Inhibition of cholesteryl ester transfer protein in normocholesterolemic and hypercholesterolemic hamsters: effects on HDL subspecies, quantity, and apolipoprotein distribution

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Abstract The effects of cholesteryl ester transfer protein (CETP) inhibition on the serum lipoprotein profile in both normocholesterolemic and hypercholesterolemic hamsters has been determined following subcutaneous injection of 12.5 mg/kg of the CETP neutralizing monoclonal antibody, TP2. Inhibition of CETP activity was greater than 60% and resulted in a 30-40% increase in high density lipoprotein (HDL) in both normal and hypercholesterolemic animals. These HDL effects were observed 1 day post-injection, were maximal by 4 days, and returned to control values by 14 days. Inhibition of CETP activity resulted in a decrease in both low density lipoprotein (LDL) and very low density lipoprotein (VLDL) cholesterol concomitant with HDL increase, and in hypercholesterolemic animals resulted in increased total serum cholesterol. In addition to the quantitative differences in LDL and HDL, there were significant increases in the size of the HDL, a shift to smaller LDL particles, and changes in apolipoprotein (apo) composition as evaluated by FPLC and Western blot analysis. Large apoA-I-poor and apoE-containing HDL became prevalent in hypercholesterolemic hamsters after CETP inhibition. In addition, the size of the CETP-containing HDL particles increased with inhibition of transfer activity. While these effects were apparent in normocholesterolemic animals, the changes in apolipoprotein distribution and HDL subspecies as detected on native gels were more significant in the hypercholesterolemic animals. The changes in the HDL profile and apolipoprotein distribution after CETP inhibition in hamsters were similar to those reported in CETP-deficient Japanese subjects, suggesting the utility of the hypercholesterolemic hamster as an in vivo model for the understanding of the lipoprotein changes associated with CETP inhibition.—**Evans, G. F., W. R. Bensch, L. D. Apelgren, D. Bailey, R. F. Kauffman, T. F. Bumol, and S. H. Zuckerman.** Inhibition of cholesteryl ester transfer protein in normocholesterolemic and hypercholesterolemic hamsters: effects on HDL subspecies, quantity, and apolipoprotein distribution. *J. Lipid Res.* 1994. **35:** 1634-1645.

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Cholesteryl ester transfer protein (CETP) has been implicated in the process of reverse cholesterol transport through studies of CETP-deficient families (1, 2), in rabbits in which CETP activity has been inhibited (3, 4), and in murine CETP transgenic models (5, 6). CETP, a 74 kDa hydrophobic glycoprotein, promotes the transfer of cholesteryl ester from HDL to acceptor apoB-containing lipoproteins and a reciprocal transfer of triglycerides to HDL (7, 8). Cholesteryl ester transfer can result in hepatic clearance under normocholesterolemic conditions or possibly in LDL-cholesteryl ester accumulation during hypercholesterolemia. The reciprocal relationship between CETP activity and HDL levels has important implications in therapeutic strategies designed to increase HDL cholesterol.

Increased CETP has been associated with dyslipidemias (9, 10), nephrotic syndrome (11), and with some patients on probucol (12). Serum cholesterol and lipoprotein profiles reflect the increase in CETP activity by an overall decrease in HDL cholesterol, and a shift in the ratio of HDL_2 to HDL_3, with an increase in the amount of the smaller HDL_{3b} particles (13). Expression of CETP in C57BL/6 transgenic mice also results in a decrease in HDL cholesterol (5, 6) and the appearance of atherosclerotic lesions after maintenance on a cholesterol-rich diet (6).

While increased CETP activity is associated with decreased HDL cholesterol and a shift to smaller HDL particles, the converse has been investigated in rabbits (3, 4) and in CETP heterozygote and homozygote deficient Japanese subjects (1, 2, 14, 15). Infusion of polyclonal (3) and monoclonal (4) antisera against CETP resulted in increased HDL cholesterol and a shift to larger HDL parti-

Abbreviations: CETP, cholesteryl ester transfer protein; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; FPLC, fast protein liquid chromatography.

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cles in normocholesterolemic rabbits (3). These changes occurred without changes in apolipoprotein composition. Significant increases in the ratio of HDL_2 to HDL_3 particles were observed in Japanese subjects homozygous for the splicing defect in intron 14 of the CETP gene (1, 15). HDL_1 particles containing apoE were also apparent in these individuals as was a decrease in VLDL and LDL cholesterol (1, 2, 14–17). The shift in cholesterol mainly to the HDL fraction in homozygous and to a lesser extent in CETP-deficient heterozygotes suggests that pharmacologic inhibition of CETP activity may result in a more favorable serum cholesterol and lipoprotein profile. These concepts require verification in small animal models which lend themselves to drug discovery efforts.

The Syrian golden hamster is unique among the rodents in having a significant amount of serum cholesterol associated with the LDL fraction (18–20). Hypercholesterolemia in this model results in a significant increase in both LDL and VLDL cholesterol as well as a 2- to 3-fold increase in plasma CETP activity (18–22). Furthermore, these animals respond to standard clinical therapies for hypercholesterolemia with appropriate reductions in LDL and VLDL cholesterol (23, 24).

In the present study, the quantitative and qualitative changes in serum lipoproteins following CETP inhibition in both normocholesterolemic and hypercholesterolemic hamsters were determined and compared with the lipoprotein profile reported in CETP-deficient individuals. Inhibition of transfer activity with the CETP specific monoclonal antibody TP2 resulted in an increase in both the size and amount of HDL particles. Significant shifts in the distribution of apoE and CETP were detected in the serum lipoproteins from TP2-treated hypercholesterolemic hamsters. The qualitative and quantitative changes in serum lipoproteins in both normocholesterolemic and hypercholesterolemic hamsters after CETP inhibition reflects the serum lipoprotein profile observed clinically with CETP deficiency.

METHODS

Hamster model

Male Syrian hamsters weighing 120–175 g (Charles River Laboratories) were maintained on a normal (Purina 5001 rodent chow, Purina Labs) or cholesterol-rich diet (normal chow supplemented with 10% saturated coconut oil and 0.12% cholesterol) for a minimum of 1 week prior to use. Under these conditions normal hamsters had a total serum cholesterol between 100 and 150 mg/dl while the hypercholesterolemic animals had greater than 200 mg/dl. Hamsters were divided into three groups prior to the start of the experiment, those injected subcutaneously at days 0 and 4 with 10–15 mg/kg TP2 monoclonal antibody or a control IgG (Cappel, West Chester, PA), as well as non-injected controls, and at designated intervals sera samples were obtained by retro-orbital bleeding. TP2 antibody (25) was used as an ascites prepared by injection of pristane-primed Balb/c mice with the TP2 hybridoma cell line (provided by Dr. Allan Tall, Columbia University, NY).

CETP assay

Donor [^{3}H]cholesteryl ester-labeled HDL were prepared with greater than 90% of the labeled cholesterol being esterified and a specific activity of 14,000 dpm/μg protein (26). CETP activity in hamster sera was quantitated using the transfer assay described by Tall et al. (9) with minor modifications. Hamster sera, 5–10 μl, were incubated with ^{3}H-labeled HDL for 3–5 h in Tris-EDTA buffer, pH 7.4, containing 40 mg/ml BSA in the presence of 150 μg of human LDL. The percent transfer of [^{3}H]cholesteryl ester to the LDL acceptor was determined by heparin-manganese chloride precipitation of the LDL and quantitation of counts remaining with the HDL supernatant fraction. Nonspecific transfer in the absence of hamster sera was 26.7% and this value was subtracted from the calculations for total transfer activity.

Serum lipoproteins

Total serum cholesterol was quantitated by an enzymatic assay (Boehringer Mannheim, Indianapolis, IN). HDL cholesterol in normal animals was determined similarly after removal of non-HDL cholesterol by magnesium phosphotungstate precipitation. In CETP-inhibited hypercholesterolemic animals, HDL cholesterol could not be determined by this approach as significant amounts (up to 40%) of the total HDL were associated with the LDL and VLDL precipitate. Accordingly, changes in HDL cholesterol in hypercholesterolemic hamsters were evaluated by FPLC (Pharmacia, Bromma, Sweden) analysis using tandem Superose 6 columns (Pharmacia) (27). Sera pools of 250 μl were resolved by FPLC with 150 mM NaCl, 1 mM EDTA, 0.02% sodium azide, pH 8.2, as the column buffer. Fractions, 0.5 ml, were collected after disposal of the first 12 ml of void volume and assayed for total cholesterol.

Electrophoresis and immunoblot analysis of FPLC fractions

Sequential fractions from the VLDL, LDL, and HDL peaks were subjected to both denaturing and native gradient gels. For the denaturing gels, aliquots from each fraction were boiled in SDS sample buffer containing dithiothreitol and electrophoresed on 10–20% gradient gels. Native 3–27% gradient gels (Integrated Separation Systems, Natick, MA) were run in a Tris-borate buffer (28). Prestained molecular weight standards, 14.3–200 kDa (Amersham International, Buckinghamshire,

England), were run on SDS gels and large molecular weight standards, 67–669 kDa (Pharmacia), on native gels. After electrophoresis, gels were stained with Coomassie blue and parallel gels were electroblotted, 500 milliamps for 40 min, onto nitrocellulose using semi-wet blotting conditions (ABN Polyblot, American Bionetics, Inc., Emeryville, CA). Nitrocellulose blots were probed with polyclonal goat antisera against human apoA-I (Calbiochem, La Jolla, CA), apoE (Chemicon, Temecula, CA), a peroxidase-conjugated polyclonal rabbit anti-hamster sera (Rockland, Gilbertsville, PA), and with the TP2 monoclonal against CETP. Detection of immunoreactive bands was by enhanced chemiluminescence, ECL (Amersham), using the appropriate peroxidase-conjugated secondary antibody and the procedure described by the suppliers. Each nitrocellulose filter was probed, stripped by heating the filter at 50°C for 60 min in 100 mM 2-mercaptoethanol, 2% SDS in 62.5 mM Tris-HCl, pH 6.7, and reprobed following washing and blocking of the stripped filter. This procedure was repeated up to 5 times for each nitrocellulose blot. Film (Hyperfilm-ECL, Amersham) was exposed to the chemiluminescent signal from 5 sec to 20 min depending on the primary antibody used and the resulting band intensity. Bands were quantitated by densitometry and expressed as integrated optical density units using a Bio Image scanner and Visage software (Bio Image Products, Ann Arbor, MI). While differences in film exposure precluded quantitative comparisons of apoproteins in different FPLC runs or in relative amounts of the different apoproteins within a single FPLC run, the distribution and amount of each apoprotein relative to other fractions from the same sera were evaluated by using film exposures that were in the linear adsorption range. The identification of hamster apoA-I was based on electrophoretic mobility and sequence determination (data not shown). Hamster apoE and CETP were identified by immunologic cross-reactivity and electrophoretic mobility.

RESULTS

CETP activity was detected in both normal and hypercholesterolemic hamster sera as evidenced by the transfer of [^{3}H]cholesteryl ester from human HDL to LDL as acceptor. Using 10 μl of hamster sera, transfer activity was linear over a 5-h incubation period (**Fig. 1A**). Furthermore, in agreement with previous studies in hamsters (12, 21, 22, 29), rabbits (30), and man (9, 10), hypercholesterolemia was associated with an approximate 2-fold increase in transfer activity. Accordingly, all subsequent experiments were based on quantitation of the amount of labeled cholesteryl ester transferred over a 3- to 4-h incubation period.

While TP2 has been reported to inhibit transfer activity in rabbits and man (4, 25), it was necessary to determine its efficacy towards hamster CETP. In vitro experiments with normal hamster sera suggested that addition of 500 ng of TP2 monoclonal antibody inhibited cholesteryl ester transfer by 60–70% (Fig. 1B). Addition of 10-fold excess of antibody did not result in any further inhibition. Partial inhibition was still observed with 50 ng

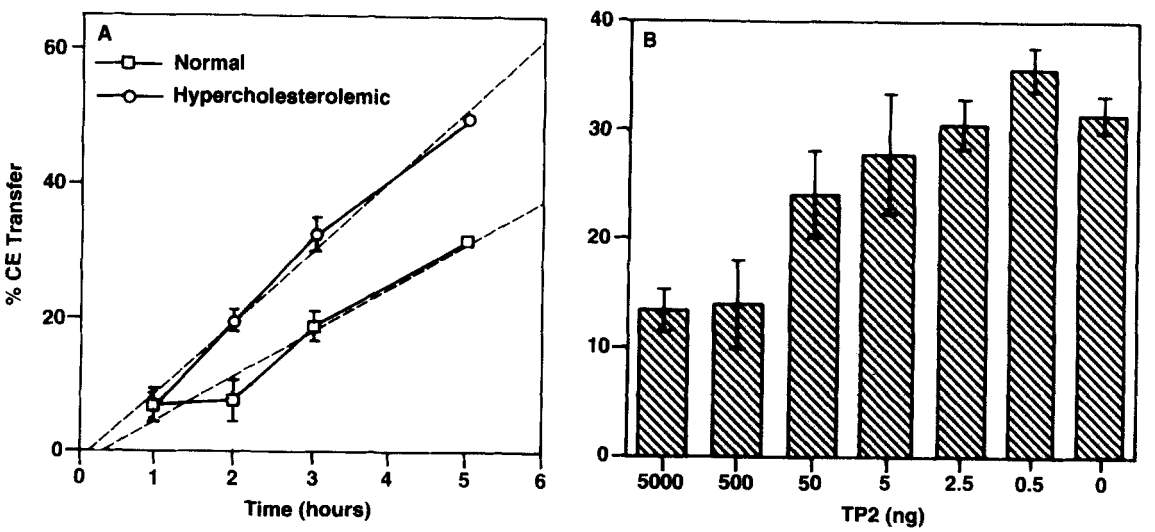

Fig. 1. In vitro hamster sera CETP assay. Normal or hypercholesterolemic hamster sera were assayed in triplicate by incubating 10 μl of sera with labeled human HDL as the cholesteryl ester donor and LDL as the acceptor. At designated intervals, LDL were precipitated and the cpm remaining in the supernatant were quantitated and expressed as percent cholesteryl ester transfer. A: Kinetics of assay were linear for both normal (r^2 = 0.952) and hypercholesterolemic (r^2 = 0.990) sera. B: CETP activity in normal hamster sera was inhibited in a dose-dependent manner with TP2 monoclonal antibody using a 3-h incubation period for cholesteryl ester transfer. The inhibition of transfer activity was statistically significant at 50 ng ($P < 0.02$) and at 500 and 5000 ng ($P < 0.001$) by a Student's two-tailed t test. Representative experiment of three. Brackets indicate SD.

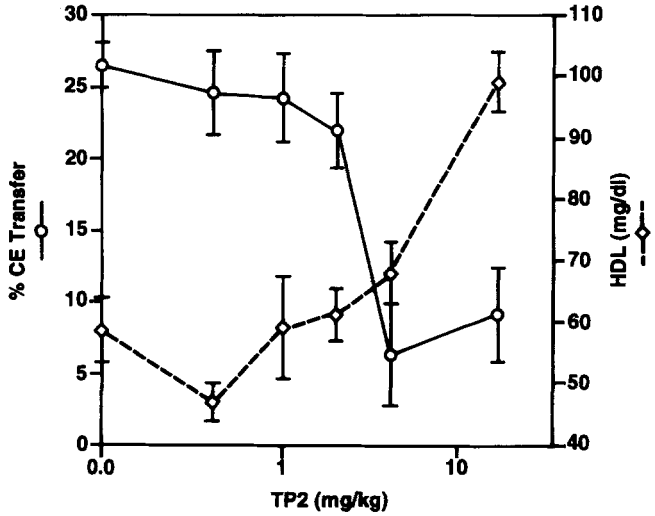

Fig. 2. Titration of TP2 in normal hamsters. Hamsters in groups of three were injected with 0–16.7 mg/kg of TP2 subcutaneously and were bled 2 days later. Percent cholesteryl ester transfer and serum HDL cholesterol levels are plotted on the left and right y axes, respectively. Brackets indicate SEM. Increases in HDL were statistically significant by a two-tailed Student's t test at the 16.7 mg/kg TP2 dose ($P < 0.01$) while inhibition of transfer activity was significant ($P < 0.01$) at both the 16.7 and 4.2 mg/kg doses.

of antibody while lower concentrations were without any significant effect. These results demonstrated that TP2 inhibited hamster CETP activity in a concentration-dependent manner, with an approximate IC_{50} of 10 μg/ml.

In vivo inhibition of CETP activity was also apparent after subcutaneous injection of TP2 in normal hamsters. As demonstrated in **Fig. 2,** hamsters injected with 0.5–16.7 mg/kg of TP2 had reduced CETP activity 48 h after injection. The decrease in CETP activity was dose-dependent and associated with a concomitant increase in HDL cholesterol. As was previously demonstrated in vitro, maximal inhibition of serum cholesteryl ester transfer activity did not exceed 60–75%.

As 12.5 mg/kg of TP2 consistently resulted in maximal inhibition of CETP activity, all subsequent experiments involved subcutaneous injection with this dose. Furthermore, for kinetic studies TP2 was again administered 4 days after the first injection and hamsters were followed for an additional 10 days with retroorbital bleeds at 3-day intervals. Using this protocol, TP2 inhibited CETP activity in both normal and hypercholesterolemic hamsters (**Fig. 3**). The inhibition in CETP activity mediated by TP2 was reversible and control levels of transfer activity were detected by 14–18 days. Although CETP activity was approximately 2-fold greater in the hypercholesterolemic hamsters, TP2 resulted in a comparable degree of inhibition of transfer activity. Furthermore, as with the normocholesterolemic hamsters, maximal inhibition of transfer activity in hypercholesterolemic hamsters was apparent 24 h after the initial antibody injection (data not shown). Injection of a pooled mouse IgG control at 12.5 mg/kg did not result in any significant effect on CETP activity in normo- or hypercholesterolemic hamsters.

Inhibition of CETP activity in normocholesterolemic animals resulted in a significant increase in HDL cholesterol (**Table 1**). Maximal increases in HDL cholesterol

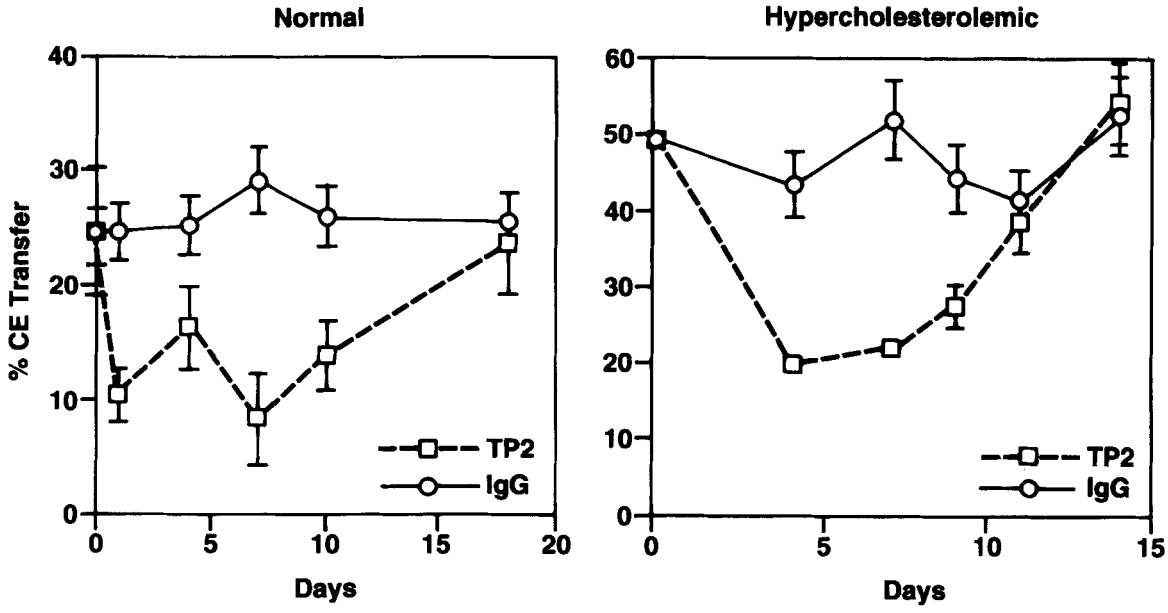

Fig. 3. In vivo inhibition of CETP activity in normal and hypercholesterolemic hamsters. Hamsters were injected at days 0 and 4 with 12.5 mg/kg of TP2 or an irrelevant mouse IgG. At intervals between 1–18 days, hamsters in groups of 3–6 were bled and CETP activity was determined using the 3-h activity assay. Brackets indicate SD. The inhibition of transfer activity in the TP2-injected normal hamsters at 1–10 days was significant by a two tailed Student's t test ($P < 0.001$). In the hypercholesterolemic hamsters, the inhibition of transfer activity by TP2 relative to the control or day 0 was statistically significant by a two-tailed Student's t test ($P < 0.001$ at days 4 and 7 and $P < 0.01$ at day 9).

TABLE 1. Effect of CETP inhibition on serum cholesterol in normal hamsters

Days[a]	Total Cholesterol[b]	HDL	LDL + VLDL	% CE Transfer
0	126.4 (8)[c]	82.7 (6.52)	43.67 (8.99)	24.73 (2.34)
2	132.54 (10.6)	102.94 (9.36)[d]	29.61 (4.86)[d]	10.38 (2.9)[e]
4	116.41 (6.83)	98.42 (4.47)[e]	17.98 (9.23)[e]	16.42 (2.53)[e]
7	124.29 (13.46)	92.91 (9.33)[f]	31.38 (9.89)[g]	8.37 (2.41)[e]
10	118.88 (11.2)	85.63 (7.85)	33.25 (5.94)[g]	14.09 (3.69)[e]
18	105.84 (7.77)	71.3 (7.78)	34.54 (3.98)[g]	23.57 (3.68)

[a]Days post TP2 injection.
[b]Total cholesterol, HDL, and LDL + VLDL cholesterol determinations were based on six animals.
[c]Parentheses indicate standard deviation. All tests for statistical significance were based on a two-tailed Student's t test relative to the day zero control. The following values were determined to be significant: [d]$P < 0.01$; [e]$P < 0.001$; [f]$P < 0.1$; and [g]$P < 0.05$.

were observed 2-4 days after antibody injection and were no longer apparent by 10 days. Associated with the increase in HDL cholesterol was a significant reduction in LDL + VLDL cholesterol. The increase in HDL cholesterol resulted in a nonstatistically significant trend towards increased total serum cholesterol in CETP-inhibited hamsters. A statistically significant increase in total serum cholesterol was, however, apparent after CETP inhibition in hypercholesterolemic hamsters (**Table 2**).

Coprecipitation of HDL with the total LDL fraction in CETP-inhibited hypercholesterolemic hamsters (data not shown) necessitated that qualitative or quantitative changes in serum lipoproteins in this model be analyzed after serum fractionation by FPLC. Accordingly, sera pools from hypercholesterolemic hamsters injected with TP2 or the control IgG were separated by FPLC at day 0, prior to injection, or at 4, 7, 9, 11, and 14 days post injection (**Fig. 4**). Inhibition of CETP caused a significant increase in both the amount and the size of the HDL particles (Fig. 4). An increase in HDL particles of larger size was detected by a shift in their elution with the well-defined valley between the LDL and HDL peaks no longer apparent. These effects, maximal at 4 and 7 days post injection, were associated with a significant decrease in VLDL cholesterol. Similar changes in both LDL and HDL particles were still observed 9 days post injection and by 14 days, the lipoprotein profiles between the TP2- and IgG-injected animals were similar with a partial reduction in the LDL peak still detected.

The increase in HDL particles of larger size after CETP inhibition in hypercholesterolemic hamsters was also apparent, although to a lesser extent in normal hamsters. As demonstrated in **Fig. 5,** at both 1 and 7 days after TP2 injection, the well-defined valley between the LDL and HDL peaks was less obvious with the shift in HDL towards a larger particle (Fig. 5). These effects, as with the hypercholesterolemic hamsters, were reversible and by 18 days post injection, the serum lipoprotein profiles were similar to the control.

An analysis of the quantitative and qualitative changes in apoproteins between the serum lipoprotein particles was then performed by subjecting fractions from the VLDL, LDL, and HDL peaks to SDS gel electrophoresis followed by electrophoretic transfer of the resolved apolipoproteins and immunoblot detection by enhanced chemiluminescence. Total proteins associated with each lipoprotein fraction were evaluated by both Coomassie blue stain (data not shown) and by immunoblot using a polyclonal rabbit anti-hamster sera. In each experiment, the same nitrocellulose blot was probed for apoA-I, apoE, CETP, or for total hamster serum proteins with the relevant antisera and, after detection, the nitrocellulose was stripped and reprobed with the next primary antisera. The utility of this approach and the resolution of the immunoblots is presented for a CETP-inhibited hypercholesterolemic hamster pool, 4 days after TP2 injection. As demonstrated in **Fig. 6,** anti-hamster sera revealed multiple serum proteins across the LDL peak, fractions 18-26, and in fractions 30-40 corresponding to the HDL peak. ApoA-I was present in fractions 28-40 with the maximal intensity colocalizing with the HDL cholesterol peak. The additional higher molecular weight bands detected be-

TABLE 2. Total serum cholesterol in CETP inhibited hypercholesterolemic hamsters

Days[a]	TP2	Control
0	318.64[b] (20)[c]	305.07 (23)
1	365.62 (11)	325 (19)
4	365.18 (21)	307 (35)
7	411.11 (25)	322 (37)
9	399.72 (29)	282 (32)
11	374.87 (31)	282 (32)

[a]Days post injection with 12.5 mg/kg of TP2, injections at days 0 and 4.
[b]Total serum cholesterol, mg/dl, after maintenance on the cholesterol-rich diet for 2 weeks prior to antibody injection.
[c]Parentheses indicate the SEM based on three animals per group. Differences in serum cholesterol between the control and TP2-injected groups were significant by a two-tailed Student's t test at days 1, 7, 11 ($P < 0.1$) and 9 ($P < 0.05$).

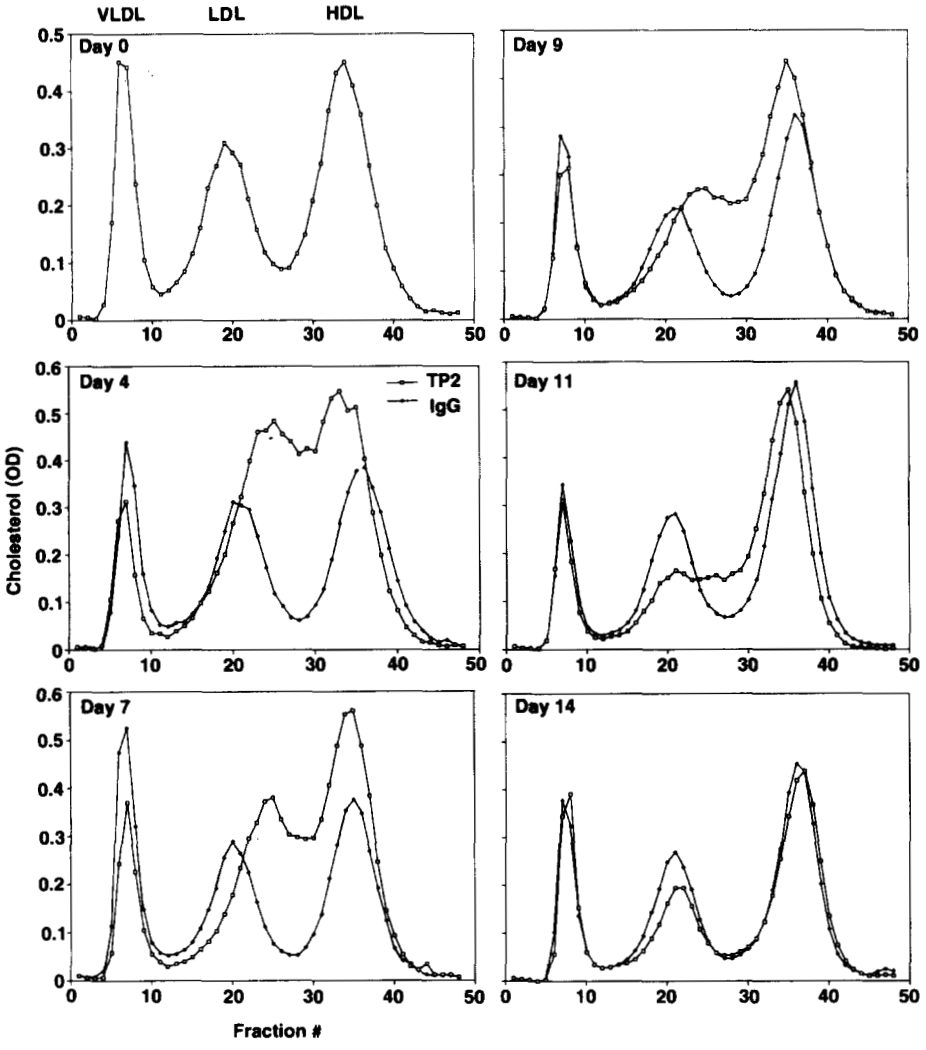

Fig. 4. Effect of CETP inhibition on serum lipoprotein cholesterol profiles in hypercholesterolemic hamsters. Hypercholesterolemic hamsters with an initial serum cholesterol between 180–200 mg/dl were injected with 12.5 mg/kg of TP2 at days 0 and 4. At 0, 4, 7, 9, 11, and 14 days post initial injection, sera were pooled from each group of six animals and 250-μl aliquots were chromatographed by FPLC on tandem-linked Superose 6 columns. The positions of the VLDL, LDL, and HDL peaks were determined by use of labeled standards. Chromatographic runs were for 75 min and 0.5-ml fractions were collected and assayed for total cholesterol by enzymatic techniques. The locations of the LDL, HDL, and VLDL peaks in the IgG-treated control hamsters were identical to that observed in parallel noninjected controls at each of the time points. Representative experiment of three.

tween fractions 34 and 36 have not been identified but may represent apoA-I multimers. ApoE did not localize in the same fractions as A-I and appeared as a doublet between fractions 20–28 and in the VLDL fraction 7. The appearance of two discrete bands has been previously reported and may reflect posttranslational modifications of apoE (31). Finally, CETP was detected in the ascending shoulder of the HDL region and in the cholesterol-containing fractions between the HDL and LDL peaks that appeared after CETP inhibition. The 28 kDa band observed in the apoE and CETP immunoblots was apoA-I which, due to its abundance, was frequently detected by other antisera when blocking conditions were not optimal.

Densitometric scans of the immunodetected apolipoproteins in the FPLC fractions permitted graphical depiction of changes in apolipoprotein distribution across the lipoprotein peaks. As demonstrated in hypercholesterolemic hamsters 7 days after injection of TP2 (**Fig. 7A, C, E**) or IgG (Fig. 7B, D, F) significant shifts in HDL cholesterol were associated with changes in apolipoprotein distribution. The overall increase in the size of the HDL particles seen after CETP inhibition, occurred without a significant shift in apoA-I (Fig. 7A, B). CETP (Fig. 7C, D) demonstrated a more significant shift in distribution with the peak in the control animals occurring between fractions 30–32 which still represents the larger HDL particles, while CETP was localized between frac-

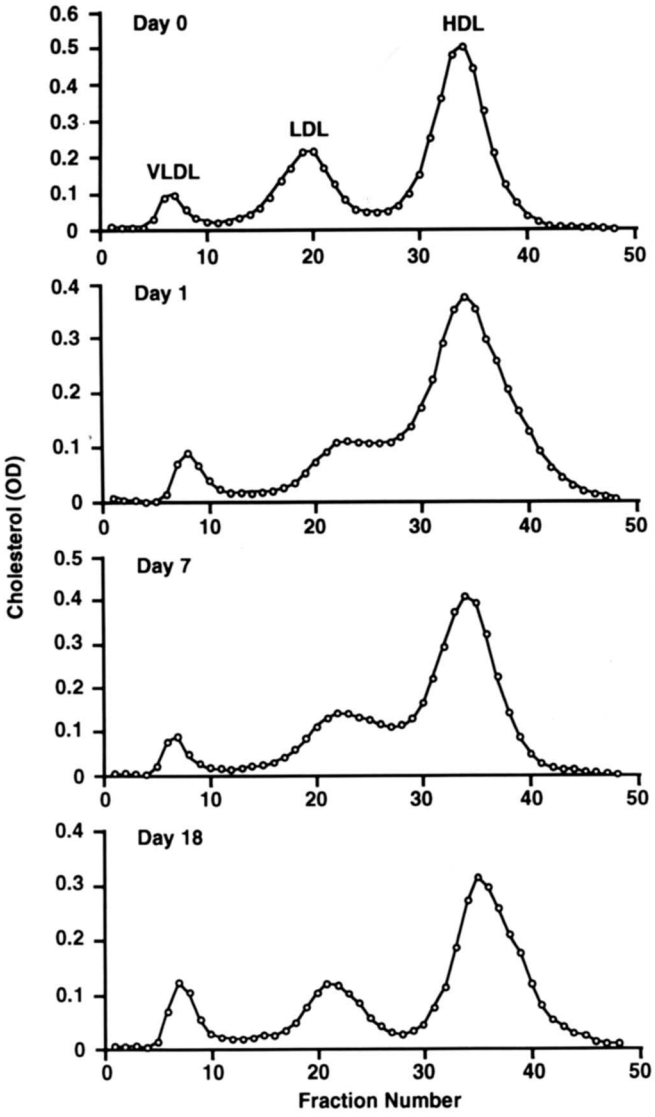

Fig. 5. Effect of CETP inhibition on serum lipoprotein cholesterol profiles in normal hamsters. Control hamsters with serum cholesterol values between 120–150 mg/dl were injected with 12.5 mg/kg of TP2 on days 0 and 4 and pooled sera samples from days 0, 1, 7, and 18 were chromatographed by FPLC. Total cholesterol on the FPLC fractions was quantitated by enzymatic assay. Note the increase in cholesterol-containing lipoprotein in particles between the LDL and HDL peaks at both days 1 and 7.

tions 22–30 in TP2-treated animals with peak activity detected at fraction 26. The immunolocalization of CETP to the larger HDL particles in the control hamsters was consistent with transfer activity assays performed on each of the fractions (data not shown). Transfer activity assays also demonstrated a second peak of CETP activity localized to fractions 48–54 which represented CETP not associated with a cholesterol-containing particle (data not shown). Finally, apoE (Fig. 7E, F), which was only observed in VLDL (data not shown) and not in the HDL or LDL fractions, became apparent within fractions 22–28 in TP2-injected animals.

Whether the significant shifts in the serum apolipoprotein and cholesterol profiles after in vivo inhibition of CETP activity in hypercholesterolemic hamsters reflected quantitative or qualitative changes in HDL species was evaluated by native gel analysis of the FPLC-resolved lipoprotein fractions (**Fig. 8**). Coomassie blue-stained native gels identified particles with a size range similar to LDL extending from fractions 20–32 and smaller HDL-like particles between fractions 30–40. ApoA-I-containing

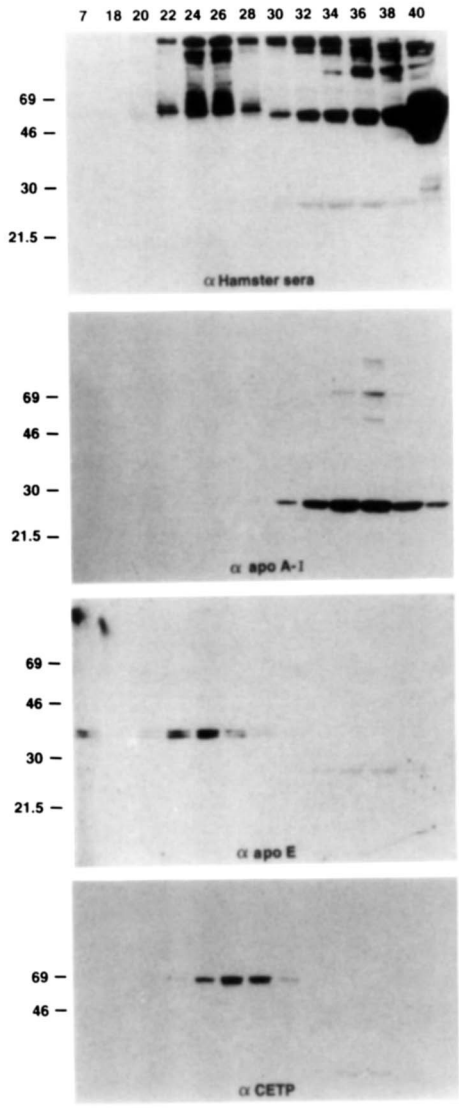

Fig. 6. Immunodetection of apolipoproteins in FPLC resolved serum lipoproteins. Hypercholesterolemic hamsters 7 days after TP2 injection were bled, sera were pooled from groups of six and chromatographed by FPLC. Aliquots, 100 μl, from each of the cholesterol-containing fractions were diluted with 5× SDS sample buffer, boiled, and electrophoresed on 10–20% denaturing gradient gels. Gels were electroblotted to nitrocellulose and probed with polyclonal antisera specific for hamster sera, human apoA-I, human apoE, and with TP2 monoclonal antibody specific for CETP. Detection of immunospecific bands was by enhanced chemiluminescence and exposure times from 10–13 min are presented. Fraction numbers and molecular weight markers are indicated.

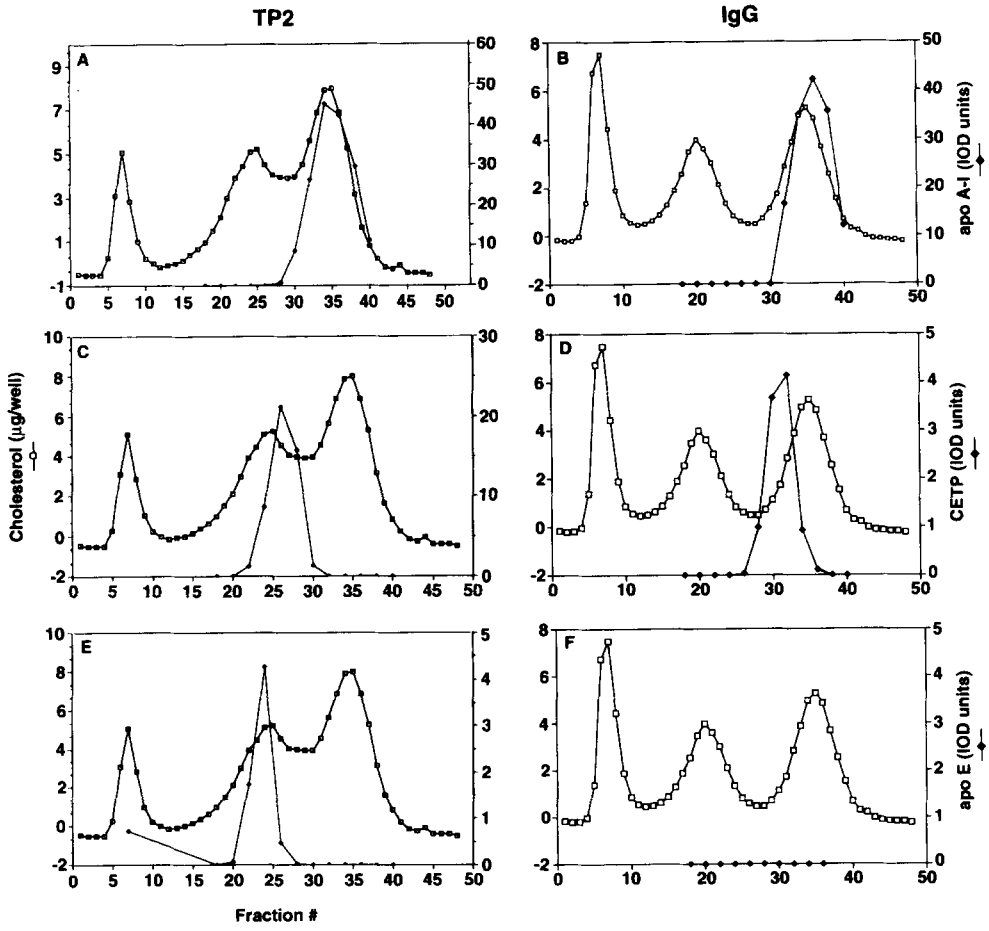

Fig. 7. Distribution of apolipoproteins in FPLC fractions. Sera from TP2- (A, C, E) and IgG (B, D, F)-treated hypercholesterolemic hamsters, 7 days post initial injection, were separated by FPLC and fractions were analyzed for cholesterol as well as for apolipoproteins by immunoblot analysis of the SDS gels. The gels for the TP2-treated hypercholesterolemic hamsters are presented in Fig. 6 and the scans are superimposed over the cholesterol values for each fraction. TP2- and IgG-treated hamster lipoprotein fractions were evaluated for the presence of apoA-I (A, B), CETP (C, D), and apoE (E, F). ECL exposures within a given panel were constant across the gel but varied among the six separate immunoblots presented. Accordingly, while the position of the apolipoprotein is accurate, the relative amounts of each of the apolipoproteins evaluated cannot be determined from these scans. Note the absence of apoE in fractions 18–36 from IgG-treated hamsters (F).

particles were detected within fractions 26–40 and migrated between the 140 and 440 kDa markers. Further antibody dilution and changes in substrate incubation conditions did not significantly improve the resolution of the A-I immunoblots on the native gels. CETP was detected within fractions 26–34 and appeared to colocalize with A-I particles resolved on the ascending shoulder of the HDL peak. ApoE-containing particles were not detected in any of the LDL- or HDL-containing fractions (data not shown).

Similar native gel analysis of the serum lipoproteins from hypercholesterolemic hamsters 7 days after the primary TP2 injection demonstrated significant differences in the apolipoprotein distribution (**Fig. 9**). Coomassie blue-stained gels revealed a qualitative pattern similar to the IgG controls, although the amounts of several discrete species in fractions 34–40 were increased. In addition, an increase in Coomassie blue staining material was apparent in fractions 28–32 with a molecular weight between 440 and 669 kDa. ApoA-I-containing particles were apparent between fractions 24–40 with the size of the A-I-containing particles increasing in fractions extending between the LDL and HDL peaks. ApoA-I-containing particles were present within the 440–669 kDa molecular weight range in contrast to the control IgG hamsters. CETP was shifted to fractions 22–28 and as with A-I appeared to localize to larger particles in the TP2-treated hamsters.

Finally, in distinction to the IgG controls, apoE was detected in larger particles with molecular weights greater than 669 kDa within fractions 23–32. The apoE-rich particles detected within fractions 23–27 were apoA-I-poor in

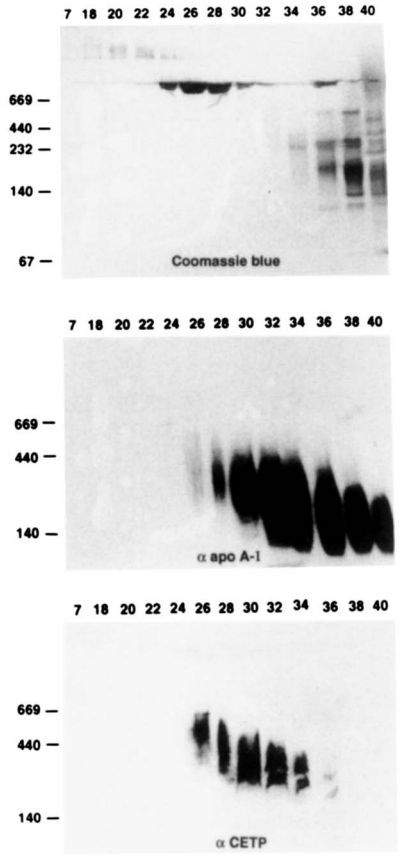

Fig. 8. Distribution of HDL species in hypercholesterolemic hamsters. Day 7 hypercholesterolemic hamster sera resolved by FPLC was electrophoresed on 3–27% native gels. Parallel gels were stained with Coomassie blue. Electroblots from fractions 7 and 18–40 were probed for apoA-I and CETP. The presence of apoE-containing particles could not be detected on these immunoblots. Representative gel from three separate experiments.

comparison to the apoA-I detected within the HDL peak fractions 30–40. Therefore, the inhibition of CETP in hypercholesterolemic hamsters results in an increase in larger apoE-rich HDL particles with a discrete size distribution between the control LDL and HDL peaks.

DISCUSSION

The Syrian hamster has served as a useful model for the understanding of cholesterol metabolism and transport as many aspects of these metabolic processes are similar to those in humans. These include similarities in hepatic cholesterol synthesis rates, the relative importance of receptor dependent and independent cholesterol transport, and the response of plasma LDL cholesterol to dietary manipulations (18–24). In addition to increases in LDL cholesterol in response to a cholesterol-rich diet, CETP activity also increases 2- to 3-fold over normocholesterolemic animals (12, 21, 22, 29). While adipose tissue in hamsters, rabbit, and humans contains significant quantities of CETP mRNA, hamster differs from human in that liver is not an abundant source for CETP mRNA in hamsters (12, 29). However, in both species adipose CETP mRNA and plasma CETP levels increase with high cholesterol diets. Hamster CETP at the protein level is 81.7% identical with the human sequence (29) and this may explain TP2 inhibition of hamster serum CETP activity observed in the present study.

Inhibition of hamster CETP activity resulted in three significant changes in the serum lipoprotein profile. First was the increase in HDL cholesterol in both normal and

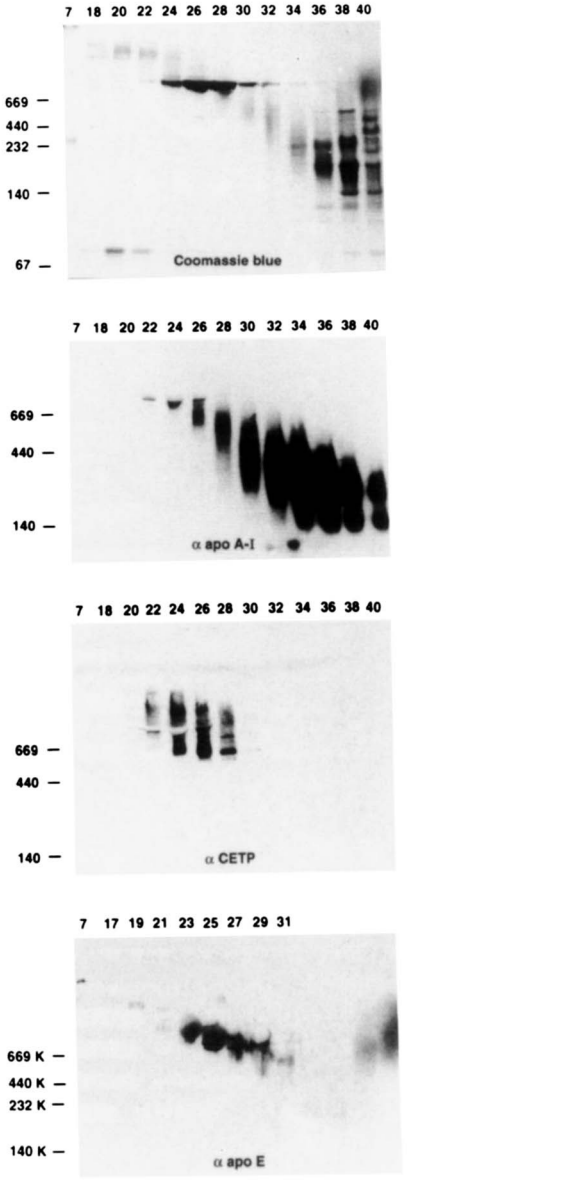

Fig. 9. Effect of CETP inhibition on HDL species in hypercholesterolemic hamsters. Sera pools from hypercholesterolemic hamsters 7 days after the initial TP2 injection were separated by FPLC and fractions were analyzed on native gels as indicted in Fig. 8. Gels were Coomassie blue stained or electroblotted and probed for apoA-I, CETP, and apoE. Representative gel from three separate experiments.

hypercholesterolemic hamsters. Increases in HDL cholesterol were apparent 24 h after TP2 injection and remained elevated for approximately 10 days. In these experiments, there was no attempt to perform parallel studies with only a single TP2 injection or with suboptimal antibody concentrations. The elevation in HDL cholesterol in the hypercholesterolemic animals was determined by FPLC fractionation as these larger HDL particles precipitated with LDL using standard precipitation procedures. The degree of HDL cholesterol elevation in TP2-injected hamsters was similar to the increase observed in heterozygote CETP-deficient patients (1, 14–17). To this extent, it is important to note that in both in vitro and in vivo studies, attempts to inhibit 100% of the transfer activity were unsuccessful suggesting that the difference in sequence homology between hamster and human was sufficient to reduce TP2 affinity or alternatively that transfer can occur by a second lipid transfer protein (32). The increase in HDL cholesterol was associated with a decrease in LDL and VLDL cholesterol after CETP inhibition in normal hamsters. As CETP has been proposed to play a central role in reverse cholesterol transport (7, 8), inhibition of transfer activity would be anticipated to result in an increase in total serum cholesterol, primarily through the accumulation of HDL-cholesterol. The reduction in cholesteryl ester transfer to LDL and VLDL would contribute to a decrease in the rate of hepatic and extrahepatic cholesterol clearance and result in an increase in total serum cholesterol as has been described in CETP-deficient subjects (1, 2, 14, 15) and as evident in the total serum cholesterol measurements in the CETP-inhibited hamsters (Table 2).

The second significant change was the shift in lipoprotein size after CETP inhibition in both normal and hypercholesterolemic hamsters. Whereas LDL and HDL were clearly resolved as two distinct peaks by FPLC, the area between these two peaks becomes cholesterol-rich after CETP inhibition. Inhibition of transfer activity resulted in both the appearance of larger HDL particles as well as a shift in the LDL peak to smaller size particles. The changes in HDL were more pronounced in the hypercholesterolemic hamsters and presumably reflect the appearance of HDL_c-like particles with a density between apoB- and apoA-I-containing lipoproteins. The accumulation of these large HDL particles presumably reflects the decreased catabolism of HDL in the CETP-inhibited hypercholesterolemic hamster. An increase in a similar size HDL species was also apparent in normal hamsters after CETP inhibition. In both dietary models, the appearance of the larger HDL particles was observed 1–2 days after inhibition of transfer activity and the loss of these larger HDL particles reflected the return of normal transfer activity by 2 weeks post TP2 injection. The shift to smaller LDL particles after CETP inhibition was also more significant in the hypercholesterolemic animals and may be related to inhibition of cholesteryl ester transfer to the LDL fraction.

The third major observation in the present study was that changes in the size of the HDL particles were associated with significant differences in apolipoprotein composition across the lipoprotein fractions. Whereas apoA-I did not show a significant shift in relative distribution within the major HDL peak after CETP inhibition, the larger HDL particles did contain apoE and CETP. These changes were observed in three separate experiments with hypercholesterolemic hamsters 4–7 days after inhibition of transfer activity. The changes in apolipoprotein distribution observed are in contrast to earlier studies performed in normal cholesterolemic rabbits (3, 4). In those studies, the only significant change was the presence of serum amyloid A on HDL, presumably reflecting the induction of an acute phase response in antibody-injected rabbits (3). Whether the shifts in apolipoprotein distribution in the present study were related to the longer interval during which CETP-inhibited animals were monitored or reflect differences in species or serum cholesterol levels remains to be determined.

The appearance of larger apoE-rich, apoA-I-poor HDL particles in CETP-inhibited hypercholesterolemic hamsters reflects the lipoprotein profile observed in CETP-deficient patients with the accumulation of HDL_c (1, 2, 14–17). In an attempt to further resolve the HDL subspecies present within the FPLC fractions, native gels were run and apolipoprotein distribution within the HDL subspecies was evaluated. The appearance of apoA-I within multiple-sized particles was similar to the pattern observed for A-I in human lipoproteins resolved by native gradient gels (28, 33). In addition, in both native and denaturing gels, CETP was associated with larger HDL particles in the non-TP2-injected hypercholesterolemic hamsters. This is in contrast to human CETP which has been reported to be localized to HDL_3 and VHDL with less than 10% associated with HDL_2 (13, 34, 35). The relative contributions of hypercholesterolemia, species, and methods of HDL preparation are all factors in explaining the differences observed in the present study and require further consideration.

The results of the native gels also demonstrated the presence of larger A-I particles following CETP inhibition. Furthermore, the native gel distribution of CETP after inhibition of transfer activity suggests that the antibody-HDL immune complexes were not selectively cleared from the circulation but rather these particles increased in size and became A-I poor. Finally, antibody treatment may also induce a shift in the association of CETP to other lipoproteins such as with LDL and this may also explain part of the CETP distribution observed on the native gels from the TP2-treated animals.

CETP deficiency in man is associated with an increase in serum apoA-I, apoA-IV, and apoE, and reduced apoB as well as a shift towards an increase in the ratio of HDL_2/HDL_3 (1, 2, 14–17). These increases in HDL_2 and apoA-I presumably reflect the decreased catabolism of HDL in the absence of functional CETP activity. The shifts in apoE to larger HDL particles in CETP-deficient homozygotes, as well as the appearance of cholesterol-containing lipoproteins between the HDL and LDL peaks, have been reported for homozygous CETP-deficient individuals (2, 14–17) and are similar to the changes observed in the hamster lipoprotein profile after CETP inhibition. However, as 30–40% of the original CETP activity is observed in vivo despite antibody excess, the hamster model would more closely reflect the lipoprotein profile associated with CETP-deficient heterozygotes. The slight shift in apoA-I in heterozygotes relative to the more significant shift in apoA-I towards larger particles in the homozygote state (17) is consistent with this interpretation. However, in contrast to the CETP-deficient heterozygotes (15), a 50% reduction in hamster CETP activity results in a HDL and LDL size shift comparable to that reported for CETP-deficient homozygotes. In other studies, incubation of human sera with TP2 for up to 6 h resulted in an increase in apoA-IV associated with HDL and the appearance of apoE on larger HDL particles (36). Clearly, the in vitro studies demonstrating a shift in apoE with inhibition of human transfer activity as well as the increase in large apoE-containing HDL particles with CETP deficiency are consistent with the apoE distribution observed in the present study.

Cholesterol and lipoprotein metabolism in the Syrian hamster has served as an important model for understanding cholesterol homeostasis in man. The results of the present study demonstrate that the hamster may also represent a useful model for understanding the consequences of blocking reverse cholesterol transport during hypercholesterolemia through the inhibition of CETP. Whether CETP inhibition also results in changes in LDL heterogeneity (37), in increased hepatic and extrahepatic synthesis of apoE, and alters the ability of the HDL fraction to mediate cholesterol efflux or foam cell formation in vitro are studies that are currently underway.

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