Characterization of dog peripheral lymph lipoproteins: the presence of a disc-shaped "nascent" high density lipoprotein

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Abstract The distribution, chemical, and apoprotein composition of plasma and peripheral lymph lipoproteins were compared in control and cholesterol-fed dogs. In both groups of animals, the agarose electrophoretic patterns of plasma and lymph lipoproteins were similar. In hypercholesterolemic dogs, β-very low density lipoprotein, β-migrating intermediate density lipoprotein, and HDL_c were major components both in plasma and lymph, providing evidence for a potential interaction of these atherogenic particles with macrophages and other peripheral cells. The chemical composition and physical appearance of peripheral lymph HDL was markedly different from that of plasma HDL (high density lipoprotein), especially in the cholesterol-fed animals. Lymph HDL had a higher cholesterol to protein ratio and a markedly increased free cholesterol content (free cholesterol to cholesteryl ester ratio of 1.7 as opposed to 0.2 in plasma HDL in cholesterol-fed animals). The phospholipid content of lymph HDL was higher than that of plasma HDL, while the protein content was lower. A significant proportion of lymph HDL obtained from cholesterol-fed dogs was in the form of disc-shaped particles stacked in rouleau structures. Changes in plasma apolipoprotein concentrations due to cholesterol feeding were reflected in peripheral lymph to different degrees, depending largely on the relative size of the lipoproteins containing the individual lipoproteins. A considerable enrichment of lymph HDL with apoE and apoA-IV was observed by both immunochemical and electrophoretic methods. In lymph HDL from control and cholesterol-fed dogs, the apoE/apoA-I and apoA-IV/apoA-I ratios were several-fold elevated, compared to those of plasma HDL. It is concluded, therefore, that during cholesterol feeding a substantial portion of interstitial HDL is assembled de novo in the periphery as a crucial stage of reverse cholesterol transport to the liver. It is likely that further modification occurs upon entry to plasma and exposure to lecithin:cholesterol acyltransferase, possibly leading to generation of HDL_c. Alternatively, these particles may be directly and rapidly removed by the liver.—**Sloop, C. H., L. Dory, R. Hamilton, B. R. Krause, and P. S. Roheim.** Characterization of dog peripheral lymph lipoproteins: the presence of a disc-shaped "nascent" high density lipoprotein. *J. Lipid Res.* 1983. **24:** 1429–1440.

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The liver is the major organ responsible for cholesterol catabolism and excretion. High density lipoproteins have been hypothesized to play an essential role in returning peripheral cell cholesterol to the liver by a process known as reverse cholesterol transport (1–5). Much of the evidence supporting the concept of reverse cholesterol transport, however, has been obtained from in vitro experiments using cultured cells (6–8). To our knowledge, recent studies in man by Reichl and associates (9) represent the only direct in vivo attempt to investigate aspects of reverse cholesterol transport using peripheral lymph, an accepted model for interstitial fluid (10). Although these studies are extremely important, the information obtained is limited by the amounts of human lymph available and the inherent limitations in human investigation.

We recently reported an animal model of interstitial fluid lipoprotein and apolipoprotein metabolism (11) in which we have partially characterized the plasma and lymph lipoproteins. Peripheral lymph from control and cholesterol-fed dogs contained lipoproteins that resembled their respective plasma lipoproteins in size and electrophoretic mobility. Our studies also suggested modi-

Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; HDL_i, interstitial high density lipoproteins; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; EDTA, ethylene diamine tetraacetic acid; apo, apolipoprotein; LCAT, lecithin:cholesterol acyltransferase.

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fication of plasma HDL by the peripheral cells. This model allows us to overcome many limitations encountered in human studies while retaining the in vivo approach. In the present experiments, we further characterized peripheral lymph lipoproteins and apolipoproteins and report the presence of an unusual lymph high density lipoprotein particle distinctly different from HDL found in plasma which, we postulate, plays an important role in reverse cholesterol transport.

METHODS AND MATERIALS

Animals and diets

Heartworm-free mongrel dogs were fed dog chow (Allied Meals, Inc., Chicago, IL) or chow supplemented with 10% sucrose, 0.3% cholic acid, 3% cholesterol, 20% lard, and 0.5% propylthiouracil (cholesterol-fed), which is a modification of the diet used by Mahley, Weisgraber, and Innerarity (12). Diets were supplied ad libitum for 30–40 days, and dogs were fasted overnight before surgery.

Peripheral lymph collection

Dogs were anesthetized (pentobarbital, 30 mg/kg IV), intubated with an endotracheal tube, and infused intravenously with saline (0.2–0.5 ml/min). Prepopliteal peripheral lymphatics of both hindlimbs were cannulated near the saphenous vein, as previously described (13, 14). Lymph flow was promoted by passive flexion of the hindlegs produced by a motor-driven piston. Lymph samples were collected into chilled vials containing 0.1% sodium azide-EDTA and 1.0 mM DTNB, final concentrations. As previously reported, lymph flow reached a steady state after 1 hr (11). Lymph collected before the attainment of steady state was discarded.

Analytical methods

Lipoproteins of peripheral lymph and plasma were separated by sequential ultracentrifugation (15) using an SW-41 rotor in a Beckman L5-50 preparative ultracentrifuge. The densities chosen were as follows: VLDL, $d < 1.006$ g/ml; IDL, 1.006–1.030 g/ml; LDL, 1.030–1.063 g/ml; HDL, 1.063–1.21 g/ml. In control dogs, a combined VLDL-IDL fraction was obtained ($d < 1.030$ g/ml). After potassium bromide was removed by dialysis, protein (16) and cholesterol (17) contents were measured in all lipoprotein fractions. The procedures used to produce antibodies against dog apolipoproteins were described in detail previously (11). Briefly, delipidated apolipoproteins were separated on Sephacryl-S200 and further purified by preparative isoelectric focusing (18). The purity of the apolipoproteins was checked by SDS-polyacrylamide gel electrophoresis (19) before injection into rabbits. The specificity of the antibodies was confirmed by immunodiffusion (20) and immunoelectrophoresis (21). In addition, specificity was further established by immunoblotting onto cellulose nitrate sheets (22). The apolipoprotein content in the various lipoprotein fractions was determined by electroimmunoassay (23). For the assays of apoA-I and apoE, Nonidet P-40 was added to samples and agarose, as previously described (24). Apolipoprotein concentrations were expressed as a percentage of a standard plasma pool (i.e., arbitrary units). Agarose electrophoresis (25) and SDS-polyacrylamide gel electrophoresis (19) were also performed on the lipoprotein fractions of lymph and plasma. Neutral lipids of HDL were separated by thin-layer chromatography (26), and free and esterified cholesterol were eluted from the plates and assayed directly (17).

RESULTS

Protein and cholesterol distribution

In control dogs, more than 90% of the total lipoprotein-protein mass was in the HDL fraction both in plasma and lymph (**Table 1**). During cholesterol feeding, about 20% of the total plasma lipoprotein-protein was present in HDL, whereas, more than 75% was in VLDL-IDL. Expressed as percent of the plasma concentration, lymph HDL-protein values increased from 8% in the control to 14% in the cholesterol-fed dogs. Seventy-seven percent of total cholesterol was found in

TABLE 1. Distribution of lipoprotein-protein among the lipoprotein fractions in plasma and lymph of control and cholesterol-fed dogs

Fraction	Compartment	Protein Concentration	
		Control (n = 4)	Cholesterol-Fed (n = 4)
		mg/dl	
VLDL	Plasma	2.9[a] ± 0.4[b]	95 ± 33
	Lymph	0.3 ± 0.1	3 ± 0.4
	Lymph (% of plasma)	10	3
IDL	Plasma	[a]	92 ± 23
	Lymph	[a]	2 ± 0.3
	Lymph (% of plasma)		2
LDL	Plasma	19 ± 4	7 ± 0.7
	Lymph	1 ± 0.2	0.5 ± 0.1
	Lymph (% of plasma)	5	7
HDL	Plasma	260 ± 25	47 ± 27
	Lymph	20 ± 1	6 ± 4
	Lymph (% of plasma)	8	13

[a] Due to the small amount of material available in the VLDL and IDL fractions of control animals, only a combined (VLDL + IDL) fraction was isolated.
[b] Values ± SEM.

HDL and only 2% was in the combined VLDL-IDL fraction in both lymph and plasma of control dogs (**Table 2**). In cholesterol-fed animals, on the other hand, only 2% of the total plasma cholesterol was present in HDL and 97% in VLDL + IDL. In the lymph from cholesterol-fed dogs, 81% of the cholesterol was in VLDL-IDL and 15% in HDL. Although total peripheral lymph HDL cholesterol decreased in cholesterol-fed dogs, peripheral lymph HDL-cholesterol values increased from 9% (in controls) to 24% (in cholesterol-fed dogs) when expressed as percent of plasma concentration. The cholesterol/protein ratio of peripheral lymph HDL was twofold higher than that of plasma HDL in cholesterol-fed dogs.

TABLE 2. Distribution of total cholesterol in plasma and lymph of control and cholesterol-fed dogs

Fraction	Compartment	Cholesterol Concentration	
		Control (n = 4)	Cholesterol-Fed (n = 4)
		mg/dl	
VLDL	Plasma	3.0[a] ± 0.9[b]	684.8 ± 288.0
	Lymph	0.3 ± 0.2	13.4 ± 3.2
	Lymph (% of plasma)	10	2
IDL	Plasma	[a]	372.2 ± 93.3
	Lymph	[a]	11.7 ± 1.7
	Lymph (% of plasma)		3
LDL	Plasma	27.5 ± 5.7	16.0 ± 2.1
	Lymph	2.1 ± 0.4	1.0 ± 0.2
	Lymph (% of plasma)	8	6
HDL	Plasma	102.2 ± 9.4	19.9 ± 11.2
	Lymph	9.0 ± 0.6	4.7 ± 0.1
	Lymph (% of plasma)	9	24

[a] Due to the small amount of material available in the VLDL and IDL fractions of control animals, only a combined (VLDL + IDL) fraction was isolated.
[b] Values ± SEM.

Agarose electrophoresis

Isolated lipoproteins of plasma and lymph were subjected to agarose electrophoresis. In general, the pattern for each lipoprotein fraction in lymph in both dietary groups was similar to the pattern in plasma (**Fig. 1**). The combined VLDL-IDL fraction of both lymph or plasma of control animals contained very little stainable material (Fig. 1a). The LDL fraction contained a β- and a preβ-migrating band, with the latter likely corresponding to HDL_1. Most particles in the HDL fraction were β-migrating. On the cholesterol-containing diet, in hyporesponders (plasma cholesterol <750 mg/dl), a substantial amount of β-migrating VLDL (β-VLDL) and preβ-migrating particles (most likely HDL_c) were present in VLDL and IDL fractions in plasma and lymph (Fig. 1b). In the hyperresponders (plasma cholesterol >750 mg/dl), however, even more material was found in VLDL, IDL, and LDL, all having a broad β mobility in both plasma and lymph (Fig. 1c). The decrease in α-migrating particles (HDL), as plasma cholesterol increases, is consistent with the data in Tables 1 and 2.

Apolipoprotein composition

The apolipoprotein composition of lymph and plasma lipoproteins was compared by SDS-polyacrylamide gel electrophoresis. In general, the apolipoprotein composition of lymph lipoproteins was similar to their plasma counterparts with the exception of HDL, especially in the cholesterol-fed animals (**Fig. 2**). In plasma HDL, only apoA-I could be detected, whereas in peripheral lymph HDL, a significant enrichment in apoE and apoA-IV was observed.

Lymph and plasma apolipoproteins were quantitated by electroimmunoassay of the individual lipoprotein fractions. **Table 3** indicates that, in control dogs, the bulk of plasma apoB and apoE was found in LDL, apoA-I in HDL, and apoA-IV in the d>1.21 g/ml fraction, with only small amounts in LDL and HDL. In peripheral lymph, on the other hand, apoE and apoA-I were mostly in HDL, whereas the distribution of lymph apoB and apoA-IV was similar to that of plasma. When lymph apolipoprotein concentration is expressed as a percentage of the plasma apolipoprotein concentration, the values for HDL-apoE (70%) and HDL-apoA-IV (23%) are much higher than that of apoA-I (7%), the latter being the major HDL apolipoprotein.

In cholesterol-fed dogs, all plasma apolipoproteins, except apoA-I, increased greatly in the VLDL and IDL fractions (**Table 4**). The distribution of plasma apoA-I was similar to that of control animals, although the total amount of apoA-I in each fraction was considerably decreased. Changes in lymph VLDL and IDL apolipoprotein concentrations due to cholesterol feeding were in the same direction as those in plasma. In agreement with the observed apoprotein composition by SDS-PAGE, there was an increase in the apoE and apoA-IV content of peripheral lymph HDL. For apoE, the peripheral lymph HDL concentration equalled the plasma HDL concentration (100%). For HDL-apoA-IV, however, the lymph concentration was more than twofold higher (233%) than that of plasma HDL, in contrast to the peripheral lymph HDL-apoA-I concentration which remained at about 10% of the plasma value (Table 4). These data suggest that plasma HDL cannot be the only source of lymph HDL-apoE or apoA-IV.

Chemical composition of HDL from control and cholesterol-fed dogs

Calculated cholesterol/protein ratios and the apolipoprotein data indicate that the chemical composition of peripheral lymph HDL differs from that of plasma

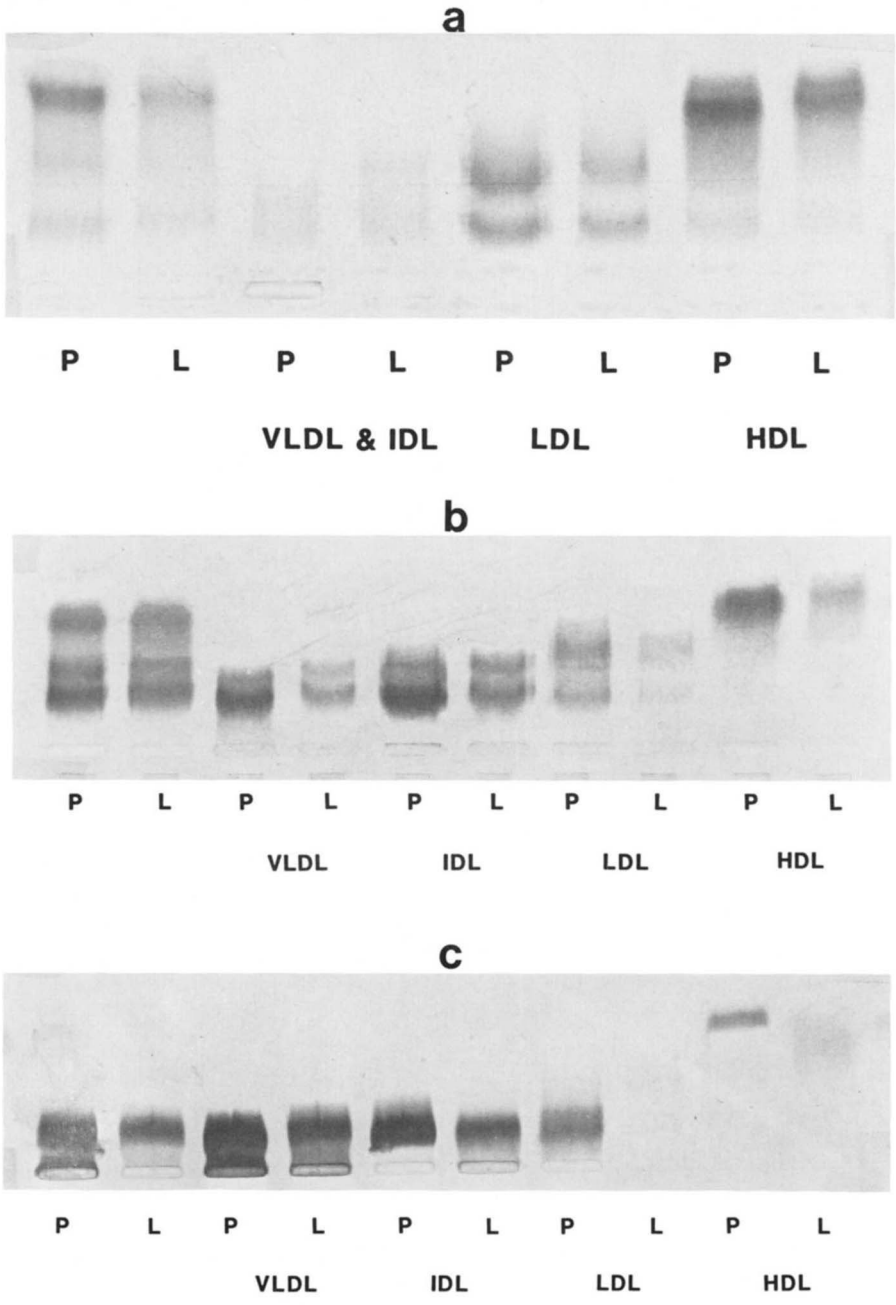

Fig. 1. Agarose electrophoresis of whole plasma and various isolated plasma lipoproteins obtained from control (a), cholesterol-fed hyporesponding (b), and cholesterol-fed hyperresponding (c) dogs.

HDL. Therefore, a more complete chemical analysis of lymph and plasma HDL was done in control as well as in cholesterol-fed dogs (**Table 5**). Peripheral lymph HDL from both control and cholesterol-fed dogs contained less protein and increased amounts of total cholesterol, consistent with data presented in Tables 1 and 2. Significantly, however, peripheral lymph HDL contained a much higher proportion of unesterified cholesterol than plasma HDL, especially in the cholesterol-fed dogs. Free cholesterol content of control peripheral lymph HDL was twice that of plasma HDL (42% vs. 25% of total cholesterol) and over three times higher in the peripheral lymph HDL of cholesterol-fed dogs, when compared to plasma HDL (63% vs. 18% of total cholesterol, respectively). Peripheral lymph HDL (both in control and cholesterol-fed dogs) had a consistently higher phospholipid content than plasma HDL. Triglyceride was a minor component in each case.

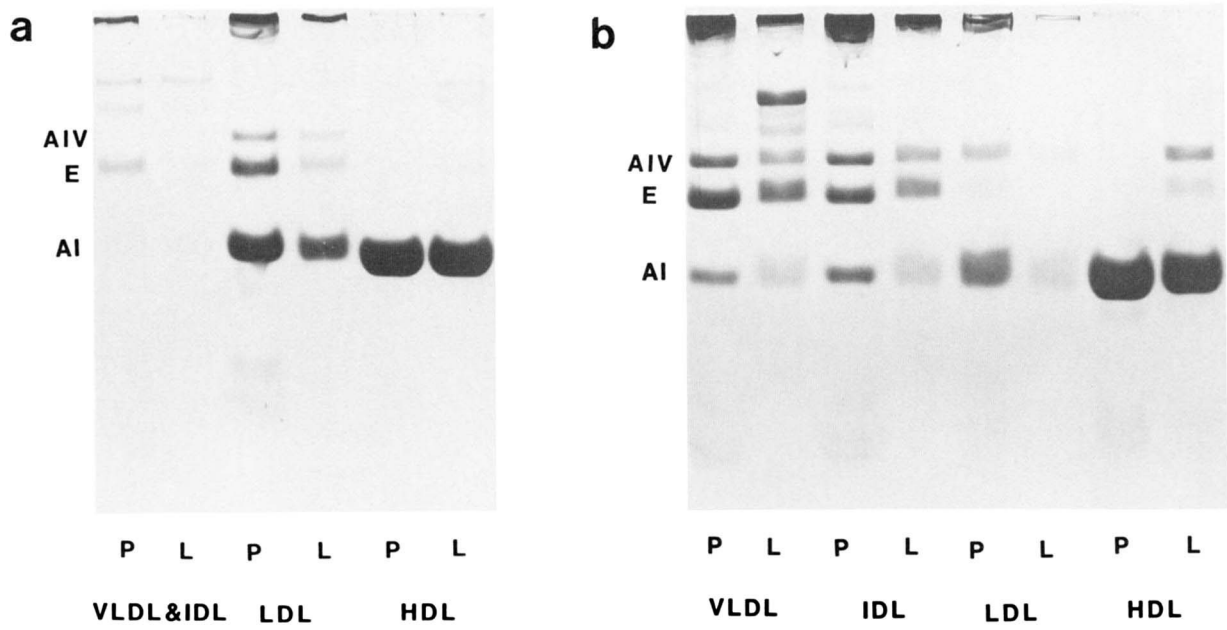

Fig. 2. SDS-polyacrylamide gel electrophoresis of delipidated lipoprotein fractions obtained from control (a) and cholesterol-fed (b) dogs. The following amounts of protein were loaded on the gels. a) VLDL + IDL: plasma, 20 μg; lymph, 10 μg; LDL: plasma, 100 μg; lymph, 40 μg; HDL: plasma, 50 μg; lymph, 50 μg. b) VLDL: plasma, 100 μg; lymph, 70 μg; IDL: plasma, 100 μg; lymph, 55 μg; LDL: plasma, 55 μg; lymph, 10 μg; HDL: plasma, 50 μg; lymph, 50 μg.

Electron microscopy

VLDL plus IDL density fractions from plasma of control dogs appeared the same in size and morphology as those fractions from peripheral lymph (**Fig. 3,** top). Plasma LDL fractions were also indistinguishable from lymph LDL in that most particles were typical of LDL, measuring about 225 Å in diameter. However, LDL from both sources contained a few smaller particles (about 120 Å), consistent with the presence of HDL_1 (Fig. 3, middle). Plasma HDL fractions from control dogs appeared quite similar to plasma HDL from other species with a mean diameter of about 90 to 100 Å, although there were always a few particles about two-fold larger (Fig. 3, bottom left). We consistently found that HDL fractions from peripheral lymph had more

TABLE 3. Distribution of apolipoproteins in plasma and lymph of control dogs[a]

Fraction	Compartment	Apolipoprotein Concentration (A.U.)[b] ApoB	ApoE	ApoA-I	ApoA-IV
VLDL + IDL	Plasma	9.0 ± 1.1[c]	2.7 ± 0.5	ND[d]	0.05 ± 0.04
	Lymph	0.5 ± 0.1	0.2 ± 0.1	ND	ND
	Lymph (% plasma)	6	7		
LDL	Plasma	49.4 ± 11.2[c]	15.0 ± 5.1	2.3 ± 0.6	4.6 ± 1.5
	Lymph	3.1 ± 0.9	0.9 ± 0.3	0.2 ± 0.1	0.5 ± 0.1
	Lymph (% plasma)	6	6	9	11
HDL	Plasma	13.4 ± 3.4	2.0 ± 0.2	122.6 ± 13.1	2.6 ± 0.7
	Lymph	0.8 ± 0.2	1.4 ± 0.1	8.4 ± 0.7	0.6 ± 0.1
	Lymph (% plasma)	6	70	7	23
d > 1.21	Plasma	ND	ND	11.3 ± 1.2	44.5 ± 6.8
	Lymph	ND	ND	3.5 ± 0.4	7.4 ± 1.2
	Lymph (% plasma)			31	17

[a] Unless otherwise indicated, the values represent the average of four dogs ± SEM.
[b] Arbitrary units; concentrations are expressed as a percentage of a standard plasma pool.
[c] n = 3.
[d] Not detectable.

TABLE 4. Distribution of apolipoproteins in plasma and lymph of cholesterol-fed dogs[a]

Fraction	Compartment	Apolipoprotein Concentration (A.U.)[b] ApoB	ApoE	ApoA-I	ApoA-IV
VLDL	Plasma	397.9 ± 127.1	112.2 ± 52.7	0.2 ± 0.1[c]	14.0 ± 6.5[d]
	Lymph	4.9 ± 1.5	3.9 ± 1.0	0.05 ± 0.02	0.8 ± 0.3
	Lymph (% plasma)	1	3	3	6
IDL	Plasma	377.3 ± 112.8	57.2 ± 16.7	0.4 ± 0.1[c]	16.0 ± 4.0
	Lymph	6.5 ± 0.8	1.8 ± 0.3	0.1 ± 0.03	1.4 ± 0.4
	Lymph (% plasma)	2	3	25	9
LDL	Plasma	17.8 ± 5.4	2.6 ± 1.1[d]	0.9 ± 0.6[d]	3.1 ± 0.8[c]
	Lymph	0.4 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	1.3 ± 0.7
	Lymph (% plasma)	2	15	22	42
HDL	Plasma	0.9 ± 0.4[c]	2.0 ± 1.4[d]	20.4 ± 13.8	1.2 ± 0.7[d]
	Lymph	0.1 ± 0.05	2.1 ± 0.1	2.1 ± 1.5	2.8 ± 0.9
	Lymph (% plasma)	11	105	10	233
d > 1.21	Plasma	ND[e]	ND	9.8 ± 3.3[d]	39.3 ± 23.3[c]
	Lymph	ND	ND	2.3 ± 1.2	12.9 ± 3.2
	Lymph (% plasma)			23	33

[a] Unless otherwise indicated, the values represent the mean of four dogs ± SEM.
[b] Arbitrary units; concentrations are expressed as a percentage of a standard plasma pool.
[c] n = 2.
[d] n = 3.
[e] Not detectable.

particles of the larger diameter compared to plasma HDL. In some negative stain preparations, these larger particles stacked to form rouleaux, showing that many of them were discoidal HDL (Fig. 3, bottom right). These discoidal HDL measured about 220 Å mean diameter by about 50 Å in edge thickness.

As in control dogs, VLDL, IDL, and LDL fractions from plasma in cholesterol-fed dogs did not appear to differ in size or shape from those obtained from peripheral lymph (**Fig. 4**). The larger particles (about 1000 Å) in VLDL fractions from both sources may be artifacts because they were not seen in whole lymph. The angular particles in VLDL and IDL were characteristic of fractions that have an abnormally high content of free cholesterol (27). Only very small amounts of LDL were recovered from cholesterol-fed dogs, and these fractions from both plasma and lymph contained a few large discs and some angular particles of LDL size (not shown). HDL fractions from cholesterol-fed dogs differed in morphology compared to normal plasma HDL. Most HDL particles from the plasma of cholesterol-fed dogs were similar in size (90–100 Å) to normal plasma HDL, but more of the second type of larger (about 225 Å) particles were present and some of these were discs (Fig. 4). However, peripheral lymph HDL from cholesterol-fed dogs always contained a proportionately greater number of discoidal particles than typical spherical HDL particles. This increased ratio of discs to spheres favored the formation of rouleaux in negative stains (Fig. 4). The discs appeared to have the

TABLE 5. Chemical composition of plasma and lymph HDL (1.063–1.21 g/ml) obtained from control and cholesterol-fed dogs

	Control (n = 3)		Cholesterol-Fed (n = 3)	
	Plasma	Lymph	Plasma	Lymph
	% (by weight)			
Protein	43.2 ± 0.4[a]	37.1 ± 0.3	40.7 ± 0.9	35.7 ± 1.2
Cholesteryl ester	12.8 ± 1.5	11.0 ± 0.5	19.1 ± 0.8	9.0 ± 0.3
Free cholesterol	4.4 ± 0.4	8.1 ± 0.4	4.2 ± 0.4	15.3 ± 0.4
Phospholipid	39.1 ± 1.1	41.9 ± 1.8	35.2 ± 1.1	40.0 ± 0.8
Triglyceride	0.5 ± 0.1	1.9 ± 0.3	0.8 ± 0.3	ND[b]
Free cholesterol/ester cholesterol	0.36 ± 0.06	0.74 ± 0.07	0.23 ± 0.04	1.70 ± 0.02

[a] Values ± SEM.
[b] Not detectable.

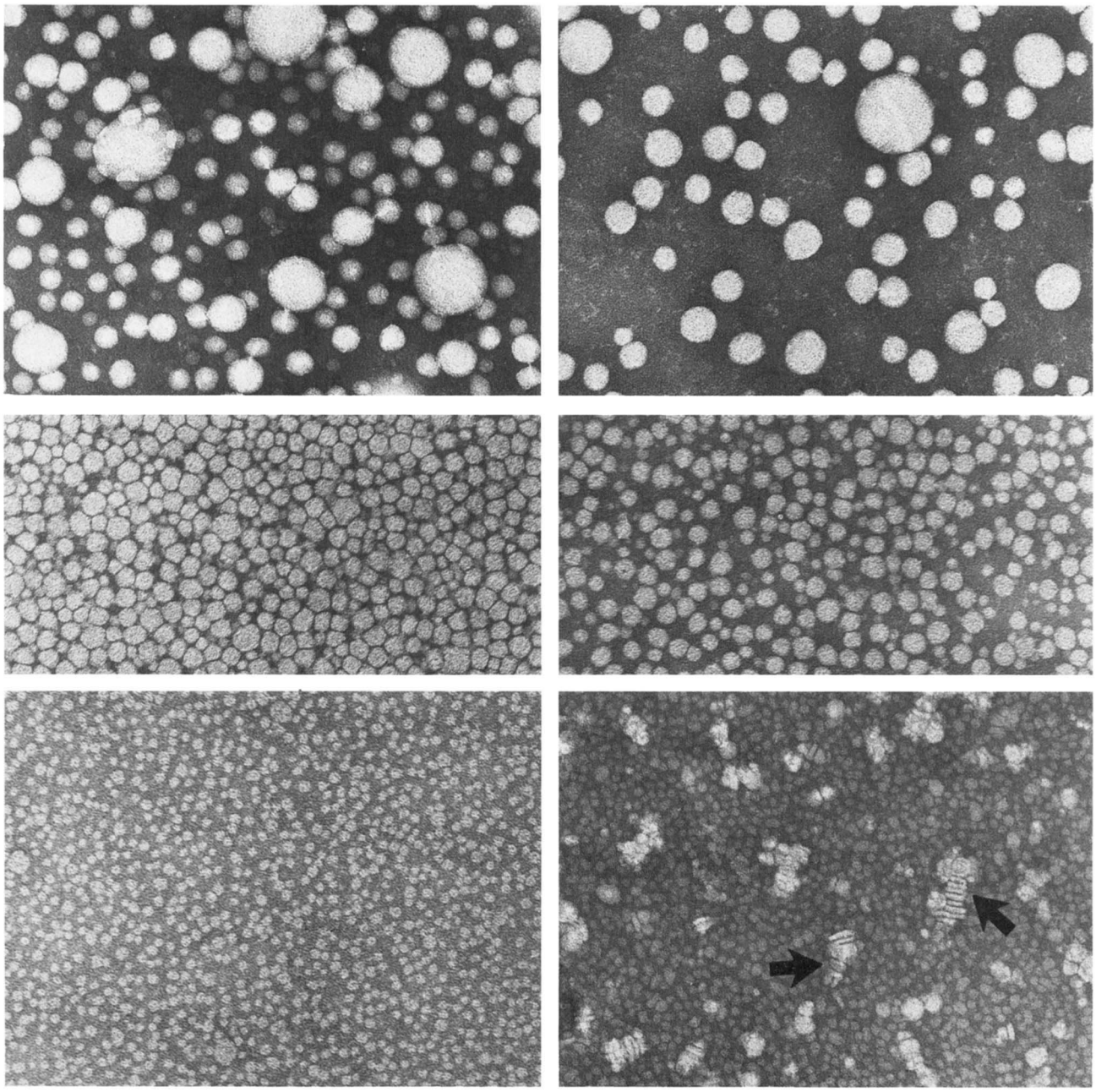

Fig. 3. Magnification, 150,000×. Plasma lipoproteins (left column) and peripheral lymph lipoproteins (right column) from control dogs. Top: VLDL plus IDL, d < 1.030 g/ml; middle: LDL, 1.03 < d < 1.063 g/ml; bottom: HDL, 1.063 < d < 1.21 g/ml. LDL fractions contain some HDL_1-sized particles, characteristic of the dog. Some discoidal particles aggregate to form rouleaux in lymph HDL fractions (arrows).

same dimensions as those in the lymph of the control dogs (i.e., about 220 Å diameter by about 50 Å edge thickness). The smaller HDL particles in lymph of the cholesterol-fed dogs that did not form rouleaux appeared more like the spherical plasma HDL of normal control dogs and were seen between the stacked discs (Fig. 4, bottom right). These particles were on the average a little larger (about 105 Å) than plasma HDL, but some of these could have been discs that were not on edge.

When whole peripheral lymph was examined by the technique of negative staining in the absence of ultracentrifugation, particles were observed with staining properties and sizes that corresponded to each of the fractions isolated by ultracentrifugation, with the exception of the very large particles (>1000 Å) in the VLDL

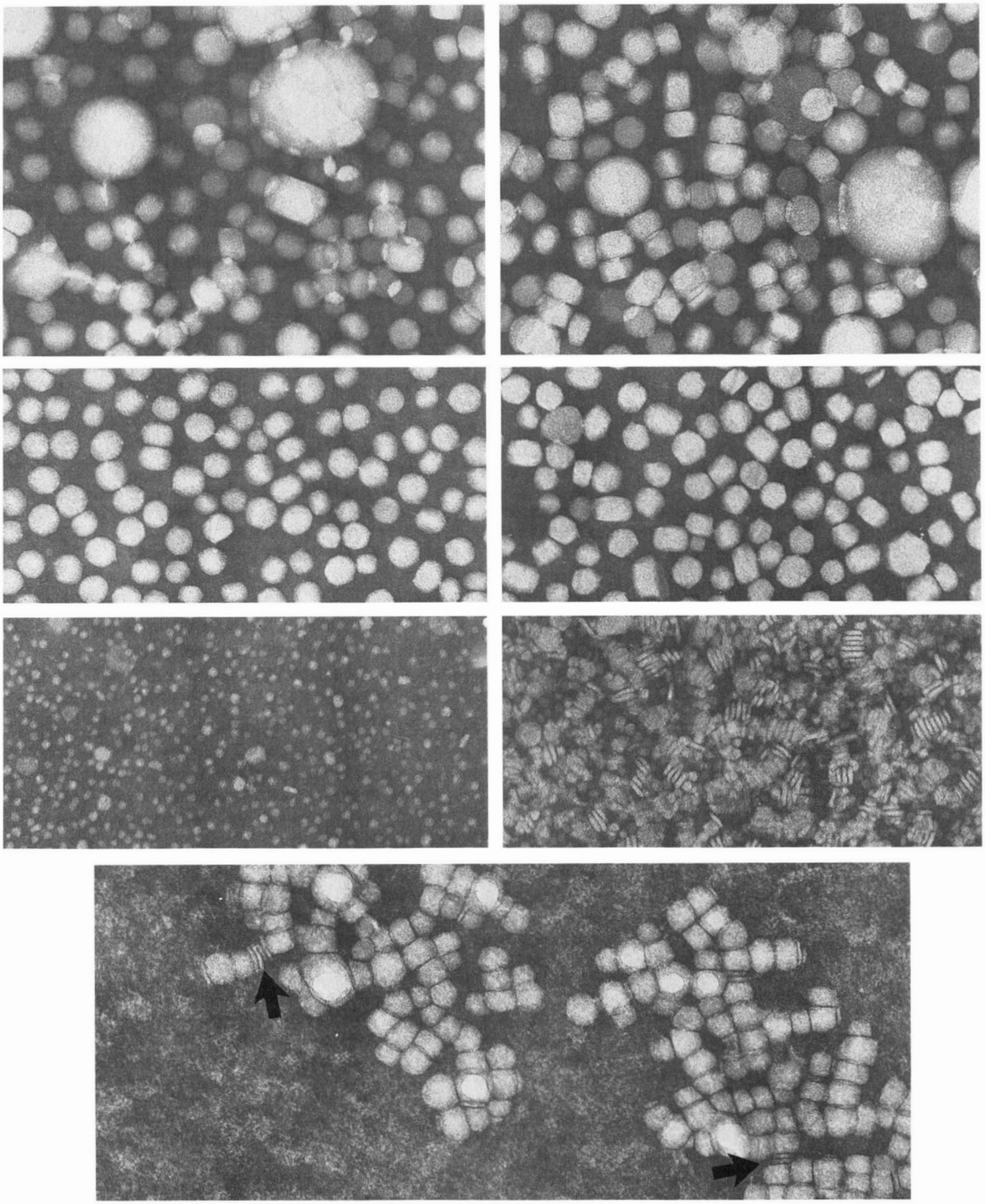

Fig. 4. Magnification, 150,000×. Plasma lipoproteins (left column) and peripheral lymph lipoproteins (right column) from cholesterol-fed dogs. Top: VLDL, $d < 1.006$ g/ml; middle: IDL, $1.006 < d < 1.030$ g/ml; bottom: HDL, $1.063 < d < 1.21$ g/ml. (LDL fraction not shown). Bottom image is of whole uncentrifuged peripheral lymph from cholesterol-fed dogs showing presence of most particles in above centrifugal fractions, including discoidal HDL in short stacks of rouleaux (arrows). Note absence of very large particles seen in VLDL, suggesting they are artifacts. The presence of single particles with an electron lucent core is consistent with a high triglyceride/cholesterol ester ratio.

fractions. Under appropriate conditions, particles in aggregates showed the presence of discs (sometimes even in short rouleau) with the same dimensions as those discoidal HDL obtained by ultracentrifugation of lymph (Fig. 4, bottom).

DISCUSSION

In a previous report we demonstrated the presence of all major plasma lipoproteins and apolipoproteins in prenodal, peripheral lymph of the dog hindlimb (11). We validated the model by showing that after the first hour, a steady state was achieved in which the lymph and plasma albumin concentration and lymph flow remained constant during the subsequent 8–10 hr of sampling. In the present study, we have used ultracentrifugation to fractionate the various plasma and corresponding lymph lipoproteins, and examined some of their chemical properties and morphology by electron microscopy. As reported by others (28), we have found HDL to be the predominant lipoprotein species in the plasma of control dogs. Cholesterol feeding resulted in a profound redistribution in lipoprotein mass in favor of the less dense, cholesteryl ester-rich lipoproteins, with the appearance of β-VLDL and HDL_c, as noted earlier by Mahley et al. (12). It also resulted in a several-fold increase in plasma apoB and apoE concentrations and a significant decrease in plasma apoA-I concentrations. Changes in plasma apolipoprotein concentrations due to cholesterol feeding were reflected in peripheral lymph to different degrees, depending largely on the relative size of the lipoproteins containing the individual apolipoprotein. Thus, the increase in plasma apoB concentrations (an apolipoprotein associated with large lipoproteins) was not reflected in lymph to the same extent as the increase in plasma apoE concentrations (an apolipoprotein associated with large as well as smaller lipoproteins).

During cholesterol feeding, β-VLDL and IDL become the major lipoproteins in both plasma and lymph. The size, apolipoprotein composition, and electrophoretic mobility of these lipoproteins in both plasma and lymph suggested little difference in the nature of these lipoproteins in the two compartments and further characterization was, thus, not pursued. The presence of high lymph concentrations of these "atherogenic" particles provides in vivo evidence for the pathophysiological significance of in vitro studies of β-VLDL-macrophage interactions (29–31). It appears likely, therefore, that in the cholesterol-fed dogs the majority of cholesterol transported to peripheral cells, including macrophages, may be, to a large extent, in the form of large apoB-containing lipoproteins as well as HDL_c.

As previously reported (12), agarose electrophoresis demonstrated the presence of HDL_c in the plasma of hyporesponding cholesterol-fed dogs. We have now clearly demonstrated the presence of this lipoprotein in the lymph of these animals. The apparent absence of HDL_c in the hyperresponders may be due to incomplete separation by agarose electrophoresis of HDL_c from large amounts of β-migrating particles (30). It has also been our experience that increasing degrees of hypercholesterolemia make the detection of HDL_c more difficult.

It is well known that ultracentrifugation alone, like any other technique, cannot yield a lipoprotein population homogeneous in all properties. Due to the scarcity of material obtainable from lymph, we have chosen ultracentrifugation as a method of lipoprotein isolation to provide data readily comparable with the bulk of existing literature. Invariably, especially in the case of less dense lipoproteins, this method yields lipoprotein mixtures of various electrophoretic mobilities, as shown in Fig. 1, and this should be kept in mind. Care was taken, however, that equivalent fractions were compared from the lymph and plasma compartments.

Lymph HDL of both control and cholesterol-fed dogs differed from plasma HDL in size as well as chemical composition. Lymph HDL consistently eluted earlier than plasma HDL in column chromatography in 10% agarose, indicating that some were larger in size than plasma HDL of the same animals (11). Alterations in the size of peripheral lymph HDL towards larger particles have been demonstrated by Reichl and Pflug (32). We have now shown that the larger size of peripheral lymph HDL was found to be associated with alterations in lipid and apolipoprotein composition, especially in cholesterol-fed dogs. The relative excess of polar (surface) components in peripheral lymph HDL, reflected by high free cholesterol and phospholipid to cholesteryl ester ratios, suggested the presence of discoidal particles, as reported by others, for intestinal lymph (33) and nascent hepatic HDL (34). Electron microscopy directly confirmed the presence of discoidal particles in peripheral lymph. A considerable enrichment of peripheral lymph HDL with apoE and apoA-IV was observed by both immunochemical and electrophoretic methods. In peripheral lymph HDL from control and cholesterol-fed dogs, the apoE/apoA-I and apoA-IV/apoA-I ratios were several-fold elevated, compared with those of plasma HDL. Simple filtration does not adequately explain these changes. The increased content of apoE and apoA-IV in lymph HDL could, therefore, be partially derived from plasma HDL, as well as *a*) redistribution within the interstitium from larger, less dense lipoproteins; *b*) transfer of "free" apolipoproteins from plasma

(35); *c*) local synthesis by peripheral tissues such as macrophages (36); or, unlikely, *d*) a preferential transport of the scarce plasma discoidal HDL.

Another striking property of peripheral lymph HDL, especially in cholesterol-fed dogs, is the relative increase in cholesterol content, as measured by an increased cholesterol to protein ratio. Much of the peripheral lymph HDL cholesterol is unesterified, compatible with previous reports (9, 37) and our own observations (38) of low LCAT activity in peripheral lymph. Although no direct evidence was provided in the present report, the source of the excess free cholesterol in lymph HDL appears likely to be the peripheral tissues, as suggested by labeling experiments using human peripheral lymph (9). The properties of peripheral lymph HDL are consistent with the general concept that HDL functions in reverse cholesterol transport by taking up cholesterol from peripheral tissues. A significant percentage of plasma apoA-I and apoA-IV was found in the $d > 1.21$ g/ml, lipoprotein-free fraction. At the present time, the physiological significance of this observation is uncertain due to the possibility of ultracentrifugal artifacts; but such "free" apolipoproteins in plasma could contribute to the peripheral lymph HDL apolipoprotein pool. Others have also found the majority of plasma apoA-IV in the lipoprotein-free fraction (without ultracentrifugation) of the human (39, 40), rat (41, 42), and dog.[2]

Although some apoE may also exist in a "lipoprotein-free" form, recent studies of macrophages in tissue culture indicate that these cells have the potential to secrete apoE contained in a disc HDL particle (43). Thus, either selective filtration of comparatively lipid-free apoA-IV (and/or apoE) and newly synthesized apoE (and/or apoA-IV) by peripheral tissues may participate in cholesterol removal from peripheral cells. These apolipoproteins may combine with phospholipid and free cholesterol at plasma membrane surfaces of peripheral cells, forming the discoidal structures. The fact that apoA-IV is increased more than fourfold in peripheral lymph HDL of cholesterol-fed dogs compared to apoA-IV content in the peripheral lymph HDL of control dogs suggests that this apoprotein is associated with the disc particle structure and perhaps its formation.

Discoidal HDL, enriched in apoE, phospholipid, and unesterified cholesterol occurs in plasma of humans with LCAT deficiency (44) and in rat liver perfusates in which LCAT is largely inhibited (34). Similar discs also accumulate in perfusates of liver of guinea pigs fed cholesterol (27). In our studies, the number of HDL discs in peripheral lymph was far greater than in the blood plasma of the same animals, suggesting that LCAT was deficient in lymph and that a redistribution of disc components may occur rapidly on mixing lymph with blood plasma. Thus, we have demonstrated that the free cholesterol-enriched discoidal peripheral lymph HDL may be the precursor of apoE-enriched, less dense HDL_c particles in cholesterol-fed dogs (38). It has been proposed that these particles represent an exaggerated accumulation of HDL particles that may normally transport cholesterol from peripheral tissues to the liver through the apoE receptors (45).

Our present study indicates that, together with other model systems such as tissue culture, the analyses of peripheral lymph components can be very useful in revealing important components and mechanisms of reverse cholesterol transport. We have integrated these observations with present concepts of lipoprotein metabolism and, specifically, reverse cholesterol transport, as advocated by other laboratories (1, 43, 45) (**Fig. 5**). Many aspects of this overall scheme remain speculative and, therefore, are subject to further investigation. Under normal conditions, filtered plasma HDL_3, which is apoA-I-rich, may combine with free cholesterol and phospholipid from peripheral tissues in the interstitium to form free cholesterol phospholipid-enriched HDL which is found in peripheral lymph. For the purpose of our model, we assumed that peripheral lymph HDL is a good representation of interstitial fluid HDL (HDL_i) and refers to it as HDL_i in this figure. The proposed events occurring within the interstitium are, thus, analogous to the in vitro observations that free cholesterol efflux from cultured cells is enhanced by the addition of HDL apolipoproteins and phospholipid (7). Once in plasma, HDL_i may be converted by the action of LCAT to HDL_2 which is further metabolized by the liver (46, 47).

During cholesterol feeding, a different situation may be present. Since HDL and apoA-I are significantly reduced in either plasma or lymph of cholesterol-fed dogs, other apolipoproteins must participate in the removal of tissue-free cholesterol. Under these conditions, apoA-IV and apoE (derived from increased de novo synthesis in the periphery or liver and intestine) may directly combine with tissue-free cholesterol and phospholipids to form HDL_i particles similar in chemical composition and shape to the discoidal "nascent" HDL. In plasma, action of LCAT on such an HDL particle may result in production of a spherical HDL_c (lower in density) containing primarily apoE (38), which would be rapidly recognized and removed by the hepatic E receptor (48), and/or HDL_2 containing primarily apoA-I. Alternatively, HDL_i, upon entering plasma, may also be rapidly removed by the liver.

It should be pointed out that peripheral lymph of control dogs contains some disc-shaped HDL while nu-

[2] Roheim, P. S., L. Dory, and C. H. Sloop. Unpublished observations.

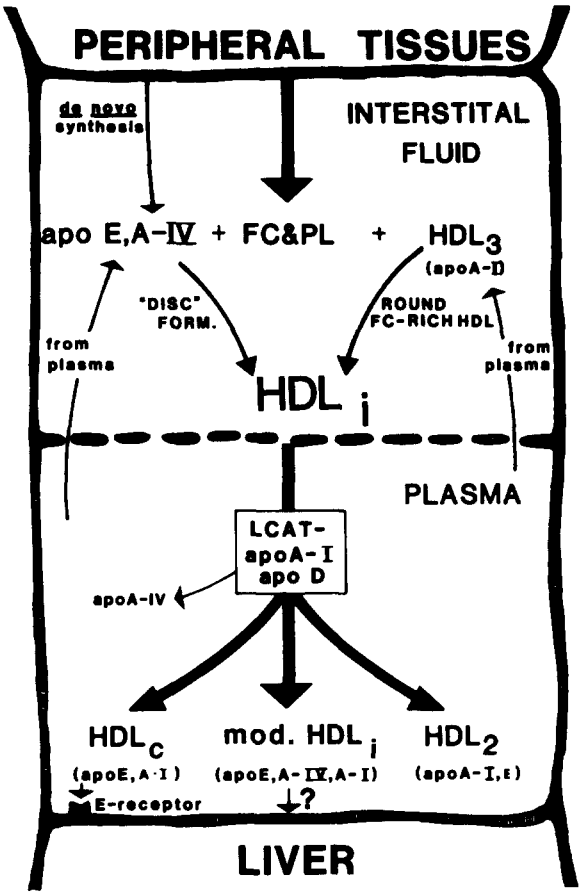

Fig. 5. Proposed scheme for the role of interstitial HDL in "reverse cholesterol transport." Two possible mechanisms for the formation of interstitial HDL (HDL_i) are possible. The status of the animal, i.e., cholesterol feeding, may influence the extent to which these two pathways are operational. The upper right side quadrant depicts the first possible mechanism: filtered plasma HDL_3, which is apoA-I-rich, may combine with free cholesterol and phospholipid from peripheral tissues in the interstitium to form free cholesterol phospholipid-enriched interstitial HDL (HDL_i). The upper left side quadrant illustrates the second possibility: apoA-IV and apoE (derived from de novo synthesis in the periphery or from free apoproteins or redistribution of apoproteins present in lipoproteins of the plasma filtrate) may directly combine with tissue-free cholesterol and phospholipids to form HDL_i particles similar in chemical composition and shape to the discoidal "nascent" HDL. The lower half of the figure illustrates the possible events taking place in the plasma. Once in the plasma, spherical HDL_i may be converted by the action of LCAT to HDL_2, which could be further metabolized. The action of LCAT on such a discoidal HDL_i particle may result in production of a spherical HDL_c (lower in density), containing primarily apoE, which would be rapidly recognized and removed by the hepatic apoE receptors. Alternatively, HDL_i, upon entering plasma, may also be rapidly removed, possibly by the liver. From discoidal HDL_i, LCAT action may also produce HDL_2, containing primarily apoA-I.

merous round HDL are seen in the lymph of cholesterol-fed dogs. It is doubtful that either of the two pathways outlined above function independently. More likely, the relative contribution of the two pathways for reverse cholesterol transport may vary, depending on the availability of the various apolipoproteins in the interstitial fluid as acceptors for free cholesterol and phospholipid.

In conclusion, studies of the dog peripheral lymph provide an in vivo model for the study of reverse cholesterol transport. We identified and partially characterized an HDL particle in peripheral lymph that is likely to be the vehicle for cholesterol transport from peripheral tissues to the liver. The various metabolic steps involved in the processing of this unusual lipoprotein species are presently under study.

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REFERENCES

1. Glomset, J. A. 1968. The plasma lecithin:cholesterol acyltransferase reaction. *J. Lipid Res.* **9:** 155–167.
2. Tall, A. R., and D. M. Small. 1980. Body cholesterol removal: role of plasma high-density lipoproteins. *Adv. Lipid Res.* **17:** 1–51.
3. Nicoll, A., N. E. Miller, and B. Lewis. 1980. High density lipoprotein metabolism. *Adv. Lipid Res.* **17:** 53–106.
4. Witztum, J., and G. Schonfeld. 1979. High density lipoproteins. *Diabetes.* **28:** 326–333.
5. Fielding, C. J., and P. E. Fielding. 1982. Cholesterol transport between cells and body fluids. *Med. Clin. N. Am.* **66:** 363–373.
6. Werb, Z., and Z. A. Cohn. 1971. Cholesterol metabolism in the macrophage. I. The regulation of cholesterol exchange. *J. Exp. Med.* **134:** 1545–1569.
7. Stein, O., and Y. Stein. 1973. The removal of cholesterol Landschütz ascites cells by high-density apolipoprotein. *Biochim. Biophys. Acta.* **326:** 232–244.
8. Bates, S. R., and G. H. Rothblat. 1974. Regulation of cellular sterol flux and synthesis by human serum lipoproteins. *Biochim. Biophys. Acta.* **360:** 38–55.
9. Reichl, D., N. B. Myant, D. N. Rudra, and J. J. Pflug. 1980. Evidence for the presence of tissue-free cholesterol in low density and high density lipoproteins of human peripheral lymph. *Atherosclerosis.* **37:** 489–495.
10. Yoffey, J. M., and F. C. Courtice. 1970. Lymphatics, Lymph and the Lymphomyeloid Complex. Academic Press, Inc., London. 1–868.
11. Sloop, C. H., L. Dory, B. R. Krause, C. Castle, and P. S. Roheim. 1983. Lipoproteins and apolipoproteins in peripheral lymph of normal and cholesterol-fed dogs. *Atherosclerosis.* In press.
12. Mahley, R. W., K. H. Weisgraber, and T. Innerarity. 1974. Canine lipoproteins and atherosclerosis. II. Characterization of the plasma lipoproteins associated with atherogenic and nonatherogenic hyperlipidemia. *Circ. Res.* **35:** 722–733.
13. Renkin, E. M., P. D. Watson, C. H. Sloop, W. L. Joyner, and F. E. Curry. 1977. Transport pathways for fluid and

large molecules in microvascular endothelium of the dog's paw. *Microvasc. Res.* **14:** 205–214.
14. Renkin, E. M., C. H. Sloop, and W. L. Joyner. 1981. Influence of venous congestion on blood-lymph transport of fluid and large molecules in the heat-injured dog's paw. *Lymphology.* **14:** 113–117.
15. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **293:** 265–275.
17. Allain, C. C., L. S. Poon, C. S. G. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20:** 470–475.
18. Marcell, Y. L., M. Bergseth, and A. C. Nestruck. 1979. Preparative isoelectric focusing of apolipoproteins C and E from human very low density lipoproteins. *Biochim. Biophys. Acta.* **393:** 175–183.
19. Shapiro, A. L., E. Vinuela, and J. V. Maizel. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem. Biophys. Res. Commun.* **28:** 815–820.
20. Ouchterlony, O. 1968. Handbook of Immunodiffusion and Immunoelectrophoresis. Ann Arbor Science Publishers, Ann Arbor, MI.
21. Grabar, P., and P. Burtin. 1964. Immunoelectrophoretic Analysis. Elsevier, Amsterdam.
22. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76:** 4350–4354.
23. Laurell, C. B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem.* **15:** 45–52.
24. Roheim, P. S., and G. Vega. 1979. Electroimmunoassay of apolipoprotein A-I. *In* Report of the High Density Lipoprotein Methodology Workshop. K. Lippel, editor. NIH Publication No. 79-1661. 241–251.
25. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.
26. Pocock, D. M. E., S. Rafal, and A. Vost. 1972. Separation of mammalian neutral lipids by chromatography on glass-fiber paper impregnated with silica gel. *J. Chromatogr. Sci.* **10:** 72–76.
27. Guo, L. S. S., R. L. Hamilton, R. Ostwald, and R. J. Havel. 1982. Secretion of nascent lipoproteins and apolipoproteins by perfused livers of normal and cholesterol-fed guinea pigs. *J. Lipid Res.* **23:** 543–555.
28. Mahley, R. W., and K. H. Weisgraber. 1974. Canine lipoproteins and atherosclerosis. I. Isolation and characterization of plasma lipoproteins from control dogs. *Circ. Res.* **35:** 713–721.
29. Goldstein, J. L., Y. K. Ho, M. S. Brown, T. L. Innerarity, and R. W. Mahley. 1980. Cholesteryl ester accumulation in macrophages resulting from receptor-mediated uptake and degradation of hypercholesterolemic canine β-very low density lipoproteins. *J. Biol. Chem.* **255:** 1839–1848.
30. Mahley, R. W., T. L. Innerarity, M. S. Brown, Y. K. Ho, and J. L. Goldstein. 1980. Cholesteryl ester synthesis in macrophages: stimulation by β-very low density lipoproteins from cholesterol-fed animals of several species. *J. Lipid Res.* **21:** 970–980.
31. Innerarity, T. L., R. E. Pitas, and R. W. Mahley. 1982. Modulating effects of canine high density lipoproteins on cholesteryl ester synthesis induced by β-very low density lipoproteins in macrophages. *Arteriosclerosis.* **2:** 114–124.
32. Reichl, D., and J. J. Pflug. 1982. The concentration of apolipoprotein A-I in human peripheral lymph. *Biochim. Biophys. Acta.* **710:** 456–463.
33. Green, P. H. R., A. R. Tall, and R. M. Glickman. 1978. Rat intestine secretes discoid high density lipoprotein. *J. Clin. Invest.* **61:** 523–534.
34. Hamilton, R. L., M. C. Williams, C. J. Fielding, and R. J. Havel. 1976. Discoidal bilayer structure of nascent high density lipoproteins from perfused rat liver. *J. Clin. Invest.* **58:** 667–680.
35. Roheim, P. S., L. Miller, and H. A. Eder. 1965. The formation of plasma lipoproteins from apoprotein in plasma. *J. Biol. Chem.* **240:** 2994–3001.
36. Basu, S. K., M. S. Brown, Y. K. Ho, R. J. Havel, and J. L. Brown. 1981. Mouse macrophages synthesize and secrete a protein resembling apolipoprotein E. *Proc. Natl. Acad. Sci. USA.* **78:** 7545–7549.
37. Reichl, D., L. A. Simons, N. B. Myant, J. J. Pflug, and G. L. Mills. 1973. The lipids and lipoproteins of human peripheral lymph, with observations on the transport of cholesterol from plasma and tissues into lymph. *J. Clin. Sci. Mol. Med.* **45:** 323–329.
38. Dory, L., C. H. Sloop, L. M. Boquet, R. L. Hamilton, and P. S. Roheim. 1983. LCAT-mediated modification of discoidal peripheral lymph HDL: a possible mechanism of HDL_c formation in cholesterol-fed dogs. *Proc. Natl. Acad. Sci. USA.* **80:** 3489–3493.
39. Utermann, G., and U. Beisiegel. 1979. Apolipoprotein A-IV: a protein occurring in human mesenteric lymph chylomicrons and free in plasma. *Eur. J. Biochem.* **99:** 333–343.
40. Green, P. H. R., R. M. Glickman, J. W. Riley, and E. Quinet. 1980. Human apolipoprotein A-IV. Intestinal origin and distribution in plasma. *J. Clin. Invest.* **65:** 911–919.
41. Roheim, P. S., D. Edelstein, and G. Pinter. 1976. Apolipoproteins in rat serum and renal lymph. *Proc. Natl. Acad. Sci. USA.* **73:** 1757–1760.
42. Van Lenten, B. J., and P. S. Roheim. 1982. Changes in the concentrations and distributions of apolipoproteins of the aging rat. *J. Lipid Res.* **23:** 1187–1195.
43. Basu, S. K., Y. K. Ho, M. S. Brown, D. W. Bilheimer, R. G. W. Anderson, and J. L. Goldstein. 1982. Biochemical and genetic studies of the apoprotein E secreted by mouse macrophages and human monocytes. *J. Biol. Chem.* **257:** 9788–9795.
44. Norum, K. F., J. A. Glomset, A. V. Nichols, and T. Forte. 1971. Plasma lipoproteins in familial lecithin:cholesterol acyltransferase deficiency: physical and chemical studies of low and high density lipoproteins. *J. Clin. Invest.* **50:** 1131–1140.
45. Mahley, R. W. 1982. Atherogenic hyperlipoproteinemia. *Med. Clin. N. Am.* **66:** 375–402.
46. Van Tol, A., T. Van Gent, and H. Jansen. 1980. Degradation of high density lipoprotein by heparin-releasable liver lipase. *Biochem. Biophys. Res. Commun.* **94:** 101–108.
47. Shirai, K., R. L. Barnhart, and R. L. Jackson. 1981. Hydrolysis of human plasma high density lipoprotein$_2$-phospholipids and triglycerides by hepatic lipase. *Biochem. Biophys. Res. Commun.* **100:** 591–599.
48. Hui, D. Y., T. L. Innerarity, and R. W. Mahley. 1981. Lipoprotein binding to canine hepatic membranes: metabolically distinct apoE and apoB, E receptors. *J. Biol. Chem.* **256:** 4646–5655.