Interconversion of prebeta-migrating lipoproteins containing apolipoprotein A-I and HDL

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Abstract Mouse plasma from strains C57BL/6J and C3H/HeJ includes a high density lipoprotein (HDL) fraction containing apolipoprotein A-I which migrates in the prebeta region upon agarose gel electrophoresis, similar to the prebeta HDL previously reported in humans. This prebeta A-I lipoprotein species has a buoyant density of 1.080–1.210 g/ml and has two molecular weight species, 65,000 and 71,000. It is lipid-poor and deficient in apolipoprotein E. When mice are fed a high fat and high cholesterol diet, the quantity of prebeta A-I increases in both strains as determined by quantitative densitometry of agarose gel immunoblots. Prebeta A-I species are highly unstable in plasma at 37°C. Initially (0–1 h) levels decreased and with further incubation (1–8 h) levels increased. Nondenaturing polyacrylamide gel electrophoresis (PAGE) demonstrated that the prebeta HDL formed during prolonged incubation (1–8 h) was identical in size to HDL in unincubated samples. The initial decrease of prebeta HDL observed during the first hour of incubation, phase I, was inhibited by DTNB, suggesting that phase I is dependent on lecithin:cholesterol acyltransferase (LCAT); however, the subsequent increase, phase II, was unaffected by DTNB and appears LCAT-independent. The prebeta A-I species formed in plasma containing DTNB after a 4-h incubation resulted in a polydisperse particle size distribution. The two strains, the atherosclerosis-susceptible C57BL/6 and -resistant C3H, displayed a similar elevation and induction of prebeta HDL during a dietary switch from laboratory chow to an atherogenic diet with a transient peak occurring at 7 days even when total HDL in the susceptible strain was greatly reduced. —**Ishida, B. Y., D. Albee, and B. Paigen.** Interconversion of prebeta-migrating lipoproteins containing apolipoprotein A-I and HDL. *J. Lipid Res.* 1990. **31:** 227–236.

Supplementary key words inbred mouse strains • prebeta-migrating HDL • LCAT • atherosclerosis • immunoblotting • gradient PAGE

Since the inverse correlation between high density lipoprotein (HDL) levels and atherosclerosis was reported, numerous investigations have focused on the mechanism through which HDL prevents lesions. These studies sought to elucidate the ability of HDL to remove excess cholesterol from peripheral tissue and to transport cholesterol directly or indirectly with other lipoproteins to the liver for clearance. Since HDL itself is not homogeneous, but a collection of particles differing in size, lipid, protein composition, and metabolic stability, the process of isolating the role of HDL is a difficult task. In fact, the major HDL subfractions on which many studies have been based may poorly reflect the flux of cholesterol in plasma. Rather, other less stable forms present in relatively small amounts may be functionally more important. The observation that a fraction composed of 5% of plasma apolipoprotein A-I is required for the net efflux of cholesterol has stimulated interest in some of the minor forms of HDL (1).

In normal human subjects, 4 to 10% of plasma apolipoprotein A-I occurs in a lipid-poor form that electrophoreses with prebeta mobility in agarose gels. This lipoprotein, called prebeta A-I by some (2–4) and "free" A-I by others (5) is small with a molecular weight of approximately 75,000 and with a density characteristic of HDL. In hyperlipidemic subjects, the quantity of prebeta HDL increases to 15–40% of total plasma apolipoprotein A-I, and multiple forms of prebeta-migrating HDL may accumulate (2). The fact that this lipoprotein is elevated in hyperlipidemic subjects raises questions both of its potential beneficial role in reverse cholesterol transport and its inverse relationship with the major HDL apolipoprotein A-I pool.

In order to address these questions, we turned to the mouse as an animal model. In this report, we show that mice also have prebeta HDL particles, that these particles are elevated when mice are fed a high fat diet, that multiple forms of prebeta HDL appear, and that the presence of this lipoprotein is influenced by LCAT activity and perhaps a second plasma factor.

Abbreviations: DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); LCAT, lecithin:cholesterol acyltransferase (EC 2.3.1.43); PAGE, polyacrylamide gel electrophoresis; HDL, high density lipoprotein; RID, radial immunodiffusion; HF-plasma, plasma from mice fed a high-fat (atherogenic) diet.

METHODS

Animal procedures and diet

Female C57BL/6J and C3H/HeJ mice 4–6 weeks in age were obtained from an animal laboratory maintained at the University of California (Berkeley, CA). Mice used from this source were never more than two generations removed from breeding mice obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in a temperature-controlled room operating under a 12-h light/dark cycle. Diets consisting of Purina rodent chow (control containing 4% fat) or a high-fat atherogenic chow (HF) were fed ad libitum. The atherogenic diet as previously described (6) contained 15% fat (P/S ratio of 0.7), 1.25% cholesterol, and 0.5% sodium cholate. Blood from mice fasted overnight was obtained from the tail vein after the tail was briefly warmed under an infrared lamp. Blood (0.2–0.3 ml) from each mouse was gently mixed with anticoagulant (1 mM Na_2-EDTA (pH 7.4), 50 μg/ml gentamycin sulfate, 0.05% NaN_3), cooled in ice-water immediately upon collection, and microfuged at 14,000 *g* for 5 min at 5°C. Plasma pooled from three to five mice was stored in tightly stoppered microfuge tubes at ice-water temperature (0°C) for use in the experiments described.

Analytical techniques

Prebeta-migrating HDL was quantitated as previously described (2) with the following modifications. Plasma was electrophoresed at 10°C directly, without removal of IgG, and the resolved plasma components were transferred from the agarose medium containing 0.25% bovine serum albumin (Sigma Chemical Co. St. Louis, MO) by capillary blotting. Each analysis (blot) consisted of duplicate measurements of samples and a set of standards consisting of chow-fed mouse plasma as a source of apolipoprotein A-I, or purified mouse apolipoprotein A-I, all diluted in 8 M urea. After electrophoresis, a dry nitrocellulose membrane (Schleicher and Schuell, BA 83) was rolled onto the agarose surface in a manner that allowed for uniform hydration of the membrane as contact was established. A 2-cm layer of absorbent filter paper was placed over the membrane and evenly pressed for 30 min using a 1 kg weight. Transfer of plasma proteins using this method resulted in quantitative recovery as demonstrated by the absence of Coomassie-staining material in the pressed gels. Blots were fixed, blocked (7), and incubated at 37°C for 60 min in rabbit antisera raised against mouse apolipoproteien A-I purified as described below. Immunodetection of apolipoprotein A-I was subsequently localized indirectly with horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L) (Bio-Rad), and visualized by staining with 0.04% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 0.01% H_2O_2 in buffer containing 100 mM Na_2HPO_4, 50 mM citric acid, pH 5.0. Immunostained blots were washed for 30 min in distilled H_2O and the hydrated blot was quantitated for apolipoprotein A-I by scanning reflectance densitometry (Helena, Model R/D). The absorbance (relative integration units) was a function of apolipoprotein A-I mass with an interassay variation of 10% (n = 5).

Plasma apolipoprotein A-I was quantified by radial immunodiffusion assay (8) at 37°C for 24 h using a 0.05 M Tris (pH 7.4)-buffered 8 M urea diluent for all samples. Plasma apolipoprotein A-I was estimated against a reference calibrator consisting of apolipoprotein A-I purified from delipidated mouse HDL by gel-sieving (1.5 × 200 cm Sephacryl S-200, Pharmacia) and ion exchange-column (DEAE-Sepharose, Pharmacia) chromatography. Purified apolipoprotein A-I electrophoresed in SDS-PAGE (9) as a single Coomassie R250-staining band of molecular weight 24,000. Gels were calibrated with a mixture of proteins of known molecular weights (Bio-Rad, LMW kit). Purified apolipoprotein A-I was assayed for protein by a modified method (10) of Lowry et al. (11) using human serum albumin as a calibrator (Cohn Fraction V, Sigma) and stored at −70°C. Antisera raised in New Zealand White rabbits were monospecific for mouse apolipoprotein A-I. Rabbit antiserum to rat apolipoprotein E which recognizes the mouse analog was kindly provided by Dr. J. Elovson.

Lipoproteins were isolated by ultracentrifugation of plasma adjusted to a solvent density of 1.210 g/ml with solid KBr using a Beckman SW 50.1 rotor at 45,000 rpm at 10°C for 48 h. For density characterization of prebeta HDL by single-spin ultracentrifugation, 50 μl plasma adjusted to the appropriate density with solid KBr was pipetted beneath 180 μl of the corresponding KBr density solution. Separation was achieved by centrifugation in a Beckman 42.2 Ti rotor for 18 h at 10°C. The top fraction (50 μl) was carefully recovered using a microliter pipetter and was dialyzed 4 h at 5°C against 0.15 M NaCl, 1 mM EDTA (pH 7.4), and 0.05% NaN_3. Molecular weights of lipoproteins were determinated by nondenaturing a 3–30% linear gradient polyacrylamide electrophoresis in a slab gel apparatus (Bio-Rad, Protean II) as previously described (2). Gels were calibrated for molecular weight and Stokes radii by coelectrophoresis of thyroglobulin (669 kDa, 8.50 nm), apoferritin (440 kDa, 6.10 nm), catalase (232 kDa, 5.20 nm), lactate dehydrogenase (140 kDa, 4.08 nm), and bovine serum albumin (67 kDa, 3.55 nm) (Pharmacia).

RESULTS

A-I-containing lipoprotein fractions

The major lipoprotein classes of plasma from chow-fed and atherogenic diet-fed mice displayed beta, prebeta,

and alpha mobilities on agarose electrophoresis as detected by Sudan Black B. When the agarose electrophoretogram was analyzed by immunoblotting, apolipoprotein A-I was present in three major bands. As expected, the major fraction containing apolipoprotein A-I was associated with material of alpha electrophoretic mobility (alpha-1 in **Fig. 1**). The slowest migrating A-I component was determined to have a mobility of R_f = 0.41 ± 0.01, relative to bovine serum albumin, and is designated prebeta-migrating HDL (2–4). The term prebeta HDL will be used to simplify the discussion. The apolipoprotein A-I mass in this fraction was elevated in plasma of C57BL/6 (B6) mice fed a high fat diet. The third A-I fraction, which had a mobility of R_f = 0.73 ± 0.01, is referred to as the alpha-2 A-I-migrating fraction in Fig. 1. This fraction also increases in B6 mice fed a high fat diet, but it is technically difficult to resolve from the apolipoprotein A-I fraction of alpha-1 mobility.

Quantitation of prebeta HDL

The prebeta HDL fraction was measured by quantitative immunoblotting as described in Methods. To insure reproducible results when measuring prebeta HDL, drawn blood was cooled rapidly in ice-water and the plasma was assayed for prebeta HDL on the same day. Fasted or unfasted mice were found to have similar levels of prebeta HDL; however, all experiments in this study involving prebeta A-I measurements used fasted mice to insure steady-state conditions.

The stability of prebeta HDL in whole plasma was tested by storing aliquots in ice-water and assaying for prebeta HDL over a 7-day period. Plasma from animals fed chow showed no significant changes in prebeta HDL during the 7 days, but plasma from mice fed the atherogenic diet showed a fourfold increase (0.018 to 0.080 mg/ml) over 4 days of storage. Thus, prebeta HDL can be formed in vitro even at 0°C.

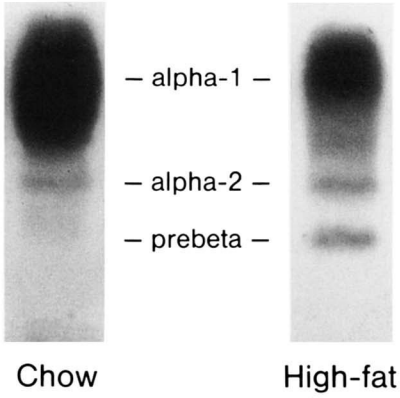

Fig. 1. Prebeta HDL in mouse plasma. Freshly drawn plasma from B6 mice fed either a control diet (chow) or an atherogenic diet (high fat) were electrophoresed in 0.5% agarose and immunoblotted for detection of apolipoprotein A-I. The electrophoretic mobilities of apolipoprotein A-I fractions are indicated.

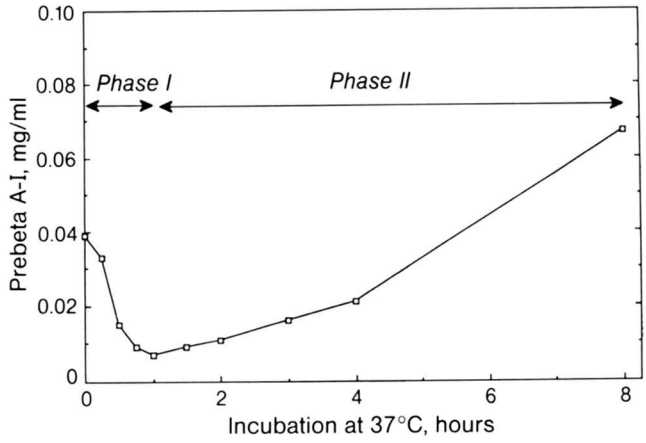

Fig. 2. The effect of plasma incubation on prebeta HDL. Aliquots of pooled plasma from B6 mice fed an atherogenic diet were incubated at 37°C for the indicated time intervals. Duplicate samples were incubated in a staggered manner, then collectively cooled in ice-water, and immediately assayed.

The unstable nature of the prebeta HDL levels was further investigated in plasma incubated at 37°C. Two reproducible phenomena occurred (**Fig. 2**). In the experiment shown, prebeta HDL levels initially decreased to 25% of the initial value (Phase I) and remained at this level for about 30 min. Subsequently, an increase in prebeta HDL levels occurred that reached 172% of the original value (Phase II). These two phases are not strain-specific since they were also observed in plasma from C3H mice (data not shown). While the magnitude of change in prebeta HDL levels varied up to twofold between experiments (n = 4), the Phase I and II changes observed in each experiment were clearly temperature-related since prebeta HDL levels did not change measurably in plasma held for up to 4 h at 0°C (**Fig. 3**). Additional experiments confirmed that no perceivable change in prebeta HDL levels at 0°C occurred for 8 h (data not shown). Thus, the increase in prebeta HDL in Phase II at 37°C appears similar to the increase that occurred at a much slower rate in HF plasma stored at 0°C.

Since apolipoprotein A-I is an enzyme cofactor of LCAT, we reasoned that the Phase I decrease in prebeta HDL levels might result from the direct action of LCAT. The result of LCAT activity on the major fraction of HDL may allow for the incorporation of prebeta HDL into HDL. This may happen if LCAT activity results in an altered HDL particle of increased surface area when free cholesterol and phospholipid fatty acyl chains become internalized within the HDL particle core as cholesteryl esters. To test this hypothesis, plasma from animals fed an atherogenic diet was incubated in the presence of DTNB to inhibit LCAT (Fig. 3). The inclusion of DTNB prevented the Phase I reaction, the disappearance of prebeta HDL, but the Phase II reaction was unimpeded suggesting that LCAT was involved in the Phase I but not the

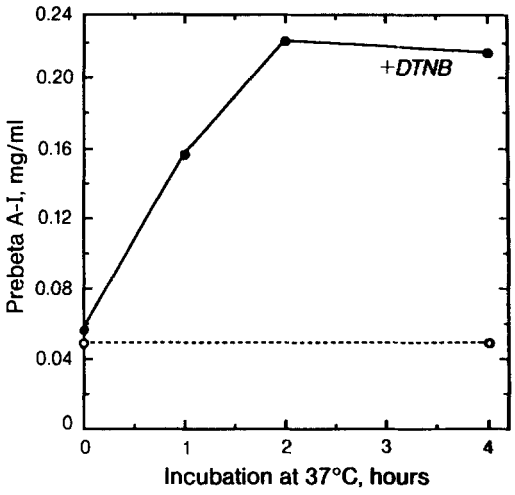

Fig. 3. The effect of incubation of plasma in the presence of DTNB. Plasma from B6 mice fed an atherogenic diet was made to 1.4 mM DTNB and incubation at 37°C. One aliquot (no DTNB) (O---O) was held on ice for the length of the incubation and all samples were measured together.

Phase II reaction. A representative result from several experiments (n = 3) is shown for B6 mice but similar results were obtained for the C3H mouse strain.

In order to further delineate the plasma requirements for the Phase I and Phase II reactions, an incubation was repeated using purified lipoproteins (d < 1.210 g/ml) isolated by ultracentrifugation (42.2 Ti rotor) rather than whole plasma (**Fig. 4**). This isolation procedure should eliminate most plasma enzymes. During incubation at 37°C, no Phase I reaction was observed further suggesting that LCAT may be involved in Phase I. However, the Phase II reaction was present, although the total increase in prebeta A-I was somewhat reduced compared to that which occurred in the presence of plasma. Thus, the absence of a Phase I reaction during the incubation of plasma + DTNB or purified lipoproteins suggest that LCAT activity is essential for the Phase I but not Phase II reaction (Figs. 3 and 4).

Reactions involving the alpha-2 A-I fraction

The third plasma A-I fraction, alpha-2 A-I, accounts for an appreciable portion of total plasma apolipoprotein A-I as shown in Fig. 1. Plasma incubation at 37°C also affected alpha-2 A-I levels; however, the degree of change was not quantitated in B6 mice due to the repeated difficulty in resolving it from the major alpha-1 A-I fraction. When plasma from C3H mice fed the atherogenic diet was used, the alpha-2 A-I component was better resolved (**Fig. 5**). The reaction kinetics of alpha-2 A-I at 37°C differed from that of prebeta HDL. Prebeta HDL levels were markedly decreased within the first 30 min of incubation while noticeable decreases in the alpha-2 fraction required up to 2 h. The magnitude of change resulting from the Phase I and II reactions for the alpha-2 A-I fraction was not as striking as that which occurred in the prebeta HDL fraction and was not quantifiable due to increased background in some lanes.

Changes in plasma apolipoprotein A-I fractions in response to consumption of a high fat diet

Since prebeta HDL was elevated in mice consuming the atherogenic diet, the relationship of prebeta HDL to total plasma apolipoprotein A-I was investigated. The quantity of prebeta HDL and total plasma apolipoprotein A-I was measured by immunoblot and RID assays, respectively, throughout a 4-week interval after a change from laboratory chow to atherogenic diet. The total plasma apolipoprotein A-I in B6 mice diminished within 48 h of the dietary switch and continuously decreased until 67% of the original value was attained after 3 weeks (**Fig. 6**). These results are consistent with the 50% decrease in HDL-cholesterol level reported earlier for the B6 strain in response to the consumption of the atherogenic diet (12), a decrease that is associated with a genetic susceptibility to diet-induced atherosclerosis. The C3H strain, which is genetically resistant to diet-induced atherosclerosis, characteristically displays higher HDL cholesterol concentrations and no reduction in levels while consuming the atherogenic diet. Fig. 6 shows that this response was also reflected by the level of total plasma

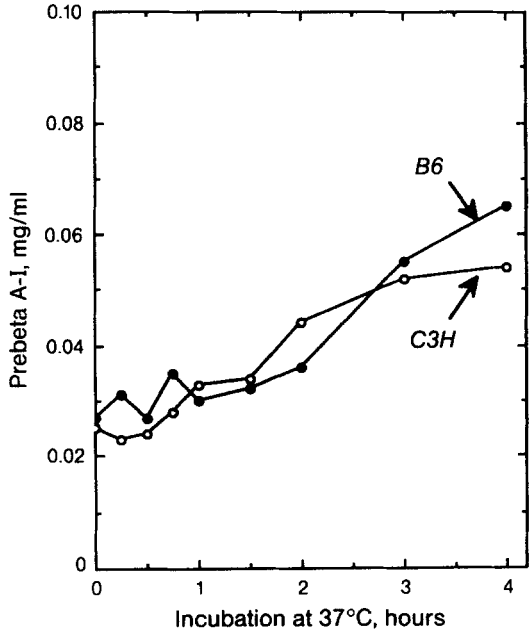

Fig. 4. Formation of prebeta migrating A-I from purified lipoproteins. The total lipoprotein fraction (d < 1.210 g/ml) from HF plasmas of B6 or C3H mice fed an atherogenic diet were isolated by ultracentrifugation and briefly dialyzed for 2 h at 5°C. Aliquots of each dialyzed fraction were incubated and assayed as described in Fig. 2. Prebeta A-I values are normalized to correct for dilution to the original plasma concentrations.

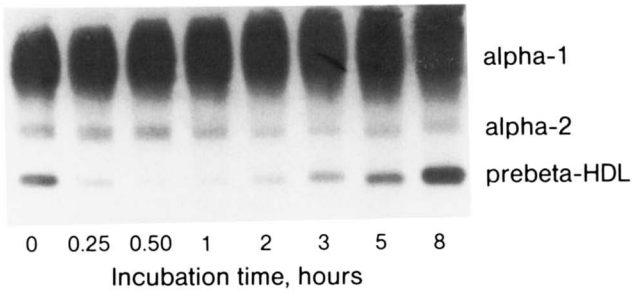

Fig. 5. Effect of incubation on alpha-2 A-I electrophoretic fraction. Plasma from C3H mice fed an atherogenic diet was incubated at 37°C and immunoblotted as described. The immunoblot depicts changes occurring in the prebeta-HDL, alpha-2, and alpha-1 fractions containing apolipoprotein A-I.

apolipoprotein A-I as might be expected since apolipoprotein A-I is the major structural protein of HDL. The apolipoprotein A-I level in C3H mice was significantly elevated (41%) at 21 days ($P < 0.05$).

The response of plasma prebeta HDL to the change in diet is shown in **Fig. 7** for B6 mice. As the total plasma apolipoprotein A-I level diminished, a dramatic induction of prebeta HDL occurred, which peaked within 1 week of atherogenic diet consumption. Prebeta HDL from B6 strain rose from undetectable levels (<0.005 mg/ml) to a maximum of 0.097 mg/ml at 7 days. Subsequently, prebeta HDL decreased to a stable level which was higher than when animals were fed a chow diet. The prebeta HDL level at 4 weeks was similar to those measured at 10 weeks (0.02 mg/ml) suggesting a steady-state level. The transient elevation observed for prebeta HDL is probably a metabolic adjustment to a drastic dietary change that we have noted for other plasma lipids such as total plasma cholesterol during the first week after the dietary switch.

Characterization of prebeta HDL

In other species, HDL containing apolipoprotein E can also display prebeta or alpha-2-like electrophoretic mobility (13) so the distribution of apolipoprotein E among agarose electrophoretic fractions was analyzed by immunoblotting. When plasma was electrophoresed and immunoblotted for apolipoprotein E, staining was largely localized to the alpha-1 and alpha-2 regions with much less staining in the prebeta region (**Fig. 8**). Apolipoprotein E was also observed in two fractions (Fig. 8, a and b) with mobilities slightly less or slightly greater but not identical to prebeta HDL.

The density of prebeta HDL and alpha-2 A-I was characterized by single-spin ultracentifugation of plasma adjusted with solid KBr at densities of 1.006, 1.019, 1.063, 1.080, and 1.210 g/ml. The floating fractions at each density were analyzed for apolipoprotein A-I fractions by agarose electrophoresis and immunoblotting (**Fig. 9**). This experiment indicated that prebeta HDL was not detectable in any density fraction less than 1.080 g/ml and distinguishes prebeta HDL from other HDL particles such as HDL-1 and HDL_c enriched in apolipoprotein E as well as other apolipoprotein E-containing lipoproteins of lesser density (13). Instead, prebeta HDL co-purifies with HDL (and the bulk of apolipoprotein A-I) in the d 1.080–1.210 g/ml HDL density interval. The average hydrated density of prebeta HDL, however, may be significantly greater than HDL since trace amounts were recovered in the d > 1.210 g/ml fraction. In contrast, alpha-2 HDL was partially distributed in the 1.080–1.210 g/ml density fraction with the majority partitioning in the d > 1.210 g/ml fraction.

It was noted that the amount of prebeta HDL recovered after ultracentrifugation was less than expected from the starting amount in plasma. Conversely, the recovery of alpha-2 A-I was greater than expected. We speculate that this may result in part from reassociation of apolipoproteins among lipoproteins during ultracentrifugation and to nonquantitative recovery after dialysis.

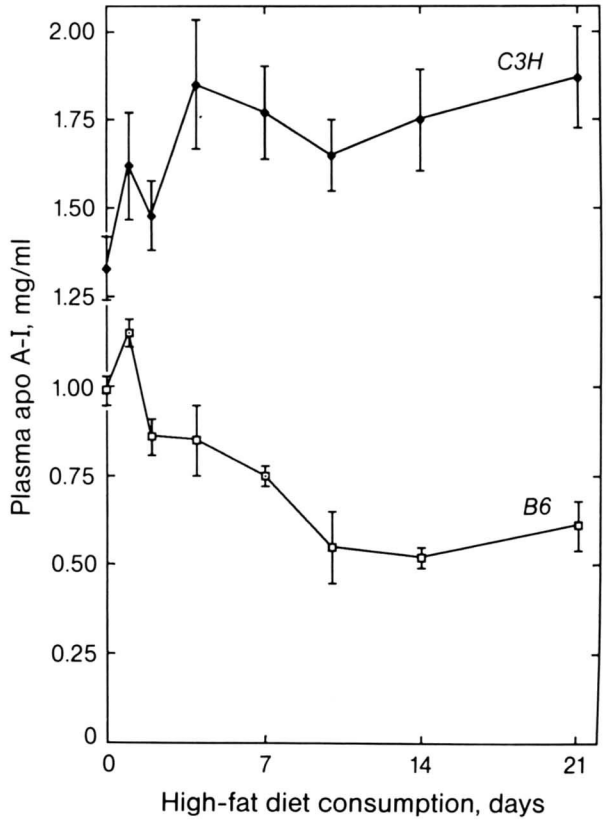

Fig. 6. Plasma apolipoprotein A-I response to an atherogenic diet. Total plasma apolipoprotein A-I in B6 and C3H mice was measured after a dietary switch from control to atherogenic diet. Unfasted plasma samples from two drops of blood obtained from the tail vein were collected over a period of 21 days and assayed by radial immunodiffusion for apolipoprotein A-I. Data points represent the mean ± SE of five mice.

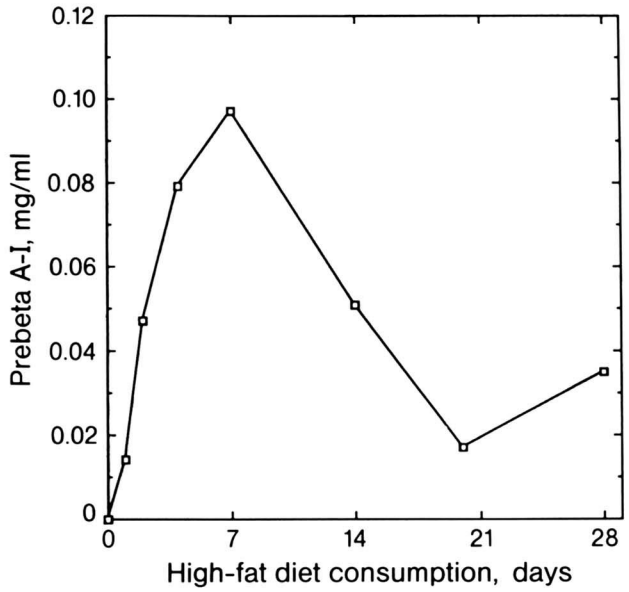

Fig. 7. Induction of prebeta HDL by consumption of a high fat diet. B6 mice consuming laboratory chow were switched to an atherogenic diet and the prebeta HDL fraction in plasma was measured over 4 weeks. Data points represent the mean of duplicate determinations obtained from a pool of five mice killed at each time point.

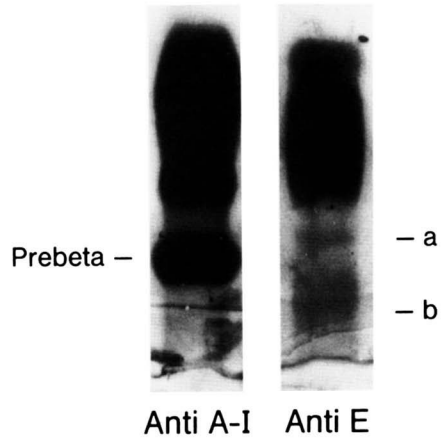

Fig. 8. Distribution of apolipoprotein E among plasma electrophoretic fractions. Pooled plasma from atherogenic diet-fed B6 mice were applied to agarose gels in excess to maximize the detection of apolipoprotein E material. Immunoblots were prepared using antisera raised against rat apolipoprotein E. A comparative blot for an equivalent amount of plasma is shown for apolipoprotein A-I. Apolipoprotein E staining fractions a and b have faster and slower, respectively, electrophoretic mobilities relative to prebeta HDL.

However, prebeta HDL and alpha-2 A-I can both be demonstrated by agarose electrophoresis of plasma indicating that these plasma components are not artefacts of the analytical technique.

The observed Phase I and II reactions suggested that a transfer of apolipoprotein A-I among different plasma fractions was occurring. As noted above, the incubation of plasma at 37°C did not result in additional agarose electrophoretic species containing apolipoprotein A-I. Instead, the prebeta HDL fractions became elevated beyond 2 h of incubation at 37°C. The agarose immunoblot data suggested that the Phase I and II reactions were affecting the prebeta HDL pool size by the transfer of apolipoprotein A-I to and from the apolipoprotein A-I pool of alpha-1 mobility. Upon closer inspection (Fig. 5), the alpha-1 apolipoprotein A-I fraction becomes progressively dispersed in mobility with increasing time of incubation. This qualitative change, while resulting in no additional electrophoretic species, suggested that a compositional change may be occurring. Unfortunately, the agarose technique does not readily discern minute changes in particle structure and possible particle size transformations could have resulted during Phase I and II but went undetected in the minimally sieving process of agarose electrophoresis. To test this hypothesis, the prebeta fractions from nonincubated HF plasma, and HF plasma incubated for 8 h at 37°C in the presence and absence of DTNB were isolated from other apolipoprotein A-I containing complexes by preparative agarose electrophoresis. The gel region containing prebeta HDL from several gel lanes was excised, collectively subjected to nondenaturing gradient PAGE to resolve molecular species, and electrophoretically transferred to nitrocellulose for apolipoprotein A-I immunoblotting (**Fig. 10**). We found that the prebeta HDL species from plasma incubated without DTNB closely resembled that of original plasma which contained two apolipoprotein A-I molecular complexes of 67 ± 4 and 74 ± 6 kDa with corresponding diameters of 6.5 and 6.8 nm. This result is consistent with similar molecular species previously described in human plasma (2). Surprisingly, plasma incubated in the presence of DTNB was found to contain a spectrum of additional prebeta migrating apolipoprotein A-I complexes varying in molecular mass with a continuum ranging from 56 to 180

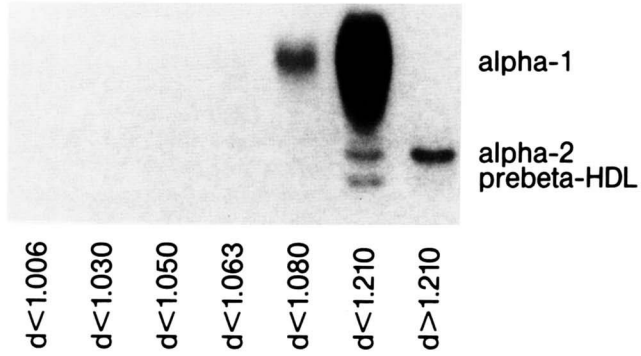

Fig. 9. Apolipoprotein A-I immunoblots from ultracentrifugal fractions. Aliquots of plasma from B6 mice fed an atherogenic diet were ultracentrifuged at the indicated KBr densities (g/ml). Equivalent volumes of each floating fraction were analyzed for prebeta HDL content.

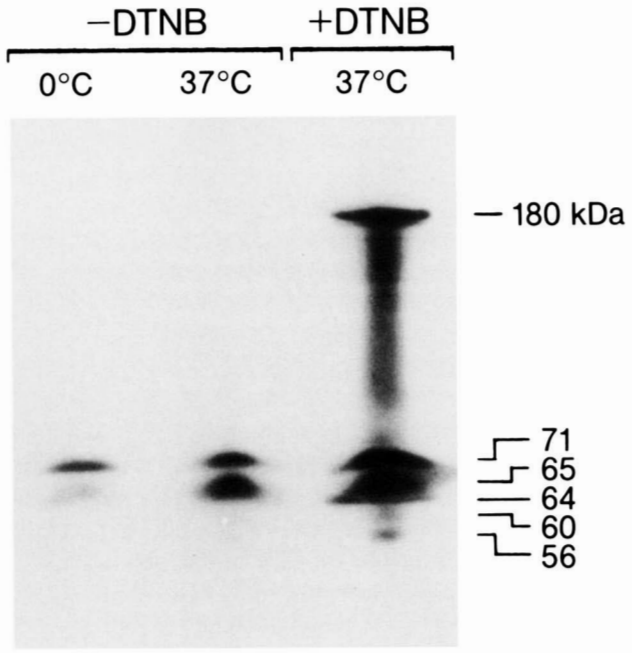

Fig. 10. Molecular size composition of prebeta-migrating apolipoprotein A-I complexes. Aliquots of plasma from B6 mice fed an atherogenic diet were incubated at 37°C with and without 1.4 mM DTNB for 8 h. A separated plasma aliquot was held in ice-water. After incubation replicate samples were electrophoresed in agarose and the prebeta region was excised from the main gel and pooled. Pooled agarose sections were carefully fused to a nondenaturing 3–30% polyacrylamide gradient gel and the electrophoretically resolved complexes containing apolipoprotein A-I were detected by immunoblotting. Molecular weight calibrator proteins were run in wells adjacent to the samples. Molecular weights of apolipoprotein A-I complexes were calculated from the relative mobility of the molecular weight reference proteins after transfer to the nitrocelluose membrane and separately stained from the immunoblot with amido black.

kDa. Included within this broad particle distribution were increased amounts of the 67 and 74 kDa species as well as distinct prebeta-migrating apolipoprotein A-I complexes of 56, 60, and 180 kDa unique to the plasma sample incubated with DTNB.

DISCUSSION

Prebeta HDL has been reported previously in human plasma (2–5, 14–16). This report confirms the presence of prebeta HDL in the mouse. Its presence in species genetically distant from man suggests some generalized function. While the role of prebeta HDL has not been elucidated, increased levels of this apolipoprotein A-I-containing fraction are associated with lipemias that affect HDL such as familial hypercholesterolemia, hypertriglyceridemia, combined hyperlipemia, and apolipoprotein C-II and LCAT deficiencies (2). As is the case with human prebeta HDL (2, 5) the mouse prebeta HDL does not appear to be a postprandial lipoprotein since its plasma concentration, which ranged from 2 to 5% of total apolipoprotein A-I, is similar in the fasted and fed states. On the other hand, the prebeta HDL level is transiently induced during a dietary change from a low fat diet (4% fat) to one containing 15% fat (predominantly saturated fatty acids) and 1.25% cholesterol in both atherosclerosis-sensitive (B6) and -resistant (C3H) strains. We believe that the modulated level of prebeta HDL measured at 3–4 weeks is a steady-state level since measurements of prebeta HDL from mice fed atherogenic diets for 10 weeks exhibit similar levels. In studying two mouse strains that differ in atherosclerosis susceptibility, we found that total plasma apolipoprotein A-I level was directly correlated to resistance. This result corroborates earlier reports for HDL-cholesterol and supports the hypothetical protective nature of HDL in humans. The level of prebeta HDL in human subjects has generally been shown to vary inversely with HDL. This study, however, indicates that prebeta HDL levels are not directly related to HDL (apolipoprotein A-I) levels since the B6 and C3H strains which differ twofold in plasma apolipoprotein A-I levels had similar prebeta HDL levels.

Storage affected the level of prebeta HDL; for this lipoprotein was found to increase in plasma over time even when stored at 0°C. Thus, for both mouse and human (5) plasmas, unfrozen storage at 0–4°C temperatures inadequately stabilizes prebeta HDL levels, presenting a technical problem for its study.

In searching for a plasma component(s) that may regulate prebeta HDL levels, two separate reproducible reactions (Phase I and II) were discovered when plasma was incubated at 37°C. The Phase I reaction proceeded during the initial hour of incubation resulting in a transient kinetic minima in the prebeta HDL level (Fig. 2). In experiments where the LCAT inhibitor DTNB was present (Fig. 3), the Phase I reaction was not detectable, which suggests that LCAT enzymatic activity plays a role in this process. This may occur through a LCAT-mediated alteration of HDL in the following manner. As LCAT esterifies surface cholesterol for internalization within the particle core, additional HDL surface area may be exposed as a result of a lower phospholipid packing density (17) allowing for the incorporation of prebeta HDL in the form of discrete 67 and 74 kDa units. Such a conversion of prebeta to alpha HDL has been demonstrated in human plasma (18).

Apparently, however, the nearly total incorporation of prebeta HDL into larger preexistent HDL particles during Phase I does not result in a favorable state. Instead, through an uncharacterized process (Phase II), small prebeta A-I-containing particles are generated. Both Phase I and II reactions may be active simultaneously; however, the Phase II reaction in plasma appears to predominate

at a specific kinetic transition point of 1 h at 37°C. Phase II does not appear coupled to the LCAT reaction since the Phase II reaction occurs in the presence of DTNB (Fig. 3) and only when purified lipoproteins ($d < 1.21$ g/ml) were incubated.

Gillette, Costa, and Owen (19) reported that maximal esterification of plasma cholesterol in the mouse occurs after 1 h of incubation, a time that coincides with the depletion of prebeta HDL. With longer periods, the lysophosphatidylcholine generated may become excessive (over the amount capable of being bound by albumin), thus destabilizing (20–22) with its detergent-like properties, and dissociating apolipoprotein A-I as prebeta HDL particles during Phase II. Some support for this hypothesis came from the work of Suzuki and Kawakami (21), who demonstrated that a lipoprotein with HDL-like characteristics becomes enriched in lysophosphatidylcholine during incubation of mouse plasma. This compositional change may affect the HDL physical structure and may be related to the acquisition by HDL of a new function to lyse red blood cells. That these observations were specific for the HDL-like particle and could not be duplicated by the exogenous addition of purified lysophosphatidylcholine further suggests that LCAT activity and not a separate plasma phospholipase is responsible for the specific enrichment.

Unusual HDL species ranging in size from very small to very large have been reported in human subjects with LCAT deficiency (23–30), and these aberrant HDL approach size and morphologic normalcy when incubated with a source of LCAT in vitro (31). Recently Nichols and co-workers (32) have reported similar findings during the incubation of human plasmas containing an enriched HDL_3 subpopulation. These investigators showed that LCAT caused a conversion of native HDL_3 into distinct populations of larger and smaller HDL particles during the incubation of plasma. When LCAT was inactivated, the HDL_3 subpopulation containing apolipoprotein A-I but without A-II was converted primarily to smaller 7.4-nm diameter particles. Under identical conditions HDL_3 (A-I + A-II) was converted primarily into larger particles. The small particles formed in these plasma incubations approach the size of the prebeta HDL particles reported here and may represent a product of a similar Phase II reaction in human plasma. In fact, when mouse plasma was incubated under conditions where LCAT is inhibited by DTNB, large prebeta A-I particles in addition to prebeta HDL are formed (Fig. 10). From these observations, LCAT activity appears necessary in maintaining the HDL structure found in normolipidemic plasma (33–35). Hence the conversion of prebeta HDL to HDL of alpha-1 mobility during the Phase I incubation of plasma may play an integrated role in these size transformations effected by LCAT.

The Phase I reactions reported here also resemble the plasma reactions involving apolipoprotein A-IV (36–38). In the rat, one-half of plasma apolipoprotein A-IV is not recoverable in the lipoprotein density (1.006–1.21 mg/ml) since it is in a lipid-free or lipid-poor state. Upon incubation at 37°C, however, apolipoprotein A-IV is rapidly redistributed among HDL particles from the lipoprotein "free" state; this suggests that the mechanisms responsible for the binding of both prebeta HDL and "free" A-IV to HDL particles are identical. Similarly, plasma A-IV may normally exist in the lipoprotein-free state due to the Phase II reaction. DeLamatre and coworkers (37) have shown in rat plasma that the apolipoprotein A-IV flux is towards the nonlipoprotein-associated form during incubation with DTNB. In attempts to explain the dissociation of lipoprotein-bound A-IV, Lefevre, Chuang, and Roheim (38) have hypothesized that during lipolysis, interparticle exchange of apolipoproteins may cause the displacement of A-IV from HDL. Work by others, however, indicates that the displacement process functions in the absence of lipolysis and may explain our observed formation of prebeta HDL during storage of unfrozen plasma and isolated lipoproteins at 0°C (39, 40). We speculate that prebeta A-I and "free" A-IV are reversibly incorporated into preexisting HDL particles by a competing process occurring between cholesterol esterification by LCAT and other uncharacterized reactions that cause the displacement of HDL apolipoproteins.

The mechanism through which the conversion process involves LCAT is uncertain. We have assumed that LCAT acts primarily upon the larger HDL species of alpha-1 mobility; however, prebeta HDL may itself be a substrate during the Phase I conversion process. In this process, prebeta HDL particles may be enlarged through the addition of surface and core material as a direct action of LCAT using other plasma lipoproteins as a source of lipids and apolipoproteins. The alpha helical content is lower in prebeta HDL than in HDL (3). Whether this change in secondary structure precludes prebeta HDL from activating LCAT remains undetermined. It is clear from the work of others (41) that a defined conformational state of apolipoprotein A-I is required for efficient LCAT activation; however, algorithms based simply upon the molar composition and the partial conformational state of apolipoprotein A-I particles have not yet been developed to adequately predict LCAT activation.

We have shown that prebeta-migrating apolipoprotein A-I complexes formed during the incubation of plasma containing DTNB can be polydisperse in molecular weight. These complexes, however, have not been characterized with respect to buoyant density. Thus, until satisfactorily determined, we suggest the designation "prebeta HDL" be reserved to describe prebeta-migrating particles containing only apolipoprotein A-I

that are present in normal plasma where LCAT activity is intact and that have a hydrated density typical of HDL (1.08–1.21 g/ml). Otherwise, the more general term "prebeta Lp-A-I" should be employed.

The clinical significance of elevated levels of prebeta HDL is largely unknown. From the studies described, we suspect that prebeta HDL may arise from a variety of sources including the bulk HDL fraction with which it exists in dynamic equilibrium. The elevation of prebeta HDL levels in LCAT-deficient plasma suggests that prebeta HDL is a sensitive indicator for cholesterol flux (2). Prebeta HDL has been reported to have a high affinity for binding cholesterol (4) a property that may allow its participation in the reverse-cholesterol transport process (1). The accumulation of prebeta HDL in various hyperlipidemias may represent a defect in some step of this process that promotes the unidirectional removal of excess peripheral tissue cholesterol. De novo synthesis may be another source of prebeta HDL as similar impaired lipoproteins have been identified in the peripheral lymph of dogs (42) and from the intestine in the rat (43). Since cultured HepG2 cells were found to secrete prebeta A-I particles (B. Y. Ishida, S. Erickson, C. J. Fielding, and P. E. Fielding, unpublished results) and since apolipoprotein A-I-containing particles secreted into the culture medium include a major subpopulation of spherical 7.4-nm diameter particles physically similar to prebeta HDL (44, 45), we are pursuing the possibility that prebeta HDL can also be a nascent synthesized lipoprotein rather than just a product of exchange involving apolipoprotein A-I among preexistent lipoproteins in circulating plasma.

This work was supported by a grant-in-aid #87-N119 from the American Heart Association, California Affiliate with funds contributed by the Alameda County Chapter, of the American Heart Association, and by the NIH grant HL-32087. The authors are extremely grateful to Jean Doty for her excellent care in preparation of the manuscript.

Manuscript received 8 May 1989 and in revised form 10 August 1989.

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