A hyperalphalipoproteinaemic family with normal cholesteryl ester transfer/exchange activity*

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Reports of two independent studies suggest that familial hyperalphalipoproteinaemia (FHALP) may be caused by a deficiency of cholesteryl ester transfer/exchange activity (CETP). We also have studied CETP in the plasma of an Italian FHALP kindred. The study group was divided into blood relatives with > 1.70 mm high-density-lipoprotein cholesterol (HDL-C) (group I, $n = 9$), with < 1.70 mm-HDL-C (group II, $n = 12$) and in spouses (group III, $n = 6$). Two different assays were performed to measure CETP activity. In method A the interfering endogenous lipoproteins in the plasma samples were removed by poly(ethylene glycol) precipitation or by ultracentrifugation at a relative density (d) of 1.180. The CETP-activity of these samples was measured in a system consisting of fixed amounts of HDL and cholesteryl [1- 14 C]oleate-labelled low-density lipoproteins (LDL). In method B, trace amounts of HDL (radiolabelled with cholesteryl [1- 14 C]oleate) were incubated with plasma for 3 h at 37 °C and the distribution of the label among lipoproteins was measured (CET activity). The results can be summarized as follows. The mean CETP activities measured by method A were 187, 213 and 243 nmol/h per ml in groups I, II and III respectively. The proband with the highest HDL-C (4.98 mm) had a CETP activity of 231 nmol/h per ml. The corresponding CET activities measured by method B and expressed as percentage transfer/h were 4.3, 8.0 and 11.2 in groups I–III. The proband with HDL-C = 4.98 mm had a value of only 1.7% /h. There was a strong negative correlation between percentage CE transfer and HDL-C concentration. Calculating these data in terms of CE exchange (nmol/h per ml), groups I, II and III exhibited mean activities of 86, 124 and 110 nmol/h per ml respectively; for the proband this value was 80 nmol/h per ml. Only a slight correlation was found between these values and the HDL-C value. Thus by both methods, (A), measuring the CETP activity *per se* and (B), measuring the activity in whole plasma (reflecting the activity of the protein and the concentration and composition of lipoproteins), no major differences could be found between the three groups. In our family, therefore, no connection between FHALP and CETP deficiency could be found. It is concluded that, for hyper- and dys-lipoproteinaemic samples, a careful selection of the assay procedure as well as the mode of calculating results is essential. Since this may not hold the previous studies, the supposed connection between FHALP and CETP deficiency is challenged.

INTRODUCTION

Familial hyperalphalipoproteinaemia (FHALP) was first described by Glueck and co-workers (Glueck *et al.*, 1975a,b). The condition seems to be associated with a relative rarity of coronary diseases and with increased longevity, consistent with the general inverse relationship between coronary risk and high-density-lipoprotein (HDL) concentration (Saito, 1984; Glueck *et al.*, 1976). The mechanism underlying the apparent protection of FHALP from coronary atherosclerosis is not known. Kinetic studies in FHALP subjects revealed significantly smaller pools of slowly exchangeable tissue cholesterol as well as lower cholesterol turnover rates as compared with subjects with normal plasma HDL concentrations (Nestel & Miller, 1980). Recently, two independent studies have been reported suggesting that FHALP is

caused by a deficiency of CETP activity (Koizumi *et al.*, 1985; Kurasawa *et al.*, 1986).

CE transfer to, and the exchange between, the various lipoproteins in human plasma is stimulated by specific lipid-transfer proteins (Zilversmit *et al.*, 1975; Barter & Lally, 1979). It is of importance to note that, in the process of CE transfer/exchange, the activity of the specific proteins and the concentration and composition of the lipoproteins in plasma independently influence the rate of CE movement. Since parts of the previous reports on CETP activity in FHALP may have been biased by this phenomenon, we investigated the CETP activity in nine FHALP individuals and in controls according to two different procedures.

Abbreviations used: VLDL, LDL and HDL, very-low-, low- and high-density lipoprotein(s); HDL-C, high-density-lipoprotein cholesterol; CE, cholesteryl ester; TAG, triacylglycerol(s); PEG, poly(ethylene glycol); (F)HALP, (familial) hyperalphalipoproteinaemia; CETP, cholesteryl ester transfer/exchange (protein); LCAT, lecithin (phosphatidylcholine):cholesterol acyltransferase; apoAI etc., apolipoprotein AI etc.; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid).

* This paper is dedicated to Professor Dr. Anton Holasek on the occasion of his 65th birthday.

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MATERIALS AND METHODS

Subjects

The study group consisted of members of an Italian family who have been investigated in detail with respect to plasma lipids, lipoproteins, LCAT activity, post-heparin lipolytic activity and other laboratory parameters. It started from the observation of a 64-year-old woman with plasma levels of total cholesterol of 8.25 mm but no familial history of atherosclerosis or myocardial infarction. In fact all the blood relatives seemed to have a high life expectancy. A closer check-up of blood lipids and lipoproteins revealed a HDL-C value of 4.98 mm and the following apolipoprotein concentrations: apoAI, 320 mg/dl (normal 130 mg/dl); apoAII, 80 mg/dl (normal 35 mg/dl). We further studied 54 family members from three generations of the propositus and found that more than 48% of the blood relatives exhibited HDL-C values which were above the 95th percentile of the local population. All other lipids, lipoproteins and notably also the post-heparin lipolytic as well as the LCAT activities of the family members were within the limits of normals (J. E. M. Groener, P. G. da Col & G. M. Kostner, unpublished work). The molecular defect causing HALP in this family remained obscure. Family members with HALP were characterized by longevity and the absence of signs of atherosclerotic diseases. In this study 21 volunteers of the HALP family were investigated. They were divided into hyper- and 'normo'-alphalipoproteinaemics (group I and group II respectively) according to their plasma HDL-C concentrations (cut-off point at the 90th percentile, 1.70 mmol/l). Six spouses of the members of groups I and II served as controls (group III).

Materials

Cholesteryl [1-¹⁴C]oleate (sp. radioactivity 50–60 Ci/mmol) was obtained from Amersham International. Porcine serum albumin and egg phosphatidylcholine were from Sigma Chemical Co, St. Louis, MO, U.S.A.; Nbs₂ was from Fluka A. G., Buchs, Switzerland, and bovine serum albumin and PEG 6000 were from Serva, Heidelberg, Germany. PEG Reagent A, a 95 g/litre solution of PEG (M_r 20000) was purchased from Immuno A. G., Vienna, Austria. All other chemicals were of analytical grade and purchased from E. Merck, Darmstadt, Germany.

Clinical chemical analyses

Blood samples were drawn into tubes containing EDTA (1 mg/ml) after subjects had fasted overnight, and plasma was obtained by low-speed centrifugation. Total cholesterol and HDL-C, after precipitation with PEG (Kostner *et al.*, 1985), were measured by enzymic methods (Boehringer kit no. 237 574), TAGs with kit no. 612318 from BioMerieux, and free cholesterol with kit no. 14106 from Merck. CE was calculated as the difference between total cholesterol and free cholesterol.

Isolation of lipoproteins

LDL and HDL were isolated from fresh human plasma by ultracentrifugation at solution densities of 1.006–1.063 and 1.063–1.210 g/ml respectively by the method of Havel *et al.* (1955). The separated lipoproteins were washed once under identical conditions and dialysed against 10 mM-Tris/HCl buffer, containing

150 mM-NaCl, 1 mM-EDTA and 0.01% NaN₃, pH 7.4 (dialysis buffer). The $d = 1.210$ infranatant was dialysed against the dialysis buffer and used as source of CETP activity for incorporating radiolabelled CE into lipoproteins.

Radiolabelling of LDL and HDL

For labelling of LDL and HDL with cholesteryl [1-¹⁴C]oleate, we essentially used the lipid-dispersion technique of Morton & Zilversmit (1981), with the modification that isolated lipoproteins were incubated with cholesteryl [1-¹⁴C]oleate/phospholipid vesicles and lipoprotein-free plasma (Groener *et al.*, 1986).

Measurement of CETP activities

Two different assays were performed in this study. In method A the CETP activity *per se* was measured. In method B the transfer/exchange of CE in plasma was measured after adding a trace amount of labelled HDL, this method thus measuring the CETP plus the concentration and composition of endogenous lipoproteins; this activity will be referred to as 'CET'. In method A, CETP activity was measured after the removal of VLDL and LDL with PEG as described by Groener *et al.* (1986). Briefly, 50 μ l of plasma were mixed with 100 μ l of PEG Reagent A (Immuno Diagnostics, Vienna, Austria), vortex-mixed and centrifuged (Kostner *et al.*, 1985). A 60 μ l portion of the supernatant (corresponding to 20 μ l of plasma) were added to 640 μ l of an incubation system in which the exchange of ¹⁴C-labelled CE from LDL to HDL was measured. In the previous investigation (Groener *et al.*, 1986) we ascertained that the measured CETP activity is not influenced by the endogenous HDL remaining in the plasma sample after PEG treatment (up to 2 mm-HDL-C). As the FHALP plasmas had HDL-C values exceeding this concentration, amounts equal to 5 and 10 μ l of FHALP plasmas were used in the assay. Alternatively, the interfering lipoproteins of plasma samples were removed by ultracentrifugation at $d = 1.180$ for 48 h at 200000 g. The infranatants were dialysed and amounts equal to 20 μ l of plasma were used in the same incubation system as described above. The incubations were performed in duplicate. Nbs₂ (2 mM) was added to each sample in order to block the LCAT activity. Control experiments were carried out without the addition of Nbs₂. CETP activity was calculated as described by Barter & Jones (1979), as outlined previously (Groener *et al.*, 1986), according to the equation:

$$S_L(t) = [S_L(0) - S_{Eq.}] \exp \left[-F \left(\frac{M_L + M_H}{M_L \cdot M_H} \right) t + S_{Eq.} \right]$$

where $S_L(t)$, $S_L(0)$ and $S_{Eq.}$ denote the specific radioactivities of CE (c.p.m./nmol) in LDL at time t (in h), at zero time and at equilibrium respectively; M_L and M_H denote the CE pool sizes (nmol/incubation) in LDL and HDL respectively; F denotes the rate of exchange (nmol/h per incubation) between LDL and HDL. From this formula F can be calculated as follows:

$$F = \frac{-\ln \left\{ 1 - \frac{C_H(t)}{M_L [S_L(0) - S_{Eq.}]} \right\}}{\left(\frac{M_L - M_H}{M_L \cdot M_H} \right) \cdot t}$$

where $C_H(t)$ is the radioactivity in HDL (c.p.m.) at time

t (in h). The CETP activity is expressed in nmol of CE/h per ml. In method B we measured the CET activity in whole plasma. This method therefore reflects the true CE transfer/exchange process occurring *in vivo* in the plasma of fasting subjects. To 300 μ l of whole plasma, trace amounts (15 μ l) of [14 C]CE-labelled HDL (3 nCi, sp. radioactivity 0.2 Ci/mol of HDL-CE) and 1 mmol of sodium iodoacetate/litre (Nbs₂ would interfere with the free cholesterol assay; see below) were added to inhibit the LCAT activity, and the mixture was incubated for 3 h at 37 °C. The total volume was 400 μ l. After incubation the samples were cooled to 0 °C. A 300 μ l portion from these samples were mixed with 30 μ l of 80% PEG 6000 (Viikari, 1976) in order to precipitate VLDL and LDL. The precipitate was washed once with 0.5 ml of 0.15 M-NaCl containing 50 μ l of a 1:1 (v/v) mixture of 2% dextran sulphate and 2 M-MgCl₂ (if PEG was used in the washing step, part of the precipitate was solubilized). Finally the precipitate was solubilized in 0.5 ml of a 10% (w/v) NaCl solution and the radioactivity was measured by liquid-scintillation counting in an LKB 1219 Rackbeta instrument. All CET assays were performed in duplicate. In control experiments the incubation was performed at 0 °C and the measured activity in VLDL and LDL of these incubations was subtracted as a blank. CET activity was calculated either as CE transfer (neglecting the back and forward movement), and is expressed as the amount of radioactivity found in VLDL+LDL after incubation (as a percentage of the total original activity added in the form of HDL) normalized for 1 h (%/h).

In addition, we calculated with these data the CE exchange by the method of Barter & Jones (1979) as described above, assuming CE exchange solely between HDL-CE and (VLDL+LDL)-CE and no mass transfer.

For theoretical considerations (see the Results and discussion section) we also calculated a possible maximal mass transfer, assuming no CE exchange, by multiplying the percentage CE transfer/h by the HDL-CE level. In the initial experiment the linearity of CE exchange/transfer with time was investigated. For this study two different plasmas, one with low HDL-C (0.68 mM) and another with high HDL-C (1.99 mM), were incubated at 37 °C and the CE exchange/transfer was measured at different time intervals. The results are presented in Fig. 1. In both plasmas a substantial amount of radioactivity is transferred from HDL to (VLDL+LDL) within 5 h. When the CE exchange was calculated as described by Barter & Jones (1979), the reaction was found to be linear with respect to time for at least 3 h. On the basis of these results all further experiments with method B were performed with a fixed incubation period of 3 h.

RESULTS AND DISCUSSION

Recent reports of Koizumi *et al.* (1985) and of Kurasawa *et al.* (1985) suggest a causal relationship of familial hyperalphalipoproteinemia with the deficiency, or with very low levels, of CETP activities. These findings do not accord with the results of the present study. Table 1 displays the values for total cholesterol, TAGs and HDL-C of the three groups of volunteers of our FHALP family. Group I, designated as FHALP, had significantly higher HDL-C values compared with group II and III respectively (2.44 compared with 1.50 and 0.89 mm). The proband which had been first recognized as

Fig. 1. Determination of the linearity with time of our CET assay method B

Trace amounts of HDL, radiolabelled with cholesteryl [1- 14 C]oleate, were added to two different plasmas, one with 0.68 mM-HDL-C (■) and another with 1.99 mM-HDL-C (●) and the CE transfer and exchange were monitored for 5 h. For details, see the Materials and methods section.

FHALP (S.R.) showed an HDL-C value of 4.98 mM. We also noticed significant differences in total cholesterol and TAGs between groups I, II and III; they are, however, of less importance for the present considerations.

The CETP activity of all plasma samples was measured by two different methods (A and B). Our experimental design allowed us to distinguish between the CETP activity *per se*, reflecting more or less the activity of the transfer/exchange protein present in the samples (method A). In addition, the process of CE transfer and/or exchange in whole plasma as a result of the CETP activity in combination with the substrate concentration and composition in the individual plasma samples (method B) was measured. We are aware of the fact that by both methods we measured activities resulting from CETP activity and a putative inhibitor, as

Table 1. Plasma concentration of total cholesterol, HDL-C and TAGs of the members of a hyperalphalipoproteinaemic family

The values are given in mM and represent means $\pm$ S.D. The range of individual values are given in parentheses. Group I, hyperalphalipoproteinaemics with HDL-C > 1.70 mM; group II, blood relatives of this family with HDL-C < 1.70 mM; group III, spouses. Abbreviation used: n.s., not significant.

Group	[Total cholesterol] (mM)	HDL-C (mM)	[TAGs] (mM)
I (n = 9)	5.42 $\pm$ 0.79 (4.66–7.20)	2.48 $\pm$ 0.98 (1.70–4.98)	0.72 $\pm$ 0.30 (0.46–1.58)
II (n = 12)	5.31 $\pm$ 1.33 (2.90–7.70)	1.47 $\pm$ 0.21 (1.12–1.68)	1.12 $\pm$ 0.59 (0.47–2.64)
III (n = 6)	4.89 $\pm$ 0.56 (3.90–5.67)	0.98 $\pm$ 0.28 (0.68–1.46)	1.09 $\pm$ 0.35 (0.63–1.88)
Significance			
I versus II	n.s.	P < 0.001	P < 0.02
I versus III	P < 0.1	P < 0.001	P < 0.02
II versus III	n.s.	P < 0.001	n.s.

Table 2. CETP activity, measured by different procedures, in the plasma of a hyperalphalipoproteinaemic family

The values represent means $\pm$ S.D., and are expressed in nmol of CE exchanged/per h ml of plasma (column 1 and 3) and as a percentage of the radioactivity present originally in HDL and transferred to VLDL + LDL within 1 h (column 2). Values in parentheses are ranges for individual values. For details of the assay procedures, see the Materials and methods section. Abbreviation used: n.s., not significant

Group or proband	CETP activity (method A) (nmol/h per ml of plasma)	CE transferred (method B) (%/h)	CE exchange (method B) (nmol/h per (method B) plasma)
Group I	187.2 $\pm$ 33.4 (141.9–244.3)	4.3 $\pm$ 1.5 (1.7–6.5)	86.3 $\pm$ 15.9 (64.5–110.5)
Proband S.R.	231.1	1.7	80.5
Group II	213.2 $\pm$ 44.5 (113.1–290.4)	8.0 $\pm$ 2.3 (4.4–13.7)	124.5 $\pm$ 33.0 (65.5–177.0)
Group III	243.9 $\pm$ 58.0 (147.9–339.8)	11.2 $\pm$ 4.1 (5.9–18.6)	110.5 $\pm$ 32.6 (73.5–161.0)
		Significance	
I versus II	n.s.	$P < 0.001$	$P < 0.001$
I versus III	$P < 0.01$	$P < 0.05$	$P < 0.001$
II versus III	n.s.	n.s.	$P < 0.02$

suggested by Son & Zilversmit (1984). None of the previous studies on FHAP, however, took such a possible inhibitor into consideration. Table 2 shows the results of our measurements. The mean CETP activities (method A, column 1) of groups I, II and III were respectively 187, 213 and 244 nmol/h per ml. Group I exhibited a 12% lower activity than group II and a 23% lower activity than group III. Only the difference between group I and III was statistically significant. The CETP activity of the proband S.R., who displayed the highest HDL-C value of all the FHAP subjects, was 231 nmol/h per ml and close to the mean value of group-III individuals. Thus there were only moderate differences in CETP activity between FHAP members and the control group (spouses). Since Koizumi *et al.* (1985) in their study measured the CETP activity in lipoprotein plasma ($d = 1.21$ infranatant), we repeated the analysis of 12 of our samples (six from group I, including proband S.R., four from group II and two from group III) after centrifugation at $d = 1.180$ g/ml. We took the $d = 1.180$ infranatant, since we knew from previous studies on normal individuals that the yield of CETP at $d = 1.210$ is only approx. 70–80% (Groener *et al.*, 1984). The yield of CETP in the $d = 1.180$ infranatant was $96.8 \pm 7.9\%$ as compared with the values found after PEG treatment of plasmas. This is in agreement with our previous report (Groener *et al.*, 1986). We note that the results with our HALP family are quite opposite to those published by Koizumi *et al.* (1985).

In another set of experiments a trace amount of CE-labelled HDL was added to the whole plasma samples and CET activity was measured by method B. The results are shown in Table 2, columns 2 and 3. By

Fig. 2. Plot of the CE transfer (a) or the CE-exchange (b) versus the plasma HDL-C concentrations

●, Blood relatives of the FHAP kindred; the vertical broken line divides them into group I (with > 1.70 mM-HDL-C) and group II (with < 1.70 mM-HDL-C); ○, spouses (group III). For CE transfer, a linear correlation coefficient of -0.701 and a logarithmic correlation coefficient of -0.893 ($P < 0.001$) were calculated. The corresponding values for CE exchange were -0.469 and -0.475 . Abbreviation: S.R., proband S.R.

calculating the data of this assay in terms of the percentage of the radioactive CE transferred from HDL to VLDL + LDL, the mean values for groups I, II and III were 4.2, 8.0 and 11.2%/h respectively. The differences between all groups were statistically highly significant. It is of importance to note that the proband S.R. exhibited a CE transfer of only 1.7%/h, thus very little radioactivity was found in VLDL + LDL after 3 h incubation. In an assay system with a high blank radioactivity in VLDL + LDL at zero time, this low value could easily escape attention. It should be pointed out that Koizumi *et al.* (1985) presented part of their data in a similar way.

In contrast, calculation of the data in terms of CE exchange between HDL-CE and (VLDL + LDL)-CE by the method of Barter & Jones (1979) resulted in

completely different values. The mean values for CE exchange in groups I, II and III were respectively 86, 124 and 110 nmol/h per ml. The highest values here were found in group II, followed by groups III and I. The difference between group I and II was statistically significant.

From the results obtained with the two methods we conclude that there is no lack of CETP activity in our FHALP subjects and that the lower values of CE transfer measured by method B mainly reflect the uneven substrate distribution in the particular samples. In order to substantiate this assumption, we correlated the CET activity from all three groups with the plasma HDL-C concentrations (Fig. 2). There was a highly significant negative correlation between the percentage CE transfer and the plasma HDL-C concentration. By linear-regression analysis a correlation coefficient of $r = -0.701$ was found. After transformation of y versus $\ln y$, an even better negative correlation of $r = -0.839$ was calculated. The HDL-C was also negatively correlated with the CE exchange, but this was of less significance.

Therefore if one calculates CET activity in terms of CE exchange and not in percentage transfer, our proband S.R. exhibited an activity which was only by 27% lower than the mean value for our control group. By estimating solely the percentage CE transfer, Koizumi *et al.* (1985) might thus have underestimated the actual activity in their two FHALP subjects. The low exchange activity in the FHALP subjects examined by Kurasawa *et al.* (1985), who applied essentially the same assay as our method B except that they used VLDL-free plasma, are most probably due to the high HDL/LDL ratio in the FHALP plasma. They therefore provide no direct information on the true CETP activity in their probands.

One may argue that the calculation described by Barter & Jones (1979) may not be appropriate for our method B, since the incubation was performed in the presence of endogenous lipoproteins and, under those conditions, a net mass transfer of CE from HDL to VLDL + LDL will probably occur. We therefore additionally calculated our data by assuming a theoretical 100% mass transfer, suggesting no exchange of CE between HDL and VLDL + LDL, and compared these values with the calculated CE exchange. The theoretical 100% mass transfer was calculated from the percentage radioactivity found in VLDL + LDL after incubation and the concentration of HDL-CE. This mass transfer is the maximum which might have been occurred and was, for all samples, $82 \pm 7\%$ of the exchange value.

Recently, Eisenberg (1985) compared mass transfer and exchange between HDL and various VLDLs and LDLs and found that mass transfer is about 10% of the exchange value for LDL and up to 90% of the exchange value for TAG-rich VLDL. In our plasmas the amount of VLDL is much smaller than the amount of LDL, so

that the actual mass transfer will probably be somewhat more than 10% from the calculated CE exchange and therefore should not greatly influence our data. Fielding *et al.* (1982), working under similar conditions, observed CE-mass-transfer rates in normal individuals of 23 nmol/h per ml.

Regardless of the method used, the present results demonstrate that, at least in our FHALP family, the high HDL values are not due to a lack of CETP, nor are they due to a major disturbance in the process of CE exchange. It further underlines the importance of choosing not only the right methods but also the right calculations for measuring CETP activities. As this is open to question in the two previous studies on the same topic, a connection of FHALP with CETP deficiency remains to be established.

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