Effect of apolipoprotein activators on the specificity of lecithin:cholesterol acyltransferase: determination of cholesteryl esters formed in A-I/C-III deficiency

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Abstract Although it is known that plasma lecithin:cholesterol acyltransferase (LCAT) is activated by several apolipoproteins (apo) including A-I, C-I, D, A-IV, and E, it is not clear what the physiological importance of having different apolipoprotein activators is. One possible explanation is that the activation by different apolipoproteins may result in the utilization of different species of phosphatidylcholine (PC), leading to the formation of different species of cholesteryl esters (CE). In order to determine this possibility, we analyzed the molecular species composition of PC and CE in two patients with familial deficiency of apoA-I and apoC-III. The LCAT activity, assayed by three different procedures, was found to be 36–63% of the control value. The lower LCAT activity, however, was due to deficiency of the enzyme rather than the absence of apoA-I. The patients' plasma was relatively enriched with *sn*-2 18:2 PC species reflecting the partial deficiency of LCAT activity. The fatty acid composition of plasma CE was not significantly different from that of controls. HPLC analysis of labeled CE formed after incubation of plasma with [C^{14}]cholesterol showed no significant difference in the species of CE synthesized by the LCAT reaction. The transfer of pre-existing as well as newly formed CE from HDL to the apoB-containing lipoproteins was accelerated compared to control plasma. These results show that the absence of apoA-I does not significantly affect either the activity or the specificity of LCAT, and that the other apolipoprotein activators can substitute adequately for it.—**Subbaiah, P. V., R. A. Norum, and J. D. Bagdade.** Effect of apolipoprotein activators on the specificity of lecithin:cholesterol acyltransferase: determination of cholesteryl esters formed in A-I/C-III deficiency. *J. Lipid Res.* 1991. **32:** 1601–1609.

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Lecithin:cholesterol acyltransferase (LCAT) reaction is believed to be responsible for the formation of most of the cholesteryl esters (CE) present in human plasma (1, 2). This enzyme acts preferentially on certain high density lipoprotein (HDL) particles, and its activity is absolutely dependent on the presence of certain apolipoprotein activators (3, 4). The most important of these activators is apoA-I, the major apolipoprotein of HDL (5). However, other apolipoproteins including A-IV, D, E, and C-I have also been shown to activate LCAT to varying degrees in vitro (6–9). It is not known, however, to what extent these apolipoproteins are physiological activators of the enzyme or whether the specificity of the enzyme with regard to the molecular species of phosphatidylcholine (PC) utilized or CE formed is affected by the apolipoprotein activators. Using isolated enzyme, Steinmetz and Utermann (7) as well as Chen and Albers (6) have shown that apoA-IV is a better activator in the presence of disaturated PC, while apoA-I was a better activator in the presence of unsaturated PC. Similarly Soutar et al. (9) reported that while apoA-I was most effective with unsaturated PC species, apoC-I activated the enzyme equally with both saturated and unsaturated PC species. It is therefore possible that different PC species are utilized in the presence of specific apolipoprotein activators, resulting in the formation of different species of CE.

We have previously shown that, in normal human plasma, LCAT preferentially utilized certain molecular species of PC (10). However, it is not known whether this specificity is affected by the presence of different apolipoprotein activators. In order to study the role of apoA-I, the primary activator of LCAT, in determining the specificity of the enzyme we determined the molecular species composition of PC and CE in the plasma of two A-I/C-III-deficient patients with no detectable apoA-I but with significant LCAT activity (11). We have also studied the

Abbreviations: apo, apolipoprotein; LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; CE, cholesteryl esters; PC, phosphatidylcholine; DTNB, 5,5'dithiobis(2-nitrobenzoic acid); CET, cholesteryl ester transfer.

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species of labeled CE formed in the patients' plasma after incubation with labeled cholesterol. The results presented here show that although the enzyme activity is lower than in normal plasma, the A-I/C-III deficiency does not affect the molecular species of CE synthesized by LCAT, indicating that apoA-I is not necessary in determining the specificity of the enzyme.

MATERIALS AND METHODS

Patients

Two sisters (aged 39 and 37) with familial deficiency of apolipoproteins A-I and C-III were studied. The clinical characteristics of these patients and their molecular defects have been described previously (11). Both patients had premature atherosclerosis, corneal clouding, and xanthomas, but were not on any lipid-lowering drugs at the time of the study. However, one of the patients (patient 2) was taking soybean lecithin capsules (1200 mg/day) as a dietary supplement. Blood was drawn in EDTA (1 mg/ml) after an overnight fast and the separated plasma samples were shipped on wet ice from Detroit to Chicago overnight. The enzyme and transfer assays were performed within 3 days of blood drawing. The analysis of lipid composition was performed on plasma samples frozen at −30°C, within 3 weeks of blood drawing. Normolipidemic controls (ages 26–40) were included in each of the studies reported.

LCAT assays

The LCAT activity was assayed by three different methods. *1*) In the Stokke and Norum procedure (12) the ^{14}C-labeled cholesterol was equilibrated with endogenous cholesterol in the presence of 1.4 mM DTNB at 37°C. Subsequently the inhibition was released by the addition of excess (12 mM) mercaptoethanol and the formation of labeled cholesteryl ester (CE) was determined after incubation at 37°C for 30 min. The lipids were extracted by the Bligh and Dyer procedure (13) and the free cholesterol and CE were separated on silica gel TLC plates using the solvent system of petroleum ether–ethyl acetate 85:15. The spots were scraped from the plate and the radioactivity was determined in a liquid scintillation counter. The fractional esterification rate was determined as percent of labeled cholesterol esterified per hour. The molar esterification rate was calculated by multiplying the fractional rate with the concentration of free cholesterol in the plasma. *2*) In the 4°C procedure, the labeled cholesterol stabilized with albumin (12) was equilibrated with free cholesterol in the plasma by incubation at 4°C for 18 h in the absence of any LCAT inhibitor. The plasma sample was then incubated at 37°C for 1 h and the LCAT activity was determined as described above. The plasma labeled by this procedure was also used for studying the distribution of newly synthesized cholesteryl esters between lipoprotein fractions, and for studying the molecular species of CE formed by LCAT reaction (see below). *3*) In the proteoliposome procedure (14) the plasma was incubated with a proteoliposome substrate containing apoA-I, [^{14}C]cholesterol, and egg PC at the molar ratio of 0.8:12.5:250 for 30 min at 37°C, and the percent of free cholesterol esterified was determined as described above. The studies of Chen and Albers (14) suggest that the activity measured by this method reflects the mass of active LCAT protein and that it is not influenced by the concentration and composition of endogenous substrates.

Assay of cholesteryl ester transfer (CET) activity

The transfer of CE from HDL fraction to the apoB-containing lipoproteins (VLDL and LDL) was studied by two different procedures *1*) Mass transfer assay. The plasma sample was incubated at 37°C in the presence of 1.5 mM DTNB in a metabolic shaker. Aliquots of the incubation mixture were withdrawn at 0, 1, 2, 4, and 6 h of incubation, chilled on ice, and the apoB-containing lipoproteins were precipitated with 0.1 volume of heparin–$MnCl_2$ (15). The supernatant containing the HDL was analyzed for free and total cholesterol using enzymatic kits (Boehringer Mannheim), and the amount of CE was calculated as the difference between total and free cholesterol. The decrease in HDL CE during incubation was taken as the net transfer of preformed CE from HDL to VLDL and LDL. *2*) Isotopic CE transfer assay. HDL from normal plasma was labeled with [^{14}C]cholesteryl oleate by the procedure of Hough and Zilversmit (16). To 450 μl of plasma 50 μl of labeled HDL (25,000 dpm and 12 μg of cholesterol) was added and incubated at 37°C. Aliquots (100 μl) of the reaction mixture were taken out at 0, 15, 30, 45, and 60 min and added to a mixture containing 50 μl of heparin–$MnCl_2$ (15) and 250 μl of 0.15 M NaCl. After being kept on ice for 30 min, the precipitate was washed once with 0.4 ml of 0.15 M NaCl and the supernatants were combined. The precipitate was then resuspended in 0.4 ml 0.15 M NaCl and transferred to a scintillation vial and the radioactivity was determined in a liquid scintillation counter after addition of 5 ml scintillation fluid (Cytoscint, ICN Biomedics, Irvine, CA). Radioactivity was also determined in an aliquot of the combined supernatants. The percent of labeled CE transferred to the VLDL + LDL at each time interval was calculated from these results. All values were corrected for the amount of label present in the precipitate at 0 min.

Because the absence of apoA-I results in abnormal lipoprotein composition in patients' plasma, it is necessary to demonstrate that the heparin–Mn^{2+} precipitation separated the VLDL + LDL fraction from HDL adequately. For this purpose, we compared the lipid composition of the lipoproteins prepared by ultracentrifugation at $d < 1.063$

g/ml with that of the lipoproteins separated by heparin-Mn^{2+} precipitation. As shown in **Table 1**, about 80% of the total phospholipid and 93% of the total cholesterol were found in the VLDL + LDL fraction prepared by either of the procedures. These results indicate that the heparin-Mn^{2+} precipitation is a valid procedure to rapidly separate the VLDL + LDL from HDL in these patients.

Analysis of molecular species of PC and CE

Plasma samples were extracted by the procedure of Bligh and Dyer (13). The phospholipids were separated on silica gel TLC plates developed in chloroform-methanol-water 65:25:4 and the PC spot was eluted from the silica gel (13). The molecular species of PC were analyzed as diacyl glycerol benzoates as described earlier (10). Another aliquot of the total lipid extract from the plasma was separated on TLC plates using the solvent system of hexane-ethyl ether-acetic acid 70:30:1. The CE spot was scraped and eluted twice with 2 ml of diethyl ether. The solvent was evaporated and the methyl esters of the fatty acids were prepared by heating with 1 ml of BF_3 in methanol (14% w/v, Alltech Associates) and 1 ml petroleum ether for 90 min at 85°C. The methyl esters were extracted twice with 2 ml of petroleum ether, and after removal of the solvent under nitrogen, redissolved in 20 μl of hexane; a 2-μl aliquot was injected into a gas chromatograph (Shimadzu GC9A). The methyl esters were separated on a Supelcowax 10 fused silica column (0.25 μm thickness, 30 meters, 0.25 mm ID) using hydrogen as carrier gas. The temperature was initially set at 172°C for 8 min then raised to 220°C at the rate of 6°C/min and maintained at 220°C for 20 min. The fatty acid peaks were identified using authentic standards, and quantitated with an electronic integrator (Shimadzu C-R3A).

HPLC of labeled CE

Total lipids extracted from the reaction mixture after 6 h incubation with labeled cholesterol were filtered, concentrated, and injected onto a C-18 reverse phase HPLC column and the various species of CE were separated as described earlier (17), using the solvent system of acetonitrile-tetrahydrofuran-water 65:35:1.5. Radioactivity was determined in the collected fractions and the percent of each CE species in the total labeled CE was calculated.

RESULTS

Assay of LCAT activity

The LCAT activity was assayed in the two A-I/C-III-deficient patients and the controls by three different procedures, two of them utilizing endogenous substrates, and the third an exogenous proteoliposome substrate. *1*) In the Stokke and Norum (12) procedure, the labeled cholesterol was equilibrated with endogenous cholesterol at 37°C in the presence of 1.4 mM DTNB, following which the formation of labeled CE was determined after releasing the inhibition with excess mercaptoethanol. With this procedure, the two patients showed 44% and 36% of the mean control activity (results not shown). To determine whether the lower LCAT activity was due to the absence of apoA-I, we added 20 μg of pure apoA-I to the reaction mixture containing the A-I/C-III-deficient plasma and determined the activity. The addition of exogenous apoA-I did not increase the LCAT activity any further. In fact the activity was inhibited by about 20%, indicating that the lack of apoA-I is not the reason for lower LCAT activity in these patients. *2*) In the 4°C procedure the [^{14}C]cholesterol was equilibrated with endogenous cholesterol at 4°C in the absence of any inhibitor

TABLE 1. Validation of the heparin-Mn^{2+} precipitation procedure for A-I/C-III-deficient plasma

Patient	Total Phospholipids		Total Cholesterol	
	Centrifugation	Precipitation	Centrifugation	Precipitation
	μmol/ml plasma		*mg/dl plasma*	
Patient 1				
HDL	0.132	0.100	6.06	6.67
VLDL + LDL	0.573	0.565	91.8	97.6
% in VLDL + LDL	81.3%	83.7%	93.8%	93.6%
Patient 2				
HDL	0.151	0.120	4.90	7.2
VLDL + LDL	0.562	0.536	93.4	99.1
% in VLDL + LDL	78.8%	81.7%	95.0%	93.2%

Freshly obtained plasma from two A-I/C-III-deficient patients was subjected to either centrifugation at $d < 1.063$ g/ml for 18 h at 100,000 *g* or heparin-Mn^{2+} precipitation as described in Materials and Methods. The total phospholipids were determined by estimation of lipid phosphorus (31) and total cholesterol was determined by using an enzymatic kit (Boehringer-Mannheim).

for 18 h and then the formation of labeled CE was determined after incubation at 37°C for 1 h. With this procedure the two patients showed 60% and 49% of the mean control values (results not shown). *3*) In the proteoliposome method, a common exogenous substrate containing apoA-I, labeled free cholesterol, and egg PC (14) was added to an aliquot of the plasma and the formation of labeled CE was determined after incubation at 37°C for 30 min. Since the enzyme activity assayed by this procedure is not influenced by the composition or concentration of the endogenous substrates and activators, it is believed to reflect the mass of active LCAT protein in the plasma (14). With this assay also, the two A-I/C-III-deficient patients showed 56% and 63% control activity (results not shown). These results therefore indicate that the lower activity found with the endogenous substrates was not due to the lack of apoA-I, but probably due to partial deficiency of the enzyme itself. The previously published results of Chen and Albers (6), showing that the LCAT mass determined by radioimmunoassay was lower in the same two patients studied here, support this conclusion.

Molecular species of plasma PC

We previously showed that the composition of molecular species of PC was significantly altered in familial LCAT deficiency because certain PC species are selectively utilized by the LCAT reaction in normal human plasma and these species accumulate in the absence of the enzyme (18). To determine the effect of partial deficiency of LCAT as well as the complete absence of apoA-I and C-III on the composition of PC species in the plasma, we determined the molecular species composition of PC in the two patients by HPLC (10, 18). The analyses were done on three different occasions and the averages of the three determinations, as well as the average of value from seven normal women, are given in **Table 2**. Both the patients showed an increase in the percentage of 18:0–18:2 and a decrease in the percentage of 16:0–20:4, compared to normal plasma. One of the patients also showed a significant increase in the percentage of 16:0–18:2. These compositional abnormalities are similar to those we reported in familial LCAT-deficient patients (18), although the changes are less severe in A-I/C-III-deficient patients. These results thus indicate that the lower LCAT activity in patients' plasma has affected the PC species composition of plasma.

Composition of CE species

In order to determine whether the deficiency of the apoA-I and C-III or the decrease in the LCAT activity resulted in the alteration of plasma CE species composition, we determined the composition of plasma CE by GLC after separation of CE from other lipids by TLC. With a few minor exceptions, the fatty acid composition of plasma CE in patients and controls was remarkably similar. Both patients showed a slight decrease in 18:0 and 18:1 (n-9) (12.2% and 11.1% compared to 14.2% in control) and an increase in 20:3 (n-6) esters (1.8% and 3%, compared to 0.6% in control). These values (except for 20:3) were within one standard deviation of mean control value (results not shown). Therefore, one can conclude that deficiency of apoA-I and C-III does not alter the CE fatty acid composition significantly.

Synthesis of CE species by LCAT reaction

In order to further study whether the absence of apoA-I affected the specificity of LCAT with regard to the types of CE synthesized, we analyzed the species of labeled CE formed in vitro by the LCAT reaction. The whole plasma cholesterol was first equilibrated with [^{14}C]cholesterol by incubation at 4°C for 18 h and then the LCAT reaction was allowed to take place for 6 h at 37°C. The labeled CE formed were then separated on reverse phase HPLC as described in Materials and Methods, and the radioactivity was determined in the collected fractions. The percentage composition of the labeled CE formed in A-I/C-III-deficient plasma did not differ significantly from that in control plasma. The major CE species formed were 18:2 (42.3%, 44.8%, and 43.5% of total) 18:1 (16.0%, 16.8%, and 18.2% of total) and 16.0 (16.6%, 15.4%, and 12.4% of total) in control, patient 1, and patient 2, respectively. There were no significant differences between patients and control in the synthesis of other CE species also (results not shown). These results suggest that the activation of LCAT by apolipoproteins other than apoA-I does not result in a change in the specificity of the enzyme with regard to the CE species formed.

TABLE 2. Molecular species composition of plasma phosphatidylcholines

Species	Patient 1	Patient 2	Control (n = 7)
		% of total	
18:1-20:5	0.04 ± 0.07	0.16 ± 0.11	0.31 ± 0.17
16:0-20:5	0.15 ± 0.11	0.06 ± 0.07	0.38 ± 0.15
16:0-22:6	3.09 ± 0.35	3.29 ± 0.54	3.68 ± 0.94
18:1-20:4	0.12 ± 0.14	0.08 ± 0.09	0.83 ± 0.31
16:0-20:4	8.72 ± 0.09	6.43 ± 0.12	9.89 ± 1.96
18:0-20:5	0.38 ± 0.06	0.21 ± 0.05	0.66 ± 0.21
18:1-18:2	2.34 ± 0.04	4.27 ± 0.17	3.76 ± 0.65
16:0-18:2	31.08 ± 0.75	43.27 ± 6.06	31.59 ± 2.24
16:0-20:3	6.32 ± 0.11	1.45 ± 2.33	4.22 ± 0.94
16:0-20:3 (n-9)	0.41 ± 0.42	0.15 ± 0.27	0.92 ± 0.45
18:0-20:4	5.83 ± 0.34	3.86 ± 0.70	4.96 ± 1.07
18:1-18:1	1.06 ± 0.11	1.19 ± 0.20	1.41 ± 0.15
16:0-18:1	13.47 ± 0.53	10.07 ± 0.95	14.46 ± 2.89
18:0-18:2	18.09 ± 0.13	21.65 ± 1.72	13.87 ± 2.89
16.0-16:0 +	3.53 ± 0.34	0.66 ± 1.31	2.07 ± 0.98
18:0-18:1	2.75 ± 0.25	2.17 ± 0.65	1.94 ± 0.28

The values given for the patients are mean ± SD of four separate determinations.

Synthesis and distribution of CE in lipoprotein fractions

In normal human plasma the cholesterol esterification takes place almost entirely on HDL particles (5). Since the amount of HDL is considerably reduced in A-I/C-III deficiency, and since the activating apolipoproteins (apoC-I, apoA-IV, and apoE) are mostly present in non-HDL lipoproteins, it would be of interest to determine whether the LCAT reaction takes place on HDL only or on the other lipoproteins also. We therefore studied the percent esterification of labeled cholesterol in VLDL + LDL and HDL fractions separated by heparin-Mn^{2+} precipitation. The labeled cholesterol was first equilibrated with plasma at 4°C for 18 h and then the plasma was incubated at 37°C for 4 h. Aliquots of plasma were taken out at 30 min, 1 h, and 4 h and the VLDL + LDL were precipitated with heparin-Mn^{2+} (15). The total lipids were extracted from both supernatant and precipitate, the free cholesterol and CE were separated by TLC, and the radioactivity was determined. As shown in **Fig. 1**, the absolute percentage of cholesterol esterified was higher in HDL compared to VLDL + LDL, in both the control and patient plasma. However, when compared to normal plasma, the percentage of esterification of VLDL + LDL cholesterol was higher in patients' plasma. The percent esterification of HDL cholesterol was lower than in normal HDL at all the time intervals studies. These results suggest that in the absence of apoA-I, either relatively more cholesterol is esterified on the apoB-containing lipoproteins or that there is a very rapid transfer of newly formed CE to VLDL + LDL.

Fig. 2 shows the distribution of newly synthesized CE between VLDL + LDL and HDL fractions after various periods of incubation. As expected, the majority of the newly formed CE in the control plasma was found in HDL at all time intervals although there was a progressive increase in VLDL + LDL fraction apparently due to the transfer of CE from HDL. On the other hand, over 90% of labeled CE in A-I/C-III-deficient plasma was found in VLDL + LDL fraction even at 30 min, the earliest time interval studied. This amount corresponds to the percentage of total cholesterol mass present in VLDL + LDL (Table 1). Interestingly, the percentage of labeled CE found in VLDL + LDL increased further with time, showing that the HDL of patients has a very limited capacity to retain newly synthesized CE.

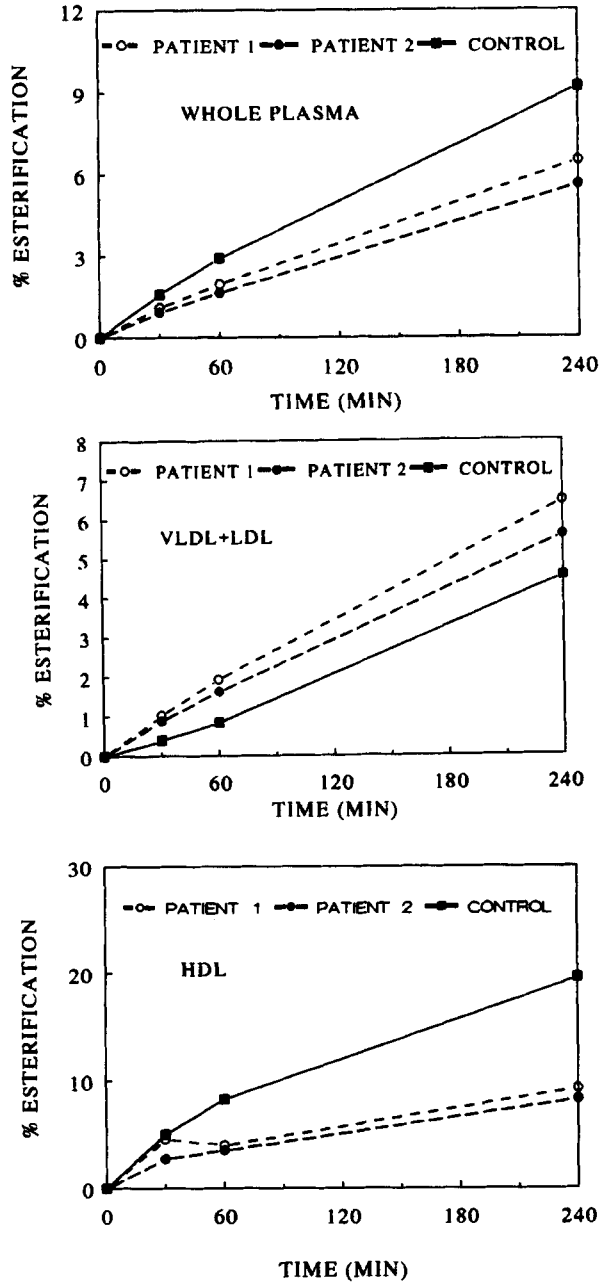

Fig. 1. Time course of cholesterol esterification in whole plasma, VLDL + LDL, and HDL in two A-I/C-III-deficient patients and one control subject. The labeled cholesterol was first equilibrated with endogenous cholesterol by incubation at 4°C for 18 h, after which the samples were incubated at 37°C. At the indicated times aliquots were removed and subjected to heparin-Mn^{2+} precipitation (15). The lipids of the precipitate (VLDL + LDL) and the supernatant (HDL) were extracted and separated on silica gel TLC plates developed in hexane-ethyl ether-acetic acid 70:30:1 (v/v). The spots corresponding to unesterified cholesterol and CE were scraped off and the radioactivity was determined in a liquid scintillation counter. The percent esterification was calculated from these results.

Assay of CE transfer

In order to study the movement of CE between plasma lipoproteins, the rate of transfer of preformed CE from HDL to apoB-containing lipoprotein was determined by two methods. In the isotopic transfer assay, normal HDL labeled with [^{14}C]cholesteryl oleate was added to the whole plasma and incubated at 37°C for 60 min. At various time intervals aliquots of plasma were withdrawn and the VLDL + LDL and HDL fractions were separated by heparin-Mn^{2+} precipitation and the radioactivity transferred to VLDL + LDL (precipitate) was determined. As shown in **Fig. 3**, the percentage of

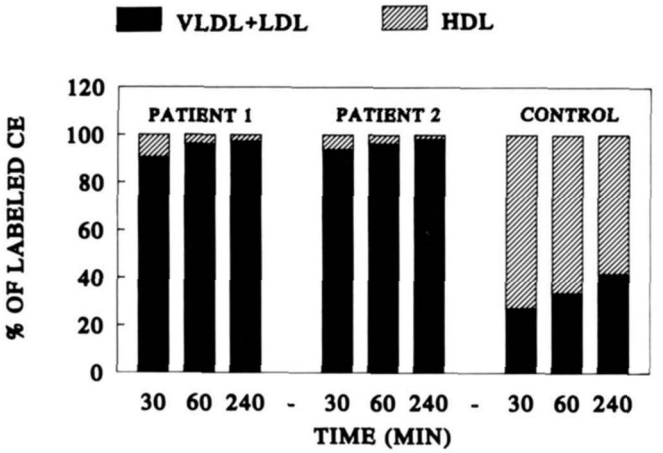

Fig. 2. Distribution of newly synthesized CE among lipoprotein fractions at various time intervals. The percent of total labeled CE found in the heparin-Mn^{2+} precipitate (VLDL + LDL) and the supernatant (HDL) was calculated from the experiment described under Fig. 1.

HDL CE transferred to VLDL + LDL is significantly higher in A-I/C-III-deficient plasma than in normal plasma. These results show that the CE transfer protein activity is increased in these patients although the concentration of the donor lipoprotein (HDL) is decreased. It should, however, be pointed out that the low HDL concentration in patients' plasma may result in an apparently accelerated rate of transfer of added labeled HDL CE because of decreased transfer to endogenous HDL.

In the mass transfer assay, the whole plasma was incubated at 37°C for 6 h in the presence of an LCAT inhibitor and the mass of HDL CE was determined in aliquots removed at various time intervals after precipitation of VLDL + LDL by heparin-Mn^{2+}. The results in **Fig. 4A** show that the total mass of CE transferred during the 6 h incubation is significantly lower in A-I/C-III-deficient plasma compared to normal plasma. This was not surprising since the amount of CE present in the patients' HDL is small. However, when the results were expressed as percent of HDL CE transferred (Fig. 4B), the A-I/C-III-deficient plasma showed a higher rate of transfer than the controls. This was especially true at the earlier time intervals when the control plasma showed a lag phase while the patients' plasma showed linear transfer rates. These results, therefore, show that although the rate of formation of CE is lower, the transfer of CE from HDL to VLDL + LDL is accelerated in A-I/C-III-deficiency.

DISCUSSION

The exact role of apolipoprotein activators in the LCAT reaction is not well understood. Since the apolipoproteins do not bind directly with the enzyme (19, 20), their role appears to be to penetrate the acyl groups of the PC molecules and facilitate their interaction with the active site of the enzyme. While apoA-I is probably the most important activator quantitatively, apoE, apoA-IV, apoC-I, and apoD have all been shown to activate LCAT in vitro with defined substrates (6, 9). It is not known, however, whether any of these activators play a significant role in vivo in the activation of the enzyme. By depleting apoA-I-containing lipoproteins from normal plasma by immunoaffinity chromatography, Cheung et al. (21) showed that about 25% of total plasma LCAT mass and activity were associated with apoA-I-free lipoproteins indicating that apolipoproteins other than apoA-I are indeed physiological activators of LCAT reaction. Furthermore, the results presented here as well as those reported by Chen and Albers (6) indicate that in the familial deficiency of apoA-I and C-III the specific activity of LCAT is not decreased much lower (6, 9). Several studies with Tangier disease patients (22–24), who are also deficient in HDL, showed a normal LCAT activity indicating that non-apoA-I activators may play an important role in LCAT activation. However, it should be noted that the low HDL levels in Tangier patients are mainly the result of the rapid turnover of apoA-I, whereas the defect in A-I/C-III-deficient patients is due to impaired synthesis of apoA-I as a result of translocation of a 6.5 kilobase pair from the apoC-III gene to apoA-I gene (25). Small but significant amounts of functionally normal apoA-I can be detected in

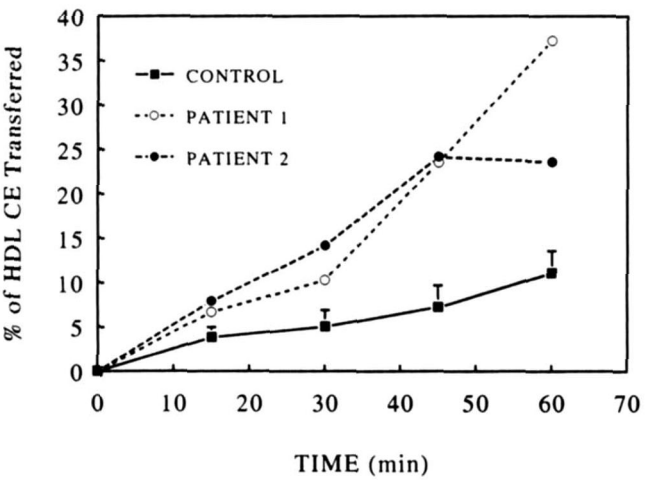

Fig. 3. Transfer of labeled CE from HDL to VLDL + LDL fraction. Fifty μl of normal HDL labeled with [^{14}C]cholesteryl oleate (16) containing 12 μg of cholesterol and 25,000 dpm was added to 450 μl of whole plasma and incubated at 37°C. At 0, 15, 30, 45, and 60 min 100-μl aliquots were removed and the VLDL + LDL fraction was precipitated by heparin-Mn_2^+. Radioactivity was determined in the supernatant (HDL) and the precipitate (VLDL + LDL) and the percent of total label transferred to the VLDL + LDL was calculated. The value obtained for the 0 min sample was subtracted from all other values. The values shown for the patients are averages of duplicate samples, while the control values represent mean ± SEM of plasma samples from six normolipemic subjects (three female, three male) analyzed under identical conditions.

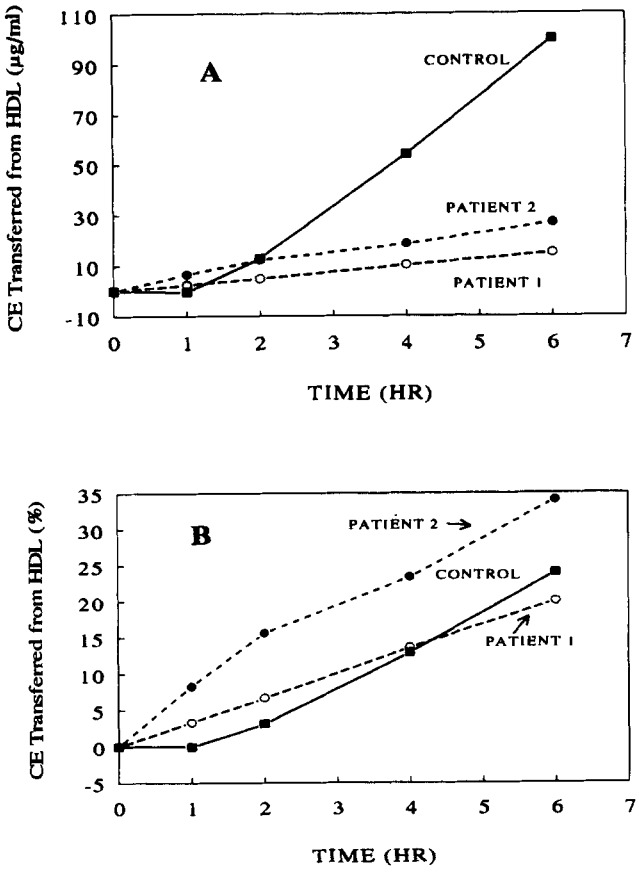

Fig. 4. Transfer of CE mass from HDL to VLDL + LDL. Plasma samples were incubated with 1.5 mM DTNB at 37°C and aliquots removed at 0, 1, 2, 4, and 6 h and precipitated with heparin-Mn^{2+}. The mass of unesterified cholesterol and total cholesterol in the HDL fraction was determined by enzymatic methods and the decrease in the mass of HDL CE was calculated as described in Materials and Methods. In the upper panel (A) the values are expressed as μg of HDL CE transferred per ml plasma, whereas in the lower panel (B) the values are expressed as percent of HDL CE transferred to VLDL + LDL. All values are averages of triplicate determinations from a single experiment.

Tangier patients' plasma (24) in contrast to the A-I/C-III-deficient plasma, where A-I is not detectable. Therefore, it is possible that the LCAT activity in Tangier plasma is for the most part activated by apoA-I. Furthermore the Tangier patients do not appear to have an increased risk of coronary disease despite decreased HDL levels, while both the A-I/C-III-deficient patients studied here have premature coronary disease.

What is the physiological importance of having several apolipoprotein activators? One possible advantage of having multiple activators is that the reaction can take place on several different lipoprotein particles simultaneously without having to transport free cholesterol and PC from one lipoprotein to another and without having to transfer the CE from the site of synthesis to other lipoproteins. This would obviously be of importance in the animal species that do not have the CE transfer activity. Another possibility is that since there are several molecular species of PC in the plasma (10), some apolipoproteins may bind specifically to certain PC species and the interaction of LCAT with such apolipoprotein-PC complexes will result in the formation of specific CE. Such a possibility is supported by the in vitro results of Steinmetz and Utermann (7) and Chen and Albers (6), which showed that apoA-IV was a better activator of LCAT in the presence of disaturated PC, while apoA-I was more efficient in the presence of the unsaturated PC species. However, when we analyzed the composition of the existing or newly formed CE in A-I/C-III-deficient patients, no significant abnormality could be demonstrated, indicating that in vivo the absence of apoA-I did not alter the specificity of the enzyme. Interestingly, the molecular species composition of plasma PC was different, possibly reflecting the partial deficiency of LCAT. Thus the increased concentration of 18:0-18:2 PC and decreased concentration of 16:0-20:4 PC resemble the abnormalities we reported in LCAT-deficient plasma (18). One of the patients (patient 2) also showed an increased concentration of 16:0-18:2 PC, although we do not know if this is due to the dietary supplementation with soy lecithin. The amount of this supplement (1200 mg/day) is probably not sufficient to change the phospholipid composition of the plasma.

The absence of apoA-I results in a marked reduction in HDL concentration in A-I/C-III-deficient plasma (11). Therefore, the bulk of the newly formed CE is present in VLDL + LDL even at the earliest time interval studied. This may be due to the formation of CE in the VLDL + LDL fraction itself, or due to a rapid transfer from the site of synthesis, or both. It is highly unlikely that these results are due to abnormal precipitation of lipoprotein by the heparin-Mn^{2+} in the A-I/C-III-deficient plasma because the same percent of phospholipid and cholesterol were present in the VLDL + LDL fraction prepared either by precipitation or by centrifugation (Table 1). The studies of Francone, Gurakar, and Fielding (26) showed that in normal plasma most of the cell-derived free cholesterol is esterified on particles containing only apoA-I and apoD, from which the CE is transferred to the bulk HDL particles as well as to the apoB-containing lipoproteins. Our present results demonstrate that CE formation can take place on A-I-free HDL particles also. It would be interesting to determine the nature of the particles that accept free cholesterol from the cells in the A-I/C-III-deficient patients with the techniques described by Francone et al. (26). There is evidence that about 10% of the CE formation in normal plasma may take place on the LDL surface (27, 28). While we do not have direct proof that the LCAT reaction takes place in the VLDL + LDL fraction in the patients' plasma, this possibility cannot be excluded. Our results also show that the VLDL + LDL fraction acquired more labeled CE from HDL during incubation,

showing that the HDL of the patients' plasma has limited capacity to retain the newly formed CE. Measurement of CE transfer rates with two different procedures in fact, showed an accelerated transfer from HDL to VLDL + LDL in the patients' plasma. The studies of Sparks, Frohlich, and Pritchard (23) showed that the transfer of CE from HDL to VLDL and LDL was also increased in Tangier disease. The increased CE transfer rates in A-I/C-III deficiency may be due to *1*) the absence of apoA-I-containing particles that form the bulk of the HDL in normal plasma and that are probably better acceptors of newly formed CE; *2*) the presence of increased amounts of CE transfer protein; or *3*) the presence of VLDL + LDL particles, which are more efficient acceptors of CE. We have previously shown that the accelerated CE transfer observed in diabetic and hyperlipidemic patients is due to the abnormal composition of VLDL (29). While this may be also true in A-I/C-III deficiency, we cannot rule out the other possibilities. Because of the possible atherogenic role of abnormal CE transfer (30) it is possible that the accelerated CE transfer may contribute to the premature atherosclerosis observed in these patients.

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REFERENCES

1. Glomset, J. A. 1979. Lecithin:cholesterol acyl transferase. An exercise in comparative biology. *Prog. Biochem. Pharmacol.* **15:** 41-66.
2. Ueno, K., N. Sakuma, M. Kawaguchi, T. Fujinami, and H. Okuyama. 1986. Selectivity and contribution of lecithin:cholesterol acyltransferase to plasma cholesterol ester formation. *J. Biochem.(Tokyo)* **99:** 541-547.
3. Dobiasova, M. 1983. Lecithin:cholesterol acyltransferase and the regulation of endogenous cholesterol transport. *Adv. Lipid Res.* **20:** 107-194.
4. Jonas, A. 1986. Synthetic substrates of lecithin:cholesterol acyltransferase. *J. Lipid Res.* **27:** 689-698.
5. Fielding, C. J. 1986. Mechanisms of action of lecithin:cholesterol acyltransferase. *Methods Enzymol.* **129:** 783-790.
6. Chen, C. H., and J. J. Albers. 1985. Activation of lecithin:cholesterol acyltransferase by apolipoproteins E-2, E-3, and A-IV isolated from human plasma. *Biochim. Biophys. Acta.* **836:** 279-285.
7. Steinmetz, A., and G. Utermann. 1985. Activation of lecithin:cholesterol acyltransferase by human apolipoprotein A-IV. *J. Biol. Chem.* **260:** 2258-2264.
8. Steyrer, E., and G. M. Kostner. 1988. Activation of lecithin:cholesterol acyltransferase by apolipoprotein D: comparison of proteoliposomes containing apolipoprotein D, A-I or C-I. *Biochim. Biophys. Acta.* **958:** 484-491.
9. Soutar, A. K., C. W. Garner, H. N. Baker, J. T. Sparrow, R. L. Jackson, A. M. Gotto, and L. C. Smith. 1975. Effect of the human plasma apolipoproteins and phosphatidylcholine acyl donor on the activity of lecithin:cholesterol acyltransferase. *Biochemistry.* **14:** 3057-3064.
10. Subbaiah, P. V., and H. Monshizadegan. 1988. Substrate specificity of human plasma lecithin:cholesterol acyltransferase towards molecular species of phosphatidylcholine in native plasma. *Biochim. Biophys. Acta.* **963:** 445-455.
11. Norum, R. A., J. B. Lakier, S. Goldstein, A. Angel, R. B. Goldberg, W. D. Block, D. K. Noffze, P. J. Dolphin, J. Edelglass, D. D. Bogorad, and P. Alaupovic. 1982. Familial deficiency of apolipoproteins A-I and C-III and precocious coronary heart disease. *N. Engl. J. Med.* **306:** 1513-1519.
12. Stokke, K. T., and K. R. Norum. 1971. Determination of lecithin:cholesterol acyltransferase in human blood plasma. *Scand. J. Clin. Lab. Invest.* **27:** 21-27.
13. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911-917.
14. Chen, C. H., and J. J. Albers. 1982. Characterization of proteoliposomes containing apoprotein A-I: a new substrate for the measurement of lecithin:cholesterol acyltransferase activity. *J. Lipid Res.* **23:** 680-691.
15. Lipid Research Clinics Program. 1974. Manual of Laboratory Operations. Vol I. Lipid and Lipoprotein Analysis. Washington, D. C., United States Government Printing Office. (DHEW Publication No. NIH 75-628).
16. Hough, J. L., and D. B. Zilversmith. 1984. Comparison of various methods for in vitro cholesteryl ester labeling of lipoproteins from hypercholesterolemic rabbits. *Biochim. Biophys. Acta.* **792:** 338-347.
17. Subbaiah, P. V., B. Banerji, R. E. Gregg, and J. D. Bagdade. 1990. Molecular species of cholesteryl esters formed in abetalipoproteinemia. Effect of apoprotein B-containing lipoproteins. *J. Lipid Res.* **31:** 927-932.
18. Subbaiah, P. V., and P. H. Pritchard. 1989. Molecular species of phosphatidylcholine in familial lecithin:cholesterol acyltransferase deficiency: effect of enzyme supplementation. *Biochim. Biophys. Acta.* **1003:** 145-150.
19. Yokoyama, S., D. Fukushima, J. P. Kupferberg, F. J. Kezdy, and E. T. Kaiser. 1980. The mechanism of activation of lecithin:cholesterol acyltransferase by apolipoprotein A-I and an amphiphilic peptide. *J. Biol. Chem.* **255:** 7333-7339.
20. Furukawa, Y., and T. Nishida. 1979. Stability and properties of lecithin:cholesterol acyltransferase. *J. Biol. Chem.* **254:** 7213-7219.
21. Cheung, M. C., D. B. Wang, K. D. Lum, and J. J. Albers. 1988. Cholesterol esterification by lecithin:cholesterol acyltransferase in A-I-free plasma. *Biochim. Biophys. Acta.* **962:** 258-264.
22. Clifton-Bligh, P., P. J. Nestel, and H. M. Whyte. 1972. Tangier disease. Report of a case and studies of lipid metabolism. *N. Engl. J. Med.* **286:** 567-571.
23. Sparks, D. L., J. Frohlich, and P. H. Pritchard. 1988. Cholesteryl ester transfer activity in plasma of patients with familial high density lipoprotein deficiency. *Clin. Chem.* **34:** 1812-1815.
24. Pritchard, P. H., R. McLeod, J. Frohlich, M. C. Park, B. J. Kudchodkar, and A. G. Lacko. 1988. Lecithin:-cholesterol acyltransferase in familial HDL deficiency (Tangier disease). *Biochim. Biophys. Acta.* **958:** 227-234.
25. Karathanasis, S. K., J. McPherson, V. I. Zannis, and J. L. Breslow. 1983. Linkage of human apolipoproteins A-I and C-III genes. *Nature (London)* **304:** 371-373.
26. Francone, O. L., A. Gurakar, and C. Fielding. 1989. Distribution and function of lecithin:cholesterol acyltransferase

and cholesteryl ester transfer protein in plasma lipoproteins. *J. Biol. Chem.* **264:** 7066–7072.
27. Barter, P. J. 1983. Evidence that lecithin:cholesterol acyltransferase acts on both high density and low density lipoproteins. *Biochem. Biophys. Acta.* **751:** 261–270.
28. Knipping, G., A. Birchbauer, E. Steyrer, J. Groener, R. Zechner, and G. M. Kostner. 1986. Studies on the substrate specificity of human and pig lecithin:cholesterol acyltransferase. Role of low density lipoproteins. *Biochemistry.* **25:** 5242–5249.
29. Subbaiah, P. V., M. C. Ritter, and J. D. Bagdade. 1990. Effects of different lipid-lowering agents on plasma cholesteryl ester transfer in hypercholesterolemic patients. *J. Drug. Dev.* **3 (Suppl 1):** 95–99.
30. Quinet, E., A. Tall, R. Ramakrishnan, and L. Rudel. 1991. Plasma lipid transfer protein as a determinant of the atherogenicity of monkey plasma lipoproteins. *J. Clin. Invest.* **87:** 1559–1566.
31. Marinetti, G. V. 1962. Chromatographic separation, identification, and analysis of phosphatides. *J. Lipid Res.* **3:** 1–20.