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# Cholesteryl Ester Transfer Protein Reaction between Plasma Lipoproteins[1]

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**The rate of the non-directional transfer of cholesteryl ester and triglyceride by human cholesteryl ester transfer protein (CETP) was measured between human plasma lipoproteins by monitoring fluorescence spectrum of pyrene-labeled lipid. The transfer rates between high density lipoproteins (HDLs) and between low density lipoproteins (LDLs) were both directly proportional to the substrate lipid concentration within the physiological range of the lipoprotein concentration. Higher preference of cholesteryl ester transfer to triglyceride was demonstrated with HDL than LDL. Although the highly selective binding of CETP to HDL was observed in the electrophoretic analysis, the transfer rate was only moderately higher with HDL for cholesteryl ester and not so at all for triglyceride. In addition, the rate of cholesteryl ester transfer between LDLs was uninfluenced by the presence of a small amount of HDL that is just sufficient to absorb all the CETP in the reaction mixture. The results indicated the preferential transfer of cholesteryl ester over triglyceride by CETP in the interaction with HDL in non-directional lipid transfer reaction among lipoproteins. However, the apparent binding of CETP to HDL does not seem to play an essential role in this type of lipid transfer by CETP.**

**Key words: cholesteryl ester transfer protein, fluorescence, high density lipoprotein, low density lipoprotein, pyrene.**

Cholesteryl ester transfer protein (CETP; plasma lipid transfer protein, LTP) catalyzes the transfer of non-polar and polar lipid between plasma lipoproteins (*1, 2*). It transfers cholesteryl ester and triglyceride between the core of lipoproteins and phospholipid between the surface of lipoproteins, and the transfer of the core lipids is random in direction (*3-5*). In the normal condition of human plasma, the rate of cholesteryl ester transfer is much higher than cholesteryl esterification (*6*) and faster than the turnover rate of low density lipoprotein (LDL) and high density lipoprotein (HDL) (*7*), whereby the reaction nearly equalizes the distribution of core lipids between these lipoproteins (*3*). On the other hand, the clearance rate of triglyceride in very low density lipoprotein (VLDL) is generally faster than the CETP-mediated lipid transfer rate. Therefore, the steady state distribution of cholesteryl ester and triglyceride is in dynamic equilibrium between VLDL and other lipoproteins in the bloodstream.

Since cholesteryl ester is generated mainly on HDL by lecithin: cholesterol acyltransferase and CETP transfers both cholesteryl ester and triglyceride, hetero-exchange of these lipids leads to the net transfer of cholesteryl ester from HDL (*8*). Triglyceride is kept hydrolyzed by lipases in HDL. The size of the cholesteryl ester pool in HDL is, therefore, a function of multiple factors, such as the rate of cholesteryl esterification, the rate of cholesteryl ester and triglyceride exchange, and the hydrolysis of triglyceride. Transfer selectivity of these lipids, if there is any, should also contribute to this equilibrium (*4, 5*). According to such a model, however, plasma HDL level is unlikely to be susceptible to fluctuation of the CETP activity in plasma unless the CETP activity is extremely low (*9-11*) or unless a high concentration of triglyceride exists in plasma in the steady state (*12, 13*).

On the other hand, CETP directly catalyzes the net transfer of cholesteryl ester from HDL and the interconversion of HDL particle size in the presence of lipolysis (*14-17*) or a certain level of free fatty acid (*18-20*). These conditions seem consistent with those in capillary vessels in certain organs where the lipolytic activity is high. These results indicate that CETP regulates the HDL level more directly by such a net transfer mechanism than the hetero-exchange transfer reaction in a bulk phase plasma described above. However, little is known about the mechanisms of these two types of CETP reaction and their roles in regulation of plasma HDL levels.

Human CETP has been shown to bind to HDL in human plasma (*21-23*). However, human CETP in transgenic mice does not bind to mouse HDL in the plasma, while it binds to HDL that has human apolipoprotein (apo) A-I in the plasma

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of the double transgenic mice of human CETP and human apoA-I, in non-denaturing gel electrophoresis (*24*). Interestingly, the reduction of HDL level was more remarkable by transgenic expression of the CETP activity in such double-transgenic animals (*24, 25*). These facts lead to the hypothesis that the HDL particle to which CETP binds makes a functional HDL complex for the transfer of esterified cholesterol from the HDL (*2*). However, CETP has also been shown to be associated with LDL, though to a less extent than with HDL, by gel permeation chromatography (*26*). In addition, kinetic analysis of the CETP reaction does not unambiguously supported either a carrier mechanism model (*27, 28*) or a ternary complex model (*29*) for lipid transfer between HDL and LDL. Free CETP molecules indeed can carry the core lipid between these lipoproteins (*30, 31*). Thus, it remains unknown how the binding of CETP to HDL contributes to the lipid transfer reaction between lipoproteins.

We have established a method to measure the lipid transfer reaction between lipoprotein containing pyrene-labeled lipid probes by monitoring the change of fluorescence spectrum of pyrene that indicates the decrease of dimer (excimer) in the donor lipoprotein particle by the transfer reaction (*4, 5, 32-34*). This method requires neither separation of donor and acceptor particles nor the disruption of the reaction, and hence it enables us to measure lipid transfer not only between a heterogeneous combination of donor and acceptor (such as HDL and LDL) but also between the same lipoprotein subclasses (*4, 5*). By using this method, we further investigated the transfer of cholesteryl ester and triglyceride by CETP between HDLs and between LDLs by focusing on a specific role of HDL in the CETP reaction. The results indicated that CETP has higher selectivity for cholesteryl ester over triglyceride in HDL than in LDL in the non-directional transfer reaction among lipoproteins, but the apparent binding of CETP to HDL in the electrophoretic analysis does not play an essential role in the reaction.

## MATERIALS AND METHODS

*Human CETP*—CETP was isolated from human plasma as described previously (*35*). The highly purified CETP used in the present study was a mixture of the proteins with molecular mass of 69 and 66 kDa in polyacrylamide electrophoresis in the presence of 0.5% sodium dodecylsulfate. Both isoforms had the same specific activity and the former isoform accounted for 90% of the preparation (*35*). Stock solution of CETP was 250 $\mu$g/ml in 10 mM sodium phosphate of pH 7.4.

*Incorporation of Pyrene-Lipids into Human Plasma Lipoproteins*—Human plasma lipoproteins labeled with pyrene-lipids were prepared as described previously (*4, 5*). Lipid microemulsion of egg phosphatidylcholine (Avanti), triglyceride, and cholesteryl acylester was prepared by sonication and ultracentrifugation. When lipoprotein was labeled for cholesteryl ester, cholesterol 1-pyrenehexanoate (pyrene-CE) (Sigma and Molecular Probes) and triolein (Sigma) were mixed with phosphatidylcholine in the starting milligram weight ratio of 1:1:2. When it was labeled for triglyceride, *rac*-1,2-dioleoyl-3-(1-pyrenedecanoyl)glycerol (pyrene-TG) (Molecular Probes) and cholesteryl oleate (Sigma) was mixed with phosphatidylcholine in the milligram weight ratio of 1:1:2. Control microemulsion was prepared from triolein, cholesteryl oleate, and phosphatidylcholine with the starting milligram weight ratio of 1:1:2. In order to prepare pyrene-lipid donor LDL, the emulsion containing pyrene lipid (the entire amount recovered) was incubated with human plasma from which VLDL had been removed beforehand, in the presence of 5 mM *N*-ethylmaleimide, $NaN_3$, aprotinin, and gentamycin, at 37°C for 48 h. To label HDL, the emulsion was incubated under the same conditions with human plasma from which VLDL and LDL had both been removed. LDL and HDL were isolated from the incubation mixtures by ultracentrifugation as fractions with densities of 1.006-1.063 and 1.12-1.21 g/ml, respectively. Acceptor lipoproteins were either untreated lipoproteins or those prepared by the same procedure except that the incubation was carried out with the emulsion that did not contain pyrene-lipid. The purity of the labeled and sham-treated lipoproteins was confirmed by electrophoresis in 0.5% agarose gel. Lipid compositions of typical preparations used in this study are shown in Table I. Acceptor lipoproteins (I) are untreated lipoproteins, and acceptor lipoproteins (II) are those that were sham-treated. Cholesterol decreased and phospholipid relatively increased in the surface, and cholesteryl ester decreased and triglyceride increased in the core upon the incubation with the microemulsions. These changes were somewhat more than in our previous experiments (*4, 5*) since the amount of emulsion used was increased three-fold in order to incorporate more pyrene-lipids.

TABLE I. **Lipid composition in molar percent of representative lipoprotein preparations for the transfer reaction.**

| | Choline-phospholipid | Cholesterol | Cholesteryl ester | Triglyceride |
|---|---|---|---|---|
| Acceptor LDL(I) | 21.1±1.4 | 21.9±0.3 | 48.0±0.7 | 9.0±0.5 |
| Acceptor LDL(II) | 31.9±0.2 | 15.8±2.3 | 41.3±1.0 | 11.1±1.0 |
| Donor LDL(CE) | 28.8±8.3 | 17.9±1.4 | 31.4±9.1 | 22.0±2.2 |
| Donor LDL(TG) | 32.4±2.5 | 19.1±1.0 | 40.0±2.1 | 8.5±0.6 |
| Acceptor HDL(I) | 39.4±2.5 | 17.0±0.8 | 39.7±1.9 | 3.7±0.8 |
| Acceptor HDL(II) | 65.9±1.3 | 7.7±0.3 | 18.0±0.6 | 8.3±0.6 |
| Donor HDL(CE) | 62.7±5.3 | 6.1±1.0 | 23.8±2.7 | 7.3±1.9 |
| Donor HDL(TG) | 63.7±4.5 | 9.7±1.2 | 22.7±2.8 | 3.9±0.5 |

The lipid transfer data shown in this paper are those from the experiments by using these preparations. Acceptor lipoproteins do not contain pyrene-labeled lipid. Donor lipoproteins contain either pyrene-cholesteryl ester {donor lipoprotein (CE)} or pyrene-triglyceride {donor lipoprotein (TG)}.

*Monitoring the CETP Reaction*—The CETP reaction was studied by monitoring the fluorescence spectrum of pyrene-lipid as described previously (*32, 33*). Donor and acceptor lipoproteins of the pyrene-lipid were mixed at 37°C and the reaction was started by adding CETP to the mixture. Fluorescence emission was monitored at wavelengths of 396 and 468 nm upon excitation at 320 nm. The ratio of the emission fluorescence intensities at the two wavelengths was calculated as an indicator of the pyrene-lipid content in the donor particles. The fractional rate of the reaction was calculated from the slope of a semilogarithmic plot of the data according to the equation described in our previous publication (*32, 33*). Lipid transfer rate between the same lipoprotein subclass was calculated from the fractional transfer rate and the total pool size of the lipid probed in the incubation mixture (*4*) and expressed as moles of lipid

transferred per mole of CETP per minute (M/M/min).

*Binding of CETP to Lipoproteins*—Association of CETP with lipoproteins was examined by density gradient polyacrylamide electrophoresis and immunoblotting in the absence of denaturant (*23, 24*). The CETP reaction mixture was applied to the gel after incubation for 30 min at room temperature, and the electrophoresis was performed at 4°C overnight. After electrophoretic transfer of the proteins and lipoproteins to nitrocellulose membranes, CETP was detected by immunostaining and fluorography by using monoclonal antibody against rabbit CETP 14-8H that cross-reacts with human CETP (*8*) as previously described (*8, 36*). Lipoprotein preparations used in this study were shown not to contain CETP by this method.

## RESULTS

The assay system employing pyrene-lipid and the monitoring of its fluorescence spectrum enabled us to measure the lipid transfer reaction between the same lipid carrier particles, because it does not require the separation of donor and acceptor of the tracer lipid. Our previous studies showed that the transfer of cholesteryl ester and triglyceride can be determined between the same plasma lipoprotein subfractions (*4, 5*). In this study, we used this method to measure the rate of the lipid transfer between HDL and HDL, and between LDL and LDL, in a dose-dependent manner of the substrate. Figure 1 shows the transfer of cholesteryl ester between HDLs and between LDLs. Fractional transfer rate was constant throughout the lipoprotein concentration examined (from $6\times10^{-6}$ to $1.7\times10^{-5}$ M for LDL, and from $2\times10^{-5}$ to $2.4\times10^{-3}$ M for HDL as choline-phospholipid). The rate of the lipid transfer was therefore a linear function of the substrate concentration over this range, which is consistent with its plasma concentrations and the rate was 75% higher with HDL as indicated by the slopes of the plot (Fig. 1, A and B). The rate was also higher for HDL than LDL in terms of the number of lipoprotein particles processed relative to the lipoprotein particle concentration, assuming that a single HDL and LDL particle contains 100 and 650 choline-phospholipid molecules, respectively (*37*) (Fig. 2).

The rate of triglyceride transfer by CETP was measured between HDLs and between LDLs by using the same method. The results were essentially identical to those for cholesteryl ester transfer, except that the rate was 60% higher for LDL than HDL (Fig. 3A). In terms of the number of lipoprotein particles processed *versus* lipoprotein parti-

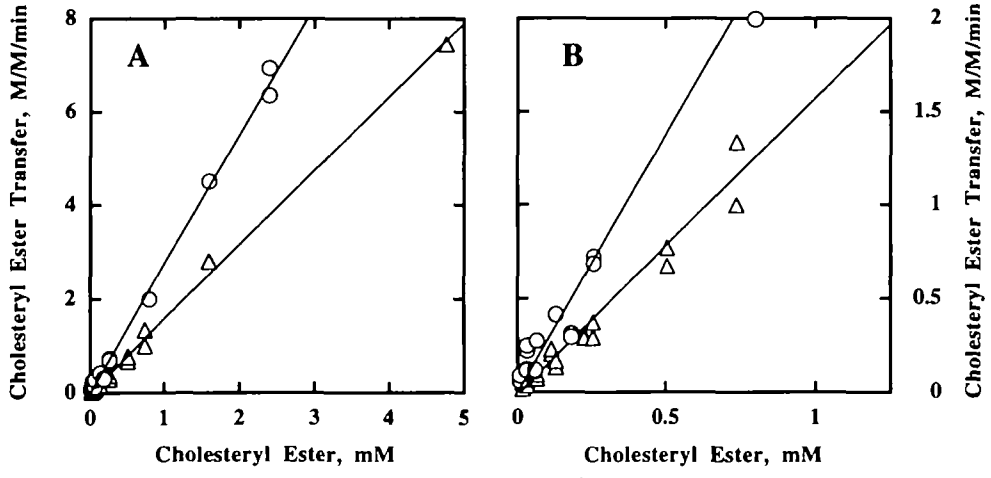


Fig. 1. **Transfer of cholesteryl ester by CETP between HDLs (circles) and between LDLs (triangles).** The ratio of pyrene-CE donor and acceptor HDLs or LDLs was maintained at 1:9 and the reaction was initiated by addition of $9.4\times10^{-9}$ M CETP. This CETP concentration was shown to be within the range in which the dose-dependency of the transfer rate was linear. Panel B is a magnification of the initial portion of panel A. The rate was calculated from fractional transfer rate and cholesteryl ester concentration in the mixture. Solid lines represent least-square regression for each set of data. Each set of data includes two series of experiments using acceptor lipoproteins listed in Table I.

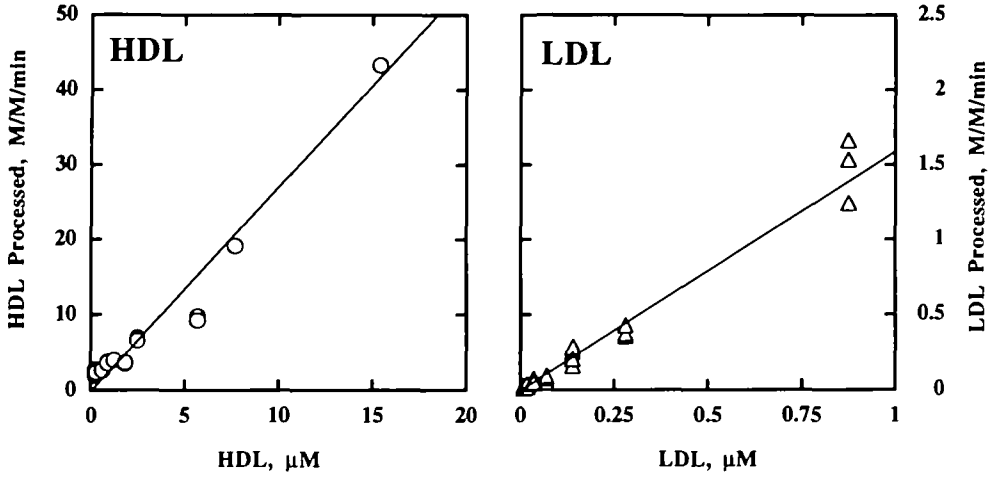


Fig. 2. **Transfer of cholesteryl ester by CETP between HDLs (left) and between LDLs (right) calculated as the number of lipoprotein particles processed, assuming that HDL contains 100 choline-phospholipid molecules and LDL contains 650 choline-phospholipid molecules per particle.**

cle concentration, the rate was same for HDL and LDL (Fig. 3B). The relative efficiencies of the transfer of cholesteryl ester and triglyceride between LDL and HDL were compared from the slopes of the plots of the rate against the substrate lipid concentration (Fig. 1, A and B and Fig. 3A). Cholesteryl ester is transferred 5.6 times more efficiently than triglyceride between HDL, and 1.7 times more efficiently between LDL. This result is consistent with the tendency indicated by our preliminary data (*4, 5*).

Interaction of human CETP with lipoproteins has been examined by immunoblotting with the anti-rabbit CETP monoclonal antibody 14-8H that cross reacts with human CETP after non-denaturing density gradient gel electrophoresis (*8*). Figure 4 shows the interaction of CETP with LDL and HDL. CETP showed apparent binding only to HDL in this analysis, in good agreement with previous reports (*22-25*). CETP seemed to bind preferentially to smaller HDL in the presence of LDL, despite the apparent absence of any change in the size distribution of HDL. The binding of CETP to lipoprotein was then examined in the

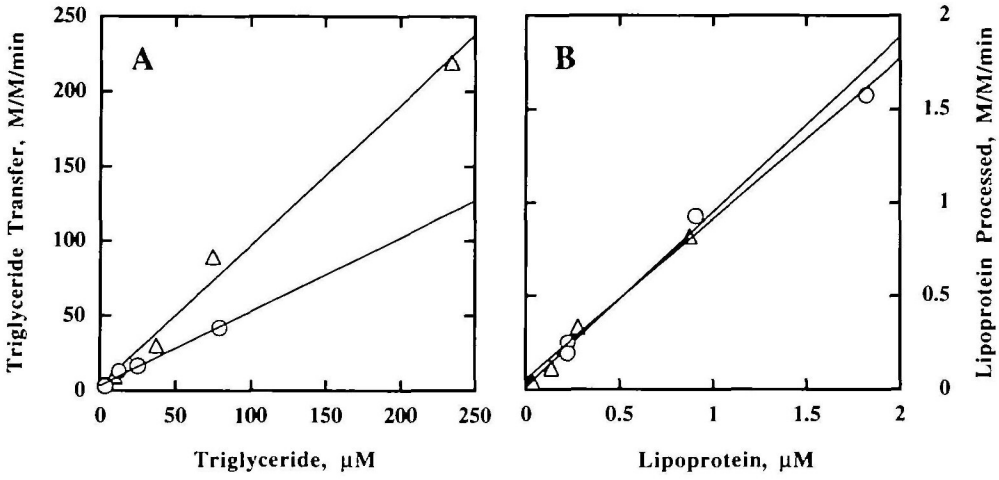


Fig. 3. **Transfer of triglyceride by CETP between HDLs (circles) and between LDLs (triangles).** The ratio of pyrene-TG donor and acceptor HDLs or LDLs was maintained at 1:9 and the reaction was initiated by addition of $2.4 \times 10^{-8}$ M CETP. This CETP concentration was shown to be within the range in which the dose-dependency of the transfer rate was linear. Panel A is the rate expressed as triglyceride transfer. The rate was calculated from fractional transfer rate and triglyceride concentration in the mixture. Panel B is the data expressed as a number of lipoprotein particles processed, assuming that HDL contains 100 choline-phospholipid molecules and LDL contains 650 choline phospholipid molecules per particle. Each set of data includes two series of experiments using acceptor lipoproteins listed in Table I.

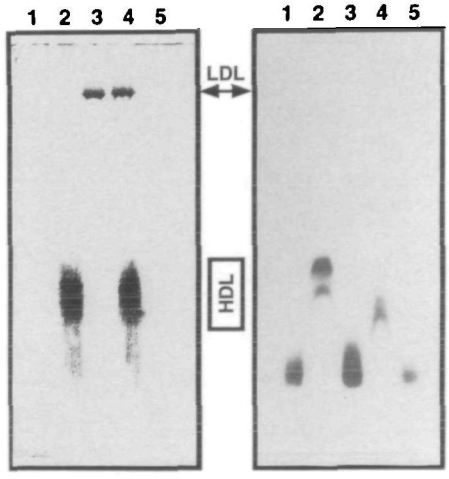


Fig. 4. **Binding of CETP to HDL.** Human lipoproteins and CETP were incubated at room temperature for 30 min in the ratio indicated below. Electrophoresis was carried out in a 4 to 12% gradient polyacrylamide gel in the absence of denaturant. After staining of protein by soaking the gel in 0.1% Ponceau Sin solution in 1% acetic acid for 1 min and washing with water, immunoblot for CETP was performed with anti-rabbit CETP monoclonal antibody 14-8H. The left panel shows the result of the protein staining and the right panel shows the immunoblot. Lane 1, CETP alone (0.13 $\mu$g) in 8 $\mu$l containing 8 $\mu$g bovine serum albumin; lane 2, CETP (0.13 $\mu$g) plus HDL (17.5 $\mu$g protein and 13.1 $\mu$g phospholipid) in 8 $\mu$l; lane 3, CETP (0.13 $\mu$g) plus LDL (15.1 $\mu$g protein and 15.8 $\mu$g phospholipid) in 8 $\mu$l; lane 4, CETP, HDL, and LDL (the same amounts, respectively as lanes 2 and 3); and lane 5, CETP alone (the same as lanes 1).

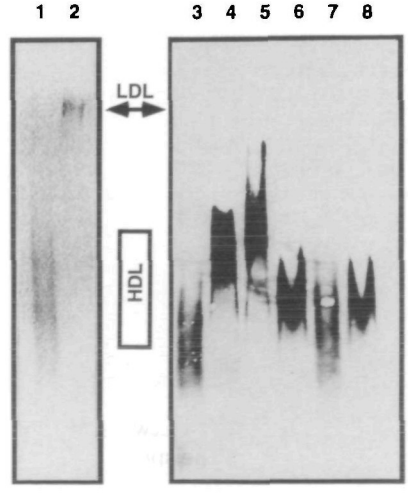


Fig. 5. **Binding of CETP to HDL in the reaction mixtures of the CETP examined in this work.** After incubation at room temperature for 30 min, electrophoresis was carried out in a 3 to 10% gradient polyacrylamide gel in the absence of denaturant and immunoblot was performed with anti-rabbit CETP monoclonal antibody 14-8H. The incubation mixtures contained the following: Lane 1, HDL 10 $\mu$g by Sudan black staining; lane 2, LDL 10 $\mu$g by Sudan black staining; lane 3, CETP 0.05 $\mu$g/20 $\mu$l; lane 4, HDL (PC 33 $\mu$g) and pyrene-CE HDL (PC 3.3 $\mu$g) plus LDL (PC 1.8 $\mu$g) with CETP 0.05 $\mu$g in 40 $\mu$l; lane 5, LDL (PC 33 $\mu$g) and pyrene-CE LDL (PC 3.2 $\mu$g) plus HDL (PC 0.5 $\mu$g) with CETP 0.05 $\mu$g in 40 $\mu$l; lane 6, HDL (2.8 $\mu$g) plus pyrene-CE HDL (0.28 $\mu$g) with 0.05 $\mu$g CETP in 20 $\mu$l; lane 7, pyrene-CE HDL (0.28 $\mu$g) with CETP in 20 $\mu$l; lane 8, HDL 2.8 $\mu$g with CETP.

TABLE II. **Fractional transfer rate of cholesteryl ester between lipoproteins.**

| Donor → Acceptor (34 μM PL) (306 μM PL) | Additional lipoprotein (17 μM PL) | Fractional transfer rate |
|---|---|---|
| HDL → HDL | No | 0.0149±0.0033 ($n$=7) |
| HDL → LDL | No | 0.0133±0.0045 ($n$=3) |
| LDL → HDL | No | 0.0086±0.0010 ($n$=4) |
| LDL → LDL | No | 0.0106±0.0010 ($n$=9) |
| LDL → LDL | HDL | 0.0123±0.0022 ($n$=3) |
| HDL → HDL | LDL | 0.0146±0.0023 ($n$=3) |

Donor lipoproteins containing pyrene-labeled cholesteryl ester were prepared as described previously. Acceptor lipoproteins were sham-treated for the incorporation of pyrene lipid. Donor lipoprotein (as 34 μM choline-phospholipid) and acceptor lipoprotein (306 μM choline phospholipid) were or were not mixed with additional acceptor lipoprotein (17 μM choline-phospholipid), and CETP was added (9.4 nM) to start the reaction.

reaction assay mixtures containing various combinations of the donor and acceptor lipoproteins of pyrene-labeled cholesteryl ester. CETP was again found associated exclusively with HDL (Fig. 5). The binding took place regardless of the HDL pretreatment for incorporation of pyrene-labeled cholesteryl ester or sham treatment. No trace of CETP binding to LDL was detected. CETP tended to bind larger HDL in the presence of LDL under the particular conditions employed, although the reason is unknown. The data were again consistent with the previous finding of the selective interaction of human CETP with human HDL in this type of analysis (*22-25*).

To examine the specific contribution of the CETP-HDL binding to the lipid transfer reaction in a bulk phase, the rate of cholesteryl ester transfer was measured with various combinations of lipoproteins in the incubation mixture. The results are shown in Table II. The fractional transfer rates of cholesteryl ester from HDL to HDL and from HDL to LDL were the same. However, the rates were slightly lower when the transfer was from LDL to LDL and from LDL to HDL, being consistent with the results demonstrated in Figs. 1 and 2. When a small amount of HDL was added to the reaction mixture for the lipid transfer between LDLs, all the CETP in the mixture was absorbed by the HDL (Fig. 5, lane 5), but the rate of the reaction was unaffected (Table II). The presence of LDL in the mixture for the reaction between HDLs (Fig. 5, lane 4) also had no effect on the transfer rate.

## DISCUSSION

Transfer rate of lipid by CETP between lipoproteins was measured in order to examine the specificity of the reaction for substrate lipids and substrate-carrying lipoproteins. The cholesteryl ester transfer rates between HDLs and between LDLs were both linearly proportional to the lipoprotein concentration within the range of their physiological concentrations in plasma. The former was greater than the latter when standardized against the cholesteryl ester concentration in the reaction mixture and also when the number of lipoprotein particles processed was standardized against the lipoprotein particle concentration. Triglyceride transfer rate was also linear with respect to the substrate concentration, and the rate between LDLs was higher than the rate between HDLs on the basis of triglyceride concentration. This difference disappeared when the rate was analyzed in terms of the lipoprotein particle processed *versus* lipoprotein particle concentration. Accordingly, cholesterol ester is more effectively transferred between HDLs than is triglyceride. This is consistent with our preliminary observation (*4, 5*).

The transfer rate was linearly proportional to the substrate concentration for both lipids and for both HDL and LDL in the concentration range where the lipoproteins greatly exceed CETP in molar terms. These results suggest the unlikelihood of a functional high-affinity interaction of CETP with lipoproteins. Although the transfer rate of cholesteryl ester was somewhat higher between HDLs, triglyceride transfer was rather faster between LDLs when standardized against triglyceride concentration, but not so on the basis of the number of lipoprotein molecules processed. Therefore, it is very unlikely that the HDL-bound CETP shown in the electrophoretic analysis is more functional than the unbound CETP for the lipid transfer between lipoproteins in the bulk phase. In addition, when a small amount of HDL was added to the reaction mixture for the cholesteryl ester transfer between LDLs, almost all CETP in the mixture was absorbed by the added HDL but the transfer rate between LDLs was unaffected. Thus, the apparently high affinity of human CETP for human HDL shown in electrophoretic analysis is not functional with respect to the lipid transfer between lipoproteins measured in the bulk phase. The moderate preference for transfer of cholesteryl ester between HDLs is perhaps due to the better accessibility to CETP of this highly hydrophobic lipid at the surface of the lipoproteins because of the smaller core of HDL than that of LDL (*37*).

These results strongly support a carrier mechanism model for the CETP-catalyzed lipid transfer between any lipoprotein. A less likely but valid model is that CETP bound to HDL is as much a functional carrier of the lipid substrate as is free CETP for the lipid transfer reaction (*1*).

The question still remains whether the binding of CETP to HDL plays a role in any other type of the reaction of CETP. It is clear that CETP regulates plasma HDL concentration partially by the hetero-exchange lipid transfer between lipoprotein in the bulk phase (*8, 12, 13, 25, 38, 39*). However, CETP also directly modifies HDL particles under such conditions as the presence of high concentration of free fatty acid (*18-20*) or lipolytic activity (*14-17*). The reactions include interconversion of HDL particles (*19, 20*) leaving apolipoprotein A-I lipid-free (*16*) and net lipid transfer from HDL to apolipoprotein B-containing lipoprotein (*14, 15, 18*). It is, therefore, conceivable that CETP directly regulates the level of HDL in the capillary vessels of certain organs where lipolysis of lipoprotein is highly active, such as muscle, adipocyte and liver. High affinity association of CETP with HDL may play a more significant role in this reaction than in the lipid transfer (exchange) reaction in the bulk phase (*40*).

The CETP-mediated non-directional lipid transfer among lipoproteins in the bulk phase is rapid enough to equalize the core lipid distribution of HDL and LDL. Unless a substantial amount of triglyceride is available for lipid exchange reaction between the HDL/LDL pool and other lipoprotein, the increase of CETP in plasma is unlikely to contribute substantially to down-regulation of plasma HDL level (*12, 13, 39*). On the other hand, the direct net lipid transfer from HDL by HDL-bound CETP in

the capillary vessels with the help of lipolysis seems a valid model to interpret the correlation of CETP and HDL in many animal models and clinical reports (*10, 11, 38, 41-43*).

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