Familial cholesteryl ester transfer protein deficiency is associated with triglyceride-rich low density lipoproteins containing cholesteryl esters of probable intracellular origin

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Abstract The net transfer of core lipids between lipoproteins is facilitated by cholesteryl ester transfer protein (CETP). We have recently documented CETP deficiency in a family with hyperalphalipoproteinemia, due to a CETP gene splicing defect. The purpose of the present study was to characterize the plasma lipoproteins within the low density lipoprotein (LDL) density range and also the cholesteryl ester fatty acid distribution amongst lipoproteins in CETP-deficient subjects. In CETP deficiency, the conventional LDL density range contained both an apoE-rich enlarged high density lipoprotein (HDL) (resembling HDLc), and also apoB-containing lipoproteins. Native gradient gel electrophoresis revealed clear speciation of LDL subclasses, including a distinct population larger in size than normal LDL. Anti-apoB affinity-purified LDL from the CETP-deficient subjects were shown to contain an elevated triglyceride to cholesteryl ester ratio, and also a high ratio of cholesteryl oleate to cholesteryl linoleate, compared to their own HDL or to LDL from normal subjects. Addition of purified CETP to CETP-deficient plasma results in equilibration of very low density lipoprotein (VLDL) cholesteryl esters with those of HDL. These data suggest that, in CETP-deficient humans, the cholesteryl esters of VLDL and its catabolic product, LDL, originate predominantly from intracellular acyl-CoA:cholesterol acyltransferase (ACAT). The CETP plays a role in the normal formation of LDL, removing triglyceride and transferring LCAT-derived cholesteryl esters into LDL precursors.—**Bisgaier, C. L., M. V. Siebenkas, M. L. Brown, A. Inazu, J. Koizumi, H. Mabuchi, and A. R. Tall.** Familial cholesteryl ester transfer protein deficiency is associated with triglyceride-rich low density lipoproteins containing cholesteryl esters of probable intracellular origin. *J. Lipid Res.* 1991. **32:** 21–33.

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The net transfer and exchange of cholesteryl esters and triglycerides among human lipoproteins is mediated by a cholesteryl ester transfer protein (CETP) (1–7). This 74 kDa glycoprotein (6, 8) also facilitates transfer of phospholipids from triglyceride-rich lipoproteins to HDL (7, 9). Although much has been learned about the in vitro properties of CETP, its role in human lipoprotein physiology has been somewhat uncertain. Families with high HDL and low CETP activity have been described (10, 11). We have recently shown that the genetic basis of CETP deficiency in one family is a point mutation in the 5' splice donor site of intron 14 of their CETP gene, a mutation known to be incompatible with normal pre-mRNA splicing (12). The purpose of the present study was to characterize further the lipoproteins in this CETP-deficient family, with particular attention to the LDL. The plasma lipoproteins from the CETP-deficient homozygous male (SY −/−) and female (KH −/−) siblings (10) and a daughter (KC +/−) of the male proband (heterozygous for the CETP gene and having approximately half her mothers' CETP activity and protein (12)) were compared to those of normal humans (NH1 +/+ through NH4 +/+) and rats (NR), which are known to have low CETP activity. We sought to determine whether lipoproteins and affinity-purified LDL from these subjects have characteristics that reflect lack of CETP activity.

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; apo, apolipoprotein; CETP, cholesteryl ester transfer protein; FAME, fatty acid methyl ester; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; Mab, monoclonal antibody; NH, normal human; NR, normal rat; SDS, sodium dodecyl sulfate; VLDL, very low density lipoprotein; CE, cholesteryl ester.

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MATERIALS AND METHODS

Sample collection

Venous blood was obtained after an overnight fast from the proband (SY −/−) and his sister (KH −/−), both with homozygous CETP deficiency, a heterozygous daughter (KC +/−) of SY −/−, and also from four unrelated normal male subjects (NH1 +/+ through NH4 +/+). Arterial blood was also obtained from three 200–250 g male Sprague-Dawley rats (NR1–3) that had been fasted overnight. Plasma (SY −/−, KH −/−, and KC +/−) was immediately prepared by low speed centrifugation, frozen, and shipped to our laboratory on dry ice. Frozen samples from additional Japanese homozygous CETP-deficient subjects were obtained. These subjects contained the same mutation as SY(−/−) and KH (−/−) (13). Plasma from the normal human subjects was either used immediately without freezing or frozen at −70°C and then thawed to simulate shipping conditions. As an additional control, we froze-thawed normal human plasma through 30 cycles and analyzed particle size by 4–30% gradient gel electrophoresis (see below). No difference in HDL particle size was observed when compared to fresh unfrozen plasma. Rat plasma used in these studies was not frozen.

Sephacryl- S-300 chromatography

Aliquots (0.5 ml) of thawed (NH4 +/+, SY −/− and KC +/−) or fresh plasma (NH4 +/+) were applied to matched Sephacryl S-300 columns (0.9 × 115 cm) and eluted with 154 mM NaCl, 0.01% EDTA, 0.02% NaN_3, 5 mM Tris-HCl, pH 7.4 (14). Fractions of 1.7 ml were collected at 4- to 6-min intervals.

Lipoprotein isolation

Lipoproteins from fresh and frozen-thawed plasma (up to 5 ml) were isolated by density flotation in the 60 Ti rotor by the addition of KBr and NaCl solutions or solid KBr. In some studies sequential fractions (top 2 ml of 24-ml tube) of $d < 1.019$ g/ml (VLDL plus IDL), 1.019–1.063 g/ml (LDL plus HDL_1), and 1.063–1.21 g/ml (HDL_2 plus HDL_3) were obtained. For other studies, d 1.019–1.050 g/ml (LDL) and 1.050–1.063 g/ml (HDL_1) were sequentially obtained in lieu of the entire d 1.019–1.063 g/ml fraction. Fractions were dialyzed extensively against 10 mM Tris-HCl, 1 mM EDTA, 0.02% NaN_3, pH 7.4. In some cases the d 1.019–1.050 g/ml fraction was further purified by anti-apoB affinity chromatography. Fractions were further characterized as described below.

Preparation of anti-apoB serum

Human LDL was isolated between d 1.030–1.050 g/ml and apoB-100 was further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15). The apoB-100 band was identified by immersing gels in cold 0.25 M KCl (16) and crushed slices (approximately 0.5–1 mg apoB) mixed with complete Freund's adjuvant were used to immunize rabbits along their backs (17). Monospecific apoB antiserum was obtained 3 weeks after two subsequent monthly booster injections of apoB in incomplete Freund's adjuvant.

Anti-apoB affinity chromatography

Total immunoglobulins of anti-apoB-100 serum were precipitated with 0–40% ammonium sulfate and covalently coupled to Sepharose CL-4B (Pharmacia) according to the manufacturer's instructions. Columns (2 ml packed beds) were cycled (1 h) at room temperature with aliquots (up to 0.5 ml) from the SY −/−, KH −/− and frozen-thawed normal d 1.019–1.050 g/ml fractions in 2 ml of 50 mM Tris-HCl, pH 8.0. After the cycling period, the columns were washed with 10 ml of cycling buffer (flow-through fraction), and then with 7.5 ml of 100 mM glycine-HCl, pH 2.2, to displace bound lipoproteins (retained fraction). One ml of 1 M Tris-HCl, pH 8.0, was added to retained fractions. Fractions were concentrated by lyophilization and subjected to SDS-PAGE (15). Relative triglyceride and cholesteryl ester content was determined from the content of fatty acids. The cholesteryl ester fatty acid composition was also determined as described below.

Fatty acid analysis

Lipoprotein fractions or affinity-purified LDL were extracted with chloroform–methanol 2:1 (v/v) according to the method of Folch, Lees, and Sloane Stanley (18). Aliquots of the lipid extract were used to isolated cholesteryl esters and triglycerides by thin-layer chromatography in hexane–diethyl ether–glacial acetic acid 80:16:4 (v/v) (19). For affinity-purified LDL, triheptadeconate (20 μg) and cholesteryl heptadeconate (20 μg) internal standards were added to the chloroform–methanol extract. Cholesteryl esters (all samples) and triglycerides (affinity-purified LDL) were transmethylated (20) and their composition was assessed by splitless capillary gas–liquid chromatography. Gas–liquid chromatography was performed using helium as the carrier gas, with the injector at 250°C and flame ionization detector at 215°C. After injection of samples in hexane (0.1–0.2 μl) the inlet was purged at 12 sec and fatty acid methyl esters were separated on a 30-m (0.32 mm ID, 0.2 μm film) SP-2340 column (Supelco) using a temperature program of 160°C for 1 min, 160°C to 180°C at 5°C/min, and a final temperature of 180°C for 5 min on a Hewlett-Packard 5890 GC interfaced with a 3390A Hewlett-Packard integrator. Retention times were determined with authentic quantitative mixes of fatty acid methyl ester standards. Cholesteryl ester fatty acid composition of lipoproteins isolated from fresh and frozen-thawed normal human plasmas were essentially identical.

Apolipoprotein analysis

Apolipoproteins A-I, A-IV, and E were determined by radioimmunoassay (21–23). Apolipoprotein B was assessed by immunoelectrophoresis (24, 25). Immunoelectrophoresis plates contained 5% anti-human apoB-100 serum in 1% agarose in 45 mM sodium barbital buffer, pH 8.8. Samples were incubated in barbital buffer containing 1% Triton X-100 for 30 min at 37°C and then electrophoresed at 1 mA/cm for 20 h.

Gradient gel and sodium dodecyl sulfate-gel electrophoresis

Sodium dodecyl sulfate (SDS)-electrophoresis was carried out on 12% gels (4% stacking gel) according to the method of Laemmli (15). Gels were stained with Coomassie Brilliant Blue R-250 or silver-stained. Non-SDS 2–16% or 4–30% gradient gel electrophoresis of lipoproteins on PAA 2/16 or PAA 4/30 gels (Pharmacia), respectively, was carried out as previously described (26). Gradient gels were stained with Coomassie Blue G-250 or Sudan Black B.

Lipid determinations

Total and free cholesterol were determined enzymatically (27). Triglycerides were determined enzymatically using a Reagentset Triglycerides-GB kit (Boehringer Mannheim Diagnostics). Phosphatidylcholine was determined by the choline oxidase assay (28). Lipoprotein compositions of subclasses isolated from either fresh or frozen-thawed normal human plasmas were virtually identical. For Sephacryl S-300 column chromatography fractions, phospholipid distribution was assessed by quantitative thin-layer chromatography (29).

Incubation studies

Plasma (300 μl) from the CETP-deficient subject YI(−/−), was incubated with 2 mM E600 to inhibit LCAT, and in the presence or absence of human CETP (150 μg Cm-cellulose fraction (6)) for 24 h at 37°C. Lipoproteins were isolated by ultracentrifugation and fatty acid composition of the cholesteryl esters was determined as described above.

Protein determination

Protein was determined by the method of Lowry et al. (30) utilizing bovine serum albumin as standard.

RESULTS

Plasma apoA-IV and apoE levels in SY−/− were elevated 2.5- and 3.3-fold, respectively, above normal levels (**Table 1**). In two other homozygous CETP-deficient subjects (from separate families), apoE levels were elevated 3.3- (YI−/−) and 2.5-(HM−/−) fold. Plasma apoA-I in SY−/− has previously been shown to be increased by 2.0–2.1 times that of normal controls, while his apoB was markedly reduced (39% of normal values) (10, 12). Similarly, KH−/−, the sister of SY−/−, had elevated plasma apoA-I and reduced apoB levels (10, 12). The level of these apolipoproteins in the heterozygous daughter (KC+/−) of SY−/− were normal (Table 1 and refs. 10, 12). Both SY−/− and KH−/− had higher than normal plasma cholesterol levels, however, most was contained in HDL; their triglyceride levels were within the low normal range (i.e., between 50–150 mg/dl). KC+/− had a relatively normal

TABLE 1. Apolipoprotein, cholesterol, and triglyceride levels of CETP-deficient plasma

Subject	ApoA-IV	ApoE	Total Cholesterol	HDL Cholesterol	Triglyceride
			mg/dl		
SY−/− (M)	93.4	13.1	318[a]	302[a]	56[a]
KH−/− (F)	ND	ND	219[a]	174[a]	62[a]
YI−/− (F)	ND	12.7	ND	ND	ND
HM−/− (M)	ND	9.8	ND	ND	ND
KC+/− (F)	37.4	3.6	149[a]	67[a]	55[a]
NH1+/+ (M)	31.2[b]	6.1[b]	205[b]	42[b]	150[b]
NH2+/+ (M)	55.4[b]	4.4[b]	179[b]	53[b]	37[b]
NH3+/+ (M)	20.7[b]	3.2[b]	171[b]	35[b]	111[b]
NH4+/+ (M)	51.6[b]	3.3[b]	148[b]	60[b]	52[b]
Normal (n = 7)	37.0 ± 4.7[b]	3.9 ± 0.4[b]	168.4 ± 11.2[b]	44.7 ± 3.5[b]	87 ± 17.3[b]

ApoA-IV and apoE were determined by radioimmunoassay; ND, not determined.
[a]From reference 10.
[b]From reference 14.

plasma cholesterol level and a slightly elevated but normal HDL cholesterol level.

We examined distribution of lipids and apoA-I, apoA-IV, apoB, and apoE by Sephacryl S-300 in a normal subject (NH4 + / +), the proband (SY – / –), and his heterozygous daughter (KC + / –) (**Fig. 1**). In SY – / – 's plasma, apoA-I had a bimodal distribution on Sephacryl S-300, with the bulk of apoA-I (79.5%) eluting in a region containing particles larger than normal HDL_2 or HDL_3 (Fig. 1, middle bottom panel). A second shallower apoA-I peak was distributed over the region where normal HDL is usually found. ApoE was distributed in a single peak that eluted between apoB-containing particles and the larger apoA-I-containing peak. This distribution was distinct inasmuch as a bimodal apoE distribution is normally found when plasma is fractionated on these columns (Fig. 1 and ref. 14). SY – / – 's plasma apoA-IV was distributed in three peaks: one (11.8% of total apoA-IV) that coeluted exactly with the single apoE peak; another (11.6% of total apoA-IV) that eluted slightly ahead of the smaller apoA-I-containing particles; and the last (76.5% of total apoA-IV) at the descending portion of the main protein peak. In contrast to normal plasma (Fig. 1, top left) in which clearly a bimodal distribution of both cholesterol and phospholipid was found associated with apoB-containing particles and HDL, these lipids in SY – / – plasma eluted as a single peak predominantly over fractions containing apoE and apoA-I (Fig. 1, top middle). ApoB (including VLDL, IDL, and LDL) in all plasmas was confined to the column void volume. Prior gel filtration studies on Bio-Gel A-5m, which separates VLDL (triglyceride-rich particles) from LDL (cholesteryl ester-rich particles), demonstrated that plasma apoB was associated with particles similar in size to LDL (10). The normal control (NH4 + / +) had HDL cholesterol concentrations similar to that of the heterozygous daughter (KC + / –), and a similar pattern of apolipoprotein and lipid distribution (Fig. 1), with the exception of KC's apoA-I distributing two fractions ahead of the elution volume of normal HDL apoA-I.

The column profile in the subject with homozygous CETP deficiency suggested that larger cholesteryl ester-rich particles containing apoE, apoA-I, and apoA-IV might overlap the distribution of apoB lipoproteins. To test this, SY – / – and NH4 + / + plasma were fractionated into traditional LDL (d 1.019–1.063 g/ml) and HDL (d 1.063–1.21 g/ml) density fractions by conventional preparative ultracentrifugation and analyzed by protein staining of native (non-SDS) polyacrylamide gradient gels containing either 2–16% of 4–30% polyacrylamide (**Fig. 2**). Electrophoresis of the d 1.019–1.063 g/ml fraction demonstrated that SY – / – 's fraction ap-

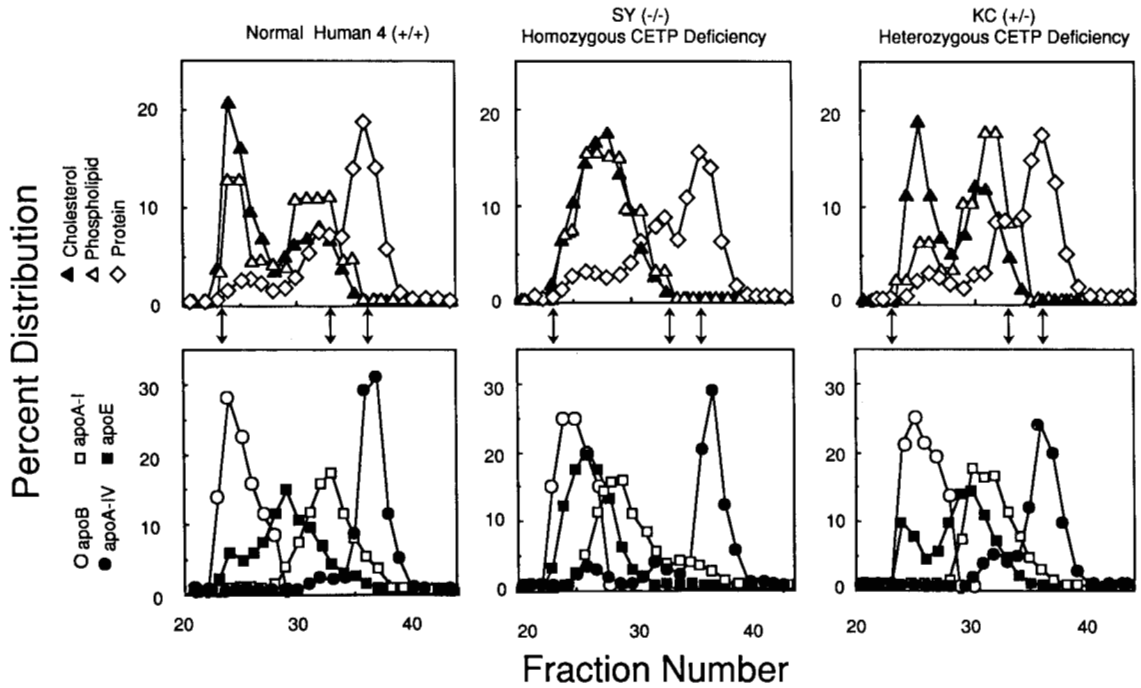

Fig. 1. Gel-filtration chromatography of normal and CETP-deficient plasma. Plasma (frozen-thawed) from normal human (NH4 + / +) (left), a CETP-deficient subject (SY – / –) (center), and his heterozygous daughter (KC + / –) (right) were gel-filtered by Sephacryl S-300 chromatography as described in Materials and Methods. Fractions were assayed for distribution of cholesterol, phospholipid, protein (three upper panels), and apoB apoA-I, apoA-IV, and apoE (three lower panels) as indicated on the figure and as described in Materials and Methods. The three arrows above each panel (left to right) represent previously characterized elution volumes of normal human ^{125}I-labeled LDL, apoA-I in HDL, and ^{125}I-labeled human serum albumin on these columns.

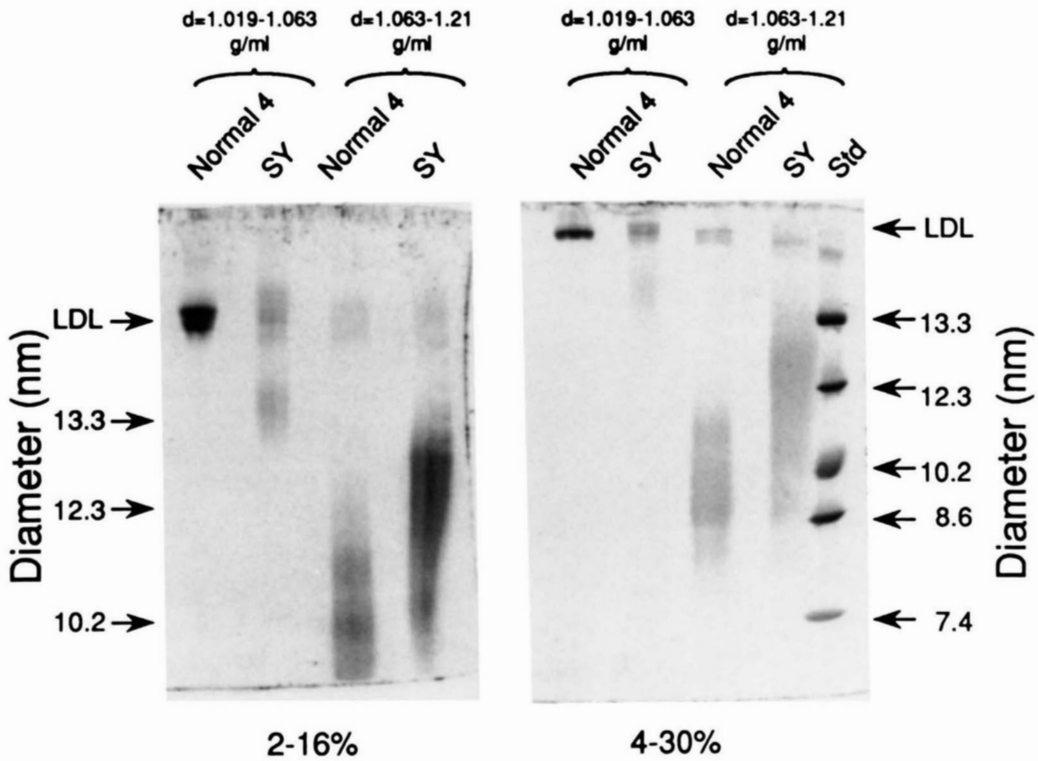

Fig. 2. Non-SDS gradient gel electrophoresis of normal and CETP-deficient lipoproteins. Lipoproteins d 1.019–1.063 g/ml (47 μg/lane) and 1.063–1.21 g/ml (98 μg/lane) were isolated from a normal (NH4 + / +) and CETP-deficient (SY – / –) plasma, and electrophoresed on 2–16% and 4–30% non-SDS gradient gels and stained for protein. Diameters (13.3 to 7.4 nm) of molecular weight standards (Pharmacia) of thyroglobulin (660 kDa), apoferritin (440 kDa), catalase (220 kDa), lactic dehydrogenase (140 kDa), and albumin (67 kDa) are indicated.

peared more heterogenous than that of NH4 + / + , including particles extending into the size range of HDL, suggesting flotation of some HDL-like particles within the LDL density range. In addition, SY's LDL contained several distinct bands, including some larger and some smaller than control LDL. SY's d 1.063–1.21 g/ml fraction contained mostly markedly enlarged HDL, in addition to some smaller HDL overlapping the size found in the normal control. Native gel electrophoresis of plasma (**Fig. 3**) using additional subjects with homozygous CETP deficiency revealed that the LDL region contained four distinct subspecies, including a prominent band above normal LDL, approximating the size of IDL_2 (31).

In an attempt to minimize HDL contamination of LDL, fractions of d 1.019–1.050 g/ml and 1.050–1.063 g/ml were prepared in lieu of a single 1.019–1.063 g/ml density fraction, in addition to d < 1.019 g/ml and a d 1.063–1.21 g/ml fractions. The apolipoprotein composition from all lipoprotein fractions was determined by SDS-PAGE (**Fig. 4**). In the normal human the d 1.063–1.21 g/ml fraction (D lanes), apoA-I was the major protein. Similarly, SY – / – and KH – / – contained predominantly apoA-I in this fraction and, in addition, contained apoA-IV, a protein not normally found associated with human HDL when isolated in the ultracentrifuge. In the d 1.050–1.063 g/ml fraction (C lanes), apoB was the major apolipoprotein in normal human subjects. However, in both SY – / – and KH – / – , this fraction contained considerable amounts of apoA-I and apoE, thus resembling the apolipoprotein composition of HDLc of cholesterol-fed animals (32, 33). This fraction in the rat predominantly contained apoE. The d 1.019–1.050 g/ml (B lanes) in the normal humans contained mainly apoB. In SY – / – and KH – / – this fraction also contained apoA-I, apoE, and apoA-IV. The d < 1.019 g/ml fraction (A lanes) in all subjects and the rat contained predominantly apoB. This fraction was also apoE-enriched in KH – / – and the rat. In order to obtain authentic LDL, aliquots of the d 1.019–1.050 g/ml fraction were further purified by anti-apoB affinity chromatography. When assessed by silver staining of SDS-PAGE gels, all HDL apolipoproteins (apoA-I, apoA-IV, and apoE) were removed from anti-apoB affinity-retained fractions (not shown). These findings establish that enlarged HDL particles (containing apoA-I, apoA-IV, and apoE) are a major component of the LDL fraction in CETP-deficient subjects. Compositional analysis of the LDL density fraction showed that it was similar in overall lipid and protein

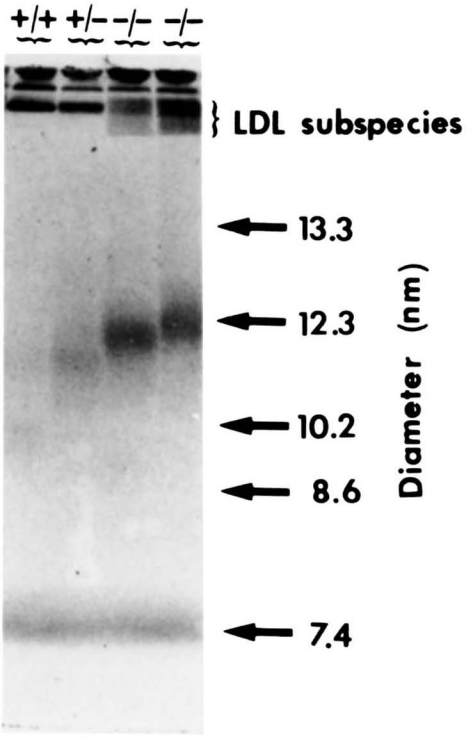

Fig. 3. Non-SDS gradient gel electrophoresis of normal and CETP-deficient plasma. Whole plasma (20 μl) from a normal subject (+/+), and a heterozygote (+/−) and two homozygote subjects (−/−) from a separate family identified with familial CETP deficiency due to the same genetic defect (12, 13) were electrophoresed on 4–30% non-SDS gradient gels and lipoproteins were identified by Sudan Black B staining. Diameters of molecular weight protein standards are indicated.

composition to normal LDL. However, the triglyceride to cholesteryl ester ratio was elevated. The affinity-purified LDL was further characterized in order to determine the possible origin of cholesteryl ester in LDL (LCAT- or ACAT-derived).

Protein and lipid composition and the triglyceride to cholesteryl ester ratios of the various density fractions, as well as selected data on affinity-purified LDL, are shown for the CETP-deficient subjects, four normal subjects, and three rats (**Table 2**). For the d 1.019–1.050 g/ml fraction, insufficient quantities of anti-apoB affinity-purified fractions were obtained to determine full compositional data. However, we were able to determine the ratio of cholesteryl ester to triglyceride of the affinity-purified LDL by gas–liquid chromatography as described in Materials an Methods. As shown in Table 2, the triglyceride to cholesteryl ester ratios for normal affinity-purified LDL (0.26 ± 0.02) are comparable to those obtained prior to affinity isolation by the enzymatic method (0.19 ± 0.05 for frozen-thawed and 0.22 ± 0.06 for fresh plasma). In the two homozygous CETP-deficient subjects, this ratio was elevated to 0.40 and 0.37. The triglyceride to cholesteryl ester ratios in the $1.050 < d < 1.063$ g/ml fraction were also increased, compared to normal humans.

The cholesteryl ester fatty acid composition of the VLDL plus IDL, apoB-affinity-retained LDL, and the HDL fractions from this experiment revealed that SY's affinity-retained LDL had a high ratio (ratio 2.2) of cholesteryl oleate to cholesteryl linoleate,[2] which was similar to the ratio of his $d < 1.019$ g/ml fraction (ratio 2.8) but different than that of HDL (ratio 0.6). This observation suggested little cholesteryl ester exchange occurred between LCAT-derived cholesteryl esters of HDL and the ACAT-derived cholesteryl esters of LDL precursors (VLDL and IDL). Unexpectedly, however, in this first analysis we observed an elevated CE 18:1/18:2 in the $d < 1.019$ g/ml fraction of NH4 +/+ plasma, although affinity-purified LDL and HDL from NH4 had the expected ratio of approximately 0.4.

To rule out artifacts due to the unusual increased CE 18:1/18:2 ratio in the $d < 1.019$ g/ml fraction of NH4 +/+ plasma, the above study was repeated with additional normal controls (including a new plasma sample from NH4 +/+), and rat plasma (i.e., low CETP activity). The cholesteryl ester fatty acid compositions of all frozen-thawed lipoprotein fractions including affinity-purified LDL are shown in **Table 3**. This compositional analysis was virtually identical whether it was performed on frozen-thawed (shown) or fresh (not shown) normal human plasma. As an index of cholesteryl ester origin, we used the cholesteryl oleate to cholesteryl linoleate ratio. Most striking, as shown in Table 3 and graphically in **Fig. 5**, was that normal human LDL (either before of following anti-apoB affinity purification) showed cholesteryl ester fatty acid composition nearly identical to HDL, whereas the ratio of cholesteryl oleate to cholesteryl linoleate in anti-apoB affinity-purified LDL of the CETP-deficient subjects was increased 2 to 3 times compared to their HDL_2 plus HDL_3 fractions. The cholesteryl oleate to cholesteryl linoleate ratio was not nearly as striking for KH −/− as they were for SY −/− or the rats. The basal level of this ratio in the $d < 1.019$ g/ml fraction was low in KH −/− although similar to that of her affinity-purified LDL. Thus, the gradient of change of this ratio in KH −/− showed a difference between all apoB-containing lipoproteins and HDL. The data from YI −/− (**Fig. 6**), as in SY −/−, also showed a markedly high ratio of cholesteryl oleate to cholesteryl linoleate in

[2]As a measure of intracellular versus plasma compartment origin, we used the ratio of cholesteryl oleate, the predominant intracellular or ACAT-derived cholesteryl ester, to cholesteryl linoleate, the predominant plasma or LCAT-derived cholesteryl ester (i.e., the CE 18:1/18:2 ratio). Thus, for a lipoprotein whose cholesteryl esters are predominantly derived in plasma, such as HDL, this ratio is approximately 0.4 (39–43). As increased ratio suggests an intracellular (secreted lipoprotein) origin. Thus, if little cholesteryl ester exchange occurs, an the lipoprotein is not an LCAT substrate, it should have an elevated ratio.

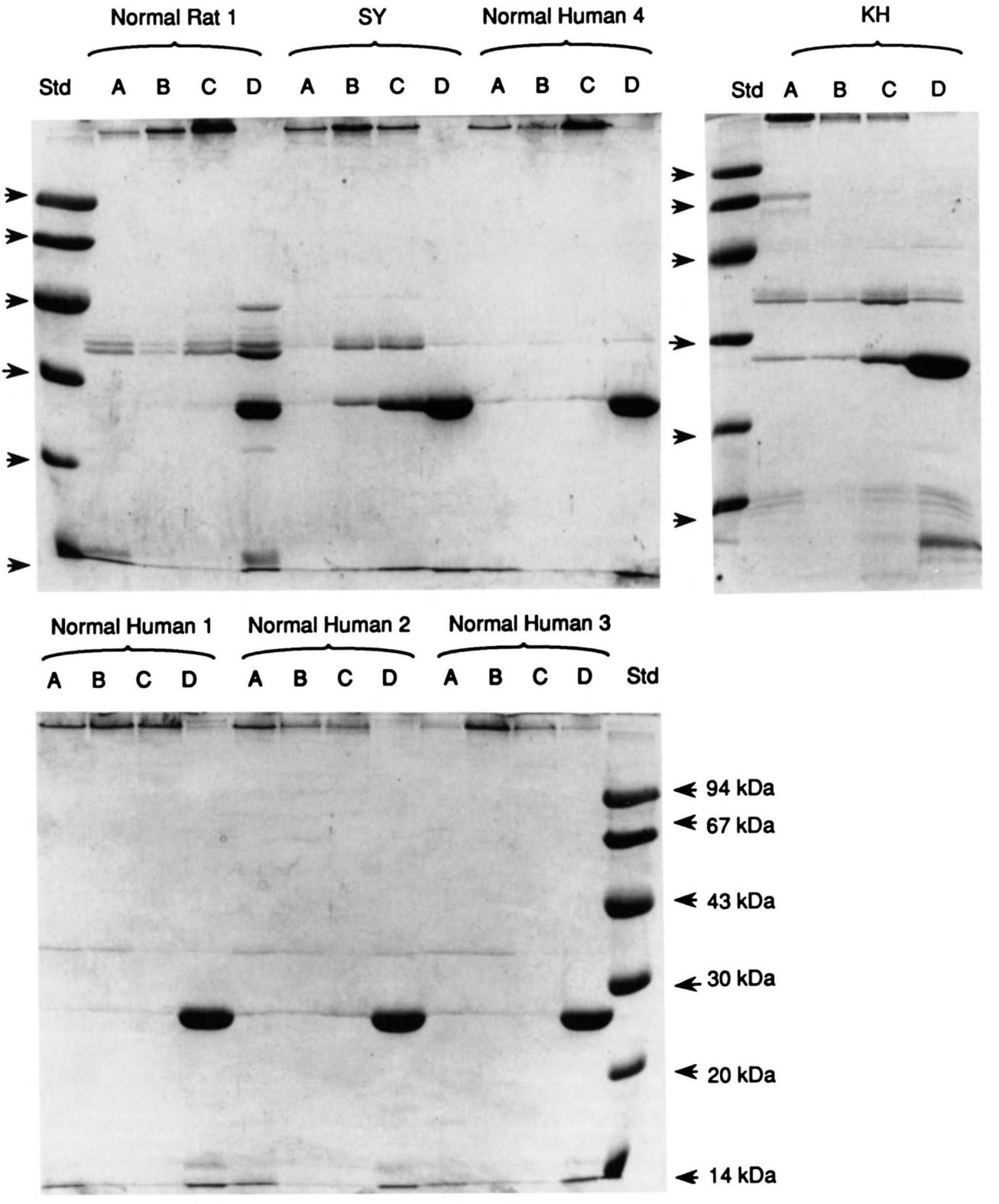

Fig. 4. Sodium dodecyl sulfate polyacrylamide electrophoresis of normal and CETP-deficient human and rat apolipoproteins. Lipoproteins (50 μg/lane) of density d < 1.019 g/ml (A), d 1.019–1.050 g/ml (B), d 1.050–1.063 g/ml (C), and d 1.063–1.21 g/m (D) isolated from a normal rat (i.e., a species that lacks CETP activity), two CETP-deficient siblings (SY −/− and KH −/−), and four normal humans (NH1 +/+ through NH4 +/+) were electrophoresed on 12% SDS-PAGE (4% stacker) under reducing conditions and stained with Coomassie Brilliant Blue R-250. Molecular weight standards (Pharmacia) or 94, 67, 43, 30, 20, and 14 kDa are indicated by arrows (top two gels) and by arrows and molecular weight (bottom gel). Apolipoproteins B-100 (512 kDa), apoA-IV (46 kDa), apoE (35 kDa) doublet), apoA-I (28 kDa) are resolved in all gels. ApoA-II (14 kDa) and C apolipoproteins (6–8 kDa) migrated near or with the tracker dye.

TABLE 2. Composition of lipoprotein fractions and TG/CE ratios

Sample	n	Percent Composition (Mass)					Mass Ratio TG/CE
		Protein	PC	TG	FC	CE	
Fraction d 1.019-1.050 g/ml							
Normal human (fresh)[a]	4	24.2 ± 3.0	18.9 ± 0.6	8.0 ± 1.7	10.5 ± 0.6	38.4 ± 2.7	0.22 ± 0.06
Normal human (frozen-thawed)[a]	4	25.8 ± 0.9	17.0 ± 0.6	7.5 ± 1.6	9.8 ± 0.2	39.8 ± 1.8	0.19 ± 0.05
Normal rat (fresh)[a]	3	34.7 ± 0.7	8.4 ± 1.0	24.5 ± 1.8	9.0 ± 0.3	23.5 ± 2.1	1.07 ± 0.18
Affinity-purified normal human (frozen-thawed)[b]	4						0.26 ± 0.02
Affinity-purified SY −/− [b]							0.40
Affinity-purified KH −/− [b]							0.37
Fraction d 1.050-1.063 g/ml							
Normal human (fresh)[a]	4	33.3 ± 0.8	18.3 ± 1.5	3.1 ± 0.2	10.9 ± 1.2	34.4 ± 2.1	0.09 ± 0.01
Normal human (frozen-thawed)[a]	4	30.9 ± 1.1	17.2 ± 1.2	2.6 ± 0.3	9.2 ± 0.5	40.1 ± 2.4	0.07 ± 0.01
Normal rat (fresh)[a]	3	40.7 ± 1.5	13.2 ± 3.5	13.3 ± 1.0	9.4 ± 1.1	23.4 ± 0.9	0.57 ± 0.03
SY −/− (frozen-thawed)[a]		35.9	15.2	10.1	9.4	29.3	0.35
KH −/− (frozen-thawed)[a]		34.9	12.5	3.6	17.2	31.8	0.11
Fraction d 1.063-1.21 g/ml							
Normal human (fresh)[a]	4	54.2 ± 0.8	21.8 ± 0.5	2.4 ± 0.1	3.1 ± 0.2	18.4 ± 0.5	0.13 ± 0.01
Normal human (frozen-thawed)[a]	4	54.4 ± 1.2	22.3 ± 0.7	2.3 ± 0.2	3.3 ± 0.2	17.7 ± 1.1	0.13 ± 0.02
Normal rat (fresh)[a]	3	48.4 ± 0.6	22.6 ± 0.7	6.6 ± 0.6	5.7 ± 0.3	16.7 ± 0.6	0.40 ± 0.04
SY −/− (frozen-thawed)[a]		44.4	24.1	2.9	5.2	23.4	0.13
KH −/− (frozen-thawed)[a]		55.4	15.6	0.6	7.0	21.4	0.03

[a]The 1.019-1.050 g/ml, 1.050 < d < 1.063 g/ml, and 1.063 < d < 1.21 g/ml fractions were prepared from fresh and frozen-thawed plasma, as indicated, by sequential ultracentrifugation. Fractions were dialyzed against 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, and protein, choline content of phosphatidylcholine, triglyceride, cholesterol content of total and free cholesterol were determined as described in Materials and Methods. For phosphatidylcholine, an average molecular weight of 775 was assumed and no corrections were made for non-choline-containing phospholipids. Esterified cholesterol was determined by difference and mass was estimated by multiplying its cholesterol content by 1.7. Values represent mean ± SEM.

[b]The 1.019-1.050 g/ml fraction isolated from frozen-thawed normal human (NH1 +/+ through NH4 +/+) and familial homozygous CETP-deficient plasma (SY −/− and KH −/−) was purified by anti-apoB affinity chromatography as described in Material and Methods. The affinity-purified fractions plus internal triheptadeconate and cholesteryl heptadeconate standards were extracted, separated by thin-layer chromatography, transmethylated, and then quantitated by gas–liquid chromatography as described in Materials and Methods. Data were converted from fatty acid methyl ester to triglyceride and cholesteryl ester mass. Data represent the triglyceride to cholesteryl ester mass ratio.

the d < 1.019 g/ml fraction. In addition, we verified that this ratio was also elevated in the d < 1.019 g/ml fraction of NH4 +/+ and also in a second control subject (NH2 +/+) (Table 3). Both these subjects[3] had very low plasma triglycerides (i.e., 37 and 52 mg/dl) while the other two controls had levels approximately 2- to 4-fold higher (See Table 1). The elevated ratio may be due to the low levels of triglyceride-rich lipoproteins in NH2 +/+ and NH4 +/+, resulting in a low cholesteryl ester transfer rate. Purified CETP addition to a CETP-deficient plasma (subject YI −/−) in the presence of an LCAT inhibitor resulted in a marked reduction in the cholesteryl oleate to cholesteryl linoleate ratio in the d < 1.019 g/ml fraction (Fig. 6). The equilibration of the cholesteryl esters contained in the d < 1.019 g/ml fraction with those of HDL, also suggest a direct role of CETP in the transfer of the cholesteryl esters from HDL to LDL precursors.

DISCUSSION

The present study further characterizes the apolipoprotein distribution and the lipid and protein composition in the genetic disorder, hyperalphalipoproteinemia due to CETP deficiency. A major finding of this study is that affinity-isolated LDL cholesteryl esters in CETP deficiency are similar in fatty acid composition to its VLDL and IDL precursors, suggesting that these LDL derive most of their cholesteryl ester from the precursors, and not through transfer from HDL. In contrast, the cholesteryl ester fatty acid composition of normal LDL more closely approximates that of HDL, probably reflecting cholesteryl ester equilibration through a CETP-mediated pathway. The equilibration of d < 1.019 g/ml cholesteryl esters with

[3]Based on earlier reports of lipoprotein cholesteryl ester fatty acid compositions (39–43), an unexpected finding of these studies was the elevated cholesteryl oleate to cholesteryl linoleate ratio in the d < 1.019 g/ml fraction of two control subjects (NH2 +/+ and NH4 +/+). Both subjects had low plasma triglyceride (Table 1), below average LDL to HDL ratios (average, 2.47; NH2, 2.24; NH4, 1.27). The two other normal subjects had above average LDL to HDL cholesterol ratios (NH1, 3.17; NH3, 3.25). Whether this relation will hold when a larger group of subjects is examined will be of interest. Taken together, the low triglyceride levels and the elevated d < 1.019 g/ml fraction cholesteryl oleate to cholesteryl linoleate ratio are consistent with our measurements of reduced transfer rates in subjects with low plasma triglycerides (A. R. Tall et al., unpublished data).

TABLE 3. Lipoprotein cholesteryl ester fatty acid composition

Fatty Acid Methyl Ester	Human Freeze-Thawed Plasma (n = 4)	SY −/− Freeze-Thawed Plasma	KH −/− Freeze/Thawed Plasma	Rat Fresh Plasma (n = 3)
Fraction d < 1.019 g/ml				
16:0	26.7 ± 3.8	36.2	22.9	26.7 ± 0.5
16:1	2.4 ± 0.4	2.7	4.1	2.2 ± 0.3
18:0	19.7 ± 4.6	27.2	19.8	25.6 ± 1.0
18:1	19.3 ± 0.4	21.3	19.6	23.6 ± 0.8
18:2	27.2 ± 7.9	8.2	30.2	15.6 ± 0.9
18:3	0.4 ± 0.1	1.5	0.9	0.9 ± 0.1
20:4	4.2 ± 0.5	2.8	2.5	5.5 ± 0.7
18:1/18:2	0.99 ± 0.33	2.59	0.65	1.52 ± 0.05
Fraction d 1.019–1.050 g/ml				
16:0	16.1 ± 0.4	26.7	16.3	21.4 ± 0.5
16:1	2.9 ± 0.6	4.9	4.7	2.5 ± 0.3
18:0	7.1 ± 0.7	18.2	4.5	16.7 ± 1.0
18:1	18.2 ± 0.5	26.4	18.7	22.3 ± 0.8
18:2	48.6 ± 2.1	18.0	50.5	24.2 ± 0.9
18:3	0.5 ± 0.1	0.8	1.1	1.2 ± 0.1
20:4	6.6 ± 1.1	4.9	4.1	11.7 ± 0.7
18:1/18:2	0.38 ± 0.02	1.47	0.37	0.93 ± 0.09
Fraction d 1.019–1.050 g/ml (anti-apoB affinity purified)				
16:0	23.0 ± 0.4	37.0	22.6	
16:1	3.2 ± 0.6	0.0	1.8	
18:0	17.4 ± 0.7	38.9	23.0	
18:1	17.5 ± 0.5	16.3	14.3	
18:2	32.2 ± 2.1	7.8	20.2	
18:3	0.4 ± 0.1	0.0	7.4	
20:4	6.2 ± 1.1	0.0	10.8	
18:1/18:2	0.55 ± 0.03	2.10	0.71	
Fraction d 1.050–1.063 g/ml				
16:0	15.3 ± 0.4	29.3	14.0	19.9 ± 1.2
16:1	3.0 ± 0.6	4.6	4.1	1.9 ± 0.5
18:0	6.6 ± 0.5	20.3	3.7	15.7 ± 1.4
18:1	17.8 ± 0.6	21.4	16.1	18.9 ± 1.3
18:2	49.3 ± 2.2	20.0	56.2	21.5 ± 1.1
18:3	0.7 ± 0.1	0.8	1.4	1.2 ± 0.2
20:4	7.3 ± 1.8	3.7	4.4	20.9 ± 1.4
18:1/18:2	0.36 ± 0.03	1.07	0.29	0.89 ± 0.09
Fraction d 1.063–1.21 g/ml				
16:0	16.2 ± 0.7	18.9	20.0	10.7 ± 0.6
16:1	3.3 ± 0.6	7.4	2.3	0.9 ± 0.3
18:0	6.4 ± 0.4	2.0	1.5	5.1 ± 0.8
18:1	17.1 ± 0.7	25.5	18.2	7.0 ± 0.8
18:2	49.7 ± 2.4	39.9	52.3	22.7 ± 0.9
18:3	0.7 ± 0.0	0.7	1.3	0.1 ± 0.1
20:4	6.7 ± 0.9	5.6	4.4	53.5 ± 1.8
18:1/18:2	0.35 ± 0.03	0.64	0.35	0.31 ± 0.04

Lipids from lipoprotein fractions and anti-apoB affinity-purified LDL were extracted and separated by thin-layer chromatography. The cholesteryl esters were extracted, transmethylated, and then quantitated by gas-liquid chromatography as described in Materials and Methods. Data represent the percent distribution fatty acid methyl ester mass of the major cholesteryl ester fatty acids. The cholesteryl ester fatty acid compositions of lipoproteins isolated from frozen-thawed plasma from NH1 +/+ through NH4 +/+ were virtually identical to those determined with fresh (never frozen) plasma (not shown).

those of HDL in LCAT-inhibited CETP-deficient plasma when incubated with CETP also suggest a specific role of CETP in remodeling LDL precursors (Fig. 6). Overall, these data imply that a major portion of LDL cholesteryl ester in normal plasma is derived via transfer from HDL to LDL precursors and not by direct LCAT activity. The recent studies of Fielding, Ishikama, and Fielding (34) suggest that both human apoE-deficient VLDL (newly secreted) and apoE-rich VLDL (metabolized) had similar cholesteryl ester to apoB ratios. This ratio was elevated in LDL, and suggests little or no LCAT-derived cholesteryl esters are transferred until VLDL become LDL. These data (34) suggest that the cholesteryl esters of VLDL and LDL are largely derived from the liver. Although some

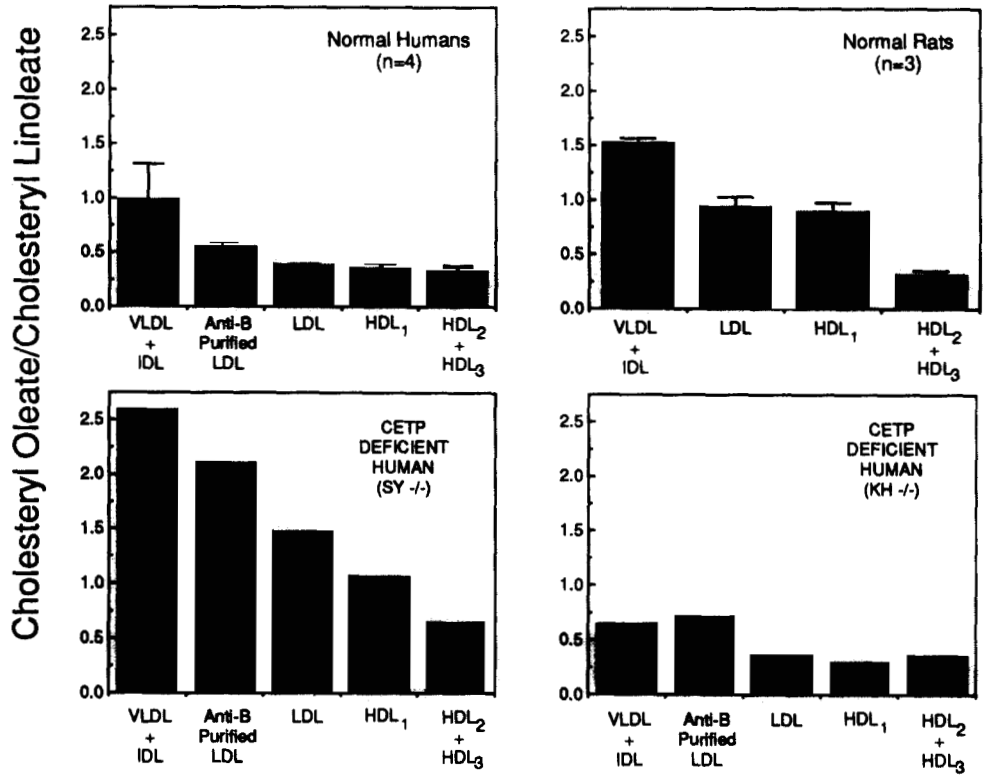

Fig. 5. Cholesteryl oleate to cholesteryl linoleate ratio of normal and CETP-deficient human and rat lipoproteins. Cholesteryl oleate to cholesteryl linoleate ratios in plasma lipoproteins isolated from the frozen-thawed plasma of the four normal subjects (NH1 + / + through NH4 + / +), the two CETP-deficient probands (SY − / − and KH − / −), and fresh plasma from three rats. Cholesteryl ester fatty acid composition of d < 1.019 g/ml (VLDL + IDL), affinity-purified apoB containing particles of d 1.019–1.050 g/ml, d 1.019–1.050 g/ml (LDL), d 1.050–1.063 (HDL_1), and d 1.063–1.21 g/ml (HDL_2 plus HDL_3) fractions was determined as described in Material and Methods. Data represent weight ratio ± SEM of fatty acid methyl esters derived from cholesteryl oleate and cholesteryl linoleate. These ratios determined in the frozen-thawed plasmas from NH1 + / + through NH4 + / + were virtually identical to those determined with fresh plasma (not shown).

reconstitution studies (35) suggest that VLDL and LDL are LCAT substrates, studies using Mabs that inhibit CETP activity (9) suggest that HDL are the preferential initial LCAT substrates. LDL were utilized as LCAT substrate only when the HDL substrate source was exhausted after prolonged incubation. The present characterization is an in vivo demonstration that LDL and its precursors probably do not become major LCAT substrates in human CETP deficiency.

In addition, our finding of lipoproteins resembling HDLc in human CETP deficiency indicates that CETP activity normally prevents formation of significant amounts of HDLc in normal humans. The extreme elevation of HDL cholesteryl esters in humans with absent CETP indicates that CETP is a major factor that influences the catabolism of HDL cholesteryl esters in humans. Presumably, the apoE-dependent removal of large HDL becomes a major route for HDL removal in the absence of CETP. In humans it appears that the HDL levels have to accumulate to very high levels before this becomes a major catabolic pathway. This could be because the HDL increases in size slowly and only attains a sufficient number of apoE molecules to enable high affinity removal by the LDL receptor at the end of its catabolic pathway. Thus, in the steady state, relatively little of the total HDL mass (5–10%) is in an apoE-rich particle within the LDL density range (Fig. 2 and 4).

In present study we also observed similarities between rat and human CETP deficiency, with regard to HDL size, apolipoprotein distribution, and cholesteryl ester fatty acid composition. We have found that CETP-deficient subjects have elevated apoE, apoA-I, and apoA-IV and reduced apoB levels, and elevated HDL cholesterol (10, 12). Similarly, additional homozygous CETP-deficient subjects, described by Yamashita et al. (11) and Eto et al. (36), had elevated apoE and apoA-I levels, a reduced apoB level, and markedly elevated HDL cholesterol. By gradient gel electrophoresis, we have shown previously that rats have large HDL (26). The present study and recent studies (11, 12) have demonstrated that plasma from

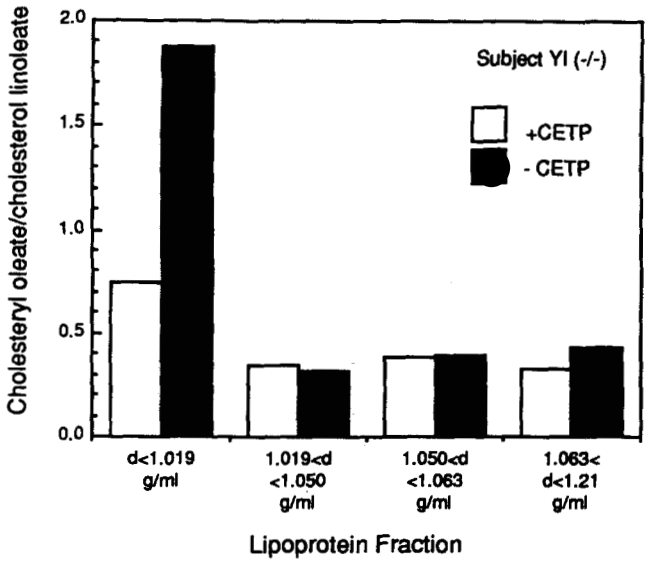

Fig. 6. Effect of CETP addition on cholesteryl ester composition of CETP-deficient plasma. YI(– / –) plasma (300 μl) was incubated with 2 mM E600 (to inhibit LCAT) in the presence (□) or absence (■) or 150 μg human CETP (CM-cellulose fraction) (6) for 24 h at 37°C. Lipoprotein fractions were isolated and assessed for cholesteryl ester fatty acid composition.

humans with CETP deficiency also have enlarged HDL. With respect to apolipoprotein distribution, SDS-PAGE of lipoproteins isolated by density flotation from rats and CETP-deficient humans demonstrates a striking, although not absolute, similarity. Human apoA-IV, known to dissociate from normal human HDL when prepared by density flotation, was present on HDL isolated from both SY – / – and KH – / – . Rat HDL isolated by density flotation contains apoA-IV (37). These observations suggest that reduced CETP levels in rats may, in part, account for the high apoA-IV content of their HDL.

One consideration for undertaking the present study was to determine whether human CETP deficiency was analogous to the observations we recorded in vitro (14). We observed (14) a modest shift of apoE from HDL to triglyceride-rich particles in incubated plasma when CETP activity was inhibited with a Mab. We suggested that this event could potentially decrease the half-life of the LDL precursors, while impeding clearance of apoE-containing HDL and contributing to the hyperalphalipoproteinemia in CETP deficiency. The resolution of the techniques used in this study (i.e., determination of apoE distribution after gel filtration or ultracentrifugal isolation of lipoproteins) may be too insensitive to detect the existence of the putative apoE-enriched LDL precursor pool in CETP deficiency, particularly since the hypothesis implies that this pool would be rapidly removed from plasma. However, it was interesting to note that the CETP-deficient subjects showed distinct subpopulations of LDL, including a prominent population of particles similar in size to IDL_2 and LDL_1 and not normally seen in LDL (Fig. 3). These particles might represent the accumulation of an LDL precursor pool which normally requires CETP for remodeling into LDL_2 or LDL_3 (31), and which is more rapidly removed by liver than LDL_2 or LDL_3 (31). The presence of polydisperse LDL has also been described in the CETP-deficient subjects studied by Yamashita et al. (11).

In summary, CETP deficiency may lead to reduced levels of LDL of altered composition and possible lower atherogenicity by one of the following mechanisms. First, a preferential partitioning of apoE from HDL to VLDL during CETP inhibition could result in an accelerated clearance of remnants and IDL, with less formation of LDL, as suggested previously (14). Second, the inhibition of cholesteryl ester transfer leads to the formation of triglyceride-rich and cholesterol-poor LDL precursors and LDL; due to their reduced cholesterol content these particles may be less atherogenic. Third, hepatic clearance of these cholesterol-poor particles or their precursors could result in up-regulation of LDL receptors, providing an explanation for reduced levels of apoB. Lastly, the reduced transfer of LCAT-derived cholesteryl linoleate from HDL to LDL precursors could potentially diminish the level of oxidative modification of LDL by substantially reducing the transfer of this lipid peroxidation substrate (38). The present report contains further evidence to suggest that the lipoprotein profile of human CETP deficiency might be anti-atherogenic, i.e., reduced levels of LDL and cholesteryl ester-poor, triglyceride-rich LDL and increased HDLc-like particles. Studies of Mahley and Holcombe (32) and Mahley (33) have previously indicated that the HDLc may participate in reverse cholesterol transport, and may be an anti-atherogenic particle.

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