paul Block, Strasbourg, France). Two days after plating, medium was replaced. Radiolabeled cholesterol ([^{3}H]cholesterol, Amersham France, Les Ulis, France) was added to the cells by adding a tracer amount (2 μCi/well) to 25% calf serum in MEM, which was then diluted to a final concentration of 5% in MEM. Then cellular cholesterol was labeled during a 48-h incubation. To allow equilibration of the label, the cells were washed with PBS and incubated for 24 h in MEM containing 0.5% bovine serum albumin, fatty acid-free (Sigma, St Louis, MO). For determination of cholesterol efflux, the cells were incubated at 37°C for 4 h in MEM containing 5% patient serum. All determinations were made in triplicate. In each assay, a standard serum previously prepared from a pool and kept frozen in aliquots was used as a reference to check for interassay homogeneity. The capacity for cholesterol efflux of isolated fresh HDL was tested following the same steps except that 5% serum was replaced by 100 μg HDL-PL/ml in medium. At the end of the efflux period, medium was removed and cleared by centrifugation. The cell monolayer was washed three times with PBS and harvested with 2 ml of isopropanol. Finally, radioactivity was measured in both medium and cells to determine the total radioactivity content in each well. Fractional cholesterol efflux, expressed as percentage, was calculated as the amount of the label released to the medium divided by the total label in each well.

Nondenaturating two-dimensional electrophoresis

Two-dimensional electrophoresis was carried out to analyze the distribution of apoA-I-containing lipoproteins in the serum samples from control and PILBD patients. As previously de-

scribed (13), the serum was first electrophoresed in 0.75% agarose gel in 50 mm barbital buffer at pH 8.6. As total apoA-I concentration was quite variable among the samples, the volume of serum applied to the gel was adjusted to a constant amount of 7.5 μg total apoA-I. Agarose gel strips containing the pre-separated lipoproteins were then transfered to a 4–30% polyacrylamide gradient gel (ALAMO Gels Inc., San Antonio, TX). Separation in the second dimension was performed at 40 volts for 17 h at 10°C in a 25-mm Tris-glycine buffer (pH 8.3). The proteins separated in the second dimension were electroblotted onto a polyvinylidine difluoride (PVDF, Bio-Rad, Ivry sur Seine, France) membrane (100 mA, 1 h) in a 25-mm Tris-glycine buffer (pH 8.3), containing 20% methanol v/v. ApoA-I-containing lipoproteins were detected by the use of a primary antibody against human apoA-I (a blend of three mouse monoclonal immunoglobulins, BIOSYS, Compiegne, France), followed by incubation with a biotinylated goat anti-mouse antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA) and then incubation with alkaline phosphatase-conjugated streptavidin (Jackson Immunoresearch Laboratories Inc.). The color reaction was started by adding the substrate solution (alkaline phosphatase-conjugate substrate kit, Bio-Rad) to the membrane and stopped by placing the membrane in distilled deionized water.

Statistical analysis

Results were expressed as mean values ± SD and comparisons of variables were performed by analysis of variance (ANOVA), *P* value <0.05 being considered statistically significant. Statistical analyses were performed using Statview II software.

RESULTS

Apolipoprotein and lipid analysis

The apolipoproteins and lipids were assayed in the serum of PILBD patients and control subjects (**Table 1**). As previously noted (2), children with severe icterus (bilirubin levels >100 μmol/l, group I) had HDL-TC and apoA-I concentrations, respectively, reduced by 40% and 42% compared to the controls, whereas children with mild icterus (bilirubin levels <100 μmol/l, group II) had significantly increased HDL-TC and apoA-I concentrations. The analysis of the serum HDL parameters confirmed that the HDL-PL and LpA-I concentrations from group I patients were not different from the controls, whereas those from group II patients were more than doubled compared to controls. Thus, in both groups of PILBD patients, HDL were apparently enriched with phospholipids, as shown by the significant increase in the HDL-PL to serum apoA-I ratio (Table 1). As a particular feature of HDL in PILBD patients, the proportion of esterified cholesterol was significantly lower than in the control HDL. This reduction was mild in group II and more pronounced in group I. Moreover, the importance of the standard deviation value for HDL-EC percentage in both groups, compared to the control, also suggested a certain heterogeneity of the biochemical modifications among the patients. Finally the calculation of the HDL-PL to FC ratio showed a nonsignificant trend for a diminution in patients from group I.

TABLE 1. Serum lipids and apolipoproteins in PILBD patients and controls

	Controls n = 14	Group I n = 13	Group II n = 12
Bilirubin (μmol/l)	<17	>100	<100
Triglycerides (mmol/l)	0.92 ± 0.51	2.74 ± 1.18[b]	1.92 ± 1.57[a]
Total cholesterol (mmol/l)	4.18 ± 0.91	25.87 ± 11.09[b]	9.13 ± 2.44[a]
Free cholesterol (mmol/l)	1.11 ± 0.35	19.75 ± 11.42[b]	3.04 ± 1.24
Phospholipids (mmol/l)	2.31 ± 0.52	20.15 ± 9.80[b]	5.14 ± 1.80[a]
ApoB (g/l)	0.71 ± 0.16	2.30 ± 0.46[b]	1.10 ± 0.32[a]
ApoA-I (g/l)	1.23 ± 0.17	0.71 ± 0.29[b]	1.71 ± 0.36[b]
LpA-I (g/l)	0.38 ± 0.10	0.46 ± 0.19	0.92 ± 0.19[b]
LpA-I:A-II (g/l)	0.86 ± 0.17	0.27 ± 0.16[b]	0.81 ± 0.33
HDL-TC (mmol/l)	0.83 ± 0.22	0.50 ± 0.17	1.94 ± 1.11[b]
HDL-PL (mmol/l)	0.54 ± 0.15	0.62 ± 0.30	1.99 ± 0.96[b]
HDL-FC (mmol/l)	0.15 ± 0.04	0.29 ± 0.16	0.68 ± 0.47[b,c]
HDL-EC (mmol/l)	0.68 ± 0.20	0.20 ± 0.13[b]	1.35 ± 0.67[b,c]
HDL-EC (%)	80.96 ± 4.97	41.69 ± 25.01[b]	68.9 ± 10.3[a,c]
HDL-PL/FC (molar ratio)	3.56 ± 1.01	2.70 ± 1.72	3.30 ± 1.17[c]
HDL-PL/apoA-I[d]	0.44 ± 0.13	0.95 ± 0.34[b]	1.11 ± 0.47[b]

Data are presented as mean ± SD.
[a]Significantly different from control $P < 0.05$.
[b]Significantly different from control $P < 0.001$.
[c]FC determination in HDL has been determined on 11 patients in group II.
[d]Serum apoA-I concentration.

Cholesterol efflux to serum

To examine the properties of serum from the two groups of PILBD patients in promoting cholesterol efflux in vitro, we measured the efflux of labeled cholesterol from Fu5AH cells after 4 h of incubation. The average fractional efflux of group II serum specimens (n = 12, 44.2 ± 6.5%) was significantly higher than that of the control sera (n = 14, 30.1 ± 4.2% ; $P < 0.001$), as were the concentrations of the HDL-related parameters assayed in these sera. On the other hand, the average fractional efflux of the patients' sera with low HDL concentrations (group I: n = 13, 23.7 ± 7.9%) was slightly decreased compared to the average value of the control sera but the difference was not significant. The lack of significance was likely due to the large deviation of the efflux individual values in the group I. In **Fig. 1**, the individual fractional efflux values are plotted with HDL-TC (1A), HDL-PL (1B), apoA-I (1C), and LpA-I (1D) concentrations. Figure 1 reveals the large range of variations in efflux values and in the HDL parameters in the two groups of patients compared with the control group, which displayed much more homogeneous values. Although all parameters were strongly correlated with fractional efflux, the best correlation was obtained with apoA-I concentrations. It was previously established that cholesterol efflux from Fu5AH best correlated with HDL-PL concentrations (17). A separate analysis, in each group taken with the controls, of the influence of HDL-PL on cholesterol efflux, including HDL-PL concentrations, HDL-PL to apoA-I ratio and HDL-PL to FC ratio, is presented in **Fig. 2**. There is a complete absence of correlation between cholesterol efflux and HDL-PL concentrations (Fig. 2A), and a striking negative correlation with the HDL-PL to apoA-I ratio (Fig. 2B)

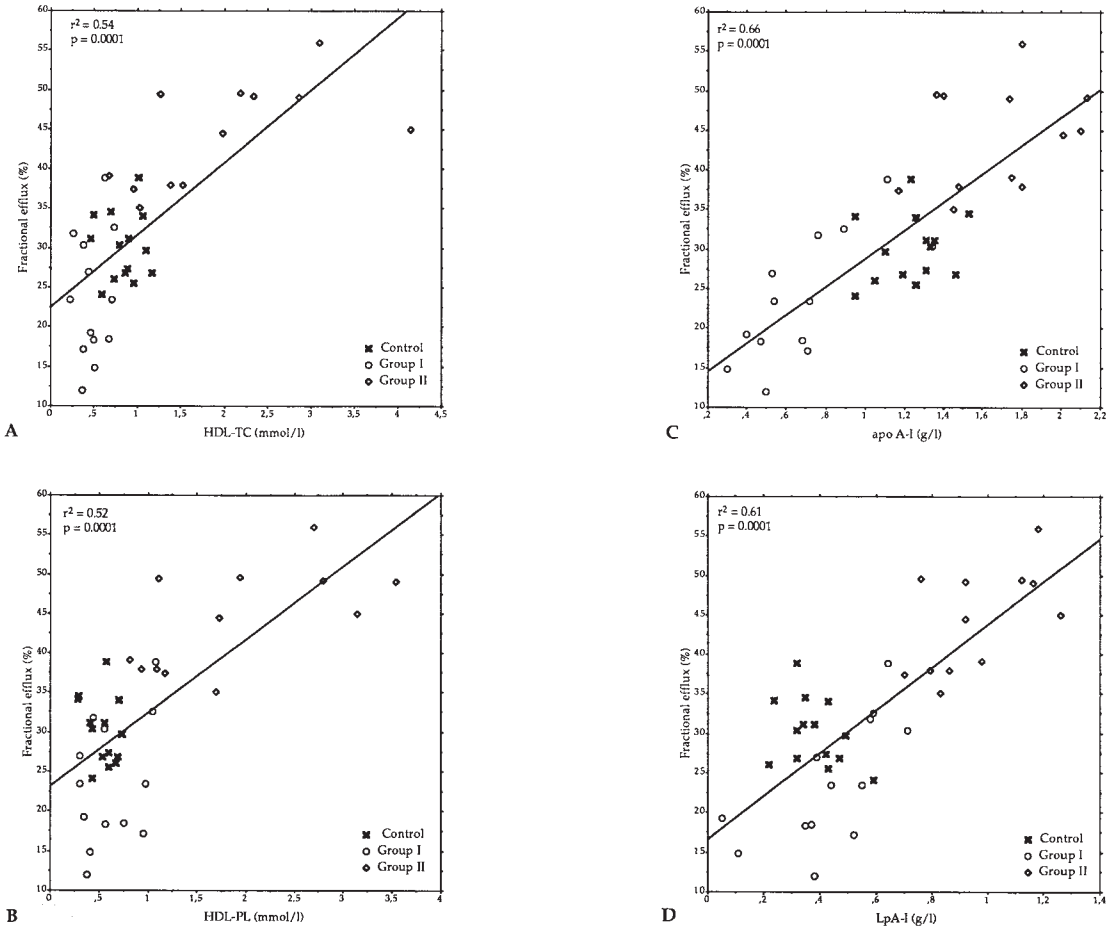


Fig. 1. Scattergrams of the correlation between the fractional efflux of cholesterol to 5% serum and the serum concentrations of HDL-TC (A), HDL-PL (B), apoA-I (C), and LpA-I (D). Black cross, control samples; circle, group I samples, diamond, group II samples.

when patients from group I and control were taken together, whereas a significant positive correlation (r^2 = 0.41; $P < 0.0004$) was found with HDL-PL to FC ratio (Fig. 2C). An opposite and more expected pattern of correlations was found when patients from group II and control were taken together, as cholesterol efflux was strongly correlated with HDL-PL concentrations (Fig. 2D) and HDL-PL to apoA-I ratio (Fig. 2E) and not with the HDL-PL to FC ratio (Fig. 2F). Thus, whereas HDL-PL remains a major determinant for cholesterol efflux in the serum of mild icteric PILBD patients, the variability of cholesterol efflux values in severely icteric PILBD patients seemed to be mainly determined by the important variability of the HDL-PL to FC ratio.

Cholesterol efflux to isolated HDL

To further characterize the efflux properties of HDL in each group, total HDL were isolated by ultracentrifugation from the serum of group I (n = 6), group II (n = 5), and control (n = 4) subjects. HDL lipid composition was analyzed and fractional efflux was measured at a constant concentration of 100 μg/ml HDL-PL. Results are presented in **Table 2** as individual values. In addition to the differences in HDL lipid concentrations mentioned above, the PILBD patients were characterized by a wide range of variations in the HDL-PL to FC ratio. These variations were in great part due to the variations in the level of esterification of the cholesterol in the HDL. This is illustrated in **Fig. 3A** showing the positive relationship between HDL-PL to FC ratio and the % of esterified cholesterol in the HDL. Cellular cholesterol efflux to isolated HDL at a constant concentration of phospholipids also exhibited important differences among the patients. Figure 3B shows that these variations in fractional efflux were strongly correlated with the PL to FC ratio in the HDL. The lowest HDL-PL to FC ratio and fractional efflux values were obtained in subjects from group I but some subjects from the same group exhibit values in the control range.

Analysis of HDL size and apoA-I distribution among particles

Figure 4 shows representative patterns of apoA-I distribution obtained after nondenaturating two-dimensional electrophoresis of samples from control, group I, and group II sera. In the control sample (Fig. 4A), most of apoA-I was found associated with α-migrating particles and distributed in HDL_{2b}, HDL_{2a}, and HDL_3 subfractions, whereas pre-β-migrating apoA-I represented only a small fraction of total apoA-I. Because of the relative heteroge-

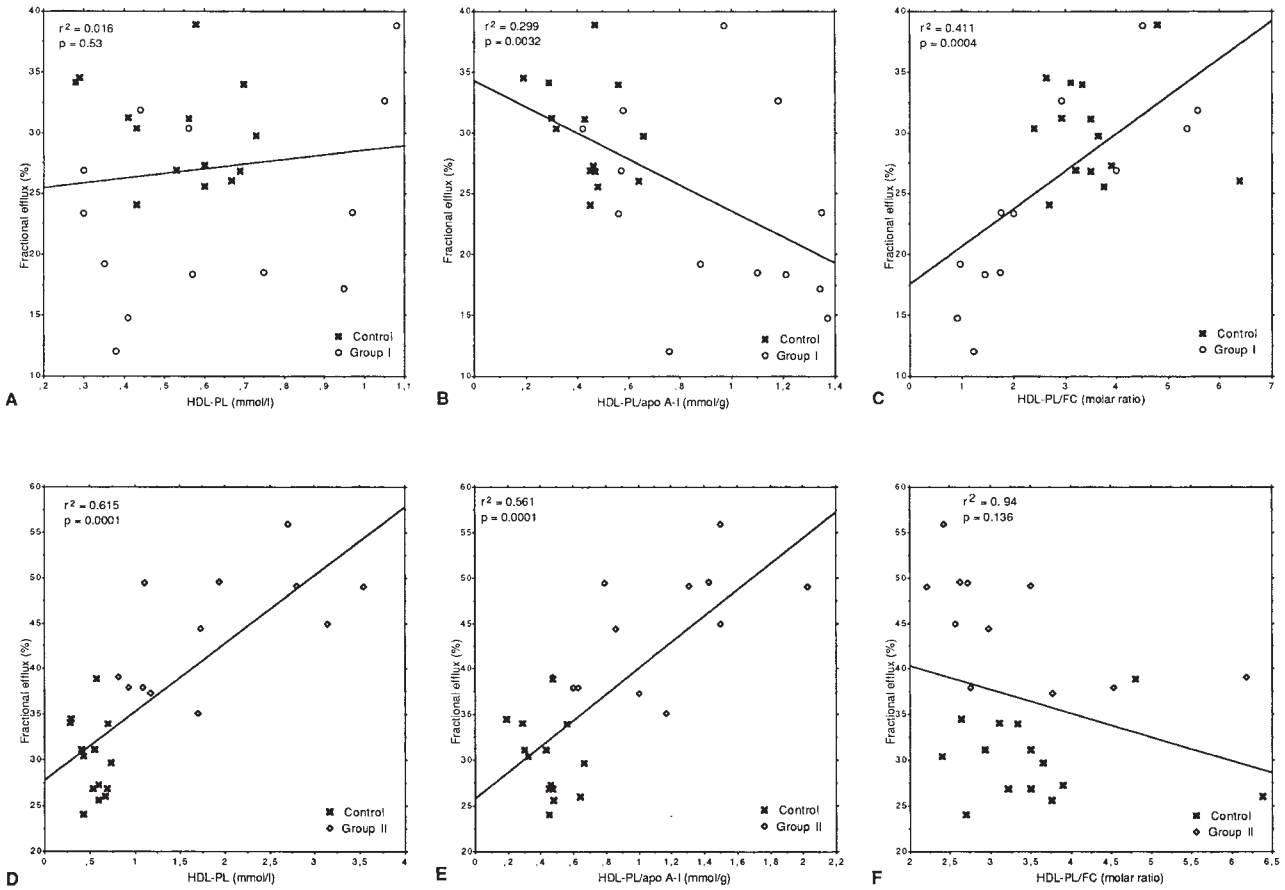


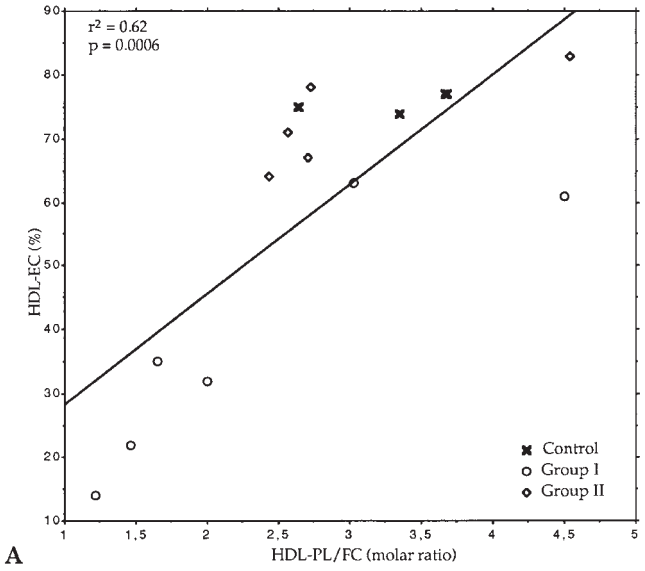
Fig. 2. Scattergrams of the correlation between the fractional efflux of cholesterol to 5% serum and the HDL-PL concentrations (A and D), the HDL-PL to apoA-I ratio (B and E), and the HDL-PL to FC ratio (C and F) in the group I and control samples (A, B, C) and in the group II and control samples (D, E, F). Black cross, control samples; circle, group I samples, diamond, group II samples.

neity of the group I, four different samples were analyzed, corresponding to patients I1, I2, I5, and I6 in Table 2. An equivalent amount of apoA-I applied to a 2D gel electrophoresis showed a distribution exclusively among HDL_3 and pre-β-migrating form in patients I1 and I2 (Figs. 4B and 4C), whereas the proportion of large α-migrating par-

TABLE 2. Lipid analysis and fractional efflux mediated by isolated HDL in individual subjects in each group

	Fractional Efflux to HDL-PL 100 μg/ml	HDL-PL	HDL-TC	HDL-FC	HDL-EC	HDL-EC[a]	HDL-PL/FC Molar Ratio
	%	mM	mM	mM	mM	%	
Controls							
C1	42.0	0.88	1.04	0.24	0.67	77	3.67
C2	36.9	0.81	0.95	0.22	0.82	77	3.68
C3	35.1	0.74	1.10	0.28	0.73	75	2.64
C4	34.2	0.77	0.90	0.23	0.80	74	3.35
Group I							
I1	10.0	0.38	0.36	0.31	0.05	14	1.22
I2	15.8	0.30	0.22	0.15	0.07	32	2.00
I3	16.7	0.56	0.52	0.34	0.18	35	1.65
I4	20.8	0.57	0.50	0.39	0.11	22	1.46
I5	29.7	0.91	0.81	0.30	0.51	63	3.03
I6	39.7	1.08	0.62	0.24	0.38	61	4.50
Group II							
II1	24.2	3.14	4.15	1.22	2.93	71	2.57
II2	28.2	1.11	1.26	0.41	0.85	67	2.71
II3	32.0	0.93	1.57	0.34	1.23	78	2.73
II4	39.5	1.09	1.38	0.24	1.14	83	4.54
II5	39.7	2.70	3.10	1.11	1.99	64	2.43

[a]Percentage calculated as (HDL-EC/HDL-TC) × 100.



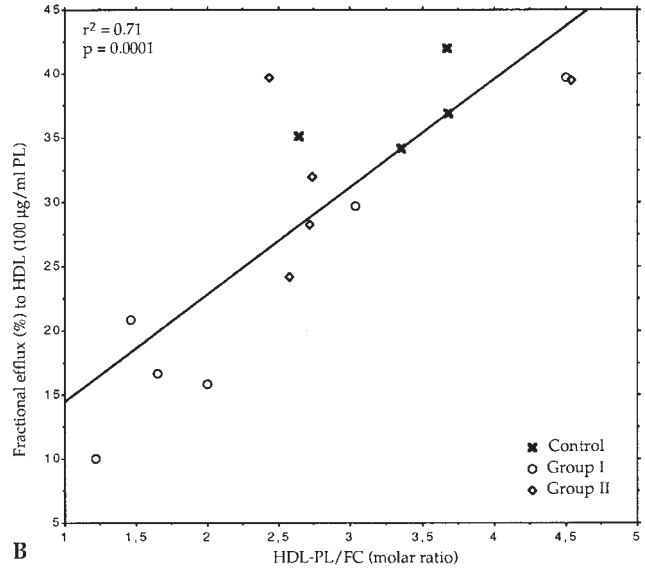


Fig. 3. Scattergrams of the relationship between the HDL-PL to FC ratio and (A) the percentage of esterified cholesterol and (B) the fractional efflux to 100 μg/ml PL of isolated HDL. Black cross, control samples; circle, group I samples, diamond, group II samples.

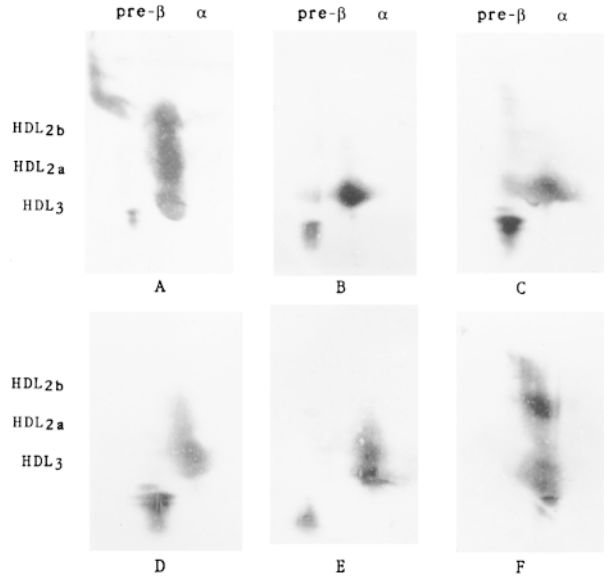


Fig. 4. Distribution of apoA-I in the HDL determined by two-dimensional gel electrophoresis of a control serum (A), group I patients' sera (B, C, D, E), and a group II patient's serum (F). The volume of serum applied on the agarose gel (first dimension) has been adjusted for each serum sample on the basis of a constant amount of 7.5 μg apoA-I.

ticles increased in patients I5 and I6 (Figs. 4D and 4E). Although no quantification of pre-β apoA-I was provided, it was shown that the proportion of apoA-I migrating in pre-β position was increased in the sera of group I patients compared to control sera and, that among them, the proportion of large α-migrating particles was more dramatically decreased when the percent of esterified cholesterol and the HDL-PL to FC ratio were low. Finally, in serum from group II patients, apoA-I was mainly distributed among the larger HDL_{2b} and the HDL_3 fractions whereas intermediate size HDL_{2a} was diminished and pre-β-migrating apoA-I was below the sensitivity threshold of our detection method (Fig. 4F).

DISCUSSION

The ability of serum from normolipidemic and PILBD children to promote cellular cholesterol efflux was determined using Fu5AH rat hepatoma cells in conditions previously validated (16). In this system, it has been established that a number of HDL-related parameters such as HDL-TC, apoA-I, LpA-I, and, especially, HDL-PL concentrations were strong determinants for the efflux capacity of serum (6, 16, 17). Moreover, it is now apparent that cholesterol efflux from Fu5AH is mediated through an interaction of extracellular acceptors with the SR-BI receptor, which is highly expressed in this cell type (18, 19).

The present study, using serum from subjects exhibiting dramatic modifications of their lipoprotein profile, allowed us to determine precisely the relative impact of HDL qualitative and quantitative features on their capacity to accept cellular cholesterol. From our previous study (2), the PILBD patients were divided into two major groups according to the degree of the icterus, the severely icteric children (group I) being characterized by a decrease in HDL concentrations and the mildly icteric children (group II) being characterized by an increase in HDL concentration. According to previous works (16, 17), elevated fractional efflux values obtained in group II patients were expected as all serum critical HDL parameters (HDL-TC, HDL-PL, apoA-I, and LpA-I) were increased. On the other hand, the slight and nonsignificant decrease in efflux values observed in the group of patients with severe icterus (group I) must result, in a first approach, from their abnormal lipid profile associating low HDL-TC and apoA-I concentrations with normal HDL-PL

and LpA-I concentrations, on average. A large range of variation in efflux values was observed in group I, which was not correlated with variations in HDL-PL concentration, as shown in Fig. 2A. This observation contrasts with the strong positive correlation obtained in group II (Fig. 2D) and also with a number of recent reports showing that HDL-PL content is a major determinant of the fractional release of cholesterol from cells expressing high levels of SR-BI receptor (19). Moreover, HDL, in both group I and group II, were enriched with phospholipids, as assessed by the increase in the HDL-PL to apoA-I ratio, but this ratio was positively correlated with the fractional efflux values only in group II, whereas in group I this correlation was strikingly negative. Thus the variations in efflux values in the group I were not driven by variations in phospholipids but rather were influenced by important variations in the HDL-PL to FC ratio. This was shown by the strong positive correlations between fractional efflux and the HDL-PL to FC ratio, obtained in whole serum (Fig. 2C), and when isolated HDL from control and patients' sera were incubated with Fu5AH cells at a constant phospholipid amount (Fig. 3B).

The abnormal large range of variation of the PL to FC ratio in the patients was partly due to the variations in the proportion of esterified cholesterol. Undoubtly, the very low PL to FC ratios found in a number of patients from group I were due to very low esterified cholesterol in relation to the decreased LCAT activity previously described in these patients (2). It is emphasized here that the reduction in LCAT activity in some stage of the PILBD disease plays a central role in modulating the lipid profile and metabolic steps such as cellular cholesterol efflux. Our data are in agreement with those of Ohta et al. (20), who have also reported a reduced efflux to isolated LpA-I and LpA-I:A-II from patients with homozygote LCAT-deficiency. In this later report, by calculating the HDL-PL to FC ratio in the isolated LpA-I and LpA-I:A-II from LCAT-deficient patients, it appears that this ratio was much lower in the homozygotes than in the heterozygotes and controls. It is not unlikely that this difference contributes to the difference in efflux observed.

Earlier studies have emphasized the role of the PL to FC ratio of extracellular acceptors in modulating the bidirectional flux of cholesterol between cells and lipoproteins (21). Basically, native or artificial particles enriched with free cholesterol, with respect to phospholipids, will behave as cholesterol donors, rather than as cholesterol acceptors, when incubated with Fu5AH cells. (22, 23). The consequent increase in intracellular cholesterol content will thus result from an increase in cholesterol influx and not from a decrease in the release of cholesterol. On the other hand, the treatment of HDL by phospholipases, PLA2 or hepatic lipase, produces PL-depleted particles without changing the cholesterol content of the lipoprotein (24). In the Fu5AH system, these particles with low HDL-PL to FC ratio will also induce an increase in the intracellular cholesterol content, by reducing the efflux of free cholesterol rather than by increasing the influx (25). These later observations are consistent with the more recent data showing the direct effect of PL enrichment on the capacity of serum to remove cholesterol from SR-BI-rich cells (19, 26). Apparently, the results obtained with the HDL of the severely icteric PILBD patients do not fit these models as the decrease in PL to FC ratio in the HDL with low efflux capacity is due to an increase in free cholesterol and not to a decrease in PL, which, according to Johnson et al. (23), should not modify the efflux capacity. It is likely that the measurement of the intracellular cholesterol content would have shown a net increase in the presence of serum or HDL from patients with low PL to FC ratio, but the explanation for the decreased efflux capacity remains to be clarified.

The last piece of information provided by this study is the variability of HDL size and apoA-I distribution. Whereas patients from group II mostly displayed a size profile quite similar to the controls, patients from group I exhibited an HDL pattern where small size HDL_3 and pre-β HDL were predominant, especially in the samples of subjects with low HDL-PL to FC ratio (Figs. 4B and C). An elevation of the pre-β HDL fraction has already been reported in the serum of LCAT-deficient patients (12). Small size acceptors, especially pre-β HDL, have been demonstrated to be the most efficient cholesterol acceptors in fresh plasma (13). Larger acceptors are less efficient in promoting cellular cholesterol efflux, but have a greater capacity to dissolve cholesterol (27). Based on these observations, recent experimental models, using macrophages or Fu5AH hepatoma cells, have demonstrated that optimal conditions for efflux can be achieved when both small and large acceptors are present through a synergistic effect of the efficiency of the small particles and of the capacity of the large phospholipid-rich acceptors (27, 28). Therefore, the deficit in large acceptors in the serum of some severely icteric PILBD patients may contribute to their low efflux capacity, in spite of the greater proportion of pre-β HDL. Although a direct regression analysis between HDL size distribution and the HDL-PL to FC ratio was not possible in this study, it appears from our illustrations (Table 2 and Fig. 4) that the deficit in large α HDL paralleled the decrease in the HDL-PL to FC ratio. Thus, a combination of a deficit in large acceptors and of an imbalance between phospholipid and free cholesterol in the acceptors seemed to be responsible for the low efflux capacity of sera from severely icteric patients. The relative mechanistic impact of these two abnormalities on the fractional release of cholesterol from Fu5AH cells remains to be clearly determined.

It is now clearly established that in PILBD disease, the degree of icterus will determine different lipoprotein profiles, which must be attributed in great part to the level of LCAT activity. However, it is also obvious from the present study that a certain heterogeneity emerged among the patients in each group, in particular in the severely icteric patients. As it has been reported that the cholestatic syndrome might improve (29) and as we have observed previously that, in such conditions, the defect in LCAT activity is reversible (2), we propose that the observed heterogeneity is a reflection of transitory stages during the disease.

The fact that, among the severely icteric patients, some exhibit a rather normal lipid profile associated with normal efflux value suggests that a restored lipid profile might precede the reduction of the cholestatic syndrome. It is also reported that in a number of patients, the severe cholestasis will not improve and secondary pathologies will appear (30, 31). In these cases it is not unlikely that the impairment in HDL metabolism that we described in some severely icteric patients would contribute to the development of cardiovascular events and thus should be considered as risk factor for complication of PILBD disease.

We would like to thank Nelly Poulain and Sophie Goyer for their valuable technical assistance. We are greatly indebted to Prof. Olivier Bernard, Pediatric Hepatology, Bicêtre Hospital, 94275 Le Kremlin-Bicêtre, France, for supplying us with the patient serum samples and for his interest throughout this investigation.

Manuscript received 6 August 1997, in revised form 23 March 1998, and in re-revised form 21 September 1998.

REFERENCES

1. Sabesin, S. M. 1982. Cholestatic lipoproteins—their pathogenesis and significance. *Gastroenterology.* **83:** 704–709.
2. Davit-Spraul, A., M. L. Pourci, V. Atger, M. Cambillau, M. Hadchouel, N. Moatti, and A. Legrand. 1996. Abnormal lipoprotein pattern in Alagille syndrome depends on icterus severity. *Gastroenterology.* **111:** 1023–1032.
3. Barter, P. 1993. High-density lipoproteins and reverse cholesterol transport. *Curr. Opin. Lipidol.* **4:** 210–217.
4. Barter, P. J., and K. A. Rye. 1996. High density lipoproteins and coronary heart disease. *Atherosclerosis.* **121:** 1–12.
5. Davidson, W. S., K. L. Gillote, S. Lund-Katz, W. J. Johnson, G. H. Rothblat, and M. C. Phillips. 1995. The effect of high density lipoprotein phospholipid acyl chain composition on the efflux of cellular free cholesterol. *J. Biol. Chem.* **270:** 5882–5890.
6. Fournier, N., M. de la Llera Moya, B. F. Burkey, J. B. Swaney, J. Paterniti, N. Moatti, V. Atger, and G. H. Rothblat. 1996. Role of HDL phospholipid in efflux of cell cholesterol to whole serum: studies with human apoA-I transgenic rats. *J. Lipid Res.* **37:** 1704–1711.
7. Hayek, T., J. Oiknine, J. G. Brook, and M. Aviram. 1994. Role of HDL apolipoprotein E in cellular cholesterol efflux: studies in apo E knockout trangenic mice. *Biochem. Biophys. Res. Commun.* **205:** 1072–1076.
8. Barkia, A., P. Puchois, N. Ghalim, G. Torpier, R. Barbaras, G. Ailhaud, and J. C. Fruchard. 1991. Differential role of apolipoprotein A-I-containing particles in cholesterol efflux from adipose cells. *Atherosclerosis.* **87:** 135–146.
9. Von Eckardstein, A., Y. Huang, and G. Assmann. 1994. Physiological role and clinical relevance of high-density lipoprotein subclasses. *Curr. Opin. Lipidol.* **5:** 404–416.
10. Fielding, C. J., and Fielding P. E. 1995. Molecular physiology of reverse cholesterol transport. *J. Lipid Res.* **36:** 211–228.
11. Rader, D. J., K. Ikewaki, N. Duverger, H. Schmidt, H. Pritchard, J. Frohlich, M. Clerc, M. F. Dumon, T. Fairwell, L. Zech, S. Santamarina-Fojo, and H. B. Brewer. 1994. Markedly accelerated catabolism of apolipoprotein A-II (apoA-II) and high density lipoproteins containing apoA-II in classic lecithin:cholesterol acyltransferase deficiency and fish-eye disease. *J. Clin. Invest.* **93:** 321–330.
12. Von Eckardstein, A., Y. Huang, S. Wu, H. Funke, G. Noseda, and G. Assmann. 1995. Reverse cholesterol transport in plasma of patients with different forms of familial HDL deficiency. *Arterioscler. Thromb. Vasc. Biol.* **15:** 691–703.
13. Castro, G. R., and C. J. Fielding. 1988. Early incorporation of cell-derived cholesterol into pre-β-migrating high-density lipoprotein. *Biochemistry.* **27:** 25–29.
14. Atger, V., F. Duval, K. Frommherz, T. Drüeke, and B. Lacour. 1988. Abnormalities in composition of uremic lipoproteins isolated by gradient ultracentrifugation: relative enrichment of HDL in apolipoprotein C-III at the expense of apolipoprotein A-II. *Atherosclerosis.* **74:** 75–83.
15. Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83:** 346–356.
16. De la Llera Moya, M., V. Atger, J. L. Paul, N. Fournier, N. Moatti, P. Giral, K. E. Friday, and G. Rothblat. 1994. A cell culture system for screening human serum for ability to promote cellular cholesterol efflux. *Arterioscler. Thromb.* **14:** 1056–1065.
17. Fournier, N., J. L. Paul, V. Atger, A. Cogny, T. Soni, M de la Llera-Moya, G. Rothblat and N. Moatti. 1997. HDL-phospholipid content and composition as a major factor determining cholesterol efflux capacity from Fu5AH cells to human serum. *Arterioscler. Thromb. Vasc. Biol.* **17:** 2685–2691.
18. Ji, Y., B. Jian, N. Wang, Y. Sun, M. de la Llera-Moya, M. C. Phillips, G. H. Rothblat, J. B. Swaney, and A. R. Tall. 1997. Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J. Biol. Chem.* **272:** 20982–20985.
19. Jian, B., M. de la Llera-Moya, Y. Ji, N. Wang, M. C. Phillips, J. B. Swaney, A. R. Tall, and G. H. Rothblat. 1998. Scavenger receptor class B type I as a mediator of cellular cholesterol efflux to lipoproteins and phospholipid acceptors. *J. Biol. Chem.* **273:** 5599–5606.
20. Ohta, T., R. Nakamura, Y. Ikeda, J. Frohlich, K. Takata, Y. Saito, S. Horiuchi, and I. Matsuda. 1994. Evidence for impaired cellular cholesterol removal mediated by apoA-I-containing lipoproteins in patients with familial lecithin:cholesterol acyltransferase deficiency. *Biochim. Biophys. Acta.* **1213:** 295–301.
21. Johnson, W. J., F. H. Mahlberg, G. H. Rothblat, and M. C. Phillips. 1991. Cholesterol transport between cells and high-density lipoproteins. *Biochim. Biophys. Acta.* **1085:** 273–298.
22. Arbogast, L. Y., G. H. Rothblat, M. H. Leshe, and R. A. Cooper. 1976. Cellular cholesterol ester accumulation induced by free cholesterol-rich lipid dispersions. *Proc. Natl. Acad. Sci. USA.* **73:** 3680–3684.
23. Johnson, W. J., M. J. Bamberger, R. A. Latta, P. E. Rapp, M. C. Phillips, and G. H. Rothblat. 1986. The bidirectional flux of cholesterol between cells and lipoproteins. *J. Biol. Chem.* **261:** 5766–5776.
24. Bamberger, M., J. M. Glick, and G. H. Rothblat. 1983. Hepatic lipase stimulates the uptake of high density lipoprotein cholesterol by hepatoma cells. *J. Lipid Res.* **24:** 869–876.
25. Bamberger, M., S. Lund-Katz, M. Phillips, and G. Rothblat. 1985. Mechanism of the hepatic lipase-induced accumulation of high density lipoprotein cholesterol by cells in culture. *Biochemistry.* **24:** 3693–3701.
26. Jian, B., M. de la Llera-Moya, L. Royer, G. H. Rothblat, O. Francone, and J. B. Swaney. 1997. Modification of the cholesterol efflux properties of human serum by enrichment with phospholipid. *J. Lipid Res.* **38:** 734–744.
27. Rodrigueza, W. V., K. J. Williams, G. H. Rothblat, and M. C. Phillips. 1997. Remodeling and shuttling. Mechanisms for the synergistic effects between different acceptor particles in the mobilization of cellular cholesterol. *Arterioscler. Thromb. Vasc. Biol.* **17:** 383–393.
28. Atger, V. M., M. de la Llera-Moya, G. W. Stoudt, W. V. Rodrigueza, M. C. Phillips, and G. H. Rothblat. 1997. Cyclodextrins as catalysts for the removal of cholesterol from macrophage foam cells. *J. Clin. Invest.* **99:** 773–780.
29. Alagille D., A. Estrada, M. Hadchouel, M. Gautier, M. Odievre, and J. P. Dommergues. 1987. Syndromic paucity of interlobular bile ducts (Alagille syndrome or arteriohepatic dysplasia): review of 80 cases. *J. Pediatr.* **110:** 195–200.
30. Hoffenberd E. J., M. R. Narkewicz, J. M. Sandheimer, D. J. Smith, A. Silverman, R. J. Sokol. 1995. Outcome of syndromic paucity of interlobular bile ducts (Alagille syndrome) with onset of cholestatis in infancy. *J. Pediatr.* **127:** 220–224.
31. Deleuze J. F., S. Dhorne-Pollet, N. Pollet, M. Meunier-Rotival, and M. Hadchouel. 1995. Le syndrome d'Alagille en 1995. Données cliniques et génétiques. *Gastroenterol. Clin. Biol.* **19:** 587–596.