Triglyceride Transfer Is Required for Net Cholesteryl Ester Transfer between Lipoproteins in Plasma by Lipid Transfer Protein

EVIDENCE FOR A HETERO-EXCHANGE TRANSFER MECHANISM DEMONSTRATED BY USING NOVEL MONOCLONAL ANTIBODIES

(Received for publication, July 25, 1994)

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In order to investigate the role of lipid transfer protein (LTP) in plasma lipoprotein metabolism, monoclonal antibodies (mAbs) have been raised against LTP isolated from rabbit plasma. Two mAbs, 3-9F and 14-8H, inhibited both [3H]cholesterol ester (CE) and [3H]triglyceride (TG) transfer from low density lipoprotein (LDL) to high density lipoprotein (HDL) mediated by LTP. Although 3-9F cross-reacted with human LTP, 2-8G was species-specific for rabbit LTP. mAb 14-8H inhibited only [3H]TG but not [3H]CE transfer and was cross-reactive with human LTP. mAbs 2-8G and 3-9F interfered with association of LTP with lipid microemulsions, again 2-8G with species specificity, whereas 14-8H did not affect LTP-microemulsion binding. Thus, mAbs 3-9F and 14-8H were used for further study in human plasma. By total inhibition of CE and TG transfer by 3-9F, LTP was shown to be responsible for net mass transfer of neutral lipids between lipoprotein classes in plasma, namely of CE from HDL to very low density lipoprotein (VLDL) and TG from VLDL to LDL and HDL. Selective inhibition of TG transfer by mAb 14-8H was also able to inhibit such net neutral lipid transfer. Such effect of these antibodies was demonstrated more remarkably in the presence of cholesterol esterification. Thus, TG transfer activity of LTP was shown to be responsible for net CE transfer, suggesting that net neutral lipid transfer in plasma between lipoproteins occurred mainly by a hetero-exchange mechanism. Inhibition of net neutral lipid transfer in plasma did not affect cholesterol esterification occurring predominantly on HDL. Consequently, mAb inhibition of TG transfer in plasma leads to CE accumulation in HDL. It is possible that hyperalophilipoproteinemia may be induced by a mutation in LTP that causes a selective defect in TG transfer activity.

In the plasma of certain vertebrates exists lipid transfer activity which can mediate the transfer of cholesteryl ester (CE) between the cores of the various classes of lipoproteins.

*This work was supported in part by Research Operating Grant MT 10607 from the Medical Research Council of Canada, a research fund provided by Sankyo Co. Ltd., and by a grant for the International Scientific Exchange Program from the Ministry of Science, Culture and Education of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§The abbreviations used are: CE, cholesteryl ester; LTP, lipid transfer protein; TG, triglyceride; mAb, monoclonal antibody; HDL, high density lipoprotein; apo, apolipoprotein; LCAT, lecithin:cholesterol acyltransferase; FCS, fetal calf serum; HAT, hypoxanthine-aminopterin-thymidine; LPDP, lipoprotein-deficient plasma; LDL, low density lipoprotein; PC, phosphatidylcholine; PAGE, polyacrylamide gel electrophoresis; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); VLDL, very low density lipoprotein.

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from studies of one mAb, TP2, originally shown to inhibit both human LTP-mediated CE and TG transfer (Hesler et al., 1988). The TP2 epitope was mapped to the COOH-terminal 26 amino acids of LTP and binding of TP2 Fab fragment to LTP affected neutral lipid binding and transfer to similar degrees (Swensen et al., 1989). Point mutagenesis studies suggest that hydrophobic residues in this region are directly involved in neutral lipid binding (Wang et al., 1993), and a basic model of how this binding site is formed has been proposed (Tall, 1993). In addition, it was shown that the transfer of CE and TG by LTP is strictly competitive, although it seems to prefer CE (Ohnishi et al., 1993). The TP2 epitope was mapped to the COOH-terminal amino acids, which may have different affinity for the two substrates. Interestingly, Fukasawa et al. (1992) have reported establishment of mAbs against human LTP, one of which is inhibitory toward both CE and TG transfer and the other novel clone, LT-31, dissociates CE and TG transfer. However, these mAbs were not further characterized for effects on other parameters of lipid transfer such as neutral lipid binding or lipoprotein binding to LTP.

Rabbit LTP is highly similar to the human counterpart in protein sequence, but there are differences, such as a 19-residue extension near the COOH-terminal in the rabbit protein (Nagashima et al., 1988) and perhaps functionally some difference in transfer specificity (Morton and Zilversmit, 1983) and distribution in plasma (Tall et al., 1987). We have purified rabbit LTP and shown that human and rabbit LTP are similar in specific activity of in vitro CE transfer (Ko et al., 1993) but slightly different in their physicochemical properties (Ko et al., 1993; Ohnishi et al., 1994b). Further effort was made to raise mAbs against rabbit LTP to study the mechanism of the LTP. Through use of different screening approaches, transfer assays, and a lipid particle binding assay, we hoped to obtain novel mAbs which might be useful probes for investigating the mechanism and physiological role of the LTP reaction further.

In this paper, we report the generation and partial characterization of novel inhibitory mAbs against rabbit LTP and the use of them to assess the role of LTP in net lipid transfer among lipoproteins in incubated human plasma. One mAb was capable of dissociating the two neutral lipid transfer functions of LTP, CE, and TG transfer by specifically inhibiting TG transfer. Using these mAbs, it was shown that the net transfer of CE between lipoprotein classes by LTP in relation to the LCAT reaction is likely to regulate plasma HDL cholesterol level. More interestingly, we tested, using the TG transfer-specific inhibitory mAb, the hypothesis that exclusive CE transfer by LTP, CE, and TG transfer by specifically inhibiting TG transfer. For this purpose, we have conducted experiments using rabbit LTP and human LTP to assess the role of LTP in net lipid transfer among lipoproteins in incubated human plasma.

**Experimental Procedures**

**Immunizations—**Female 6-week-old BALB/c mice (University of Alberta Laboratory Animal Services) were immunized with active native rabbit LTP (approximately 3 μg) isolated as described (Ko et al., 1993), combined with monophosphoryl lipid A plus trehalose dimeroyl adjuvant (Ribi Immunochem) by intraperitoneal injection. At week 3, a secondary injection (3 μg of LTP) was given and after detection in tailbled serum (control serum was negative) at week 4 of positive inhibitory activity toward rabbit LTP mediated [3H]CE (below), mice were boosted again at week 6. At week 12, final boosting intraperitoneally and intravenously (3 μg of LTP with and without adjuvant, respectively) was performed 3 days before fusion.

**Microscopy—**Mouse splenocytes and cell line P3X63 Ag8 were cultured in myeloma medium (Dulbecco’s modified Eagle’s medium (low glucose), with 8% fetal calf serum (FCS) and 50 μg/ml gentamycin) at 37°C in a CO2 (7%) incubator, splitting at 1:40. On the day of the fusion, 10^4 cells were collected (1,500 rpm for 10 min, benchtop centrifuge) in rinse medium (RPMI 1640 with 5% FCS, 50 μM 2-mercaptoethanol (Sigma), and 50 μg/ml gentamycin). The mouse was sacrificed by cervical dislocation, and the spleen was excised aseptically, placed into a Petri dish, and washed with prewarmed rinse medium. The spleen was then homogenized in 5 ml of prewarmed 0.15 M sterile distilled water, and splenocytes were collected and resuspended in 10 ml of rinse medium. The splenocytes and myeloma cells were combined in a sterile centrifuge tube (Corning), and the volume was adjusted to 50 ml with RPMI 1640 medium alone. The cells were collected and washed once more with RPMI medium before drying the pellet carefully. Then 1 ml of prewarmed 50% ethanol-glycerol (Sigma) was added with gentle shaking, followed by 1 min standing. Then 500 μl of RPMI 1640 was added over 45 s, followed by 500 μl over 15 s, 5 ml over 5 min, and 15 ml over 5 min. One ml of FCS and 25 ml of hypoxanthine-aminopterin-thymidine (HAT) medium (RPMI 1640 with 10% FCS, 50 μM 2-mercaptoethanol, 50 μg/ml gentamycin, 1 x HAT (Sigma)) were then added, and cell density was adjusted to 2 x 10^6 fused cells/ml with further HAT medium before aliquoting 100 μl/well into sterile 96 well plates (Corning). Next day, 150 μl of HAT medium was added; subsequently, media changes of 170 μl were performed twice a week with HAT medium, followed by subcloning and expansion in HT medium (HAT medium, substituting 1 x hypoxanthine-thymidine (HT, Sigma) for HAT) for 4 weeks, during which preliminary screening by inhibition assay or dot blotting (below) was performed. Positive wells were cloned by limiting dilution at least three times with a splenocyte feeder cell method. Large amounts of individual mAbs were produced by intraperitoneal injection of mice in phosphate-buffered saline (3 x 10^9 cells) into pristane (Sigma) primed BALB/c mice and harvesting of serum and ascites fluid 1–2 weeks later. Antibodies (IgG) were purified by 25–50% ammonium sulphate precipitation and protein A-Sepharose 4 (Pharmacia Biotech Inc.) chromatography under the high salt condition (Harlow and Lane, 1988).

**Inhibition Assay Screening and Immunotitration Studies—**Inhibition assay screening and immunotitration studies evaluated the ability of mAbs to inhibit [3H]CE or [3H]TG transfer mediated in vitro by rabbit or human lipoprotein depleted plasma (LPDP) as an LTP source. Rabbit 25 μg of LPDP were treated with d = 1.25 g/ml microemulsions of about 25 nm, approximating the size of LDL, from fresh rabbit or frozen rabbit LDL thus obtained was 1.27 m~ and 26,600 dpm/μl. Isotopically labeled LDL donor was prepared as described for L3HJCE transfer assay screening and immunotitration studies evaluated the ability of mAbs to inhibit [3H]CE or [3H]TG transfer mediated in vitro by rabbit or human lipoprotein depleted plasma (LPDP) as an LTP source. Rabbit 25 μg of LPDP were treated with d = 1.25 g/ml microemulsions of about 25 nm, approximating the size of LDL, from fresh rabbit or frozen rabbit LDL thus obtained was 1.27 m~ and 26,600 dpm/μl.

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Gel Electrophoresis and Immunoblotting—Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was performed as described previously (Ko et al., 1993). Non-denaturing PAGE was performed in the absence of SDS using the same mini-gel apparatus and a 4% acrylamide stacking, 8% separating gel run in 89 mm 2x Tris-HCl, pH 8.5, 2% acrylamide, 0.1% bisacrylamide, 0.1% SDS, and 10% glycerol at 100 V for 2 h. The gel was reacted at 4 °C with 1:1(v:v) TBE (pH 8.3) and 10% acetic acid for 2 h. After the gel was washed with water, it was stained with Coomassie blue dye and destained in water. The positions of the protein bands were visualized by fluorography after either procedure were performed as described previously (Ko et al., 1993), substituting various mAbs as the primary antibody.

Microemulsion Binding Analysis—The effects of mAb treatment on LTP binding to small PC/TG microemulsions (Tajima et al., 1983; Ko et al., 1993) was assessed by preincubating 2 µg of rabbit or human LTP with 20 µg of various IgG fractions for 30 min at 4 °C before addition of LTP, 0.5% ovalbumin (Sigma) with or without microemulsion (100 µg in TG and 70 µg in PC) in total volume of 200 µl and incubation at 37 °C for 15 min. The samples were then centrifuged and separated as described previously (Ko et al., 1993) before 10-µl aliquots were analyzed by SDS-PAGE immunoblotting as described above, using mAb 14-8H (recognizes native and denatured rabbit LTP) as a primary antibody (30 µg of IgG/ml) to immunostain LTP in all fractions.

Radioisotopic Assays of Neutral Lipid Transfer in Plasma—Human plasma mediated isotopic neutral lipid transfer from LDL to HDL was measured employing our in vitro assay for [1H]CE or [1H]TG transfer activity (Dall et al., 1997). The transfer of [1H]labeled neutral lipids from HDL within human plasma was determined using [1H]CE and [1H]TG-labeled HDL isolated as the flow-through material from the dextran sulfate-cellulose column during preparation of the respective labeled LDL (Francis et al., 1991) after ultracentrifugation (d > 1.21 g/ml fraction) and dialysis. Either radiolabeled HDL (30 µg total cholesterol) was added to 0.5 ml of human plasma, thus increasing HDL cholesterol of the sample by about 10%. At the indicated times, 150-µl aliquots were removed and placed on ice before total and HDL radioactivity were measured. HDL radioactivity was determined in the supernatant following treatment of plasma with heparin (250 units/ml) and KCN (0.5 mM) for 2 h at 4 °C, and apoB containing lipoprotein associated radioactivity was the difference between total and HDL counts.

Plasma Incubation Experiments—Fasting (12 h) plasma was obtained from 6 healthy subjects (three male, three female) by collection of blood into 0.1% EDTA and 4 °C centrifugation. Cure was taken to cool the blood as rapidly as possible, including immersion of the catheter of blood into 0.1% EDTA and 4 °C centrifugation. Care was taken to cool lipoprotein associated radioactivity was the difference between total times, 150-pl aliquots were removed and placed on ice before total and increasing HDL cholesterol of the sample by about 10%. At the indicated times, 150-µl aliquots were removed and placed on ice before total and HDL radioactivity were measured. HDL radioactivity was determined in the supernatant following treatment of plasma with heparin (250 units/ml) and KCN (0.5 mM) for 2 h at 4 °C, and apoB containing lipoprotein associated radioactivity was the difference between total and HDL counts.

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and 3-9F were hardly detected (Fig. 2). Apparent also are the slight differences in \( M_e \), between rabbit (74,000) and human LTP (66,000–69,000) (Ko et al., 1993; Ohnishi et al., 1990). Recognition of denatured rabbit and human LTP by TP2, an mAb raised against human LTP (Hesler et al., 1988) also validates that 14-8H recognizes LTP authentically (Fig. 2, lanes 11 and 12). In contrast, nondenaturing PAGE immunoblotting was able to demonstrate interaction of all three clones to rabbit LTP, as well as the lack of recognition of the human LTP by 2-8G (Fig. 3). The interaction of 3-9F was slightly weaker toward human LTP than rabbit LTP. In nondenaturing PAGE, both LTPs migrated slower than bovine serum albumin (66 kDa) but faster than transferrin (81 kDa), behavior consistent with LTP being a monomer under these conditions in agreement with our sedimentation equilibrium result (Ohnishi et al., 1994b). Overall, the PAGE results showed that 14-8H binds to an epitope resistant to denaturation by SDS. The lack of binding of 2-8G and 3-9F to denatured antigen suggests that their interaction with LTP may be toward more complex epitopes formed by LTP in its native protein conformation.

We previously showed that both rabbit and human LTP bind to lipid microemulsions strongly (Ko et al., 1993; Ohnishi et al., 1994b). Therefore, the effects of our mAbs on the association of LTP with microemulsion was studied in order to examine the modes of their inhibitory action. Microemulsion binding of LTP was estimated by SDS-PAGE immunoblotting (Fig. 4). Consistent with the previous results, a major portion of rabbit or human LTP partitions to the bottom half fraction when centrifuged in the absence of lipid (Fig. 4, leftmost columns). In the presence of the microemulsion, LTP becomes almost entirely recovered in the top half fraction upon centrifugation (Fig. 4, second from left columns), also consistent with previous results (Ko et al., 1993; Ohnishi et al., 1994b). When rabbit LTP is pretreated with 2-8G, appreciable LTP was recovered in the

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**FIG. 1. Immunotitration of CE (left two panels) and TG (right two panels) transfer mediated by rabbit (top two panels) and human (bottom two panels) LTP.** Rabbit (25 µl) or human (50 µl) LPDP were treated with the indicated amounts of IgG fraction containing mAb 2-8G (squares), 3-9F (diamonds), or 14-8H (circles) before performing in vitro [³H]CE or [³H]TG transfer assay between lipoproteins on the mixtures. In the absence of mAb, control rabbit LTP CE transfer activity was 2403 dpm/25 µl/h and control human LTP CE transfer activity was 2352 dpm/50 µl/h, and control rabbit LTP TG transfer activity was 1736 dpm/25 µl/h, and control human LTP TG transfer activity was 1223 dpm/50 µl/h. The results are representative of three separate experiments.

**TABLE I**

Apparent first order rate constants for the transfer of pyrene-lipids by human and rabbit LTP between lipid microemulsions in the presence of mAbs

| Condition          | Rabbit LTP | Human LTP |
|--------------------|------------|-----------|
|                    | Pyrene-CE  | Pyrene-TG |
|                    | Pyrene-CE  | Pyrene-TG |
| ApoA-I with no LTP | 1.5        | 3.0       |
| ApoA-I with LTP    | 28.8       | 9.3       |
| Plus mAb 2-8G      | 3.3        | 3.2       |
| mAb 3-9F           | 0.6        | 3.4       |
| mAb 14-8H          | 4.0        | 4.5       |

| Rate constant \((k/L) \times 10^4\) |
|-------------------------------------|
| ApoA-I with no LTP                  | 1.5 \((k/L) \times 10^4\) |
| ApoA-I with LTP                     | 28.8 \((k/L) \times 10^4\) |
| Plus mAb 2-8G                       | 3.3 \((k/L) \times 10^4\) |
| mAb 3-9F                            | 0.6 \((k/L) \times 10^4\) |
| mAb 14-8H                           | 4.0 \((k/L) \times 10^4\) |

**FIG. 2. SDS-PAGE immunoblotting of LTP.** LTP (1 µg) was subjected to SDS-PAGE, followed by Coomassie Blue (CB) staining of the gel (lanes 1–4) or immunostaining of the LTP following electrophoretic transfer to nitrocellulose membrane (lanes 5–12). Lanes 1 and 4 contain Bio-Rad SDS-PAGE molecular weight standards (low range) and prestained SDS-PAGE Standards (low range), respectively (Ko et al., 1993), and the lines mark the respective positions of the prestained standards between the gel and the nitrocellulose membrane. For immunostaining, 30 µg of IgG/m of the indicated mAbs were used as the primary antibody to detect LTP by fluorography. R and H denote the source species of LTP rabbit and human, respectively. The results are representative of three separate experiments.

**FIG. 3. Native PAGE immunoblotting of LTP.** LTP (1 µg) was subjected to native PAGE followed by Coomassie Blue (CB) staining of the gel (lanes 1 and 2) or immunostaining of LTP (lanes 3–10) as described in the legend for Fig. 2. Once again, R and H denote rabbit and human LTP, respectively, and the migration positions of catalase (C, 220 kDa), transferrin (T, 81 kDa), bovine serum albumin (B, 66 kDa), and ovalbumin (O, 43 kDa) in the same gel are as indicated. The results are representative of three separate experiments.
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Fig. 4. Effects of mAbs on interaction of LTP with microemulsions. Following treatment of rabbit (A) or human (B) LTP with various mAbs as indicated, microemulsions were added to the mixtures also as indicated. Following a brief incubation, an aliquot was removed to serve as the unspun (U) fraction and the remainder was subjected to centrifugation followed by separation into top (T) and bottom (B) half fractions. All fractions were then analyzed for LTP by SDS-PAGE immunoblotting with mAb 14-8H. The R and H on the sides of the fluorography denote the positions of rabbit and human LTP, respectively, and the lower M bands are mAb heavy and light chains, also recognized by the secondary anti-mouse IgG antibody during the immunoblotting procedure. The results are representative of two separate experiments.

Effects of Monoclonal Antibodies on Radioisotopic Neutral Lipid Transfer in Human Plasma—Using the in vitro assay to measure \([^3H]CE\) and \([^3H]TG\) transfer between isolated lipoprotein classes, 3-9F was found to inhibit totally \([^3H]CE\) and \([^3H]TG\) transfer from radiolabeled LDL to HDL mediated by human plasma and 14-8H almost completely inhibited \([^3H]TG\) transfer while decreasing \([^3H]CE\) transfer only 28% (Fig. 5), consistent with the results of titration (Fig. 1). Also, DTNB treatment of human plasma in order to inhibit LCAT did not appear to alter either \([^3H]CE\) or \([^3H]TG\) transfer. Radioisotopic transfer of lipid in the opposite direction (HDL to apoB-containing lipoproteins) in whole plasma was investigated by spiking samples with radiolabeled HDL. mAb 3-9F completely prevented \([^3H]CE\) and \([^3H]TG\) from being chased out of HDL, whereas 14-8H effectively blocked \([^3H]TG\) transfer and reduced \([^3H]CE\) transfer by 40% (Fig. 6). In this experimental condition, VLDL in plasma provided an additional exchangeable pool of TG for CE in HDL, approximately 35 molar percent of the CE in apoB-containing lipoproteins, expanding capacity of the acceptors for radiolabeled CE in this assay. Eliminating this additional pool by 14-8H may have lead to less appearance of \([^3H]CE\) in the acceptor fraction from HDL. Nevertheless, 14-8H allowed CE transfer to occur between lipoproteins while completely inhibiting TG transfer, whereas 3-9F completely blocked both transfer of CE and TG. From the rate of \([^3H]CE\) transfer, it can be estimated that much more than 90% of CE molecules in each lipoprotein fraction should undergo the LTP-mediated transfer during an incubation for 24 h (below), regardless of the presence of 14-8H (Barter and Jones, 1979, 1980; Kurasawa et al., 1985).

Effect of Monoclonal Antibodies on Net Neutral Lipid Transfer in Human Plasma—Human plasma, with LCAT inactivated by DTNB treatment, was incubated at 37 °C for 24 h to demonstrate net CE and TG transfer among the various lipoprotein classes in absence of new CE synthesis, compared with 4 °C controls (Table II). Following the incubation period, although HDL-CE decreased, this was more than compensated for by increases in VLDL and LDL-CE, both of which may be overestimated as suggested by the larger errors associated with these measurements. The higher error in LDL CE values in all the conditions is unexplained and occurred in a similar study (Yen et al., 1989) and would account largely for the apparent increase in total CE values of all the incubated groups. The lack of change in free/total cholesterol ratios among all the groups would suggest that LCAT was effectively inhibited in this experiment. A decrease in VLDL TG, on the other hand, was well compensated for by increases in LDL and HDL TG levels. As well, overall net increase of core neutral lipid content was observed in LDL, but not in VLDL or HDL, where an increase in one neutral lipid was compensated by an equimolar decrease in the other, following plasma incubation. Pretreatment of DTNB treated human plasma with either mAb 3-9F or 14-8H practically abolished the neutral lipid transfer among the lipoprotein classes normally seen upon incubation, including the changes in LDL total core lipid content. Thus, LTP activity in the absence of CE generation by LCAT leads to net transfer of CE from HDL to VLDL and perhaps LDL, with TG transfer from VLDL to LDL and HDL. mAb 3-9F, which inhibits both radioisotopic CE and TG transfer mediated by LTP, was able to abolish these changes. mAb 14-8H, which permits transfer of CE molecules and not TG among different lipoprotein classes in plasma, also abolishes such net transfer of CE and TG among the lipoproteins.

Effect of Monoclonal Antibodies on Net Neutral Lipid Transfer in Plasma with Active LCAT—Table III shows the results of a similar experiment as described in Table II, performed in the absence of DTNB. Following 37 °C incubation for 24 h of intact plasma, compared with the 4 °C control, VLDL-TG dropped markedly, accompanied by increases in LDL and HDL-TG levels. CE levels increased in all the lipoprotein classes, although the change in LDL was not significant. The increase in HDL-CE reflects the net result of synthesis of CE by LCAT and removal of CE by LTP. This role of LTP in mediation of net CE
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mass transfer out of HDL is further illustrated when lipid transfer is blocked by either mAb 3-9F that inhibits both CE and TG transfer or 14-8H that selectively inhibits TG transfer. Both antibodies practically abolished any net neutral lipid changes in VLDL and LDL as well as changes in HDL-TG levels. In the presence of either antibody, the increase of CE was almost exclusively in HDL resulting in its marked build up in HDL, showing that CE transfer out of HDL is prevented. Thus, the experiments in the presence of cholesterol esterification demonstrated in a more prominent manner than its absence that permitting LTP to transfer only CE but not TG between lipoproteins prevents net removal of CE from HDL. Again, core neutral lipid content was increased in LDL upon plasma incubation with LTP active, and both mAbs inhibited this change.

Cholesterol esterification was observed upon incubation of intact human plasma at 37 °C for 24 h (Table III). The results also indicated that no cholesterol esterification activity on VLDL or LDL, since the increase of CE was only in HDL when LTP was completely inhibited, in contrast to a previous report by Yen et al. (1989). However, the presence of either mAb did not affect the total cholesterol esterification in plasma, indicating that LTP-mediated neutral lipid transfer, including CE transfer out of HDL, does not regulate the LCAT reaction. This was further demonstrated by an initial time course of cholesterol esterification for 3 h (data not shown). Finally, the results in Table III are consistent with the notion that DTNB treatment in Table II was effective in specifically inhibiting LCAT without affecting LTP function, which may not be the case for all LCAT inhibitors, such as p-chloromercuri phenylsulfonate (Hopkins and Barter, 1980; Morton and Zilversmit, 1983).

DISCUSSION

Three mAbs were generated against rabbit LTP: 2-8G, 3-9F, and 14-8H. Characterization of their effects on neutral lipid transfer between lipoproteins by LTP revealed that 3-9F inhibited both isotopic CE and TG transfer, whereas 14-8H inhibited only TG transfer, both cross-reacting with human LTP. These two antibodies were therefore used to study total inhibition and dissociation of CE and TG transfer by LTP in human plasma. The conclusions from the present study are: 1) LTP is responsible for net transfer of CE from HDL to VLDL and of TG from VLDL to LDL and HDL; 2) inhibition of CE and TG transfer completely blocked net transfer of these neutral lipids; 3) inhibition of only TG transfer but not CE transfer also resulted in almost complete blockade of all net transfer of the neutral lipids among lipoproteins; and 4) LCAT esterifies cholesterol only in HDL, and neither types of LTP inhibition influenced the esterification reaction. Thus, it was demonstrated that TG transfer activity of LTP is essentially required for LTP to mediate net CE transfer from HDL.

Our combined CE/TG transfer inhibitory mAbs 2-8G and 3-9F appear to be novel in that they recognize a native epitope, and their binding to LTP interferes with its association with lipid microemulsions. mAb 2-8G is species-specific for rabbit LTP in this reaction as well. In contrast, the well studied mAb TP2 which both inhibits CE and TG transfer mediated by LTP recognizes an epitope stable to denaturation and binding of mAb increases LTP binding to lipoproteins (Tall, 1989). More information about the epitope location of any of our mAbs is unavailable at this moment, but the difference in effects of our mAbs on binding of LTP to lipid particle surfaces compared with TP2 suggest that they may bind in a different location than TP2. Thus, our mAbs may not recognize the COOH-terminal region of LTP identified as the TP2 epitope, but perhaps some other region crucial for lipid transfer function. One candidate is a putative "interfacial recognition region" away from the TP2 epitope (Swenson et al., 1989) involved in lipoprotein
Net Lipid Transfer Mechanism by LTP

Table II
Effects of incubation of human plasma with LCAT inhibited, and with or without anti-LTP mAb 3-9F or 14-8H, on lipoprotein lipid composition

Plasma samples (n = 6) were pretreated with DTNB and antibody, then incubated and treated as described under "Materials and Methods." All values are in milligrams/dl as mean (± S.E.) and statistically significant differences between 4 °C control values are shown.

| Incubation conditions | 4 °C control | 37 °C alone | 37 °C + 3-9F | 37 °C + 14-8H |
|-----------------------|--------------|-------------|--------------|--------------|
| VLDL                  |              |             |              |              |
| Free cholesterol      | 7.1 (1.2)    | 7.9 (1.4)p  | 8.7 (1.6)p   | 8.3 (1.4)p   |
| Cholesteryl ester     | 12.5 (5.3)   | 22.0 (11.4)p| 13.9 (6.5)   | 14.4 (6.0)p  |
| Triglyceride          | 58.2 (15.2)  | 37.3 (10.5)p| 60.2 (16.0)  | 55.4 (13.4)  |
| LDL                   |              |             |              |              |
| Free cholesterol      | 20.3 (0.9)   | 22.9 (1.4)p | 20.6 (1.3)   | 20.9 (1.6)   |
| Cholesteryl ester     | 75.3 (9.7)   | 82.6 (12.8) | 81.3 (11.2)p | 80.0 (14.3)  |
| Triglyceride          | 13.9 (2.4)   | 28.1 (6.4)p | 15.9 (1.8)   | 12.9 (3.7)   |
| HDL                   |              |             |              |              |
| Free cholesterol      | 19.2 (0.6)   | 19.0 (0.5)  | 19.7 (0.5)   | 19.1 (0.3)   |
| Cholesteryl ester     | 38.7 (5.9)   | 33.9 (4.2)p | 40.8 (4.9)   | 37.8 (5.1)   |
| Triglyceride          | 26.9 (1.9)   | 36.7 (1.6)p | 27.1 (2.0)   | 31.0 (2.7)   |
| Total                 | 46.6         | 49.9        | 48.9         | 48.3         |
| Free cholesterol      | 126.5        | 138.4       | 135.9        | 132.2        |
| Cholesteryl ester     | 99.0         | 102.1       | 103.2        | 99.2         |

Table III
Effects of incubation of human plasma containing active LCAT, and with or without anti-LTP mAb 3-9F or 14-8H, on lipoprotein lipid composition

Plasma samples (n = 6) were pretreated with antibody, then incubated and treated as described under "Materials and Methods." All values are in milligrams/dl as mean (± S.E.) and statistically significant differences between 4 °C control values are shown.

| Incubation conditions | 4 °C control | 37 °C alone | 37 °C + 3-9F | 37 °C + 14-8H |
|-----------------------|--------------|-------------|--------------|--------------|
| VLDL                  |              |             |              |              |
| Free cholesterol      | 7.0 (1.3)    | 3.1 (0.8)p  | 4.8 (0.9)p   | 4.4 (0.7)p   |
| Cholesteryl ester     | 12.5 (2.2)   | 19.0 (3.5)p | 13.7 (2.0)   | 15.2 (2.2)p  |
| Triglyceride          | 58.2 (15.5)  | 29.7 (10.4)p| 61.2 (17.8)  | 53.1 (14.2)p |
| LDL                   |              |             |              |              |
| Free cholesterol      | 20.3 (1.6)   | 7.8 (2.9)p  | 5.7 (1.3)    | 5.7 (1.2)p   |
| Cholesteryl ester     | 75.3 (4.5)   | 83.5 (5.8)  | 76.0 (4.9)   | 77.3 (5.2)   |
| Triglyceride          | 13.9 (3.1)   | 25.7 (6.6)p | 13.1 (2.9)   | 16.7 (4.3)   |
| HDL                   |              |             |              |              |
| Free cholesterol      | 19.2 (1.0)   | 14.8 (0.6)p | 15.0 (0.5)   | 15.0 (0.3)p  |
| Cholesteryl ester     | 38.7 (2.3)   | 46.0 (3.3)p | 55.9 (4.3)p  | 52.1 (3.8)p  |
| Triglyceride          | 26.9 (2.0)   | 36.4 (2.5)p | 26.3 (1.8)   | 28.3 (1.3)   |
| Total                 | 46.6         | 25.7        | 25.4         | 25.1         |
| Free cholesterol      | 126.5        | 148.4       | 145.6        | 144.6        |
| Cholesteryl ester     | 99.0         | 91.8        | 100.7        | 98.2         |

binding (Pattnaik and Zilversmit, 1979; Morton, 1985), which involved in lipid transfer is associated with the COOH-terminal region of LTP (Tall, 1993). The fact that CE and TG compete for transfer (Morton and Zilversmit, 1983; Ohnishi et al., 1994a) is consistent with such a hypothesis. Dissociation of CE and TG transfer by LTP was somehow demonstrated by treating with mercurial compounds (Hopkins and Barter, 1980, 1982; Morton and Zilversmit, 1982) and by TP2 Fab fragments (Swenson et al., 1989). In light of the information about the role of the LTP COOH-terminal, a likely explanation is that transfer of the larger substrate, TG, is more susceptible to inhibition by certain treatments for steric reasons. This may be supported by the finding that the CE molecule has higher apparent affinity for LTP than TG (Ohnishi et al., 1994a). Thus, our 14-8H and the LT-JI of others (Fukasawa et al., 1992) may act like TP2 Fab fragment in a more extreme manner. Pyrene-CE transfer was inhibitible by 14-8H, likely due to the increased bulk of the pyrene group limiting its access to the neutral lipid binding site. mAb 14-8H did not interrupt association of LTP with lipid microemulsions, a condition possibly necessary (but not sufficient) for LTP-mediated lipid transfer within the pyrene assay system. Presumably, 14-8H does not decrease binding of lipoprotein by LTP and allows the transfer mechanism to proceed, except that only transfer of CE can occur. The use of these novel inhibitory mAbs raised against rabbit plasma LTP permitted us to investigate the role of LTP in human plasma lipoprotein metabolism and the basic mechanism of neutral lipid transfer mediated by LTP. The two mAbs employed in this study were 3-9F and 14-8H, which both
recognize and inhibit human as well as rabbit LTP.

In normolipidemic fasting plasma incubated at 37°C, there was net transfer of CE from HDL to VLDL (and perhaps LDL) and of TG from VLDL to LDL and HDL. These changes indicate that the neutral lipid pools distributed among the various lipoprotein classes in plasma are subject to net redistribution by some specific (protein-mediated) or nonspecific (i.e. collision or diffusion) processes when isolated plasma is incubated. The directions of the neutral lipid mass changes are consistent with the reverse cholesterol transport pathway, as well as with an equilibration of the cores of the various lipoprotein classes according to their neutral lipid (i.e. TG/CE) compositional ratios (Table II). Treatment of plasma with 3-9F, the mAb which totally inhibits isotopic CE and TG transfer, blocked the net transfer of neutral lipid between the lipoprotein classes normally seen upon plasma incubation, thus implicating plasma LTP entirely in this process.

mAb 14-9H, which strongly inhibited isotopic TG transfer, also blocked the net transfer of both CE and TG (Table II). The fact that selective inhibition of TG transfer is sufficiently able to abolish net mass transfer of CE (and TG) is consistent with a mechanism whereby CE and TG transfer are both required for net neutral lipid transfer as the result of reciprocal hetero-exchange (Morton and Zilversmit, 1983). Thus, when one transfer function, TG transfer, is inhibited, and assuming that the mAb treatment did not affect the mechanism of LTP, LTP is converted to being a transfer protein only able to mediate CE homo-exchange and thus no longer CE/TG hetero-exchange, resulting in no net neutral lipid transfer by LTP. In this condition isotopic CE transfer strictly represents exchange transfer of CE molecules that can occur without net CE mass transfer.

The precise mechanism of LTP remains controversial, with kinetic evidence for either carrier (Barter and Jones, 1980) or ternary complex-mediated processes (Im et al., 1992). mAb 14-9H blocked pyrene-lipid transfer between lipid microemulsions but did not affect LTP-microemulsion interaction. It has also been shown that the presence of activator (apoA-I) of the transfer reaction does not affect LTP binding to lipid (Ohnishi et al., 1992b). Therefore, the apparent stable association of LTP with lipid, which otherwise may support a ternary complex mechanism, is not enough for the transfer of lipid.

The results of this study also provided strong evidence for inter-relationship of CE and TG transfer mediated by LTP, which was first indicated by using reconstituted lipoproteins (Morton and Zilversmit, 1983). It has been clearly demonstrated in this work that, by selectively eliminating TG transfer activity of LTP, net transfer of CE was almost completely inhibited between lipoproteins in human plasma, especially between HDL and VLDL. In other words, the net CE transfer function of LTP within plasma is closely linked to its TG transfer function, since loss of TG transfer activity (and not of CE transfer activity) is accompanied by loss of net neutral lipid transfer capability under the conditions of this study. Presumably, specifically inhibiting CE transfer would also have the same result, although this has not been experimentally demonstrable using chemical modification reagents or mAbs to date.

Somewhat inconsistent results were seen for the LDL fraction, in which net expansion of core neutral lipid content, mainly due to an increase in TG, seemed to occur as a result of LTP action during plasma incubation (Tables II and III). For this to occur, LTP must be able to mediate direct mass transfer under some conditions, whereby it is able to deliver TG to LDL without picking up another lipid. Nevertheless, this process was also inhibitable by either mAb 3-9F or 14-9H.

Our mAbs also allowed us to investigate the effects of inhibition of net neutral lipid transfer on plasma LCAT activity. It was demonstrated that inhibition of net neutral lipid transfer between lipoproteins did not influence plasma LCAT activity, which was shown to reside predominantly on HDL. During incubation of plasma with active LCAT, increase of CE was found in all lipoprotein fractions but relatively more in HDL, indicating that the lipid transfer does not completely overcome the esterification reaction. However, a large portion of the CE increment generated by LCAT is transferred to apoB-containing lipoproteins and would thus increase clearance of HDL-CE from plasma in vivo. The fact that TG transfer-specific inhibition of LTP causes CE accumulation in HDL suggests that screening of hyperalphalipoproteinemia should be done through both CE and TG transfer assays, because there is a possibility for a genetic defect of LTP selectively in TG transfer causing such a lipoprotein abnormality.

Acknowledgments—We acknowledge Drs. Tim Mosmann, Jean-Marie Haslan, and Rita Marcotte for their invaluable assistance in preparing the mAbs and the technical assistance of Lisa Main, and of Cedric Tan, an Alberta Heritage Foundation for Medical Research summer student.

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