paper on methodology

Use of fluorescent cholesteryl ester microemulsions in cholesteryl ester transfer protein assays

Charles L. Bisgaier,[1] Laura L. Minton, Arnold D. Essenburg, Andrew White, and Reynold Homan

Department of Pharmacology, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48105

Abstract In the present report we describe a simple and practical method to assess CETP activity in a defined system by use of microemulsions containing a fluorescent cholesteryl ester analog. The microemulsions are stable, simple to prepare, and can be made to defined composition. Initial transfer rates are easily determined by monitoring changes in fluorescence. We have used the fluorescent cholesteryl ester analog, cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacenedodecanoate (BODIPY-CE), to demonstrate the utility of this assay. The assay takes advantage of the concentration-dependent self-quenching of BODIPY-CE, when this analog is incorporated into microemulsions. We have used this new assay to demonstrate fluorescent lipid transfer facilitated by rabbit and human d>1.21 g/ml plasma fraction and recombinant human CETP. A known inhibitory monoclonal antibody (Mab) to human CETP blocked BODIPY-CE transfer in a dose-dependent manner. We have also used BODIPY-CE microemulsions to measure CETP activity in whole plasma.—**Bisgaier, C. L., L. L. Minton, A. D. Essenburg, A. White, and R. Homan.** Use of fluorescent cholesteryl ester microemulsions in cholesteryl ester transfer protein assays. *J. Lipid Res.* 1993. **34:** 1625–1634.

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Cholesteryl ester transfer protein facilitates the transfer of neutral lipids between plasma lipoproteins (1–6). A high degree of amino acid sequence homology exists between human, rabbit, and cynomolgus monkey CETP (1–3). A variety of species, including rats, dogs, and mice, have little or no CETP activity (4, 5). The role of CETP in lipoprotein metabolism has been elucidated, in part, by characterization of the human deficiency (6–9) and of transgenic mice (5, 10). Overall, these data suggest a reduction in CETP activity results in HDL elevation, diminution of atherogenic lipoproteins, and reduced risk of atherosclerosis.

CETP activity appears to be dependent on its plasma concentration (11) and the total plasma lipid pool (12, 13). In general, CETP activity is assayed with a lipid-free fraction (i.e., the crude or purified fraction from the d > 1.21 g/ml plasma fraction) in the presence of lipoprotein acceptors with lipoprotein donors containing radiolabeled cholesteryl ester or triglyceride. Donor and acceptor lipoproteins are separated at various time intervals by physical means (i.e., ultracentrifugation, precipitation, column chromatography) to assess transfer (14–18). The separation methods are laborious or problematic (19, 20). In addition, the initial isolation of lipoprotein pairs, radiolabeling one of the pairs, its reisolation, and the stability of lipoproteins makes their use and maintenance of quality control impractical. Assays performed in whole plasma supplemented with trace amounts of [^{3}H]cholesteryl ester HDL_3, are also somewhat limited since the ratio of HDL/LDL, the CETP level, and the endogenous apoE levels cannot be controlled (19). Precipitation with heparin/Mn^{2+} is used frequently to separate donor from acceptor lipoproteins for CETP assays. The apoE content and its distribution among lipoproteins, especially in non-human samples, may be problematic in assays that use heparin/Mn^{2+} precipitation. When the acceptor lipoproteins are precipitated (i.e., VLDL, IDL, LDL, and apoE-containing HDL), supernatant radioactivity is determined (i.e., [^{3}H]cholesteryl ester HDL_3). As a relatively small decrease in donor radioactivity is determined, the accuracy in measurement of initial velocity is compromised (i.e., small change in a large number). As the core lipid concentration of apoB-containing lipoproteins is much greater than that of HDL it is not appropriate to use apoB-containing lipoprotein as the radiolabel donor in a whole plasma assay. To circumvent this problem, some laboratories have used scintillation proximity

Abbreviations: BODIPY-CE, cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacenedodecanoate; CETP, cholesteryl ester transfer protein; Mab, monoclonal antibody; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; 1×DB buffer, 50 mM Tris, 150 mM NaCl, 2 mM EDTA, pH 7.4.

[1]To whom correspondence should be addressed.

methods, while others have used LDL radiolabeled with cholesteryl esters as donors in component assays to assess CETP activity (10).

In this report we present a simple and sensitive fluorescence method to detect and characterize CETP activity. No separation of donor and acceptor particles is required for analysis. Transfer kinetics are continuously monitored in reaction volumes as small as 250 μl by detecting the fluorescence emission of a fluorescent cholesteryl ester analog (BODIPY-CE) containing 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacenedodecanoate in place of the normal fatty acid. This is a useful spectroscopic probe for cholesteryl ester transfer because of the fluorescence self-quenching that occurs with increasing concentrations of this analog in a lipid phase. This cholesteryl ester analog was incorporated into a stable, aqueous dispersion of lipid particles containing POPC, triolein, and cholesteryl oleate. The advantages of the method, described in this report, that distinguish it from others previously reported include: *1)* development of a novel solvent injection technique for emulsion preparation that is simpler than existing methods for preparation of aqueous lipid dispersions based on sonication (21) or solvent injection (22); *2)* utility of fluorescence detection in a 96-well format with front-face optics that allows rapid analyses of transfer kinetics in turbid samples, which are often encountered when working with hyperlipidemic samples; and *3)* utility of a probe that has fluorescence excitation and emission maxima in the visible range lending to its suitability for screening pharmacological inhibitors that frequently cause spectroscopic interference at shorter wavelengths.

MATERIALS AND METHODS

Microemulsion preparation

Stock lipid solutions of triolein (Sigma Chemical Co., St. Louis, MO), cholesteryl oleate (Sigma Chemical Co., St. Louis, MO), [1,2-^{3}H]cholesteryl oleate (Amersham Corporation, Arlington Heights, IL), and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) (Avanti Polar-Lipids, Inc., Alabaster, AL) in chloroform and BODIPY-CE (Molecular Probes, Inc., Eugene, OR) in ethanol were combined in the desired proportions and the solvent was evaporated under a stream of nitrogen. Residual solvent was removed by 4 h of vacuum. The dried lipid mixture was dissolved in dioxane (40 nmol lipid/μl dioxane). The microemulsions were prepared by slowly injecting an aliquot of the dioxane-solubilized lipids from a Hamilton syringe (26-gauge needle) below the liquid surface of 50 mM Tris, 150 mM NaCl, 2 mM EDTA, pH 7.4 (1×DB) contained in a 20-ml glass scintillation vial that was immersed in a water bath sonicator (Cole-Parmer Model 8851). Generally, 4–20 μl of lipids in dioxane was injected per ml of buffer. Microemulsions stored for over 6 months remained stable (i.e., solutions remained clear with no apparent aggregation or precipitation or particle size change as determined by gel-filtration). In addition, fluorescence changes initiated by CETP addition were essentially identical for fresh emulsion preparations and those stored up to 6 months at 4°C. Preliminary incubation studies in the absence (i.e., dialyzed emulsions) or presence of up to 3.6% dioxane resulted in fluorescence yields that were indistinguishable both at zero time and at intervals following addition of CETP. Therefore, in our studies, the residual dioxane, which could account for up to 1% of the final volume, was not removed from the microemulsions prior to incubations. Microemulsions contained 46–48 mole percent cholesteryl esters (cholesterol oleate plus BODIPY-CE), 44–46 mole percent POPC, and 8 mole percent triolein. Microemulsions lacking BODIPY-CE or 20% Intralipid (Kabi Vitrum, Inc., Alameda, CA) were used as acceptors.

Preparation of CETP

The d > 1.21 g/ml fraction was isolated from rabbit (Pel-Freez Biologicals, Rogers, AR) or human plasma and dialyzed against 1×DB buffer. Aliquots were stored frozen at −20°C. Chinese Hamster Ovary cells transfected with human recombinant CETP (23) were obtained by license agreement from Drs. Alan Tall and Suke Wang (Columbia University, New York). In some studies media from these cells (grown in 10% fetal bovine serum in Hams F-12) were also used as a source of human CETP without further purification. In other studies recombinant CETP (from media of cells grown in EX-CELL 301, JRH Biosciences, Lenexa, KS) was purified by an approximate 20,000-fold (over human plasma) by phenyl-Sepharose chromatography (24). The human CETP inhibitory monoclonal antibody TP2 (Mab TP2) (25) was a kind gift from Drs. Ross Milne and Yves Marcel (University of Ottawa Heart Institute). Mab TP2 is also known to inhibit rabbit CETP (26).

Spectroscopy

Absorption maxima of BODIPY-CE in ethanol or isopropanol were determined in a DU-64 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Fluorescence spectra were obtained with a Model LS-3 fluorescence spectrophotometer (Perkin-Elmer, Oak Brook, IL). The excitation and emission maxima of BODIPY-CE in ethanol were 503 nm and 518 nm, respectively.

Studies with BODIPY-CE-labeled emulsions were done in flat-bottom 96-well plates (Costar Corporation, Cambridge, MA) containing up to 250 μl of sample per well. The 96-well plates were covered and kept in a humidified 37°C incubator in the time intervals between plate scans. BODIPY-CE fluorescence was detected with a Titertek Fluoroskan II 96-well plate reader (Labsystems, Inc., Research Triangle Park, NC) equipped with 485 nm and

538 nm band pass filters in the excitation and emission paths, respectively. The Fluroskan II contained a 40 W xenon lamp as the excitation source. All fluorescence readings were calibrated to a fluorescent solid located in the plate carrier. Thus, variations in instrument performance were corrected for. The optical path was such that excitation and fluorescence detection took place at the upper surface of the well contents. This enabled accurate measurement of fluorescence from turbid samples.

To determine relative transfer rates for BODIPY-CE and cholesteryl oleate, microemulsions containing both BODIPY-CE (8% of total CE) and [1,2-^{3}H]cholesteryl oleate (92% of total CE) were incubated with a 10-fold excess (i.e., core lipids) of human HDL (d 1.063–1.21 g/ml) and purified CETP, at 37°C. At intervals, aliquots of mixtures were separated by Superose 6 HR 10/30 (Pharmacia LKB Biotechnology, Piscataway, NJ) gel-filtration chromatography using an HPLC (Rainin Instrument Company, Inc., Woburn, MA) in which post-column eluant was mixed on-line with 3 volumes of isopropanol and directed through a FL2000 fluorescence detector (excitation 490, emission 530) (Spectra-Physics Analytical, Inc. Fremont, CA). Eluant was also collected and assessed for radioactivity by liquid scintillation counting.

Analysis of fluorescence data

The relationship between BODIPY-CE concentration in the microemulsions and fluorescence yield was nonlinear (**Fig. 1**) and could not be fitted to the standard Stern-Volmer equation for fluorescence quenching (27). Instead, the self-quenching of BODIPY-CE was well fit by a monoexponential function of the following form:

$$f = (f_{max} - f_{min}) \exp(-k\ B/C) + f_{min} \qquad Eq.\ 1)$$

Here f is the fluorescence yield per mole (RFU/mol), B is the moles of BODIPY-CE in the microemulsion, C is the total moles of neutral core lipids in the microemulsion, f_{max} is the maximum fluorescence intensity obtained when C is infinitely large, f_{min} is the fluorescence observed when B is equal to C, and k is a constant. The preexponential term is a constant and can simply be denoted as Δf. The fluorescence intensity for B moles of BODIPY-CE is then

$$F = B(\Delta f \exp(-k\ B/C) + f_{min}) \qquad Eq.\ 2)$$

A mathematical description of the fluorescence changes detected in the transfer experiments is obtained by substituting equation 2 into the derivation described by Nichols (28) for analysis of fluorescent phospholipid transfer. The total observed fluorescence in the transfer experiments is the sum of the fluorescence from the donor and acceptor emulsions as follows:

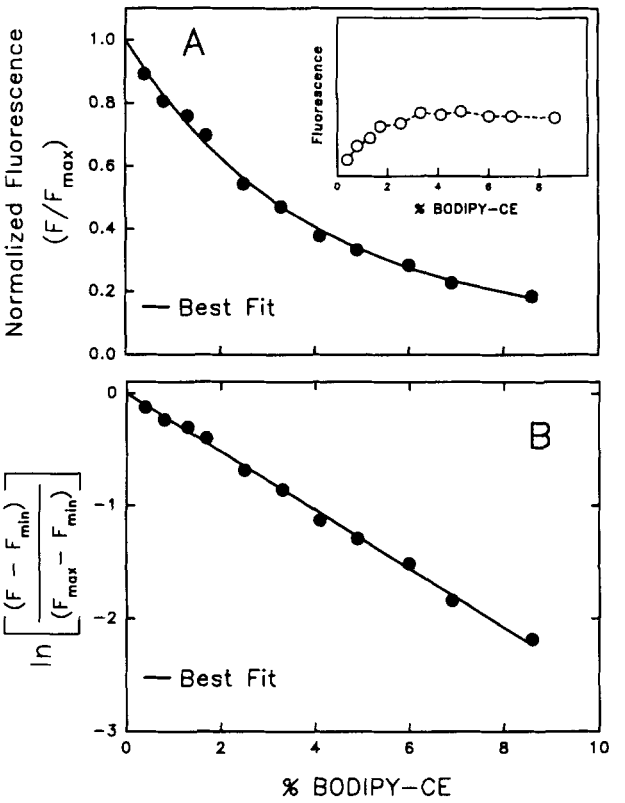

Fig. 1. BODIPY-CE fluorescence yield as a function of concentration in lipid microemulsions. (A) Microemulsions containing various concentrations of BODIPY-CE (mol% of total core lipids) were prepared. The total BODIPY-CE content in all preparations was kept constant at 1.3 μM while the amount of the other lipid constituents was varied. The mol percents of cholesteryl esters (cholesteryl oleate and BODIPY-CE), triolein, and POPC were 46, 8, and 46, respectively. The solid line represents the best fit of the data to equation 1 (Materials and Methods) by nonlinear regression. (A, inset) The inset shows the same relation between fluorescence yield and percent BODIPY-CE (mol percent of total core lipids), but in this case the amount of BODIPY-CE was varied as the other lipid constituents were held constant. (B) Normalized fluorescence semi-log representation of the data in A.

$$F = B_D(\Delta f \exp(-k\ B_D/C_D) + f_{min}) + B_A(\Delta f \exp(-k\ B_A/C_A) + f_{min}) \qquad Eq.\ 3)$$

Here the D and A subscripts signify the parameters for donor and acceptor emulsions, respectively. Initially, at time t = 0, before any transfer takes place,

$$F_o = B_T(\Delta f \exp(-k\ B_T/C_D) + f_{min}) \qquad Eq.\ 4)$$

where B_T is the total BODIPY-CE in the sample. The increase in the observed fluorescence as a result of transfer of BODIPY-CE from donor to acceptor particles is simply

$$\Delta F = F - F_o \qquad Eq.\ 5)$$

Substitution of equations 3 and 4 into equation 5 yields

$$\Delta F = B_D \Delta f \exp(-k\ B_D/C_D) + B_A \Delta f \exp(-k\ B_A/C_A) - B_T \Delta f \exp(-k\ B_T/C_D) \quad \textit{Eq. 6)}$$

At equilibrium, when BODIPY-CE is completely equilibrated between donor and acceptor particles, the observed fluorescence is expressed as

$$F_{eq} = B_T(\Delta f \exp(-k\ B_T/(C_A + C_D)) + f_{min}) \quad \textit{Eq. 7)}$$

and the maximum change in fluorescence is expressed as

$$\Delta F_{eq} = F_{eq} - F_o \quad \textit{Eq. 8)}$$

Substitution of equations 4 and 7 into equation 5 gives

$$\Delta F_{eq} = B_T \Delta f \exp(-k\ B_T/(C_A + C_D)) - B_T \Delta f \exp(-k\ B_T/C_D) \quad \textit{Eq. 9)}$$

The ratio of equation 6 over equation 9 represents the fractional change in fluorescence and is defined as

$$\frac{\Delta F}{\Delta F_{eq}} = \frac{B_D \exp(-k\ B_D/C_D) + B_A \exp(-k\ B_A/C_A) - B_T \exp(-k\ B_T/C_D)}{B_T \exp(-k\ B_T/(C_A + C_D)) - B_T \exp(-k\ B_T/C_D)} \quad \textit{Eq. 10)}$$

Immediately following mixing of donor and acceptor particles, only a small portion of BODIPY-CE is transferred such that B_D is essentially equivalent to B_T. If, in addition, acceptor particles are in excess such that $C_A >> C_D$, as in our experiments, then equation 10 reduces to

$$\frac{\Delta F}{\Delta F_{eq}} = \frac{B_A}{B_T \exp(-k\ B_T/C_A)} \quad \textit{Eq. 11)}$$

Thus, in the initial stages of BODIPY-CE transfer to an excess of acceptor particles, the fractional change in fluorescence is directly proportional to the amount of BODIPY-CE taken up by the acceptor particles.

An example of how the loss of BODIPY-CE by the donor particles does not significantly contribute to the observed change in sample fluorescence is given in the inset of Fig. 1. The data graphed in the inset were obtained by preparing a series of microemulsions in which the total amounts of each lipid class were kept constant but the ratio of BODIPY-CE (B) to total neutral lipid (C) was varied by changing the amount of BODIPY-CE. As can be seen, up to a 50% decrease in the BODIPY-CE content of the microemulsion particles can occur without any major change in the fluorescence yield. This is the result of the opposing effects of fluorophore depletion and dilution on total fluorescence yield.

The self-quenching of BODIPY-CE was evaluated by measuring the fluorescence of a series of microemulsion preparations that all contained the same amount of BODIPY-CE but varied in the amounts of other lipid components. The relative composition of the preparations was held constant at 46 mol percent cholesteryl ester (cholesteryl oleate plus BODIPY-CE), 46 mol percent POPC, and 8 mol percent triolein but the total mass of POPC, cholesteryl oleate, and triolein was varied such that the BODIPY-CE constituted between 0.4 and 8.6 mol percent of the neutral core lipids (i.e., cholesteryl esters and triolein). Fluorescence yields were measured in the Fluoroskan II. All samples contained 1.3 μM BODIPY-CE. The fluorescence data were fit to equation 1 by nonlinear regression (Fig. 1) (29). The fit yielded a value of 26 for the exponential constant k and a value of 0.08 for the ratio of F_{min} to F_{max}.

The value of F_{eq} must be known to analyze the transfer data. The value of F_{eq} approaches F_{max} as the amount of acceptor emulsion is increased. In our experiments, conducted with a 10-fold excess of acceptor emulsion, F_{eq} was approximately 83% of F_{max}. This was calculated from the curve in Fig. 1B by substituting the values for F_{max}, F_{min}, and the final expected dilution of BODIPY-CE. F_{max} was estimated by measuring the fluorescence yield of an aliquot of donor emulsion diluted 10-fold in isopropanol. The value obtained was within 10% of that predicted by the nonlinear fit. F_{min} was derived from F_{max} according to the nonlinear fit of the standards.

RESULTS

Characterization of BODIPY-CE microemulsions

BODIPY-CE was incorporated into lipid microemulsions composed of 44 mole percent POPC, 8 mole percent triolein, and 48 mole percent cholesteryl esters, by dioxane solvent injection. Multiple microemulsion preparations, including fresh preparations that were dialyzed to remove residual dioxane, and preparations stored up to 6 months at 4°C, had essentially identical gel-filtration profiles with an elution volume centered around a single peak characteristic of VLDL size. **Fig. 2** (zero time lower graph) illustrates a typical microemulsion gel-filtration profile (although this particular preparation was mixed with HDL and CETP prior to chromatography).

The fluorescence emitted by these preparations was inversely dependent on the BODIPY-CE content (Fig. 1). The fluorescence excitation and emission maxima were unaffected by the concentration-dependent self-quenching (**Fig. 3**).

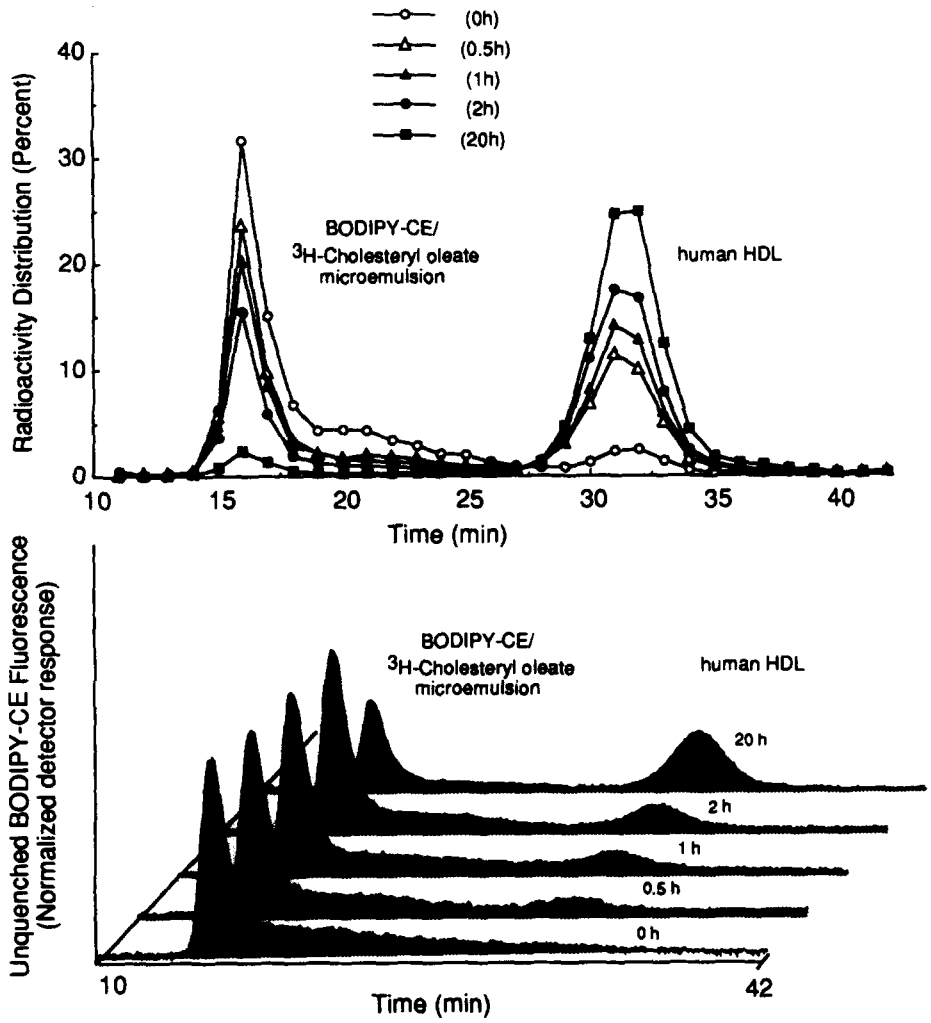

Fig. 2. Relative transfer of cholesteryl oleate and BODIPY-CE from microemulsions to HDL. Microemulsions containing both BODIPY-CE (8% of total CE) and 4 × 10^5 dpm [1,2-^{3}H]cholesteryl oleate (92% of total CE) were incubated with a 10-fold excess (i.e., 44 nmol core lipids) human HDL and 2.5 μg recombinant human CETP (phenyl Sepharose fraction) at 37°C in 0.4 ml of 50 mM Tris, 150 mM NaCl, 2 mM EDTA, pH 7.4. At intervals up to 20 h, aliquots (100 μl) were separated by Superose 6 HR 10/30 gel-filtration chromatography. Column eluant was monitored for unquenched fluorescence by on-line post-column mixing with 3 volumes of isopropanol before passing through a fluorescence detector. Eluant fractions were also collected and monitored for radioactivity by liquid scintillation counting. In control incubations (not shown), BODIPY-CE and [1,2-^{3}H]cholesteryl oleate transferred at 1/10 the rate of incubations with CETP (i.e., distribution in 20 h control was equivalent to 2 h CETP) and may be due to residual amounts of CETP on human HDL.

The relative transfer rate of cholesteryl oleate to BODIPY-CE was assessed by incubating microemulsions with a 10-fold excess of human HDL (with respect to core lipids) in the presence of highly purified human recombinant CETP. Microemulsion cholesteryl esters consisted of 8% BODIPY-CE, 92% cholesteryl oleate, and trace amounts of [^{3}H]cholesteryl oleate. At intervals, incubation mixtures were gel-filtered to separate microemulsion donors from HDL. Unquenched fluorescence and radioactivity in the column effluent were determined by on-line fluorescence detection and liquid scintillation spectroscopy of collected fractions (Fig. 2). The ratio of percent of total [^{3}H]cholesteryl oleate in HDL to percent of total BODIPY-CE in HDL was 2.8, 2.8, and 3.1 at 0.5, 1, and 2 h, respectively, indicating a 3-fold preferential transfer of cholesteryl oleate over BODIPY-CE. By 20 h, this ratio was 1.8 and reflects complete equilibration of [^{3}H]cholesteryl oleate (90.9% in HDL) and the incomplete equilibration of BODIPY-CE (50.4% in HDL).

The effect of CETP concentration, on BODIPY-CE transfer rates was assessed at 37°C with various concentrations of rabbit or human d>1.21 g/ml plasma fractions, and an acceptor to donor microemulsion ratio of 10 (**Fig. 4**). Fluorescence yield was determined for incubations with rabbit and human plasma fractions (d>1.21 g/ml) up to 9 h (Fig. 4, top panel). The analysis method

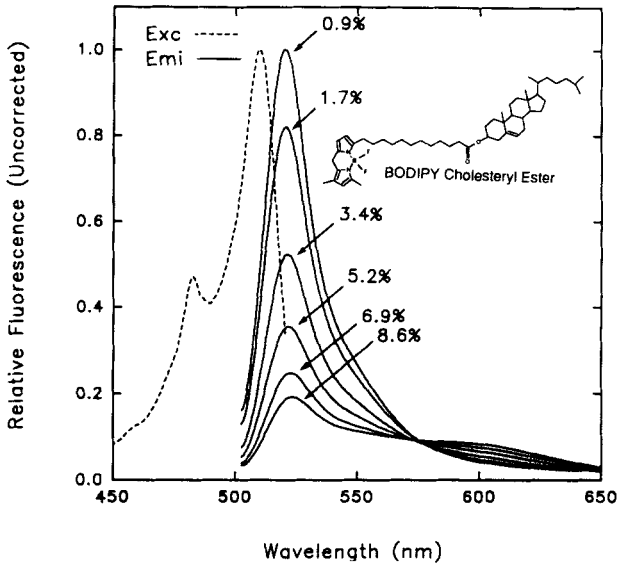

Fig. 3. Fluorescence spectra of BODIPY-CE in lipid microemulsions. Fluorescence spectra were obtained for microemulsions containing 0.86–8.6 mol percent BODIPY-CE. Emission spectra (line) were collected at a fixed excitation of 490 nm. The excitation spectrum (dashes) was obtained for a microemulsion preparation containing 0.86 mol percent BODIPY-CE. The emission was fixed at 530 nm. The structure of BODIPY-CE is shown.

described in Materials and Methods was used to convert the fluorescence data to BODIPY-CE mass transferred (Fig. 4, middle panel). The relation between d>1.21 g/ml fractions and initial velocity is shown in the lower panel (Fig. 4). In each case, initial transfer velocity was linear up to 50 μl of human or rabbit d>1.21 g/ml plasma; above 50 μl d>1.21 g/ml plasma, microemulsion substrates became limiting. Similar data were obtained for human recombinant CETP (not shown).

Using the BODIPY-CE microemulsion system, under conditions where microemulsion substrates became limiting (i.e., approaching the apparent K_m), inhibition of BODIPY-CE transfer was demonstrated with Mab TP2 (**Fig. 5**), suggesting CETP specificity in facilitating transfer of the fluorescent probe.

Consideration of endogenous lipoproteins must be made to assess transfer activity in whole plasma, since these lipoproteins will effect initial transfer rates. Thus, if an exogenous CETP-free lipoprotein or emulsion source is added in excess to whole plasma, activity could be assessed under maximal velocity conditions independent of the endogenous lipoproteins. To determine whether a fluorescence assay could be used to determine CETP activity in whole plasma, we used mixtures of normal human plasma, or normal or hypercholesterolemic rabbit plasma with Intralipid and BODIPY-CE microemulsions.

In the presence of various concentrations of Intralipid (280, 560, or 840 μg triglyceride/200 μl well) turbidity was

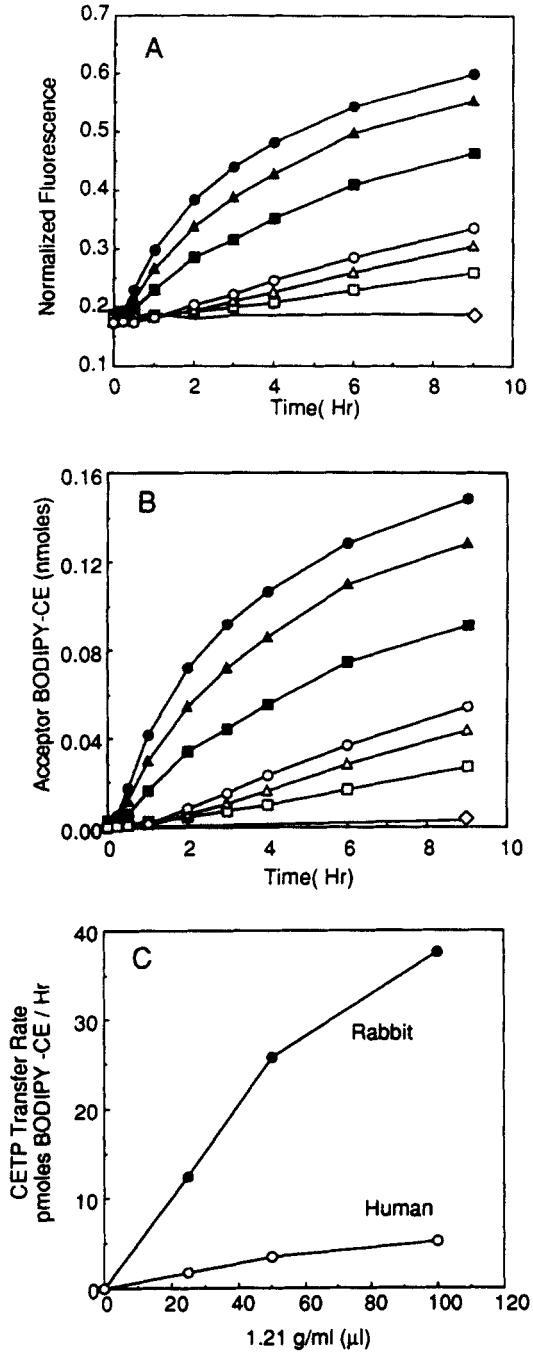

Fig. 4. Use of microemulsions and fluorescence yield to determine CETP-facilitated transfer of BODIPY-CE. (A) Microemulsions containing 8.6 mol percent BODIPY-CE (0.385 nmol BODIPY-CE) were mixed with a 10-fold excess of nonfluorescent microemulsions and incubated in the absence (◇) or presence of 25 (■, □), 50 (▲, △), or 100 μl (●, ○) of rabbit (■, ▲, ●) or human (□, △, ○) d>1.21 g/ml plasma fractions in 96-well microtiter plates in a total volume of 250 μl 1 × DB buffer. Plates were incubated at 37°C, and fluorescence at 485 nm excitation and 538 nm emission was periodically determined up to 9 h. (B) The mass of BODIPY-CE transferred was calculated from the fluorescence data as described in Materials and Methods. (C) Initial transfer rate per μl of 1.21 g/ml fraction was calculated using 1 and 4 h data for rabbit (●) and human (○) fractions, respectively. Data shown are from a representative experiment.

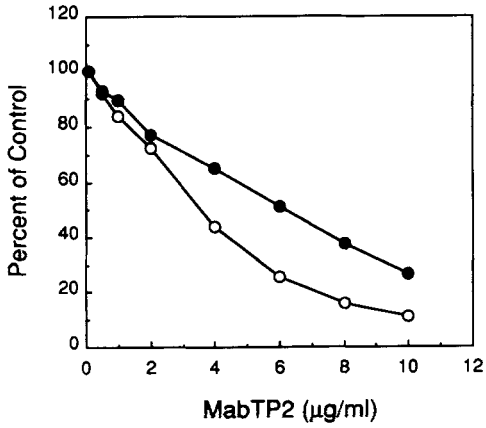

Fig. 5. Inhibition of microemulsion BODIPY-CE transfer by monoclonal antibody TP2. Microemulsions containing 6.9 mol percent of their core lipids as BODIPY-CE were mixed with nonfluorescent microemulsions in a 1 to 10 ratio with or without Mab TP2 at the indicated concentrations. The concentration of rabbit d > 1.21 g/ml plasma fraction (●) and recombinant human CETP (○) were chosen near the apparent K_m such that substrate would be limiting. Plates were incubated at 37°C for 60 min and fluorescence was determined at excitation of 485 nm and emission of 538 nm.

apparent. Fluorescence yields were slightly higher at the two highest Intralipid concentrations used. However, when normalized fluorescence standard curves (like those in Fig. 1) were generated in the presence of Intralipid and fit to equation 1, the fit yielded k and f_{min}/f_{max} constants that were essentially identical to those obtained in the absence of Intralipid. Thus, turbidity does not effect changes in fluorescence yield when detected with the front-face optical design of the Fluoroskan II plate reader. Therefore, the measurement of transfer kinetics in the presence of turbid samples and under V_{max} conditions is possible. Furthermore, in any assay in which excess Intralipid is added at a single concentration (relative to plasma lipoprotein lipid concentration), F_o values are essentially identical (e.g., In one typical experiment using 2.5–20 μl plasma/well from a variety of species, $F_o = 66.4 \pm 0.1$, $n = 360$). **Fig. 6** demonstrates how this observation can be used to determine relative transfer kinetics for normal human plasma, and normal and hypercholesterolemic rabbit plasma in the absence or presence of Intralipid. For all plasma, the CETP transfer rate was not maximal, in the absence of exogenous Intralipid. However, a maximal transfer rate could be demonstrated when Intralipid was added at 150 and 300 μg triglyceride/250 μl well, suggesting that the CETP transfer process was saturated. The relation between maximal transfer rate (relative to human plasma) was approximately 4 times greater for normal rabbit plasma and 12 times greater for hypercholesterolemic rabbit plasma. As a practical measure, determination of activity at various plasma dilutions should be made to ensure linearity between plasma volume and CETP activity, and periodically, to ensure assessment of

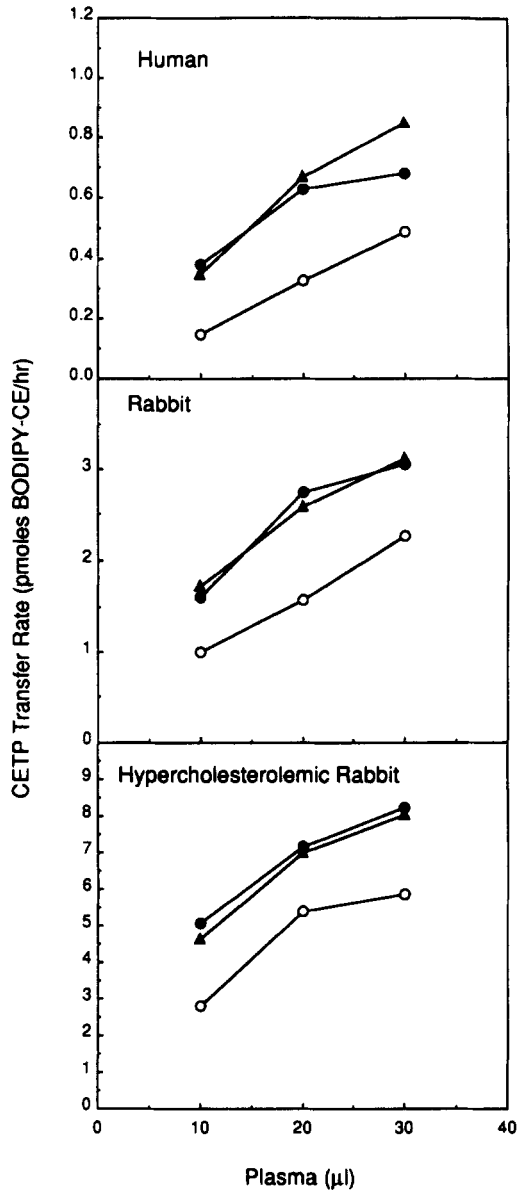

Fig. 6. Determination of CETP activity in whole plasma. Normal human and rabbit and hypercholesterolemic rabbit plasma (10–30 μl) were incubated in the absence (○) or presence of 169 nmoles (●) or 339 nmoles (▲) Intralipid triglyceride, with donor microemulsions containing 8.6 mol percent BODIPY-CE (1.15 nmol BODIPY-CE in 13.39 nmol total core lipids) in a total volume of 250 μl in 1 × DB buffer at 37°C in a humidified chamber. Each well contained 0.24% dioxane. Fluorescence yields were determined at intervals up to 19 h, and converted to mass of BODIPY-CE transferred. CETP transfer rate is representative of initial velocity and was calculated at 19 h for the human samples, and from 1–3 h for normal rabbit plasma, and 0.25–3 h for hypercholesterolemic rabbit plasma. For the data shown here, calculations reflect consideration of an acceptor pool consisting of Intralipid and endogenous lipoproteins. Practically, the endogenous lipid pool is negligible relative to added Intralipid and therefore its contribution to the acceptor can be ignored.

initial velocity. As a further demonstration of the utility of the fluorescent assay, we determined the CETP activity in plasma from a variety of species that were fed normal or

TABLE 1. Determination of CETP activity in whole plasma

Group	n	CETP Activity
		pmol BODIPY-CE/ml/h
Cynomolgus monkey (normal diet)	10	539 ± 67
Hamster (chow diet)	3	not detected
Hamster (0.5% cholesterol in 10% peanut oil/2 weeks)	3	23 ± 12
Human (normal lipidemic)	3	61 ± 4
Rabbit (chow diet)	3 pools of n>10 each	190 ± 26
Rabbit (0.5% cholesterol in 3% coconut oil plus 3% peanut oil/3 weeks)	8	769 ± 110

Plasma samples (1.25–20 μl) were incubated with 678 nmol Intralipid triglyceride in the presence of BODIPY-CE donor microemulsions (0.37 nmol BODIPY-CE in 5.36 nmol total core lipids) in a total volume of 220 μl in 1 × DB buffer at 37°C. Fluorescent yield (excitation 485 nm, emission 538 nm) was periodically determined for duplicate or triplicate incubations at the various plasma dilutions up to 20 h. The fluorescence data were converted to mass of BODIPY-CE transferred. The endogenous lipid pool was negligible relative to added Intralipid and therefore its contribution to the acceptor pool was ignored in calculating the mass of BODIPY-CE transfer to the acceptor pool. The results represent initial velocities ± SEM (i.e., the conditions were such that the mass of BODIPY-CE transferred was linearly dependent on time and plasma concentration).

cholesterol-supplemented diets (**Table 1**). The relation of CETP activity among these species and dietary treatments is consistent with reported results (4, 30, 31).

DISCUSSION

Quantification of CETP activity in both component and whole plasma systems is shown in the present report. A unique advantage of this assay is that the transfer kinetics can be continuously monitored (in a single cuvette or well) without using complicated and time-consuming separation techniques. The assay also eliminates the need for radioisotopes. In addition, we describe a novel method for the preparation of lipid microemulsions that are used to assess CETP activity. The microemulsions are simple to prepare and quite stable even when stored at 4°C for over 6 months. The use of solvent injection to prepare stable aqueous suspensions of lipid microemulsions containing CE has been described previously by Via et al. (22). They used isopropanol as the injection solvent, which had to be kept heated with a specialized heating apparatus during the injection process to prevent phase separation of lipids and solvent. We also found that isopropanol had limited capacity to dissolve neutral lipids, particularly those containing the pyrene fluorophore. We found that dioxane, which is 100% miscible with water, was much more effective for dissolving all lipids, without heating.

In preliminary studies (not shown) we also assessed lipid transfer kinetics using cholesteryl 12-(1-pyrenyl) dodecanoate microemulsions made by dioxane injection. Previously, pyrene-labeled cholesteryl analogs that were incorporated into emulsions by sonication were used by Milner et al. (32) and Dousset and Douste-Blazy (33) to specifically assess CETP-facilitated transfer. Data we obtained were qualitatively similar to those reported by Milner et al. (32).

BODIPY-CE was transferred about one-third as efficiently as cholesteryl oleate. Whether CETP relative affinity for BODIPY-CE is less or the differences in transfer rate reflect the differential packing of core lipids within the particles used was not addressed further. In this regard, Morton (34) has shown specificity differences of CETP for naturally occurring cholesteryl esters, therefore it was not surprising to find difference in specificity for BODIPY-CE. Nonetheless, despite the lower transfer rate, relative to cholesteryl oleate, this probe is useful for accessing CETP-facilitated transfer. A known inhibitory Mab to CETP blocked fluorescent lipid transfer. Using the same CETP source (stored at −20°C), essentially identical results were obtained when BODIPY-CE microemulsion preparations were stored up to 6 months. In general, for our studies, donor and acceptor microemulsions were mixed just prior to CETP addition; however, storage of mixtures did not result in spontaneous (non-CETP-facilitated) exchange of microemulsion lipids. As a one-to-one stoichiometry for exchange between cholesteryl esters and triglycerides facilitated by CETP between particles occurs (24, 35, 36), we were able to utilize either triglyceride-rich emulsions (Intralipid) or cholesteryl ester-rich microemulsions as acceptor particles. However, various studies (37–40) have suggested a preferential affinity of CETP for HDL. Whether this has functional significance in preferentially directing homologous exchange between HDL or merely suggests that ternary complex formation is necessary for exchange to occur is unclear. It is, however, clear that triglyceride-rich lipoprotein enrichment (12, 39) markedly increases CETP activity to an apparent maximal level. Thus, CETP activity in basal plasma does not facilitate maximal neutral lipid transfer, but requires a hyperlipidemic state, such as postprandial lipemia to allow maximal transfer velocity. In our in vitro model, by adding Intralipid we have attempted to mimic conditions of a hyperlipidemic state to insure measurement of a maximal transfer rate.

Our method has a variety of applications. First, due to the simplicity of the system, CETP activity can be easily and simultaneously measured in a large number of plasma samples. Second, our novel and simple method for preparation of microemulsions results in stable, uniformly sized, and consistent formation of particles. Third, the fluorophore chosen for our studies has excitation and emission maxima within the visible spectrum. This property has great utility for testing organic compounds that could inhibit CETP activity but, as is frequently the case, can also cause spectral interferences at ultraviolet wavelengths. Albeit, we have found organic compounds that do cause spectral interference in the assay. Lastly, this method has great utility for the determination of ex vivo CETP activity in animals treated with pharmacological inhibitors (C. L. Bisgaier, A. Essenburg, L. Minton, R. Homan, and A. White, unpublished results).

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REFERENCES

1. Drayna, D., A. S. Jarnagin, J. McLean, W. Henzel, W. Kohr, C. Fielding, and R. Lawn. 1987. Cloning and sequencing of human cholesteryl ester transfer protein cDNA. *Nature.* **327:** 632–634.
2. Nagashima, M., J. W. McLean, and R. M. Lawn. 1988. Cloning and mRNA tissue distribution of rabbit cholesteryl ester transfer protein. *J. Lipid Res.* **29:** 1643–1649.
3. Pape, M. E., E. F. Rehberg, K. R. Marotti, and G. W. Melchior. 1991. Molecular cloning, sequence, and expression of cynomolgus monkey cholesteryl ester transfer protein. Inverse correlation between hepatic cholesteryl ester transfer protein mRNA levels and plasma high density lipoprotein levels. *Arterioscler. Thromb.* **11:** 1759–1771.
4. Ha, Y. C., and P. J. Barter. 1982. Differences in cholesteryl ester transfer activities in sixteen vertebrate species. *Comp. Biochem. Physiol.* **71B:** 265–269.
5. Agellon, L. B., A. Walsh, T. Hayek, P. Moulin, X. C. Jiang, S. A. Shelanski, J. L. Breslow, and A. R. Tall. 1991. Reduced high density lipoprotein cholesterol in human cholesteryl ester transfer protein transgenic mice. *J. Biol. Chem.* **260:** 10796–10801.
6. Koizumi, J., H. Mabuchi, A. Yoshimura, I. Michishita, M. Takeda, H. Itoh, Y. Sakai, T. Sakai, K. Ueda, and R. Takeda. 1985. Deficiency of serum cholesteryl-ester transfer activity in patients with familial hyperalphalipoproteinaemia. *Atherosclerosis.* **58:** 175–186.
7. Brown, M. L., A. Inazu, C. B. Hesler, L. B. Agellon, C. Mann, M. E. Whitlock, Y. L. Marcel, R. W. Milne, J. Koizumi, H. Mabuchi, R. Takeda, and A. R. Tall. 1989. Molecular basis of lipid transfer protein deficiency in a family with increased high density lipoproteins. *Nature.* **342:** 448–451.
8. Inazu, A., M. L. Brown, C. B. Hesler, L. B. Agellon, J. Koizumi, K. Takata, Y. Maruhama, H. Mabuchi, and A. R. Tall. 1990. Increased high-density lipoprotein levels caused by a common cholesteryl-ester transfer protein gene mutation. *N. Engl. J. Med.* **323:** 1234–1238.
9. Bisgaier, C. L., M. V. Siebenkas, M. L. Brown, A. Inazu, J. Koizumi, H. Mabuchi, and A. R. Tall. 1991. Familial cholesteryl ester transfer protein deficiency is associated with triglyceride-rich low density lipoproteins containing cholesteryl esters of probable intracellular origin. *J. Lipid Res.* **32:** 21–33.
10. Marotti, K. R., C. K. Castle, R. W. Murray, E. F. Rehberg, H. G. Polites, and G. W. Melchior. 1992. The role of cholesteryl ester transfer protein in primate apolipoprotein A-I metabolism. Insights from studies with transgenic mice. *Arterioscler. Thromb.* **12:** 736–744.
11. Zilversmit, D. B., L. B. Hughes, and J. Balmer. 1975. Stimulation of cholesterol ester exchange by lipoprotein-free rabbit plasma. *Biochim. Biophys. Acta.* **409:** 393–398.
12. Mann, C. J., F. T. Yen, A. M. Grant, and B. E. Bihain. 1991. Mechanism of plasma cholesteryl ester transfer in hypertriglyceridemia. *J. Clin. Invest.* **88:** 2059–2066.
13. Bagdade, J. D., M. C. Ritter, and P. V. Subbaiah. 1991. Accelerated cholesteryl ester transfer in plasma of patients with hypercholesterolemia. *J. Clin. Invest.* **87:** 1259–1265.
14. Morton, R. E., and D. B. Zilversmit. 1981. A plasma inhibitor of triglyceride and cholesteryl ester transfer activities. *J. Biol. Chem.* **256:** 11992–11995.
15. Sparks, D. L., J. Frohlich, P. Cullis, and P. H. Pritchard. 1987. Cholesteryl ester transfer activity in plasma measured by using solid-phase-bound high-density lipoprotein. *Clin. Chem.* **33:** 390–393.
16. Abbey, M., G. D. Calvert, and P. J. Barter. 1984. Changes in lipid and apolipoprotein composition of pig lipoproteins facilitated by rabbit lipid transfer protein. *Biochim. Biophys. Acta.* **793:** 471–480.
17. Burstein, M., H. R. Scholnick, and R. Morfin. 1970. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. *J. Lipid Res.* **11:** 583–595.
18. Warnick, G. R., and J. J. Albers. 1978. A comprehensive evaluation of the heparin-manganese precipitation procedure for estimating high density lipoprotein cholesterol. *J. Lipid Res.* **19:** 65–76.
19. Gibson, J. C., A. Rubinstein, and W. V. Brown. 1984. Precipitation of apoE-containing lipoproteins by precipitation reagents for apolipoprotein B. *Clin. Chem.* **30:** 1784–1788.
20. Groener, J. E. M., R. W. Pelton, and G. M. Kostner. 1986. Improved estimation of cholesteryl ester transfer/exchange activity in serum or plasma. *Clin. Chem.* **32:** 283–286.
21. Ginsburg, G. S., D. M. Small, and D. Atkinson. 1982. Microemulsions of phospholipids and cholesteryl esters: protein-free models of low density lipoprotein. *J. Biol. Chem.* **257:** 8216–8227.
22. Via, D. P., I. F. Craig, G. W. Jacobs, W. B. Van Winkle, S. C. Charlton, A. M. Gotto Jr., and L. C. Smith. 1982. Cholesteryl ester-rich microemulsions: stable protein-free analogs of low density lipoproteins. *J. Lipid Res.* **23:** 570–576.
23. Wang, S., L. Deng, R. W. Milne, and A. R. Tall. 1992. Identification of a sequence within the C-terminal 26

amino acids of cholesteryl ester transfer protein responsible for binding a neutralizing monoclonal antibody and necessary for neutral lipid transfer activity. *J. Biol. Chem.* **267:** 17487–17490.
24. Pattnaik, N. M., A. Montes, L. B. Hughes, and D. B. Zilversmit. 1978. Cholesteryl ester exchange protein in human plasma isolation and characterization. *Biochim. Biophys. Acta.* **530:** 428–438.
25. Hesler, C. B., A. R. Tall, T. L. Swenson, P. K. Weech, Y. L. Marcel, and R. W. Milne. 1988. Monoclonal antibodies to the M_r 74,000 cholesteryl ester transfer protein neutralize all of the cholesteryl ester and triglyceride transfer activities in human plasma. *J. Biol. Chem.* **263:** 5020–5023.
26. Whitlock, M. E., T. L. Swenson, R. Ramakrishnan, M. T. Leonard, Y. L. Marcel, R. W. Milne, and A. R. Tall. 1989. Monoclonal antibody inhibition of cholesteryl ester transfer protein activity in the rabbit. Effects on lipoprotein composition and high density lipoprotein cholesteryl ester metabolism. *J. Clin. Invest.* **84:** 129–137.
27. Lakowicz, J. R. 1983. Principles of Fluorescence Spectroscopy. Plenum Press, New York. 257–262.
28. Nichols, J. W. 1988. Phospholipid transfer between phosphatidylcholine-taurocholate mixed micelles. *Biochemistry.* **27:** 3925–3931.
29. Schreiner, W., M. Kramer, S. Krischer, and Y. Langsam. 1985. Nonlinear least squares fitting. *PC Tech J.* **3:** 170–181.
30. Quinet, E. M., L. B. Agellon, P. A. Kroon, Y. L. Marcel, Y-C. Lee, M. E. Whitlock, and A. R. Tall. 1990. Atherogenic diet increases cholesteryl ester transfer protein messenger RNA levels in rabbit liver. *J. Clin. Invest.* **85:** 357–363.
31. Quinet, E., A. Tall, R. Ramakrishnan, and L. Rudel. 1991. Plasma lipid transfer protein as a determinant of the atherogenicity of monkey plasma lipoproteins. *J. Clin. Invest.* **87:** 1559–1566.
32. Milner, T. G., K. W. S. Ko, T. Ohnishi, and S. Yokoyama. 1991. Enhancement of the human plasma lipid transfer protein reaction by apolipoproteins. *Biochim. Biophys. Acta.* **1082:** 71–78.
33. Dousset, N., and L. Douste-Blazy. 1992. Fluorescent determination of cholesteryl ester transfer protein (CETP) activity in plasma. *Clin. Chem.* **38:** 306.
34. Morton, R. E. 1986. Specificity of lipid transfer protein for molecular species of cholesteryl ester. *J. Lipid Res.* **27:** 523–529.
35. Nichols, A. V., and L. Smith. 1965. Effect of very low-density lipoproteins on lipid transfer in incubated serum. *J. Lipid Res.* **6:** 206–210.
36. Barter, P. J., and J. I. Lally. 1979. In vitro exchanges of esterified cholesterol between serum lipoprotein fractions: studies of humans and rabbits. *Metabolism.* **28:** 230–236.
37. Pattnaik, N., and D. B. Zilversmit. 1979. Interaction of cholesteryl ester exchange protein with human plasma lipoproteins and phospholipid vesicles. *J. Biol. Chem.* **254:** 2782–2786.
38. Speijer, H., J. E. M. Groener, E. van Ramshorst, and A. van Tol. 1991. Different locations of cholesteryl ester transfer protein and phospholipid transfer protein activities in plasma. *Atherosclerosis.* **90:** 159–168.
39. Tall, A., D. Sammett, and E. Granot. 1986. Mechanisms of enhanced cholesteryl ester transfer from high density lipoproteins to apolipoprotein B-containing lipoproteins during alimentary lipemia. *J. Clin. Invest.* **77:** 1163–1172.
40. Barter, P. J., and M. E. Jones. 1980. Kinetic studies of the transfer of esterified cholesterol between human plasma low and high density lipoproteins. *J. Lipid Res.* **21:** 238–249.