Immunospecific Scintillation Proximity Assay of Cholesteryl Ester Transfer Protein Activity

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We describe a novel, immunospecific scintillation proximity assay for determining cholesteryl ester transfer protein (CETP) activity in total human serum and in reconstituted experimental mixtures. The assay is based on the measurement of radiolabeled cholesteryl esters transferred from a tracer dose of biosynthetically labeled high-density lipoprotein subtraction 3 to unlabeled apolipoprotein (apo) B-containing lipoproteins. The radioactivity content of the apo B-containing lipoprotein fraction can be evaluated without separating the donor from the acceptor lipoprotein substrates, and is measured through the formation of ternary complexes involving the radiolabeled apo B-containing lipoproteins, specific anti-apo B antibodies from sheep, and anti-sheep antibody-labeled fluoromicrospheres. Good correspondences were observed between CETP activity values obtained either with the ultracentrifugation method or the immunospecific scintillation proximity assay (n = 70; r = 0.94; P = 0.0001), and between values obtained for either fresh or frozen serum samples (n = 70; r = 0.93; P = 0.0001). Because of its potential for automation, the immunospecific scintillation proximity assay may constitute a convenient tool to measure serum CETP activity in the clinical laboratory.

Indexing Terms: lipid transfer activity/immmunoassay/fluoromicrospheres/radioassay

In several species, including humans, cholesteryl ester transfer protein (CETP) promotes the exchange of cholesteryl esters and triglycerides between different plasma lipoprotein fractions (1, 2). During the last decade, CETP has been recognized as one of the important factors determining the atherogenicity of the lipoprotein profile in human plasma (1, 2). Recent studies in transgenic mice significantly increased the formation of atherosclerotic lesions in comparison with control animals, which normally lack plasma cholesteryl ester transfer activity (3). Furthermore, several pathological conditions involving lipoprotein disorders are associated with significant alteration of cholesteryl ester transfer activity in plasma (2), underlying the determinant role that CETP may play in lipoprotein metabolism in vivo. Because both dietary and hypolipidemic drug interventions reportedly alter cholesteryl ester transfers in human plasma (2), measurements of CETP activity can be of clinical utility. Taken together, these observations have raised considerable interest in evaluating cholesteryl ester transfer activity in biological samples. To this end, several assays, based on the measurement of radiolabeled cholesteryl esters transferred from one lipoprotein donor to one lipoprotein acceptor, were set up (4). Whereas those assays constitute some convenient and sensitive methods for evaluating cholesteryl ester exchange activity in various biological samples, a major concern is their requirement for separation of donor from acceptor lipoprotein substrates before counting the radioactivity in the liquid scintillant (5).

Here, we report a new immunospecific scintillation proximity assay (SPA), set up to measure the rate of radiolabeled cholesteryl esters transferred from high-density lipoprotein (HDL) to the apolipoprotein (apo) B-containing lipoprotein fractions, very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL). We have applied the assay to the measurement of cholesteryl ester transfers in total human serum and in experimental mixtures containing purified CETP and isolated lipoprotein fractions.

Principle of the Assay

The SPA technology has been proposed to overcome some of the problems inherent in the use of radioisotopes in biological assays. SPA involves the use of fluoromicrospheres coated with acceptor molecules to which a specific radiolabeled ligand can bind selectively and generate light emission at the surface of the fluorosphere (6–8). In the present study, the immunospecific SPA of cholesteryl ester transfer activity is based on the measurement of radiolabeled cholesteryl esters transferred from biosynthetically labeled 3H-CE-HDL₃₀ to unlabeled apo B-containing lipoproteins. As shown in Fig. 1A, radiolabeled cholesteryl esters are transferred during incubation at 37°C of lipoprotein substrates in the presence of a source of CETP. At the end of the incubation, anti-apo B polyclonal antibodies from sheep and fluoromicrospheres coupled with anti-sheep antibodies are added to the mixtures (Fig. 1B), and the samples are then maintained overnight at 4°C to allow the formation of immunospecific lipoprotein–fluorosphere complexes (Fig. 1C). Because the tritium and fluorophore are brought into proximity in the lipoprotein–fluorosphere complexes, electrons released during the
decay of tritium can excite the fluorophore; the resulting fluorescence can be measured directly in a scintillation counter.

Materials and Methods

Reagents

\([1\alpha,2\alpha(n)\text{-}^3\text{H}]\text{cholesterol} \) (specific activity, 46 kCi/mol) and SPA anti-sheep antibody-labeled fluorospheres were purchased from Kodak (Amerham, Bucks, UK). Sheep anti-sheep B \(\gamma\)-globulin fraction was from Boehringer Mannheim (Mannheim, Germany). Bovine serum albumin was purchased from Sigma (St. Quentin Fallavier, France).

Blood Samples

Venous blood was collected after an overnight fast. Blood samples were drawn into plain glass tubes and placed without delay in 4°C storage. Sera were collected at the same temperature by a 5-min centrifugation at 3000g.

Procedures

Lipoprotein preparation. Human lipoprotein fractions were ultracentrifugally isolated from fasting normolipidemic sera at 350 000g in a 100.2 rotor in a TL-100 ultracentrifuge (Beckman, Palo Alto, CA). Densities were adjusted by adding solid KBr and checked with a DMA 35 digital densitometer (Paar, Graz, Austria). VLDL were isolated as the plasma fraction of density <1.006 kg/L by two 4-h centrifugations. VLDL+LDL were isolated as the plasma fraction of density <1.055 kg/L by two 5-h centrifugations. LDL were isolated as the plasma fraction of density 1.019–1.055 kg/L by one 4-h centrifugation at the lower density and two 5-h centrifugations at the higher density. HDL\(_3\) were isolated as the plasma fraction of density 1.13–1.21 kg/L by one centrifugation of 7 h at the lowest density and two for 10 h each at the highest density. The lipoprotein fractions, recovered by tube slicing, were dialyzed overnight against a pH 7.4 buffer of 10 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L Na\(_2\) EDTA, and 3 mmol/L Na\(_3\) Pi.

Radiolabeling of HDL\(_3\). HDL\(_3\) were biosynthetically labeled by incubating the fraction >1.13 kg/L from 20 mL of human plasma with 40 nmol of \([1\alpha,2\alpha(n)\text{-}^3\text{H}]\text{cholesterol} \) for 24 h at 37°C to allow cholesterol esterification by lecithin:cholesterol acyltransferase (9). At the end of the incubation, HDL\(_3\) containing tritiated cholesterol esters \([\text{^3H-CE-HDL}_3] \) were recovered by sequential ultracentrifugation as described above. As judged by thin-layer chromatography, >95% of total radioactivity resided in the cholesteryl ester moiety.

Partial purification of human CETP. CETP was partially purified from human plasma by using the general chromatographic procedure previously described (9) except that we included an additional heparin-column chromatographic step to separate plasma phospholipid transfer protein from CETP. Briefly, 2.5 L of citrated human plasma were precipitated with ammonium sulfate saturated between 35% and 55% and ultracentrifuged at a density of 1.25 kg/L. The resulting fraction >1.25 kg/L was then subjected successively to hydrophobic interaction chromatography on a phenyl-Sepharose CL-4B column (Pharmacia, Uppsala, Sweden), to cation-exchange chromatography on a carboxymethylcellulose column (Whatman, Kent, UK), to affinity chromatography on a heparin-Ultrogel A4R column (IBF-Biotechnics, Villeneuve-la-Garenne, France), and to anion-exchange chromatography on a MonoQ HR 5/5 column (Pharmacia). All the chromatographic steps were performed on a Fast Protein Liquid Chromatography system (Pharmacia). MonoQ fractions containing cholesteryl ester transfer activity were pooled, aliquoted, and stored at –20°C. The CETP preparation used in the present experiments was deficient in both lecithin:cholesterol acyltransferase activity and phospholipid transfer activity, as checked with the cholesteryl esterification assay of Piran and Morin.
Incubations. Cholesteryl ester transfer activity was determined by measuring the rate of radiolabeled cholesteryl esters transferred from $^3$H-CE-HDL₃ to apo B-containing lipoproteins, either in experimental mixtures containing isolated LDL, $^3$H-CE-HDL₃, and purified CETP, or in total human sera supplemented with $^3$H-CE-HDL₃. The reconstituted mixtures contained LDL (cholesterol concentration, 200 μmol/L), $^3$H-CE-HDL₃ (cholesterol concentration, 50 μmol/L), and purified CETP in a final volume of 50 μL. The total serum samples contained 25 μL of human serum, iodoacetate (1.5 mmol/L), and $^3$H-CE-HDL₃ (total cholesterol, 50 μmol/L) in a final volume of 50 μL. After the mixtures incubated at 37 °C in a shaking water bath, we immediately placed the samples on ice for 15 min and centrifuged for 5 min at low speed in an Eppendorf centrifuge to recover condensed water. Subsequently, we measured the radioactivity content of the apo B-containing lipoproteins either in ultracentrifugally isolated lipoprotein fractions or directly in total incubation mixtures by the SPA system.

Liquid scintillation counting of ultracentrifugally isolated lipoprotein fractions. The ultracentrifugation assay of CETP activity was conducted as previously described (12). We added 45 μL of incubated mixtures to 2 mL of a 1.07 kg/L KBr solution in Beckman centrifugation tubes, sealed the tubes, and ultracentrifuged the samples for 7 h at 269 000 g in a 50.4 Ti rotor in an L7 ultracentrifuge, both from Beckman. The fractions of <1.068 and >1.068 kg/L were subsequently recovered in a volume of 1 mL after tube slicing and transferred into counting vials. We added 2 mL of scintillation fluid (Optiscint Hisafe 3; Pharmacia) to each vial and counted the radioactivity for 5 min with a Wallac 1410 liquid scintillation counter (Pharmacia). The recovery of radioactivity after fractionating the <1.068 and >1.068 kg/L plasma fractions was >95%. In nonincubated controls, the radioactivity recovered in the fraction <1.068 kg/L was <5%.

Radioactivity counting of apo B-containing lipoproteins in the SPA. We further incubated 10 μL of radiolabeled incubated mixtures overnight at 4°C in 2-mL Eppendorf tubes with 50 μL of a 50-fold dilution of anti-apo B antibodies from sheep and 230 μL of anti-sheep antibody-labeled fluorospheres. The fluorospheres were reconstituted as recommended by the manufacturer and supplemented with bovine serum albumin (final concentration, 20 g/L) to reduce the nonspecific interaction between radiolabeled compounds and the microspheres. These samples were shaken end-over-end in an orbital shaker during the overnight incubation to keep the fluorospheres in suspension. At the end of the 4°C incubation, we immediately placed the samples in a Wallac 1410 liquid scintillation counter for a 5-min radioactivity counting.

Because various serum samples vary in their apo B content, we supplemented all sera, before the overnight period at 4°C, with the fraction <1.055 kg/L isolated from human serum to adjust the apo B content of the samples to the same value (2.5 mg of total apo B per milliliter of initial serum sample). The addition of lipoproteins <1.055 kg/L, anti-apo B antibodies from sheep, and anti-sheep fluorospheres were all added to the incubated serum samples simultaneously.

Calibration. CETP activity was determined in pooled sera from normolipidemic subjects by the previously described ultracentrifugation method (13). This pool, constituting the serum calibrator (cholesteryl ester transfer activity, 363% transferred per hour per milliliter of sample), was aliquoted into 100-μL samples and stored in liquid nitrogen. CETP activity in various serum samples was expressed in comparison with the serum calibrator, which was processed simultaneously.

Lipoprotein components assays. All chemical assays were carried out on a Cobas-Fara centrifugal analyzer (Roche, Basel, Switzerland). Total cholesterol, unesterified cholesterol, and triglyceride concentrations were measured by using enzymatic kits from Boehringer Mannheim. Plasma HDL cholesterol concentrations were measured after selective precipitation of apo B-containing lipoproteins with Boehringer Mannheim phosphotungstic acid/MgCl₂ reagent as recommended by the manufacturer. Apo B concentrations were determined by immunoturbidimetry with use of anti-apo B antibodies and apo B standard from Behring (Marburg, Germany) (14). Total protein concentrations were measured by using bichinchoninic acid reagent (Pierce Chemical Co., Rockford, IL) (15).

Statistical analysis. Correlations were analyzed by linear regression analysis. Paired-sample t-test was used to determine the significance of the difference between two means.

Results
Measurement of Cholesteryl Ester Transfers Between Isolated Lipoprotein Fractions

Isolated LDL (cholesterol concentration, 200 μmol/L) and $^3$H-CE-HDL₃ (cholesterol concentration, 50 μmol/L) were incubated for 3 h at 37 °C in the presence of increasing concentrations of purified CETP. As shown in Fig. 2, the amount of radiolabel measured in apo B-containing lipoproteins increased as the amounts of CETP added increased from 0 to 2.8 μg. In the absence of anti-apo B antibodies, the radioactivity counted remained constant, independent of CETP concentration (Fig. 2).

Measurement of Cholesteryl Ester Transfers in Total Human Serum

In fasting human sera, CETP activity was evaluated by determining the transfer of radiolabeled cholesteryl esters from $^3$H-CE-HDL₃ (total cholesterol, 50 μmol/L) to serum apo B-containing lipoproteins. As shown in Fig. 3, the radioactivity content of the apo B-containing lipoproteins, which was determined by adding anti-apo B antibodies and anti-sheep antibody-labeled fluoromi-
microspheres, increased progressively with incubation time from 0 to 6 h. When fluoromicrospheres were added alone, without anti apo-B antibodies, the non-specific radioactivity count remained constant over the 6-h period (Fig. 3).

To compare the time courses of the transfer of radiolabeled cholesteryl ester from $^3$H-CE-HDL$_3$ to serum apo B-containing lipoproteins in either the SPA assay or an independent ultracentrifugation method, we incubated human serum at 37°C for as long as 12 h in the presence of $^3$H-CE-HDL$_3$ (Fig. 4). Every hour, we determined the radioactivity content of the apo B-containing lipoproteins—either directly by using the SPA or after separation of VLDL+LDL and HDL fractions (densities <1.068 and >1.068 kg/L, respectively) by sequential ultracentrifugation. Over the 12-h period, the radioactivity content of the serum apo B-containing fraction increased progressively when measured by either method (Fig. 4). In both cases, the radioactivity content of the plasma apo B-containing lipoproteins increased linearly during the first 6 h of incubation, then tended to plateau after longer incubation times (Fig. 4).

To check further the validity and accuracy of the SPA of serum CETP activity, we determined the rate of cholesteryl ester transfer from $^3$H-CE-HDL$_3$ to the serum apo B-containing lipoprotein fraction during a 3-h incubation at 37°C for 70 human sera (24 normolipidemic, 21 hypertriglyceridemic, 5 hypercholesterolemic, and 20 with combined hyperlipidemia), using three methods: ultracentrifugation, SPA without apo B normalization, and SPA with apo B normalization. In each assay, cholesteryl ester transfer activity, expressed as the percentage of total radiolabeled cholesteryl esters transferred per hour per milliliter of serum, was determined by interpolation from a calibration curve constructed with a frozen serum calibrator of known activity processed simultaneously. As shown by the paired-sample t-test, the mean CETP activity measured with the ultracentrifugation method (405% ± 184% · h$^{-1}$ · mL$^{-1}$) differed significantly from the mean CETP activity value obtained with the immunoassay SPA without apo B normalization (342% ± 97% · h$^{-1}$ · mL$^{-1}$; $P = 0.0001$), but not from the mean value obtained with the immunoassay SPA with apo B normalization (396% ± 142% · h$^{-1}$ · mL$^{-1}$).

Among the 70 normo- and hyperlipidemic sera studied, ultracentrifugation values (x) correlated better with the SPA values (y) obtained after normalization of apo B content ($y = 0.72 ± 0.03x + 103.20; r = 0.94; P = 0.0001$) (see Fig. 5) than with the SPA values obtained without normalization ($y = 0.75 ± 0.06x + 295.43; r = 0.81; P = 0.0001$). The conventional statistical method described by Burnett (16) and polynomial regression

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**Fig. 2.** CETP-mediated transfer of radiolabeled cholesteryl esters between isolated lipoprotein fractions as measured by the immunoassay SPA. Each point represents mean of triplicate determinations.

**Fig. 3.** Time course of the transfer of radiolabeled cholesteryl esters from a tracer dose of $^3$H-CE-HDL$_3$ towards the apo B-containing lipoprotein fraction in total normolipidemic serum as measured by the immunoassay SPA. Symbols as in Fig. 2.

**Fig. 4.** Comparison of the time-dependent transfer of radiolabeled cholesteryl esters as measured in total normolipidemic serum by (top) the previously described ultracentrifugation method (10) or (bottom) the immunoassay SPA. Samples were 45-μL (top) and 10-μL (bottom) aliquots of the incubation mixtures. Each point represents mean of triplicate determinations.
transferred from donor towards acceptor lipoprotein substrates with distinct physicochemical properties (4). Whereas this general procedure provides a sensitive and reliable tool to evaluate cholesteryl ester transfer rates, it requires the physical separation of acceptor particles from donor ones. This latter step—previously done by using either solid-phase-bound lipoprotein substrates (17), precipitation techniques (18, 19), sequential ultracentrifugation (6, 20), or polyacrylamide gradient gel electrophoresis (21)—is time consuming and exposes researchers to the hazards of manipulating radioactive compounds. Other nonisotopic cholesteryl ester transfer assays, measuring the transfer fluorescent cholesteryl ester analogs, have been recently proposed (22–24). However, although this latter approach did not involve radioisotope handling and time-consuming separation techniques, the fluorescent probes did not adequately trace cholesteryl ester molecules. For instance, the transfer of cholesteryl oleate was threefold that of a fluorescent analog (24).

Recently, an SPA system to measure CETP activity was proposed by Amersham International. In this assay, [3H]cholesteryl esters transferred from HDL to biotinylated LDL are detected through the addition of streptavidin-coated fluoromicrospheres. Although that assay also avoided separation of donor from acceptor lipoprotein substrate, the biotinylation of LDL is time consuming and can modify the surface properties of lipoprotein particles; most importantly, the Amersham approach does not measure cholesteryl ester transfers from HDL to native, endogenous VLDL and LDL in total plasma. In the present study, we avoided the biotinylation of LDL particles, replacing the interaction of biotinylated LDL with streptavidin-coated fluoromicrospheres with use of an immunospecific reaction based on the recognition of apo B antigens by specific polyclonal antibodies from sheep. The concurrent addition of anti-sheep antibodies, covalently linked to fluoromicrospheres, allows the radiolabeled apo B-containing lipoproteins and fluoromicrospheres to be so close that the low-energy-electrons released during the decay of tritium can excite the fluorophore, which consequently emits light.

In the present study we first applied the immunospecific CETP–SPA to the measurement of cholesteryl ester transfers between radiolabeled HDL and unlabeled LDL fractions. We demonstrated that appropriate conditions could be arranged in which the immunospecific CETP–SPA conveniently showed the ability of purified CETP to promote the transfer of radiolabeled cholesteryl ester between isolated lipoprotein fractions.

Some studies have evaluated plasma CETP activity by determining the ability of lipoprotein-deficient plasma to promote the transfer of radiolabeled cholesteryl esters between isolated HDL and apo B-containing lipoprotein fractions of exogenous origin (6). Although this approach allows one to evaluate plasma CETP concentration, it may not reflect exactly cho-

Fig. 5. Correlation between cholesteryl ester transfer activity values obtained in 70 human sera by either the ultracentrifugation method, (x) or the immunospecific SPA with normalized apo B values (y). Study samples were 24 normolipidemic sera (total cholesterol, 1.85 ± 0.43 g/L; HDL-cholesterol, 0.57 ± 0.16 g/L; triglycerides, 0.84 ± 0.17 g/L), 21 hypertriglyceridemic sera (total cholesterol, 1.95 ± 0.42 g/L; HDL-cholesterol, 0.39 ± 0.14 g/L; triglycerides, 2.18 ± 1.16 g/L), 5 hypercholesterolemic sera (total cholesterol, 2.72 ± 0.30 g/L; HDL-cholesterol, 0.65 ± 0.19 g/L; triglycerides, 0.92 ± 0.12 g/L), and 20 sera with combined hyperlipidemia (total cholesterol, 2.82 ± 0.36 g/L; HDL-cholesterol, 0.45 ± 0.12 g/L; triglycerides, 2.42 ± 0.92 g/L). Values are expressed in percentage of total radiolabeled cholesteryl ester transferred per hour per milliliter of serum as compared with a frozen serum standard of known activity (383% ± h⁻¹/mL⁻¹). Each point represents mean of triplicate determinations.

analysis showed that CETP activity values obtained with either the SPA or the ultracentrifugation methods were linearly related over a wide range of cholesteryl ester transfer activity values (ranging from 167% to 849% · h⁻¹ · mL⁻¹). CETP activity values obtained with fresh and frozen serum correlated well (r = 0.93; P = 0.0001; n = 70).

To investigate the effect of alterations in the relative proportions of added VLDL and LDL on CETP activity values obtained with the immunospecific SPA method, we normalized the apo B concentrations in the 70 serum samples studied by adding to the samples either the fraction <1.006 kg/L (containing mainly VLDL particles) or the fraction <1.055 kg/L (containing mainly LDL particles), which had been isolated from a pool of normolipidemic sera. The resulting two sets of values correlated positively (r = 0.96; P = 0.0001), with the lighter fraction giving slightly but significantly greater values for mean CETP activity (420% ± 141% vs 396% ± 142% · h⁻¹ · mL⁻¹, respectively; P = 0.0001).

The imprecision and reproducibility of the CETP-SPA assay were evaluated by determining cholesteryl ester transfers from [3H]-CE-HDL₃ to apo B-containing lipoproteins after a 3-h incubation at 37°C of three serum samples with low (255% · h⁻¹ · mL⁻¹), intermediate (400% · h⁻¹ · mL⁻¹), and high (849% · h⁻¹ · mL⁻¹) CETP activity. Assaying the samples 10 times in the same experiment gave the intraassay CVs of 6.1%, 5.7%, and 8.9%, respectively. The interassay CV, determined by assaying one serum sample (intermediate activity) six times over a 6-day period, was 9.4%.

Discussion

In the past, CETP activity has been measured by determining the rate of radiolabeled cholesteryl esters...
olesteryl ester transfers as they actually occur in vivo. Indeed, recent studies demonstrated that both the composition and concentration of endogenous lipoprotein particles can influence plasma cholesteryl ester transfer activity (2). In the present study, we applied the immunospecific CETP-SPA to the measurement of cholesteryl ester transfer activity in serum samples containing endogenous lipoprotein substrates. The normalization of apo B concentrations was necessary before assessing the radioactivity content of serum apo B-containing lipoproteins, and serum cholesteryl ester transfer activity values so obtained correlated well with data from an independent ultracentrifugation method previously used in our laboratory to determine CETP activity in total human plasma (10). The immunospecific SPA of CETP activity was applicable to both fresh and frozen serum samples, making it interesting for clinical studies. However, linear regression analysis of the relationship between the ultracentrifugation and the SPA methods showed the slope deviating significantly from 1.00 and the y-intercept from zero. The reasons for these alterations are uncertain, but it is tempting to ascribe them, at least in part, to differences in the criteria used to evaluate the radioactivity content of the VLDL + LDL fraction—i.e., either the lipoprotein density with the ultracentrifugation method, or theapolipoprotein content with the SPA. For instance, the radioactivity content of lipoprotein(a) could conceivably be part of the HDL-containing, ultracentrifugally isolated fraction >1.068 kg/L in the former case, but might be measured as part of the apo B-containing lipoprotein family in the apolipoprotein-specific approach.

In conclusion, the experimental observations outlined above suggest that the immunospecific CETP-SPA provides a simple, specific, and accurate method for quantifying cholesteryl ester transfer activity in various biological samples. In particular, because of its potential for automation, the immunospecific SPA we describe may constitute a convenient tool to measure plasma CETP activity in the clinical laboratory. In addition, by making use of specific anti-apolipoprotein antibodies, this method may be readily applied to the measurement of the rate of cholesteryl ester transferred—not only towards apo B-containing lipoproteins, as presented in this report, but also towards lipoprotein substrates of distinct apolipoprotein composition by use of antibodies with different specificities.

This investigation was supported by the Université de Bourgogne, the Conseil Régional de Bourgogne, the Institut National de la Santé et de la Recherche Médicale (INSERM), and the Fondation pour la Recherche Médicale (FRM).

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