Polyclonal antibody-based immunoradiometric assay for quantification of cholesteryl ester transfer protein

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Abstract Cholesteryl ester transfer protein (CETP) catalyzes the transfer of neutral lipids among plasma lipoproteins and in this way plays a prominent role in cholesterol metabolic routing and, thus, probably for atherosclerosis. Studies of this important protein in various clinical settings require the ability to accurately quantify CETP in plasma. In order to gain access to such a capability, an immunoradiometric assay (IRMA) for quantification of CETP was developed. CETP was purified from human plasma to apparent homogeneity and used for raising anti-CETP antibodies in rabbits. The specificity of the polyclonal antiserum obtained was demonstrated by inhibition assays and immunoblot analysis. Before use in the CETP-IRMA, the antibodies were affinity-purified by chromatography on CETP-Sepharose. Sensitivity of the CETP-IRMA was 0.1 ng, and intra- and interassay coefficients of variation were 2.9 and 8.0%, respectively. In 30 normolipidemic healthy subjects, the mean (± SD) CETP concentration was 1.1 (± 0.22) μg/ml of plasma; individual values ranged from 0.644 to 1.694 μg CETP/ml and agreed well with measurements of CETP activity of the same samples (r = 0.85).—**Ritsch, A., B. Auer, B. Föger, S. Schwarz, and J. R. Patsch.** Polyclonal antibody-based immunoradiometric assay for quantification of cholesteryl ester transfer protein. *J. Lipid Res.* 1993. **34:** 673–679.

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The distribution of cholesteryl esters and triglycerides among plasma lipoproteins greatly affects the metabolism of LDL and HDL, both known to constitute powerful risk factors of coronary artery disease (CAD) (1). Individuals with elevated fasting triglycerides tend to have a preponderance of small LDL, termed pattern B (2), and small HDL, i.e., HDL_3 (3). Even in normolipidemic subjects, who display high triglyceride concentrations in the postprandial state only, the transfer of postprandial triglycerides from chylomicrons and VLDL into HDL_2 is sufficient to enrich the core of the HDL_2 for conversion into HDL_3 by hepatic lipase (4). The transfer of triglycerides and cholesteryl esters among lipoproteins is catalyzed by a plasma glycoprotein, called lipid transfer protein (LTP-I) (5) or cholesteryl ester transfer protein (CETP) (6–9). The important role of this protein for the metabolism of lipoproteins and the development of CAD is demonstrated by the inherited condition CETP deficiency. So far, one molecular defect has been elucidated as a cause for CETP deficiency, where a mutation in the CETP gene prevents correct mRNA splicing and synthesis of CETP (10). Affected family members show extremely high levels of HDL-cholesterol with a preponderance of large HDL particles and a reported decreased risk for CAD resulting in longevity (11).

With the crucial role that CETP holds for the distribution of cholesteryl esters and triglycerides among plasma lipoproteins and, thus, for cholesterol metabolic routing to body tissues, extensive studies will be necessary if we are to understand how CETP is affected by diet, exercise, hormones, and drugs. For such studies, a basic requirement is the ability to accurately measure the concentration of CETP in plasma. Measurement of CETP activity has been made available (12) and has proved helpful and informative (13). However, this approach has limitations inherent in activity measurements in general, where exogenous substrates have to be prepared and partial loss of activities is difficult to control. Measuring enzyme mass directly avoids such problems and is much more feasible for everyday use. Indeed, mass measurement of CETP

Abbreviations: CETP, cholesteryl ester transfer protein; IRMA, immunoradiometric assay; RIA, radioimmunoassay; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; EDTA, ethylenediamine tetraacetic acid; BSA, bovine serum albumin; CAD, coronary artery disease.

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has been successfully developed by Marcel et al. (14) and by Fukasawa, Arai, and Inoue (15). Both methods used independently raised monoclonal antibodies against CETP for establishing solid-phase radioimmunoassay (RIA) procedures. The well-documented method of Marcel et al. (14) has provided a great deal of information on CETP, including plasma levels in normolipidemic and hyperlipidemic individuals and correlations of CETP levels with other plasma lipid transport parameters (14, 16). Also, the effects of alcohol (17) and probucol (18) were described. The focus of our research interest over recent years has been to study the effect of triglyceride-rich lipoproteins on HDL particles. As CETP is a major mediator in this relationship, the ability to quantify CETP mass is important for our studies. We therefore set out to develop a reliable and accurate method to measure CETP mass. In this report we describe the development of an immunoradiometric assay (IRMA) for CETP quantification and demonstrate its applicability and usefulness for clinical studies.

MATERIALS AND METHODS

Blood samples

Blood samples were collected from donors, after an overnight fast, into tubes containing EDTA to give a final concentration of 1.6 mg/ml. To obtain a normolipidemic plasma pool as internal standard for the IRMA, plasma samples (1 ml) from each of 100 normolipidemic donors were combined. HDL-cholesterol ranged from 40 to 60 mg/dl, low density lipoprotein (LDL)-cholesterol from 120 to 170 mg/dl, and triglycerides from 0 to 180 mg/dl. The pooled plasma was supplemented with sodium azide (NaN_3) to a final concentration of 1 mM and stored in aliquots at −80°C. Concentrations of total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides in this plasma pool were 223, 148, 48, and 132 mg/dl, respectively.

Preparation of LDL and HDL

For CETP activity assays, LDL and HDL were isolated from fresh human EDTA-plasma by zonal ultracentrifugation using Beckman Ti-14 rotors and Beckman L-8 ultracentrifuges (19). For LDL, rotor fractions under the entire LDL peak were pooled. For HDL, fractions under both the HDL_2 and HDL_3 peaks were pooled. LDL and HDL isolated in this way correspond to LDL and HDL isolated by conventional sequential ultracentrifugation of densities 1.02–1.06 g/ml and 1.07–1.20 g/ml, respectively (3, 20). Lipoproteins were dialyzed against phosphate-buffered saline (PBS), concentrated by pressure filtration in an Amicon® stirred cell (Amicon Corporation, Danvers, MA) equipped with membranes YM100 and PM30, respectively, and stored in 1 mM NaN_3 at 4°C.

Radioactive labeling of LDL

[^{14}C]cholesteryl-oleate (DuPont-New England Nuclear Research Products, Boston, MA) was incorporated into LDL by the use of synthetic [^{14}C]cholesteryl-oleate/phospholipid vesicles and lipoprotein-deficient plasma (12). The labeled LDL were purified from the labeling mixture by ultracentrifugation in a NaBr step-density gradient (12), dialyzed against PBS, and stored at 4°C. The specific radioactivity of the labeled LDL ranged from 1000 to 2000 cpm/μg cholesteryl ester.

Determination of CETP activity

To assay for CETP activity, the transfer of [^{14}C]cholesteryl-oleate from LDL to HDL was quantified. The CETP activity of plasma samples was measured as described by Groener, Pelton, and Kostner (12). For measurement of CETP activity of various fractions in the course of the purification procedure, we used the assay described by Ohnishi, Yokoyama, and Yamamoto (21).

Purification of CETP

CETP was isolated from fresh human plasma obtained from patients after an overnight fast following essentially the procedure described by Ohnishi et al. (21) using, consecutively, Fractogel TSK Butyl-650 (M) for hydrophobic interaction chromatography, Fractogel TSK CM-650 (M) for ion exchange chromatography, and Fractogel TSK HW-55 (S) for gel permeation chromatography (all from Merck, Darmstadt, Germany). However, to assure a gentle treatment of the protein, the procedure of Ohnishi et al. (21) was modified as follows: all dialysis steps, except the one at the end, were eliminated. Furthermore, all precipitation steps were eliminated. Instead, we used small Butyl-Sepharose columns (Fractogel TSK Butyl-650, M) to concentrate the protein solutions. For this purpose, samples were supplemented with 2 M NaCl, and applied to the Butyl-Sepharose columns whose volumes ranged from 0.5 to 2.0 ml. After washing the columns with 50 mM Tris-HCl, pH 7.6, protein was eluted with 3 mM Tris-HCl, pH 7.6. At the end of the procedure the protein was dialyzed against PBS and stored at −80°C.

For estimation of protein concentrations in the course of the purification procedure we used the method of Lowry et al. (22) using bovine albumin as a standard. However, when assaying purified CETP by the Lowry method, 3.7-fold higher values were obtained when compared to those from amino acid analysis. Therefore, we used the method described by Scopes (23) which is based on the peptide bond absorbance; with this procedure, values only 6% higher than those from the amino acid analysis were obtained. Therefore, results from the Scopes method were used.

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Immunization

Two rabbits were immunized by injecting 100 μg of purified CETP in Freund incomplete adjuvant (Difco, Detroit, MI) subcutaneously at 5–8 sites. The injection procedure was repeated after 2 months and 4 months, respectively. Rabbits were bled before and after each immunization, and the sera obtained were tested for their content of CETP-specific antibodies with a dot-blot immunobinding assay using purified CETP fixed to a cellulose nitrate membrane (BA 83, Schleicher and Schuell, Dassel, Germany). Two weeks after the last boosting, the rabbits were bled and the serum was stored at −80°C.

Affinity purification of the antibody

Whole IgG-type antibodies were isolated from the antiserum by a Protein-G column (Protein G Sepharose 4 Fast Flow, Pharmacia, Uppsala, Sweden) and applied to an affinity column prepared by coupling 1 mg CETP to 3 g CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). After elution with 0.1 M glycine (pH 2.7), the IgG solution was dialyzed against PBS and stored at −80°C.

CETP-depleted plasma

For the preparation of CETP-depleted plasma, an anti-CETP column was prepared by adding 4.6 mg antibody (purified from 1 ml antiserum with a Protein-G column) to 1.5 g of CNBr-activated Sepharose 4B. Using this column, CETP could be entirely removed from a 50-ml sample by passing the plasma and the respective eluates for a total of 3 times through the column. Between passages the column was washed with 0.1 M glycine (pH 2.7).

Iodination of anti-CETP antibody

The affinity-purified anti-CETP antibody was iodinated by the Chloramin T method (24). Five μg of anti-CETP antibody was incubated with 0.5 mCi sodium ^{125}iodide (Na ^{125}I, Behringwerke AG, Marburg, Germany) and 50 μg Chloramin T in a total volume of 55 μl for 20 sec after 10 sec of gentle mixing. The reaction was terminated by the addition of 100 μg sodium disulfite and 1 mg potassium iodide. The remaining free Na ^{125}I was removed by filtration of the reaction mixture through a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). The specific radioactivity for the anti-CETP antibody was 4500 Ci/mmol. The iodinated anti-CETP antibodies were stored in aliquots at −20°C.

Immunoradiometric assay

Polystyrol tubes (12 × 75 mm, MaxiSorp® StarTubes, Nunc, Roskilde, Denmark) were coated with 250 μl affinity-purified anti-CETP antibody solution containing 10 μg/ml 0.01 M bicarbonate buffer, pH 9.6, at room temperature for 2 h. After rinsing two times with PBS, 200 μl of sample in PBS, supplemented with 2% BSA and 1% Triton X-100, was added to each tube. After an incubation of 30 min at room temperature and two washes with PBS, 200 μl of radiolabeled antibody solution (100,000 cpm) was added and incubated for 4 h. After three rinses with PBS, the radioactivity bound to each tube was measured in a gamma counter (1272 Clinigamma, Pharmacia-LKB, Uppsala, Sweden).

Other analytical methods

Plasma cholesterol and triglycerides were measured by enzymatic methods (25, 26). Quantification of HDL-, HDL_2-, and HDL_3-cholesterol, included the same enzymatic methods in combination with a stepwise precipitation procedure to remove apoB-containing lipoproteins and HDL_2, respectively (27, 28). SDS-PAGE was performed as described by Laemmli (29). Protein bands were visualized by staining the gels with Coomassie Brilliant Blue R-250 or by silver staining (Silver stain plus, Bio-Rad, Richmond, CA).

RESULTS

Purification of CETP

Repeated purification procedures were necessary to obtain CETP in quantities sufficient for the immunization of the rabbits, the preparation of the CETP-Sepharose column, and for amino acid analysis. For example, starting with 2350 ml of human plasma, we obtained 1.19 mg CETP corresponding roughly to a 30,000-fold purification. When the final protein-solution was scrutinized by SDS-PAGE, only the duplex band typical of CETP (21) was detected, even when gels were heavily loaded with as much as 1 μg of protein and subjected to silver staining (**Fig. 1A**). Furthermore, amino acid analysis assured us that it was indeed CETP that had been purified, because it was in excellent agreement with published data (30).

Production of CETP-specific IgG

As early as after the first immunization step, antibodies specific for CETP could be detected in both immunized rabbits by a dot-blot immunobinding assay using pure CETP fixed to a cellulose nitrate membrane.

The specificity of the anti-CETP antiserum obtained was ascertained as follows. *i)* In a Western blot, the duplex band of CETP was detected by the antibody (Fig. 1B); *ii)* in an inhibition study, various amounts of IgG were added to CETP-activity assays of a normal plasma pool, and the entire transfer activity of the plasma was inhibited; and *iii)* when plasma was passed through an anti-CETP IgG-Sepharose column, it lost 93% of its CETP activity.

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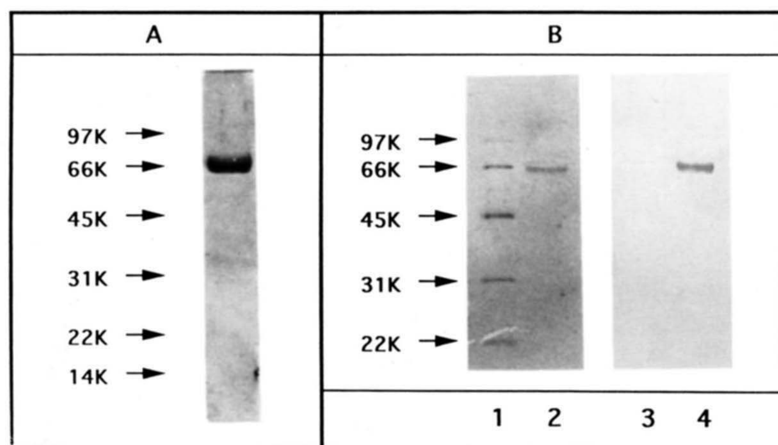

Fig. 1. A: Analysis of purified CETP by 10% SDS-PAGE. Molecular weight (MW) marker proteins used were phosphorylase B (MW 97,000), bovine serum albumin (MW 66,000), ovalbumin (MW 45,000), carbonic anhydrase (MW 31,000), trypsin inhibitor (MW 22,000), and lysozyme (MW 14,000). One μg CETP was applied to the gel and visualized by silver staining. B: Western blot analysis of CETP (lanes 3 and 4) compared to SDS-PAGE analysis of CETP (lanes 1 and 2); lane 2, 100 ng of purified CETP was applied to SDS-PAGE; lane 1, same MW standards as in Fig. 1A. A second identical gel (lane 3: molecular weight standards, lane 4: 100 ng of CETP) was blotted on a cellulose nitrate membrane, and incubated subsequently with affinity-purified anti-CETP antibodies. Thereafter, bound rabbit-IgG was visualized with anti-rabbit-IgG antibodies coupled to peroxidase (Tago Inc., Burlingame, CA).

Immunoradiometric assay

The immunoradiometric assay is a two-site binding assay. In a two-step procedure, CETP is first bound quantitatively to an anti-CETP antibody adsorbed to a solid phase (capture antibody), and subsequently is detected by the same antibody labeled with ^{125}I (detection antibody).

The concentration of the capture antibody in the coating solution was 10 μg/ml. In our hands, this solution could be used as often as six times without a noticeable decrease of capture antibody bound. Two hours were sufficient for binding of the capture antibody to the tube. Extending the coating time for up to 12 h did not have any effect. The MaxiSorp® StarTubes (Nunc, Roskilde, Denmark) proved superior to standard tubes, due probably to the larger surface area thus increasing the binding of the capture antibody, and in turn, leading to a higher binding capacity for the antigen.

With samples containing 1 ng or more of CETP, the binding of CETP reaches its maximum within 30 min, whereas the second incubation step with the detection antibody requires at least 4 h.

Intra- and interassay coefficients of variation on 10 samples and 10 subsequent measurements at ~1.2 μg CETP/ml were found to be 2.9 and 8.0%, respectively. The scale of accurate measurements ranged from 5 to 200 ng CETP with incubation times of sample and detection antibody of 30 min and 3 h, respectively. Increasing incubation time of the sample to 14 h increased the sensitivity to a detection limit of 0.1 ng CETP.

For determination of the CETP concentration in the normal plasma pool, we used purified CETP in CETP-depleted plasma to create a standard curve for the IRMA. From these data we obtained a CETP concentration value of 1.2 μg/ml for our normal plasma pool. This figure is in excellent agreement with our results from the CETP-activity assays, where our normal plasma pool showed an activity corresponding to 1.6 μg/ml purified CETP in CETP-depleted plasma (**Fig. 2**).

One of the advantages of our CETP-IRMA is its high specificity for CETP, thus affording the possibility to assay samples with high concentrations of other proteins as is the case with total untreated plasma. We assayed plasma samples of 30 normolipidemic healthy subjects, 18 males and 12 females, and determined a CETP concentration of 1.1 ± 0.22 μg/ml of plasma (mean ± SD) for the entire set of subjects. For the males, mean CETP concentration was 1.03 ± 0.18 μg CETP/ml plasma, and for the females, it was 1.18 ± 0.26 μg CETP/ml plasma. CETP activity in these samples measured according to Groener et al. (12) was 102 ± 22.6 (mean ± SD) nmol [^{14}C]cholesteryl-oleate/ml·h transferred from LDL to HDL. In the plasma samples that we assayed, there was a strong direct association of CETP mass and activity (**Fig. 3**).

A second requirement of the CETP quantification method was a precision sufficient to perform studies on

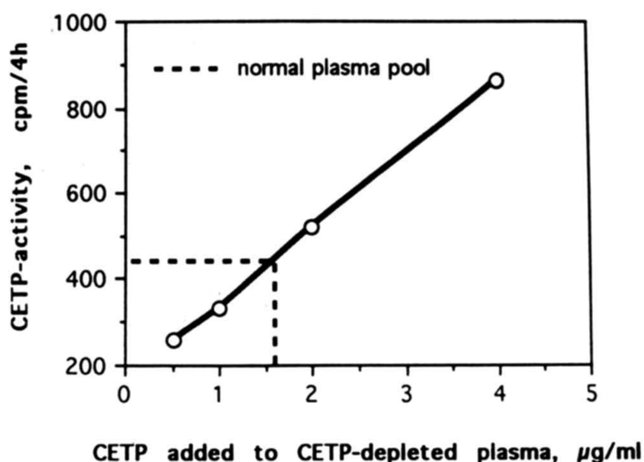

Fig. 2. CETP activity assay for the estimation of CETP protein concentration in a normal plasma pool. CETP-depleted plasma was supplemented with 0.5, 1, 2, and 4 μg of purified CETP/ml, respectively, and used to create a standard curve. Means of duplicate activity assays are shown. Mean coefficient of variation was 2.4%.

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the effect of diet, exercise, and drugs on CETP. To test for this capability of our IRMA, we followed the plasma CETP concentration of eight participants in a bicycle marathon. As a result of the strenuous exercise, we found a precipitous decrease of the plasma CETP levels (**Fig. 4**), steadily increasing in subsequent days until, by day 8, they almost reached the pre-exercise levels.

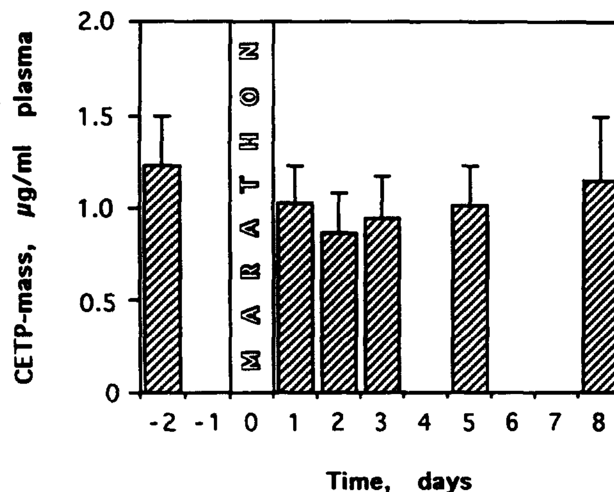

Fig. 4. Plasma CETP concentrations of eight participants (mean ± SD) in a bicycle marathon, measured 2 days prior to and 1, 2, 3, 5, and 8 days after the race.

DISCUSSION

The assay described in this report was developed because CETP affects the transfer of cholesteryl esters from particles such as HDL into triglyceride-rich lipoproteins. This transfer becomes of particular quantitative importance when triglycerides accumulate as in the postprandial phase (31). To be useful for our purposes, the assay had to fulfill the following requirements. It had to be unaffected by high amounts of proteins other than CETP in the sample, so as to allow measurement of CETP in whole untreated plasma. Precision and accuracy of the quantification method had to be sufficient for kinetic studies, where changes in CETP concentration on the order of 10% would be clearly definable. Also, the assay had to be simple so as to be feasible for everyday use. A further desirable feature was a sensitivity high enough to study the effect of postprandial lipoproteins on CETP production by cells in culture.

We first approached our goal through setting up a competitive RIA, a methodology successfully used by others (14, 15). Our RIA did not show satisfactory results with total plasma when compared to those with purified CETP. We thought plasma components other than CETP were responsible for this discrepancy, because CETP-depleted plasma showed strong competition. Several attempts to abolish these effects were undertaken including delipidation of plasma with a mixture of n-hexane and isopropanol, precipitation of lipoproteins with sodium phosphotungstate and $MnCl_2$, removal of IgG using a protein G Sepharose column, and addition of various amounts of Triton X-100 and Tween 20 to the assay. None of these measures diminished the putative matrix effects of plasma satisfactorily. To obtain reliable data with purified CETP it was, therefore, necessary to add an excess of CETP-depleted plasma to the incubation mixture. Unfortunately, under these conditions the cpm-range of the RIA was small, resulting in an unacceptably low precision. The two solid-phase RIAs for CETP developed successfully by Marcel et al. (14) and Fukasawa et al. (15) both use monoclonal antibodies. Our antibody is polyclonal and appears unsuitable for methods with simultaneous incubation of sample and antibody. Therefore, we decided to take another approach for quantification of CETP by attempting to develop an immunoradiometric assay (IRMA).

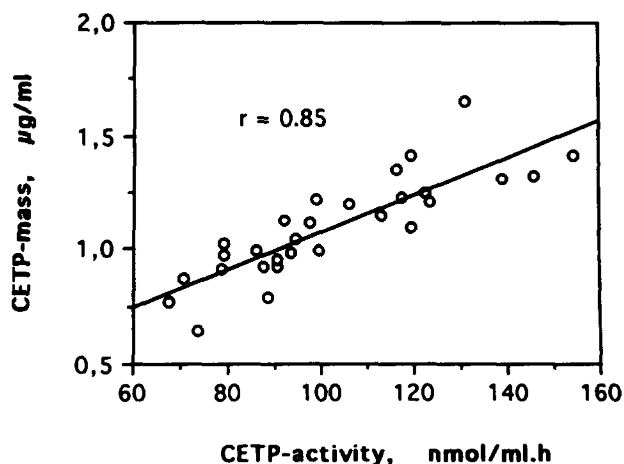

Fig. 3. Comparison of CETP activity and CETP mass in the plasma of 30 normolipidemic healthy subjects.

Generally, the IRMA does not depend heavily on antigen or antibody concentration, an advantage over the RIA where the concentration of the radiolabeled antigen strongly determines measuring range, lower limit, and precision. Crucial for the reliability of an IRMA, however, is the correct specificity of the antibody used. For the production of the polyclonal anti-CETP antibody we used only CETP whose purity was attested to by SDS-PAGE and amino acid analysis. For the purification of CETP, use of small Butyl-Fractogel columns instead of the ammonium sulfate precipitation steps helped to avoid denaturation of the protein. Also, the purification procedure was shortened considerably in that no dialysis steps for removing ammonium sulfate were necessary. In contrast to the precipitation steps, the Butyl-Fractogel columns are very well suited for highly diluted protein so-

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lutions such that almost no protein is lost. Fortunately, the troublesome background effects seen in our competitive RIA did not appear in the IRMA, due probably to the separate sequential incubation steps of sample and detection antibody, respectively.

There are several findings that attest to the correct specificity of the CETP-IRMA described. First, the polyclonal antibody used in this assay is able to inhibit CETP activity of both plasma and purified CETP. Second, the mean CETP level of our 30 controls is only 35% lower than that reported by Marcel et al. (14) and, thus, quite comparable. More importantly, we also observed the sex-related difference reported by Marcel et al. (14). In our opinion, the differences of the average CETP levels are not caused by the use of RIA or IRMA, because data from our competitive RIA were in accordance with our IRMA data. A possible reason may be differences in quantification of the CETP standard solutions, which can yield very different results depending on which method for protein estimation is used (see below). Third, if our polyclonal antibody detected proteins other than CETP, our values would be higher than those of Marcel et al. (14) and of Fukasawa et al. (15). Lastly, our IRMA data are in accordance with our CETP activity measurements.

The different results of CETP concentration observed with the methods of Lowry et al. (22) and Scopes (23) and with amino acid analysis were unexpected. However, repeated measurements using different CETP preparations were in excellent agreement. Furthermore, measurements of a BSA-solution with the Lowry method agreed well with the results obtained with the method of Scopes for the same sample. One explanation for the unexpected phenomenon with the Lowry method may be the high hydrophobicity of CETP (30).

The effect of exercise on CETP levels and the usefulness of our IRMA were demonstrated by the marathon study. Effects of other perturbations such as diet and drugs need to be studied also and our assay can be expected to prove adequate for such studies.

Finally, our observation on the severe effect of rigorous exercise on the plasma levels of CETP suggests that many more systematic studies are warranted to identify major factors and/or measures that influence CETP. We expect that the CETP-IRMA described herein will prove a valuable supplement to the methods already described for the quantification of this important protein.

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