Effect of a neutralizing monoclonal antibody to cholesteryl ester transfer protein on the redistribution of apolipoproteins A-IV and E among human lipoproteins

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Abstract The effect of inhibiting cholesteryl ester transfer protein (CETP) on the in vitro redistribution of apolipoproteins(apo) A-IV and apoE among lipoproteins in whole plasma was studied in seven normal male subjects. Plasmas were incubated in the presence of a purified monoclonal antibody TP2 (Mab TP2) that neutralizes the activity of CETP. Mab TP2 had no effect on lecithin:cholesterol acyltransferase (LCAT) activity. Prior to and following a 6-h incubation at 37°C in the presence of Mab TP2 or a control mouse myeloma immunoglobulin (IgG), plasmas were gel-filtered on Sephacryl S-300 and the distribution of apoA-IV and apoE among lipoproteins was determined by radioimmunoassay. Incubation (i.e., with active LCAT and CETP) increased the amount of apoA-IV associated with lipoproteins by 240%. When CETP activity was inhibited during incubation, the amount of apoA-IV that became lipoprotein-associated was significantly increased (315% of basal). Plasma incubation also caused a redistribution of apoE from high density lipoproteins (HDL) to larger lipoproteins (131% of basal); however, when CETP was inhibited, significantly greater amounts of apoE became associated with the larger particles (155% of basal). These effects were observed in all seven subjects. Increased movement of apoE from HDL to triglyceride-rich particles was not due to displacement by apoA-IV since loss of apoE from HDL was still observed when no movement of apoA-IV onto HDL occurred, such as during LCAT or combined LCAT and CETP inhibition. We speculate that low CETP activity (e.g., in species such as rats) may lead to an increased content of HDL apoA-IV and also to apoE enrichment of triglyceride-rich lipoproteins, augmenting their clearance.—**Bisgaier, C. L., M. V. Siebenkas, C. B. Hesler, T. L. Swenson, C. B. Blum, Y. L. Marcel, R. W. Milne, R. M. Glickman, and A. R. Tall.** Effect of a neutralizing monoclonal antibody to cholesteryl ester transfer protein on the redistribution of apolipoproteins A-IV and E among human lipoproteins. *J. Lipid Res.* 1989. **30:** 1025–1031.

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Although cross-species comparisons suggest that CETP activity is most closely correlated with the content of VLDL cholesteryl esters(1), reduced CETP activity may indirectly lead to other modifications of plasma lipoprotein constituents. For example, rats, which have low CETP activity, have large HDL and approximately half of their apoA-IV is HDL-associated (2-5). Furthermore, rats rapidly clear VLDL remnants from their plasma and, therefore, do not accumulate LDL (6-8). In contrast, human plasma contains higher CETP activity, has smaller HDL, and most apoA-IV is unassociated with lipoproteins (1-5, 9-15). Compared to rats, humans have higher LDL levels (1-4, 6-8). These observations prompted us to assess the effects that a newly available neutralizing monoclonal antibody to CETP (16) would have on the distribution of apoA-IV and apoE among lipoproteins in human plasma. We sought to test whether CETP inhibition would lead to additional binding of apoA-IV to HDL. Furthermore, we wanted to test whether inactive CETP might lead to apoE-enrichment of triglyceride-rich lipoproteins.

Abbreviations: apo, apolipoprotein; CETP, cholesteryl ester transfer protein; Chol, cholesterol; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); Fab, papain-generated IgG fragment; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; IgG, immunoglobulin; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; MOPC 21, purified mouse myeloma monoclonal immunoglobulin of unknown specificity; VLDL, very low density lipoprotein; TG, triglyceride; Mab TP2, purified mouse myeloma monoclonal immunoglobulin against human CETP.

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MATERIALS AND METHODS

Production of neutralizing monoclonal antibodies to CETP activity

A monoclonal antibody, Mab TP2 (formerly named 5C7), that completely neutralizes the activity of the 74,000 mol wt human CETP was produced as previously described (16). Protein A-Sepharose-purified immunoglobulins were prepared from ascitic fluid obtained from mice injected with Mab TP2-producing hybridoma cells. Fab fragments were generated by papain hydrolysis of Mab TP2 (16). A nonspecific purified mouse myeloma protein (MOPC21) used as a control immunoglobulin was obtained from Bionetics Laboratory Products, Kensington, MD.

Plasma samples

Blood of normal human males fasted for 12 h, drawn from an antecubital vein, was collected in tubes EDTA (1 mg/ml blood), and plasma was prepared by low speed centrifugation. Plasmas were used immediately for experiments.

Measurement of plasma lipid levels

Plasma cholesterol, triglyceride, and HDL cholesterol were quantitated by Lipid Research Program procedures (17) using an Abbott Biochromatic Analyzer 100 (Abbott, Chicago, IL). LDL cholesterol was estimated according to the equation of Friedewald, Levy, and Fredrickson (18). Total and free cholesterol mass in incubated plasmas were determined enzymatically according to the method of Allain et al. (19). Esterified cholesterol was calculated as the difference.

Plasma incubations

Plasmas (2 ml) were incubated in the presence of 8 μg/ml MOPC21 (control) or 8 μg/ml Mab TP2 (anti-CETP) for 6 h at 37°C. Immediately following incubation, aliquots (900 μl) were frozen for subsequent total and free cholesterol determinations. In some experiments, plasmas were also incubated for 6 h at 37°C with the lecithin:cholesterol acyltransferase (LCAT) inhibitor 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), with both 1 mM DTNB and 8 μg/ml Mab TP2, or Mab TP2 Fab fragments.

Sephacryl S-300 chromatography

To 1.1 ml of unincubated plasmas and the remaining 1.1 ml of incubated plasmas, trace amounts of human ^{125}I-labeled LDL (44,000 cpm) and ^{125}I-labeled albumin (44,000 cpm) were added, and 1-ml plasma aliquots were promptly applied to matched 115 × 0.9 cm Sephacryl S- 300 columns, equilibrated with 154 mM NaCl, 0.01% EDTA, and 0.02% sodium azide, 5 mM Tris-HCl, pH 7.4. Fractions of 1.5 ml were collected at 4.5-min intervals at room temperature. Sephacryl S-300 fractionates proteins between molecular weights 1×10^4 and 1.5×10^6. Thus, VLDL, IDL, and LDL elute in the void volume, while HDL (mol wt $\approx 2 \times 10^5$) and nonlipoprotein-associated apoA-IV (4.6×10^4) separate from each other and the larger particles. In all column runs for all subjects, the apoE in the VLDL-IDL-LDL-containing fractions were separated by a well-defined valley (a single point) from apoE-HDL containing fractions. The amount of apoE contained in this valley point ranged between 0.4% and 4.6% (average = 2.4%) for the 21 column runs. For calculations, the amount of apoE contained in this valley point was evenly divided between the VLDL-IDL-LDL and HDL peaks. Although incubation increased HDL size (see Results), VLDL-IDL-LDL and HDL apoE resolution was not compromised. HDL size changes were mainly reflected in HDL_3 (apoE-free HDL) and much less so in HDL_2 (apoE-containing HDL). Following 37°C incubations, Sephacryl S-300 resolution of VLDL- IDL-LDL from HDL-apoE was independent of HDL_2 size, since HDL_2 size increased to the same extent in the absence or presence of anti-CETP (see Results). The elution patterns of ^{125}I-labeled LDL, apoA-I (determined by immunodiffusion analysis), and ^{125}I-labeled albumin were used to calibrate each column run. For 21 column runs the elution volumes were: ^{125}I-labeled LDL, 38.5 ± 0.5 ml, apoA-I, 54.2 ± 0.4 ml, and ^{125}I-labeled human serum albumin, 60.5 ± 0.5 ml.

Quantitation of apolipoproteins

Human apoA-IV and apoE were quantitated by previously described radioimmunoassays (14, 20).

Gradient gel electrophoresis

Immediately before and following incubations with Mab TP2 and DTNB, plasma aliquots (40 μl) were mixed with 10 μl of 60% sucrose containing bromophenol blue (tracking dye) and applied to nondenaturing 4–30% polyacrylamide gradient gels (Pharmacia) (21). An aliquot of high molecular protein standards (Pharmacia) was also applied to a lane. Gel lanes containing plasma were stained for lipid with Sudan Black B (22) and the lane containing the protein standard was stained with Coomassie Blue G-250.

Data analysis

Significant difference was determined by paired *t*-test (two-sided). Data are presented as mean ± the standard error of the mean.

TABLE 1. Plasma characteristics of subjects used in study

Subject	Age	Plasma Chol	Plasma TG	HDL Chol	LDL Chol	LDL-Chol/HDL-Chol	ApoA-IV	ApoE
			mg/dl				*mg/dl*	
1	29	196	136	40	129	3.22	36.8	3.91
2	34	205	150	42	133	3.17	31.2	6.10
3	35	179	37	53	119	2.24	55.4	4.43
4	36	171	111	35	114	3.25	20.7	3.19
5	31	117	45	36	72	2.00	35.1	3.24
6	41	148	52	60	78	1.29	51.6	3.32
7	29	163	78	47	100	2.14	28.4	2.96
Mean ± SEM	33.6 ± 1.6	168.4 ± 11.2	87.0 ± 17.3	44.7 ± 3.5	106.3 ± 9.1	2.47 ± 0.29	37.0 ± 4.7	3.88 ± 0.42

RESULTS

Plasma lipid, apoA-I, and apoE levels of the seven subjects used in the studies are shown in **Table 1**. All subjects had lipid levels, apoA-IV and apoE levels within the normal range determined in our laboratory.

Prior studies (16) established that all plasma CETP activity was abolished at a concentration of 8 μg Mab TP2/ml plasma. The inhibition of cholesteryl ester transfer by monoclonal TP2 had no effect on LCAT activity in whole plasma (**Table 2**), as reported in detail elsewhere (23). In basal plasma, 70.9 ± 0.9% cholesterol was esterified. Following a control incubation for 6 h at 37°C, the amount of cholesterol esterified significantly increased to 79.6 ± 0.5%, and this amount was not different from the amount of cholesterol esterified in incubations containing the CETP monoclonal antibody (78.8 ± 0.7%).

Plasma samples from each subject were gel-filtered on Sephacryl S-300 chromatography prior to and following a 6-h incubation at 37°C with MOPC 21 (control) or Mab TP2. A typical gel-filtration profile is shown in **Fig. 1**.

Apo-A-IV was distributed among three peaks: a minor peak associated with the void volume, another with HDL-containing fractions, and the last eluting without lipoproteins. In basal plasmas of the seven subjects studied, little apoA-IV was associated with lipoproteins (12.6 ± 1.7%). This was distributed between HDL (10.4 ± 2.0%) and the VLDL-IDL-LDL fraction (2.2 ± 0.5%). After control incubations, where both LCAT and CETP were active, the amount of apoA-IV associated with lipoproteins increased to 30.3 ± 7.1%. The amount in HDL increased to 24.7 ± 5.9% of plasma apoA-IV, and the amount in VLDL-IDL-LDL increased to 5.6 ± 2.3%. The inhibition of CETP led to greater association of apoA-IV with lipoproteins (39.8 ± 8.6%). Increases in apoA-IV were found both in HDL (29.9 ± 6.3% of plasma apoA-IV) and in the VLDL-IDL-LDL (9.9 ± 3.9%). The values for individual subjects are shown in **Fig. 2**.

In all subjects apoE was distributed between two peaks: one associated with the void-containing fractions (triglyceride-rich lipoproteins) and the other with HDL (Fig. 1, bottom panel). In basal plasma, 36.2 ± 9.4% was associated with the triglyceride-rich lipoproteins. In control incubations, the apoE content of the triglyceride-rich lipoproteins increased to 47.5 ± 10.0%. With CETP inhibition, apoE content of the triglyceride-rich lipoproteins was further increased to 56.2 ± 10.3% in these fractions. In all cases, with control or anti-CETP Mab incubations, the increased amounts of apoE observed in the VLDL-IDL-LDL region were distributed over the entire peak. Data for the individual subjects are shown in **Fig. 3**.

Experiments were performed to test whether apoE was being displaced from HDL because Mab TP2 IgG bound to CETP was occupying space on the surface of HDL. Plasma (2 ml) from one subject 3) was incubated at 37°C in the presence of MOPC 21 (106 pmol), Mab TP2 (106 pmol), or Mab TP2 Fab fragments (212 pmol). In basal plasma, 71% of apoE was associated with HDL. When CETP was inhibited with intact Mab TP2 IgG, the amount of apoE associated with HDL decreased to 54%. When CETP was inhibited with Mab TP2 Fab fragments, the amount of apoE associated with HDL also de-

TABLE 2. Cholesterol esterified (percent)

Subject	Basal[a]	6-h Control Incubation	6-h Anti-CETP Incubation
1	71.8	77.9	76.3
2	71.1	80.3	78.6
3	65.8	77.8	78.7
4	72.2	81.5	80.4
5	70.5	78.9	77.2
6	71.9	1.0	82.4
7	72.8	80.1	78.3
Mean ± SEM	70.9 ± 0.9	79.6[b] ± 0.6	78.8[b] ± 0.8

[a]Total and free cholesterol were determined enzymatically as described in Materials and Methods in unincubated plasma, or plasma incubated for 6 h at 37°C in the presence of control mouse monoclonal immunoglobulin (MOPC 21) or a mouse monoclonal antibody that inhibits human CETP activity (Monoclonal TP2). Esterified cholesterol was estimated as the difference. Each value is the average of triplicate determinations.
[b]Compared to unincubated plasma $P < 0.001$.

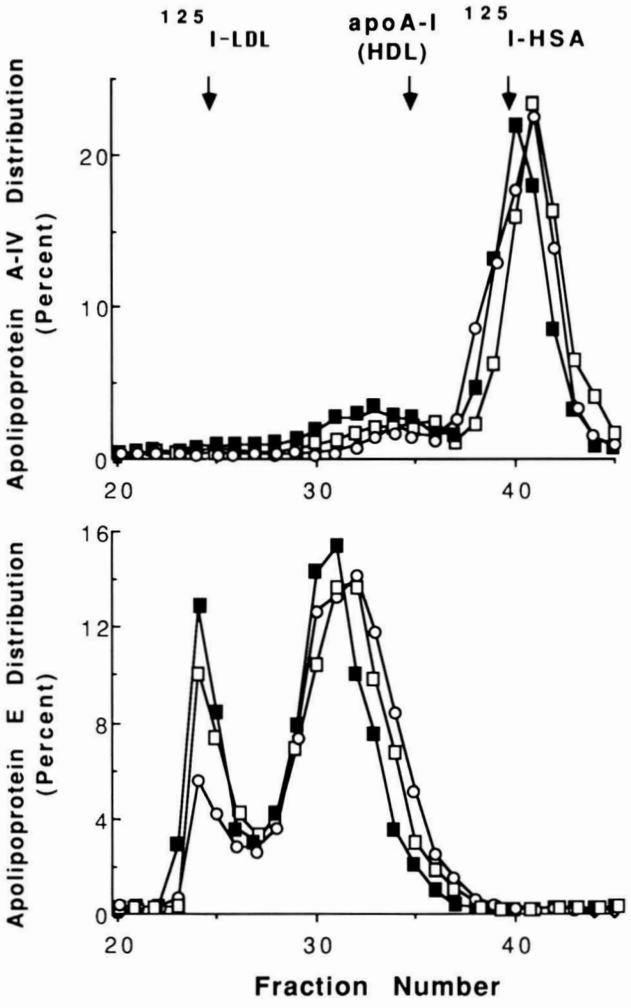

Fig. 1. Fractionation of plasma apoA-IV and apoE by Sephacryl S-300 chromatography. Unincubated plasma (○) or plasma incubated at 37°C for 6 h in the presence of Mab TP2 (8 μg/ml) (■) or a nonspecific mouse myeloma IgG (8 μg/ml) (□) was fractionated by Sephacryl S-300 chromatography as described in Materials and Methods. ApoA-IV (top panel) and apoE (bottom panel) content of fractions were determined by radioimmunoassay. The elution of ^{125}I-labeled LDL, apoA-I, and ^{125}I-labeled human serum albumin are indicated. Data from subject 5 are shown; they are representative of a typical profile.

creased to 54%. In the control incubation, as before (Fig. 3), the amount of apoE associated with HDL decreased to a lesser extent (59%). The reciprocal changes were observed in VLDL. The fact that identical results were observed with both IgG and smaller Fab fragments suggests that the size of the antibody molecule is not a factor in the redistribution of apoE associated with CETP inhibition. A separate additional control incubation study (subject 3) was conducted at 4°C in the presence of Mab TP2 (8 μg/ml), also to determine whether apoE was being displaced from HDL because Mab TP2 IgG bound to CETP was occupying space on the surface of HDL. The amount of apoE associated with HDL following a 6-h incubation at 4°C with Mab TP2 was 82%, similar to the amount found associated with HDL following (80%) a control 6-h incubation at 4°C. Control incubation at 37°C for 6 h, however, resulted in an HDL-associated apoE of 72%, which was further decreased to 63% when Mab TP2 (8 μg/ml) was present. The inability of Mab

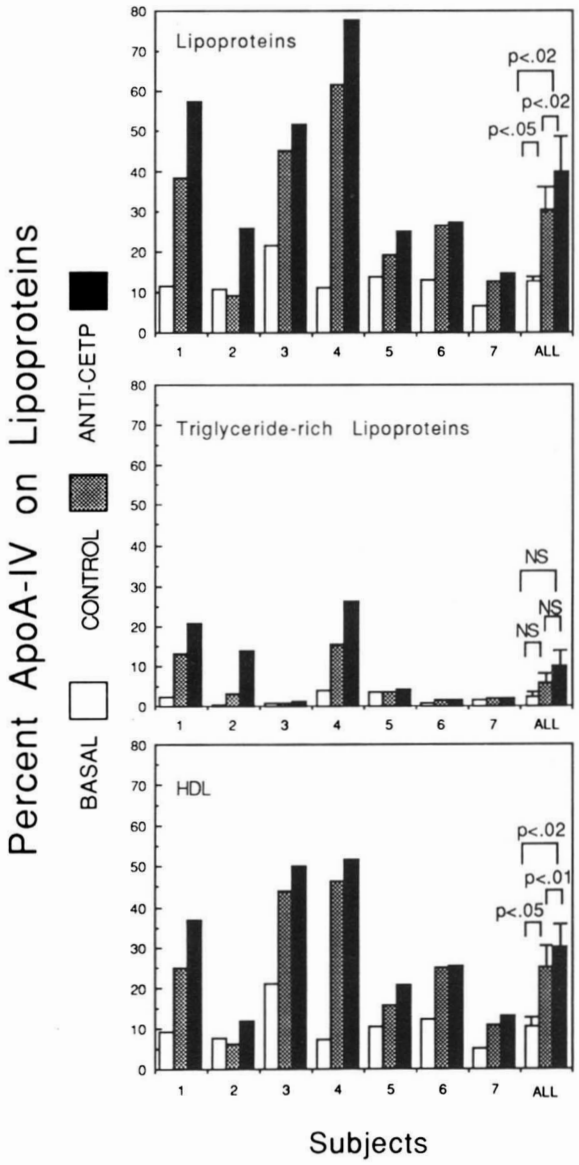

Fig. 2. Effect of cholesteryl ester transfer protein inhibition on distribution of apoA-IV among lipoproteins. Unincubated plasma or plasma incubated for 6 h at 37°C in the presence of control mouse monoclonal immunoglobulin (MOPC 21) or a mouse monoclonal antibody that inhibits human CETP activity (Mab TP2) was fractionated by Sephacryl S-300 chromatography and apoA-IV distribution was determined by radioimmunoassay as described in Materials and Methods. ApoA-IV content of each column fraction was determined from triplicate determinations. ApoA-IV eluted in three peaks, one associated with the void volume (combined VLDL-IDL-LDL peak), another with HDL-containing fractions, and the last eluting without lipoproteins. Values represent the percent of total apoA-IV contained in total lipoproteins (top figure), the VLDL-IDL-LDL peak (middle figure), and HDL (bottom figure). Probability levels for all subjects are derived from two-sided paired *t*-test analysis; NS, not significant.

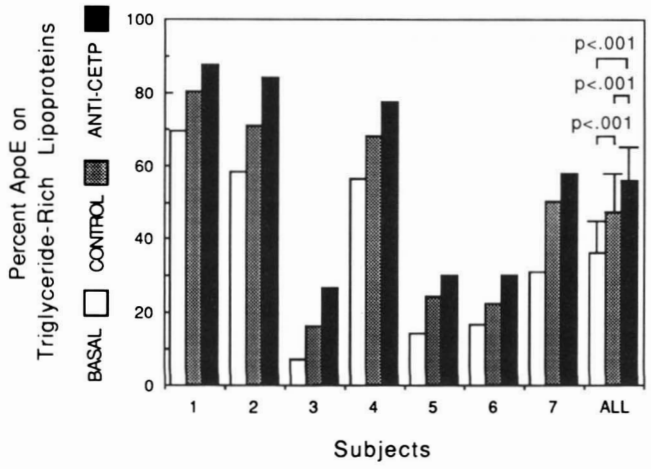

Fig. 3. Effect of cholesteryl ester transfer protein inhibition on distribution of apoE on triglyceride-rich lipoproteins. The apoE content of fractions described in Fig. 2 was determined by radioimmunoassay as described in Materials and Methods. ApoE content of each column fraction was determined from triplicate determinations. ApoE eluted in two peaks, a combined VLDL-IDL-LDL peak and an HDL peak. Values represent the percent of total apoE contained in the VLDL-IDL-LDL peak. Probability levels for all subjects are derived from two-sided paired *t*-test analysis.

TP2 to cause displacement of HDL apoE during a 4°C incubation suggests that the apoE redistribution observed at 37°C was specifically due to CETP activity inhibition and not due to the presence of Mab on the HDL surface.

To determine whether the decrease of apoE on HDL with CETP inhibition was due to displacement by apoA-IV, incubation studies were performed in the presence or absence of Mab TP2 with LCAT inactivated. Thus, additional plasma incubation studies were performed in the presence of Mab TP2 (8 μg/ml), the LCAT inhibitor DTNB (1 mM), or Mab TP2 plus DTNB. The distribution of apoA-IV and apoE among lipoproteins was determined in subject 3 (**Fig. 4**). Control incubations (active CETP and LCAT) increased HDL-associated apoA-IV, which was further increased when CETP was inactivated. When LCAT was inhibited with DTNB, HDL did not acquire any additional apoA-IV beyond the basal amounts present. However, under all incubation conditions apoE was displaced from HDL to triglyceride-rich particles. Incubation of plasma from subject 4 gave similar results (not shown). These experiments demonstrated that factors other than apoA-IV shifting to HDL are responsible for the displacement of apoE.

Nondenaturing gradient gel electrophoresis (Sudan Black B lipid-stained) of subject 3 plasma demonstrates HDL particle size distribution before and after incubations (**Fig. 5**). Under all incubation conditions both major HDL populations increased in size. When LCAT was active (lanes 2 and 3) the particles appeared to get larger than when LCAT was inactive (compare lanes 2 and 3 to lanes 4 and 5). However, when inhibited with DTNB, there was a complex change in the pattern of HDL (lanes 4 and 5). Interconversion factors (24) or HDL transformation factor (25) may have resulted in these observed changes in HDL size when LCAT was inactive. The inhibition of CETP activity had no additional effect on particle size (compare lanes 2 to 3 and lanes 4 to 5) whether LCAT was active or inactive. Thus, the changes in apoE and apoA-IV distribution produced by the monoclonal antibody were not due to a difference in the overall size or pattern of HDL subclasses.

DISCUSSION

The present studies suggest that inhibition of human plasma CETP activity increases lipoprotein-bound apoA-IV and causes a redistribution of apoE from HDL to VLDL, IDL, and LDL. Our present data show no causal relationship between apoA-IV enrichment of HDL and apoE enrichment of a VLDL, IDL, LDL fraction, since the redistributions were independent of each other during LCAT inhibition. The increased VLDL-IDL-LDL apoE content during 37°C incubations was modest; therefore it was necessary to assess a sufficient number of subject plasmas to assure this observation was statistically significant. In all subjects studied, VLDL-IDL-LDL apoE was consistently increased to a greater degree when CETP was

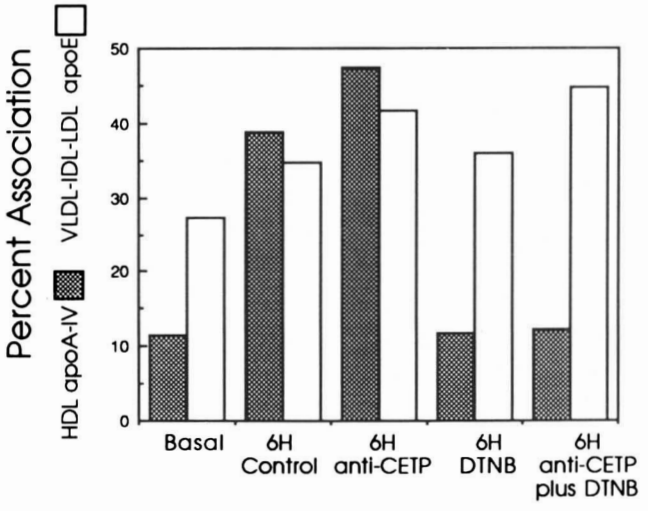

Fig. 4. Effect of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein inhibition on lipoprotein distribution of apoA-IV and apoE. Unincubated plasma (basal) or plasma incubated for 6 h at 37°C in absence or presence of a mouse monoclonal antibody that inhibits human CETP activity (Mab TP2 at 8 μg/ml plasma), DTNB (1 mM), or Mab TP2 plus DTNB from subject 3 was fractionated by Sephacryl S-300 chromatography and apoE and apoA-IV distributions were determined by radioimmunoassay as described in Materials and Methods. ApoE and apoA-IV content of each column fraction was determined from triplicate determinations. Values represent the percent of total apoA-IV contained in the HDL peak, and apoE in the VLDL-IDL-LDL peak.

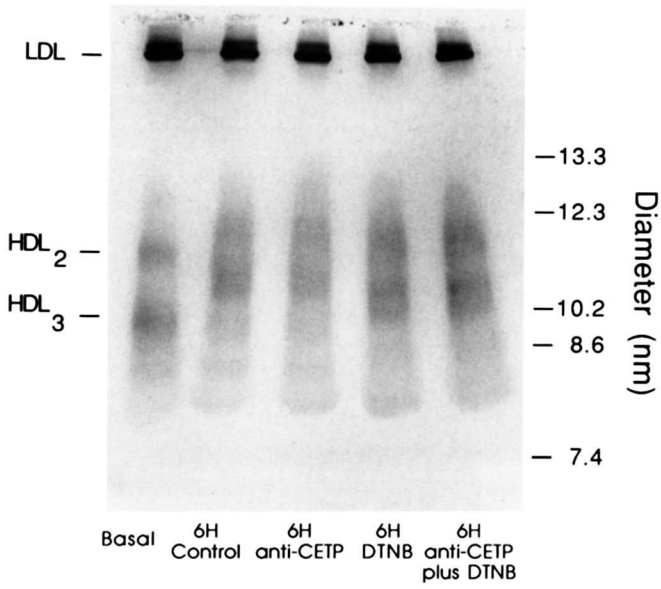

Fig. 5. Effect of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein inhibition on HDL particle size. Sudan Black B-stained gradient gel electrophoresis of plasma from subject 3. See legend to Fig. 4 for experimental conditions. To calibrate the gel, high molecular protein standards (Pharmacia) of thyroglobulin, 660K, diameter = 13.3 nm; apoferritin, 440K, diameter = 12.3 nm; catalase, 220K, diameter = 10.3 nm; lactic dehydrogenase, 140K, diameter = 8.6 nm; and albumin, 67K, diameter = 7.4 nm were applied to a lane on the gel and stained separately for protein.

inhibited. These in vitro data suggest that when CETP is continuously inhibited in vivo a new apoE steady state might be achieved leading to the lipoprotein changes observed in species-specific or human familial CETP deficiency. The apoE-enrichment of VDL-IDL-LDL could potentially facilitate their rate of clearance (26), perhaps contributing to low levels of VLDL and LDL in rats and in subjects with CETP deficiency (27, 28).

Prior studies in rats (5) and humans (15) demonstrate that LCAT activity resulted in apoA-IV enrichment of HDL; when LCAT was inhibited, apoA-IV became (5) or remained (15) essentially unassociated with lipoproteins. Thus, as the LCAT reaction progresses, surface phospholipid and cholesterol of HDL are consumed and in part redistributed to the particle core as cholesteryl esters (5, 15). The net result is the creation of enlarged particles with surface hydrophobic "gaps" and an increased affinity for apoA-IV (5, 15). In the present study, lipoprotein association of apoA-IV appeared to be largely due to LCAT action. However, when LCAT was active and CETP inhibited, significantly more apoA-IV became associated with HDL. The discriminating factor in plasma among species (i.e., rat versus human) or within species (normal versus human familial CETP deficiency) is CETP activity; LCAT is always active. In rats, in the steady state (active LCAT, inactive CETP) apoA-IV is largely associated with HDL. In humans (active LCAT and CETP) apoA-IV is largely unassociated. Perhaps, when CETP was inhibited, HDL became relatively more surface-deficient and acquired increased amounts of apoA-IV. In the recent studies of Yen et al. (23), Mab TP2, in addition to decreasing cholesteryl ester transfer from HDL to VLDL, also reduced the amount of phospholipid and cholesterol transferred from VLDL to the other lipoproteins. Perhaps, with CETP inhibition, there is less phospholipid and cholesterol available to replace that used by the LCAT reaction, leading to increased hydrophobicity of the HDL surface and increased binding of apoA-IV to HDL. Similarly, the increased content of VLDL phospholipid and cholesterol associated with CETP inhibition (23) could account for increased binding of apoE to VLDL.

The recent studies of Koizumi et al. (27) and Yamashita et al. (28) described subjects with human familial hyperalphalipoproteinemia associated with a CETP activity deficiency. These subjects have markedly elevated HDL cholesterol and HDL cholesterol to apoA-I ratios, enlarged HDL, and also low apoB levels. The present study suggests a mechanism that could link CETP deficiency and low levels of apoB-containing lipoproteins. CETP deficiency could cause apoE-enrichment of VLDL and IDL, augmenting their clearance and reducing the intravascular formation of LDL.

During lipolysis, CETP-dependent remodeling of apoB-100 VLDL and IDL leads to the production of the cholesteryl ester-rich LDL found in normal human plasma (2). CETP deficiency might lead to incomplete processing of VLDL to LDL, perhaps allowing the precursor particles to be preferentially cleared (27, 28). Efficient clearance of these precursors would result in reduced LDL levels. Since recent liposome studies (29) suggest that only apoE, and not apoA-IV or A-I, augments whole particle clearance, a CETP deficiency-induced depletion of HDL apoE could also contribute to hyperalphalipoproteinemia.

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