Composition and ultrastructure of size subclasses of normal human peripheral lymph lipoproteins: quantification of cholesterol uptake by HDL in tissue fluids

M. N. Nanjee,* C. J. Cooke,* J. S. Wong,† R. L. Hamilton,† W. L. Olszewski,*,§ and N. E. Miller[1],*

Department of Cardiovascular Biochemistry,* St. Bartholomew's and the Royal London School of Medicine and Dentistry, Charterhouse Square, London EC1M 6BQ, UK; Cardiovascular Research Institute and Department of Anatomy,† University of California, San Francisco, CA 94143; Department of Surgical Research and Transplantology,§ Medical Research Center, Polish Academy of Sciences 02-106, Warsaw, Pawinskiego, Poland

Abstract **Peripheral lymph lipoproteins have been characterized in animals, but there is little information about their composition, and none about their ultrastructure, in normal humans. Therefore, we collected afferent leg lymph from 16 healthy males and quantified lipids and apolipoproteins in fractions separated by high performance-size exclusion chromatography. Apolipoprotein B (apoB) was found almost exclusively in low density lipoproteins. The distribution of apoA-I, particularly in lipoprotein A-I (LpA-I) without A-II particles, was shifted toward larger particles relative to plasma. The fractions containing these particles were also enriched in apoA-II, apoE, total cholesterol, and phospholipids and had greater unesterified cholesterol-to-cholesteryl ester ratios than their counterparts in plasma. Fractions containing smaller apoA-I particles were enriched in phospholipid. Most apoA-IV was lipid poor or lipid free. Most apoC-III coeluted with large apoA-I-containing particles. Electron microscopy showed that lymph contained discoidal particles not seen in plasma. These findings support other evidence that high density lipoproteins (HDL) undergo extensive remodeling in human tissue fluid. Total cholesterol concentration in lymph HDL was 30% greater ($P < 0.05$) than could be explained by the transendothelial transfer of HDL from plasma, providing direct confirmation that HDL acquire cholesterol in the extravascular compartment. Net transport rates of new HDL cholesterol in the cannulated vessels corresponded to a mean whole body reverse cholesterol transport rate via lymph of 0.89 mmol (344 mg)/day.**—Nanjee, M. N., C. J. Cooke, J. S. Wong, R. L. Hamilton, W. L. Olszewski, and N. E. Miller. **Composition and ultrastructure of size subclasses of normal human peripheral lymph lipoproteins: quantification of cholesterol uptake by HDL in tissue fluids.** ***J. Lipid Res.*** **2001.** 42: **639–648.**

Supplementary key words tissue fluid • apolipoproteins • phospholipids • electron microscopy

The extracellular metabolism of lipoproteins occurs in intravascular and extravascular compartments. Although it is recognized that most cells are exposed not to plasma lipoproteins but to those of tissue fluid, there is little information about the latter. This is largely on account of the difficulties of sampling tissue fluid in sufficient quantity under physiologic conditions. The only reliable matrix is prenodal (afferent) peripheral lymph, collection of which requires cannulation of a lymph vessel. Roheim and co-workers (1–3) have shown that the lipoproteins of canine peripheral lymph differ from plasma lipoproteins in concentration, composition, and physical properties. Our knowledge of human lymph is less extensive. Essentially all the available information was provided by Reichl and colleagues (4, 5), who collected lymph from the foot. Owing to the high failure rate, low flow rate, and short cannulation life associated with this procedure, their studies were limited to a few subjects, often with hyperlipidemia, postphlebitis syndrome, or chronic lymph stasis. In only two studies (6, 7) was more than one apolipoprotein assayed in normal subjects [apolipoprotein A-I (apoA-I) and apoA-II], and no data were presented on the distributions of apoB, apoA-IV, apoE, or apoC in size subfractions. One study examined the distribution of cholesterol in size subfractions of normal lymph (8), but no similar data were reported for triglycerides (TG) or phospholipids (PL). Furthermore, there has been no study of the ultrastructure of normal human lymph lipoproteins. The only study of this type used lymphedema fluid (9), the lipoproteins of which are likely to have been altered by increases in endothelial permeability and the extravascular residence time of macromolecules (10).

Because of the problems associated with collecting foot lymph, we have adapted a procedure for cannulating an

Abbreviations: apoA-I, apolipoprotein A-I; CE, cholesteryl esters; HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoproteins; L/P ratio, lymph/plasma ratio; PL, phospholipids; TG, triglycerides; VLDL, very low density lipoproteins.

[1] To whom correspondence should be addressed.
e-mail: n.e.miller@mds.qmw.ac.uk

afferent vessel in the leg (11). As the vessels of the leg are downstream from the foot, they are larger and more reliable and provide much greater volumes. This has enabled us to examine both the ultrastructure of lipoproteins in normal lymph and the distributions of all the major apolipoproteins and lipids in size subfractions.

MATERIALS AND METHODS

Subjects

Lymph was collected from 16 healthy males (**Table 1**). Subjects were excluded if they had renal, hepatic, endocrine, or cardiovascular disease, or if they were taking a special diet or medication. The study was approved by the local ethics committee. All subjects gave informed consent.

Clinical procedures

All cannulations were performed at 8:00–11:00 AM under sterile conditions. The subjects had fasted for 12–14 h overnight, but were allowed fat-free drinks. The details of the procedure have been described (11). Briefly, an area of skin over the lower tibia was anesthetized, a 15- to 20-mm incision was made 6 cm above the ankle, and the subcutaneous lymph vessels were dissected. A second smaller skin incision was made above the first, through which was passed a tapered, sterile, siliconized, polyethylene cannula (Intramedic® PE60; Becton Dickinson, Sparks, MD). The vessel was ligated proximally and opened, and the first valve distal to the opening was destroyed. The cannula was passed into the vessel and secured with a ligature. The other end was passed into a polypropylene tube containing 2 mg of disodium ethylenediaminetetraacetic acid (Na_2EDTA). Thereafter, the subject remained in a metabolic ward, was encouraged to walk, and was given a light fat-free meal and fruit juice. Lymph flow rates were 0.4–3.3 ml/h. The collection tubes were changed every 2–3 h. In all the subjects blood was collected from a vein into Na_2EDTA (final concentration, 1 mg/ml). Lymph was routinely collected at ambient temperature, but in four subjects it was collected from the same vessel into two tubes: one at ambient temperature and another immersed in ice. In three subjects lymph and blood for electron microscopy were collected into iodoacetate (final concentration, 5.0 mM), in addition to Na_2EDTA, to inhibit lecithin:cholesterol acyltransferase (LCAT).

Laboratory procedures

Blood and lymph samples were centrifuged (1,500 *g*, 15 min, 4°C) and the supernatants were transferred to polypropylene tubes. Plasma-lymph pairs from the same subject were always processed together.

Lipids

Total cholesterol, unesterified cholesterol (UC), total glycerol (i.e., glyceride glycerol plus free glycerol), and total choline-containing PL were quantified by enzymatic colorimetric procedures (12). Cholesteryl esters (CE) were calculated by difference. Plasma total high density lipoprotein (HDL) cholesterol (data in Table 1) was assayed with polyethylene glycol 8000 (13). Precinorm L® (Boehringer-Mannheim, Mannheim, Germany) was used as calibrator.

Apolipoproteins

ApoA-I, -A-II, -A-IV, -B, -C-III, and -E were quantified by radioimmunoassays or rocket immunoelectrophoresis (13). Rabbit anti-apoA-IV was a gift from J-C. Fruchart (Lille, France). Other primary antisera were goat polyclonal IgG against delipidated human apolipoproteins (International Immunology, Murietta, CA). In radioimmunoassays the precipitating antibodies were donkey anti-goat IgG or donkey anti-rabbit IgG (Chemicon, Temecula, CA). Radioiodinated tracers were prepared with human apolipoproteins or low density lipoproteins (LDL; d 1.020–1.055 g/ml). Radioactivity in antibody-bound pellets was quantified to ⩽0.1% counting error. All assays were standardized with Precinorm L®. Coefficients of variation for replicated assays of apoA-I, -A-II, -A-IV, -B, -C-III, and -E were 3.4%, 5.6%, 11.1%, 2.1%, 2.5%, and 8.5%, respectively.

Nonlipoprotein proteins

α_2-Macroglobulin (750 kDa), total IgG (150 kDa), and albumin (67 kDa) were assayed by immunoelectrophoresis, using polyclonal antisera (International Immunology). Total protein in size subfractions was derived as the product of relative UV absorbance (280 nm) and the total protein in the starting material, measured by a bicinchoninic acid assay procedure (Pierce, Rockford, IL).

Superose 6 size-exclusion chromatography

To separate subclasses of very low density lipoproteins (VLDL), LDL, and HDL, 100 μl of 4-fold-diluted plasma or 100 μl of undiluted lymph was passed through a 10 × 300 mm Superose 6 column (HR 10/30; Pharmacia LKB, Uppsala, Sweden) (13). Recoveries of cholesterol and apoA-I were >90%. In three subjects the same procedure was applied to samples from which all apoA-II-containing particles had been removed by immunoprecipitation. Preformed immune complexes of donkey anti-goat IgG/polyclonal goat anti-human apoA-II were added in sufficient quantity to remove a 3-fold excess of apoA-II from plasma and lymph samples and incubated with vortexing for 2 h at 4°C. Absence of apoA-II in the supernatant fractions was confirmed by immunoelectrophoresis.

Superdex size-exclusion chromatography

Other aliquots of 3-fold-diluted plasma or undiluted lymph (50 μl) were passed through Superdex 200 and Superdex 75 gel-permeation columns (HR 10/30; Pharmacia LKB) in series (14). This procedure separates the apoA-I-containing particles of plasma into three size subclasses: a major population of 70- to 500-kDa particles (fractions 16–41), composed of CE-rich HDL

TABLE 1. Clinical details of subjects

	Age	Weight	Body Mass Index	Plasma Concentrations				
				Cholesterol	Triglycerides	HDL-Chol	ApoA-I	ApoB
	years	*kg*	*kg/m²*	*mM*	*mM*	*mM*	*mg/dl*	*mg/dl*
Mean	30.8	84.1	26.9	4.7	1.4	1.1	118	82
SD	13.5	13.2	4.0	1.1	0.7	0.4	32	27
Range	20–69	68–107	21–33	3.3–7.1	0.4–2.7	0.6–1.8	84–188	50–150

HDL, high density lipoproteins; ApoA-I, apolipoprotein A-I.

with α electrophoretic mobility; a minor population of >500-kDa particles (fractions 11–15); and a population of 40- to 60-kDa particles (fractions 42–56), which contain apoA-I as the only protein, contain no CE, have pre-β mobility, and appear to be a mixture of lipid-free apoA-I dimers and lipid-poor HDL similar to pre-β_1 HDL (14).

Quantification of net cholesterol uptake by HDL in tissue fluid

The concentrations of cholesterol and total protein in subfractions of plasma and lymph after Superdex high performance size-exclusion chromatography (HP-SEC) were used to calculate the net mass of cholesterol acquired by HDL in tissue fluid in vivo. This was based on four assumptions: *1*) that plasma HDL of different sizes cross endothelium at the same rates as plasma proteins of the same size, *2*) that there is no catabolism of HDL by peripheral cells, *3*) that all HDL return to plasma from tissue fluid via lymph, and *4*) that our Superdex HP-SEC procedure isolates from lymph all HDL that have acquired cholesterol since leaving plasma.

For each pair of corresponding fractions of plasma and lymph that contained apoA-I, the lymph-to-plasma (L/P) ratio of total protein concentration was multiplied by the concentration of cholesterol in the plasma fraction. This gave an estimate of the concentration of cholesterol in each lymph fraction that was derived from plasma by filtration of HDL across endothelium. Summation of these values gave the total concentration of plasma-derived cholesterol in lymph HDL. In all but one subject this value was smaller than the measured total cholesterol concentration in these fractions. The difference between the measured and expected concentrations of total HDL cholesterol in lymph was then multiplied by the lymph flow rate, to give the net transport rate of new cholesterol by HDL in the vessel.

Electron microscopy

All samples of lymph collected over 72 h were pooled, adjusted to d 1.21 g/ml with KBr, and ultracentrifuged for 60 h at 40,000 *g* and 4°C in a Beckman (Fullerton, CA) SW41 rotor. The supernatants were subjected to Superose 6 HP-SEC, as described above. The eluates were collected in 24 fractions, each of which was assayed for apoA-I, apoE, UC, and CE. Four size ranges of particles were defined: *A*) fractions 1–6 (particles larger than LDL), *B*) fractions 7–11 (LDL), *C*) fractions 12–16 (large HDL, characterized by a peak in UC/CE ratio), and *D*) fractions 17–22 (medium-sized and small HDL). The fractions in each size range were then pooled. Each of the four pools (A, B, C, and D) was concentrated by centrifugal ultrafiltration, using Ultrafree 10K MWCO tubes (Millipore, Bedford, MA), before being shipped from London to San Francisco on ice. Aliquots were examined by electron microscopy after negative staining with potassium phosphotungstate (15, 16).

RESULTS

Lipids and apolipoproteins in Superose 6 subfractions

Fasting lymph contained few VLDL and had relatively more lipid in HDL than in LDL (**Fig. 1**). Lymph also had a higher absolute concentration of total glycerol in the lipoprotein-free fraction than did plasma (not shown). Within the HDL size range the distributions of UC, CE, and PL were shifted toward larger particles (Fig. 1). The same was true for apoA-I and apoE, and for apoA-I in lipoprotein A-I (LpA-I) without A-II particles (**Fig. 2**). The distribution of

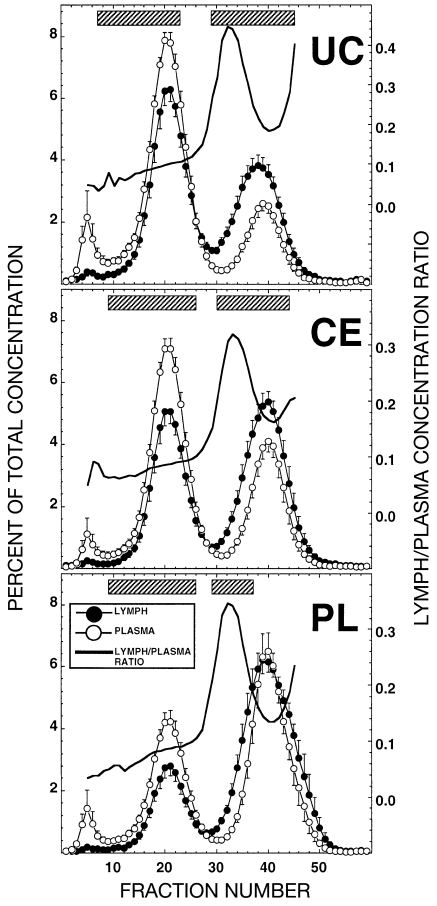

Fig. 1. Distributions of unesterified cholesterol (UC), cholesteryl esters (CE), and phospholipids (PL) in size subfractions of plasma (open circles) and lymph (solid circles) after high performance size-exclusion chromatography (HP-SEC) through Superose 6. Average results (with SEM) from 10 subjects are shown. The continuous lines represent the lymph/plasma (L/P) ratios in corresponding fractions. The shaded areas encompass those values that were significantly different at $P < 0.05$. SEM bars that are not visible are within the dimensions of the symbols.

apoA-II was also shifted toward larger particles, although to a lesser degree than apoA-I (**Fig. 3**). Throughout the size range extending from large HDL to small LDL, a substantial increase in UC/CE ratio was evident in lymph relative to plasma, the peak ratio coinciding with the nadir of total cholesterol in this region (**Fig. 4**).

In both plasma and lymph, apoA-IV was contained in two populations of particles: a major population that was mostly the size of or smaller than albumin, corresponding to lipid-poor or lipid-free apolipoprotein; and a minor population of larger particles (Fig. 3). The ratio of small to large particles was greater in lymph than in plasma. The majority of apoB in lymph was in LDL-sized particles, the size distribution of which did not differ consistently from that of plasma LDL (Fig. 3). More apoC-III coeluted with apoA-I and less with apoB in lymph compared with plasma (Fig. 3). A small amount of apoC-III in lymph eluted in fractions corresponding to lipid-free or lipid-poor apolipoprotein.

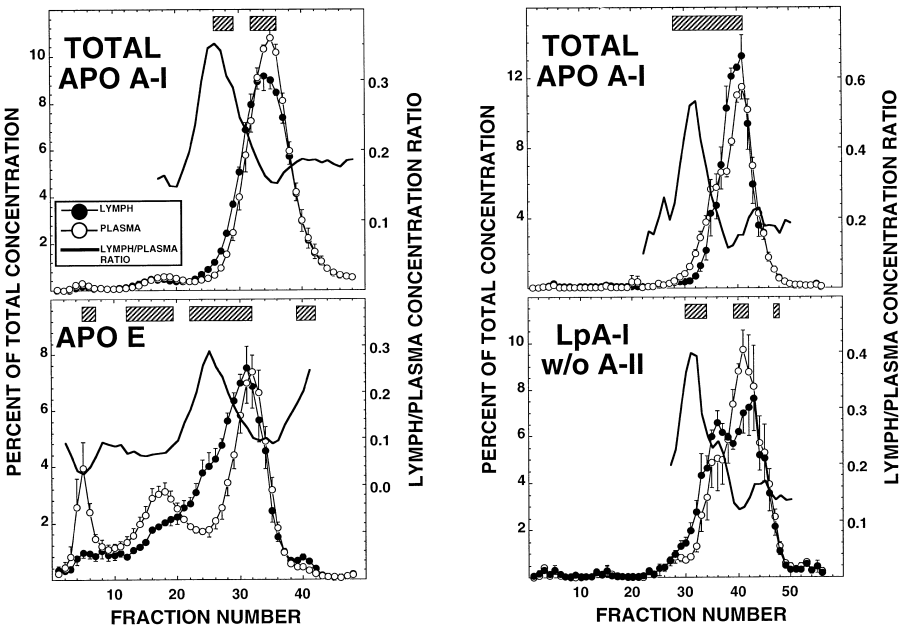

Fig. 2. Distributions of total apolipoprotein A-I (apoA-I) and apoE in three subjects (left) and of total apoA-I and apoA-I in LpA-I without A-II particles in another three subjects (right) after Superose 6 HP-SEC of lymph (solid circles) and plasma (open circles). Ave rage results (with SEM). The continuous lines represent the L/P ratios for corresponding fractions. The shaded areas encompass those values that were significantly different at $P < 0.05$. SEM bars that are not visible are within the dimensions of the symbols.

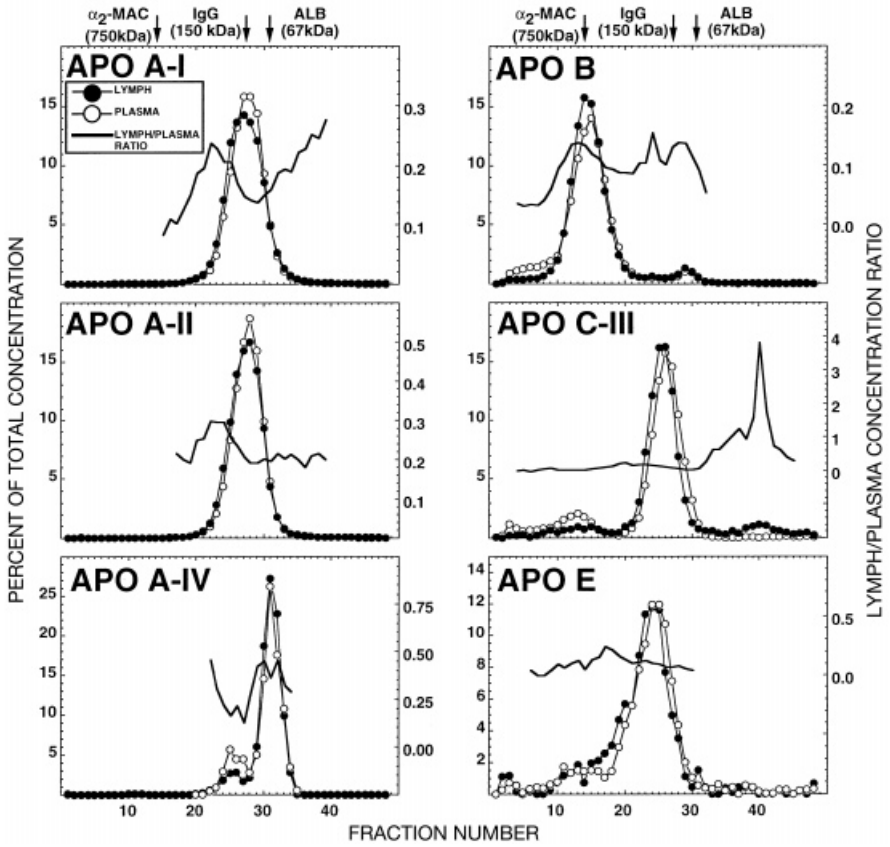

Fig. 3. Distributions of apoA-I, -A-II, -A-IV, -B, -C-III, and -E in size subfractions of plasma (open circles) and lymph (solid circles) after HP-SEC through Superose 6. Results are from a single subject. The continuous lines represent the L/P ratios in corresponding fractions. α_2-MAC, α_2-macroglobulin; IgG, immunoglobulin G; ALB, albumin.

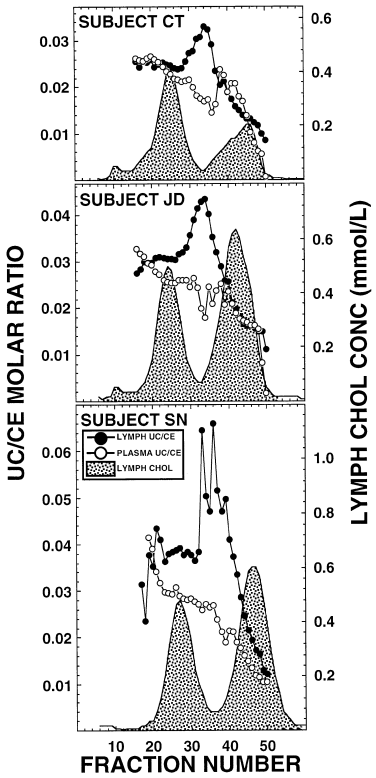

Fig. 4. Unesterified cholesterol-to-cholesteryl ester ratios in size subfractions of lymph (solid circles) and plasma (open circles) separated by HP-SEC through Superose 6. The shaded areas represent the distribution profiles of total cholesterol concentration in the samples of lymph for comparison. Results are from three different subjects.

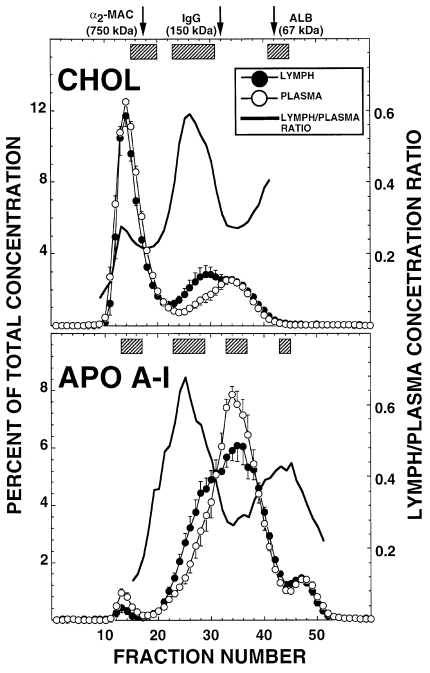

Fig. 5. Distributions of total cholesterol and apoA-I in fractions of plasma (open circles) and lymph (solid circles) separated by HP-SEC through Superdex 200 and 75 in series. Results (with SEM) represent the average of four subjects. The continuous lines represent the L/P ratios in corresponding fractions. The shaded areas encompass those values that were significantly different at $P < 0.05$. SEM bars that are not visible are within the dimensions of the symbols.

Cholesterol and apoA-I in Superdex subfractions

The distributions of cholesterol and apoA-I were also examined by HP-SEC through Superdex 200 and 75 in series, which separates the apoA-I-containing particles of plasma into three subclasses (14). As shown in **Fig. 5**, the apoA-I of lymph was also separated into three subclasses. The smallest particles (fractions 42–56), which in plasma are a mixture of lipid-free and lipid-poor apoA-I (14), contained no detectable cholesterol, and had the same size distribution as in plasma. Within the major population of apoA-I-containing particles, which in plasma are CE-rich α HDL, there was a major difference between the two matrices, proportionately more apoA-I being found in the largest particles in lymph (Fig. 5). An even greater shift was observed for cholesterol (Fig. 5).

Net uptake of cholesterol by HDL in tissue fluid

The method used to estimate net cholesterol uptake by HDL in tissue fluid was based on the results obtained by Superdex HP-SEC (which has greater resolving power than Superose over this size range), and is illustrated in **Fig. 6**. In Fig. 6 (top) the open symbols give the L/P ratios of total protein in the various chromatographic fractions. These were determined by UV spectrophotometry, but identical results were obtained by a Coomassie dye-binding method (not shown). The closed symbols in Fig. 6 (top) give the observed total cholesterol concentrations in the various plasma fractions. In Fig. 6 (bottom) the open symbols are the observed concentrations of total cholesterol in the fractions of lymph, and the closed symbols are the estimates of the cholesterol concentrations (calculated from the results in Fig. 6, top) that would have been present if filtration of HDL from plasma had been the only source of cholesterol. In four of the five subjects the observed cholesterol concentrations in these fractions were greater than could be explained by filtration of plasma HDL. Most of the excess cholesterol was in the largest HDL. For each subject a perpendicular was dropped from the nadir of the observed cholesterol concentrations in lymph (at fraction 14 or 15). Our earlier studies had shown that only a trace of apoA-I eluted to the left of this point. For each matrix the cholesterol in fractions 14 or 15 to fraction 40 inclusive was summated to give the total cholesterol in these particles (shaded areas in Fig. 6). The difference between the values in lymph and plasma was then multiplied by the flow rate in the vessel to give the net transport rate of new cholesterol by lymph HDL (**Table 2**).

Ambient versus low temperature collection of lymph

To determine whether the lipoproteins in lymph might have been altered as a consequence of cholesterol esterification and/or lipid transfers ex vivo during the collection, in four subjects lymph was collected from the same vessel into two tubes, one at ambient temperature and the other at 0–4°C, and the samples were analyzed by Superose 6

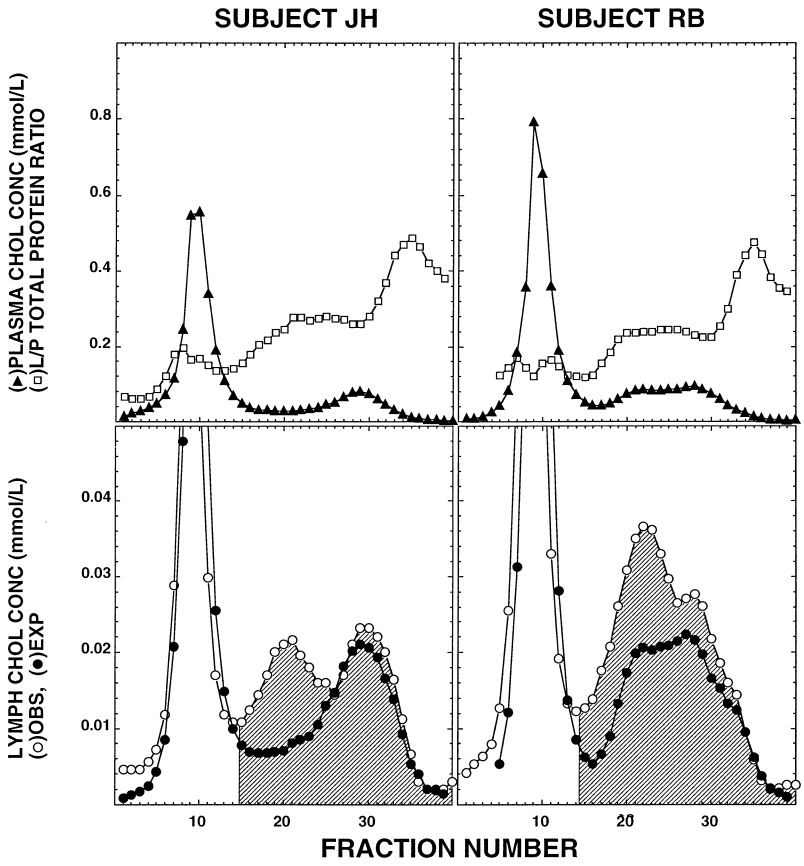

Fig. 6. Estimation of the net uptake of cholesterol mass by high density lipoproteins (HDL) in tissue fluid. Results of two representative subjects are shown. Top: L/P ratios of total protein (open squares) and cholesterol concentrations in plasma fractions (solid triangles). Bottom: expected cholesterol concentrations in lymph fractions (calculated from the data in top panel) (solid circles); observed cholesterol concentrations in lymph fractions (open circles). The shaded areas indicate the fractions that were used for the calculation of total net cholesterol uptake by tissue fluid HDL.

HP-SEC. The temperature of collection had no effects on UC/CE ratio or on the distribution profiles of total protein, UC, CE, or total glycerol (not shown).

Ultrastructure

Electron micrographs of pooled Superose 6 HP-SEC fractions of lymph are shown in **Fig. 7**. Small numbers of discoidal particles were evident in all pools except the one that contained the medium-sized and small HDL (pool D). The relative numbers of discoidal and spheroidal particles did not appear to differ greatly between pools A, B, and C. However, an accurate count was not performed, because the number of discs in each pool would be underestimated by the fact that some (probably most) are inevitably seen en face. As expected, no discoidal particles were seen in any images of the pooled fractions of plasma (not shown).

TABLE 2. Calculation of net cholesterol uptake by HDL in tissue fluid of five normal males

Subject	Observed Total Lymph HDL Cholesterol	Predicted Total Lymph HDL Cholesterol	Observed Minus Predicted	Lymph Flow Rate	Net Cholesterol Uptake By HDL in Tissue Fluid
	μM	*μM*	*μM*	*ml/h*	*μmol/h*
D.E.	400	451	−51	1.0	−0.051
A.B.	392	335	57	3.3	0.188
J.H.	375	245	130	1.0	0.130
R.B.	493	315	178	2.6	0.463
J.M.	516	383	133	0.4	0.053
Mean ± SD	435 ± 64	346 ± 77	89 ± 90	1.7 ± 1.2	0.16 ± 0.19

By both the paired *t*-test and the Wilcoxon test, the results for observed lymph HDL cholesterol concentration were significantly greater than the predicted concentrations ($P = 0.045$).

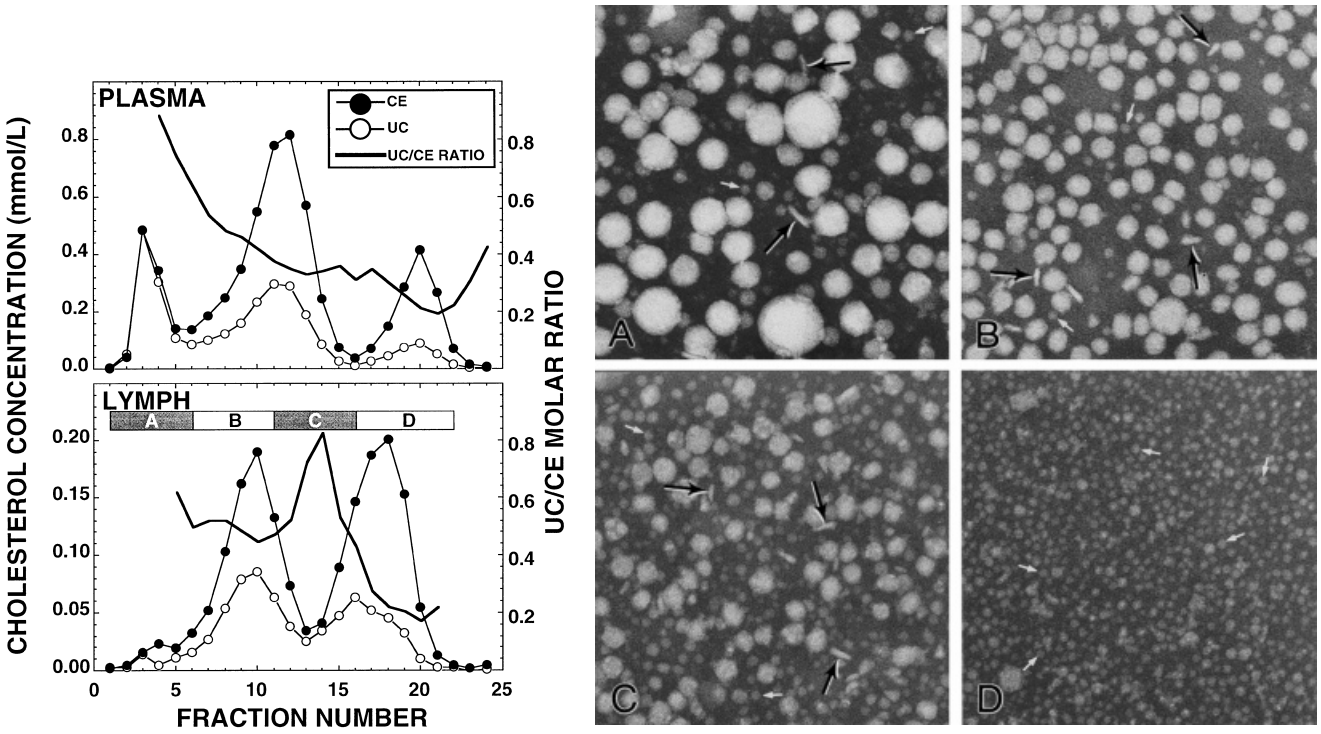

Fig. 7. Right: Electron micrographs of negatively stained human lymph d < 1.21-g/ml lipoproteins separated into four major fractions (A–D) by Superose 6 HP-SEC. Left: Elution profiles of UC concentration, CE concentration, and UC/CE ratio in corresponding fractions of plasma and lymph. Fraction A is composed largely of electron-lucent particles 27–60 nm in diameter, characteristic of apoB-containing triglyceride-rich lipoproteins. A few discoidal particles are present (black-on-white arrows in micrograph A). Fraction B is composed mostly of particles 22–33 nm in diameter, typical of apoB-containing remnants, together with considerable numbers of same-diameter discoidal particles, a few of which fortuitously stand on edge (black-on-white arrows in micrograph B). Fraction C consists of a mixture of particles ~17–33 nm in diameter, typical of LDL, and discoidal HDL together with about an equal number of HDL-sized particles (white arrows in micrograph C). Fraction D is composed largely of particles that closely resemble those typical of plasma HDL, ranging in diameter from about 7 to 12 nm. Note that HDL-sized particles (~10 nm) are present in this fraction, as well as in the background of the apoB-containing fractions A–C (white arrows in micrograph D). Original magnification: ×180,000.

DISCUSSION

The components of lymph lipoproteins are derived mostly from plasma lipoproteins, which cross endothelium by filtration (1, 5, 17, 18) and possibly also by transcytosis (19, 20). Both processes favor small over large particles (16, 18, 21). This explains the overall positive association between HP-SEC fraction number and the L/P ratio of total protein concentration. Once outside the intravascular space, lipoproteins are likely to undergo remodeling as a consequence of interactions with cells, lipid transfer proteins, and enzymes. Previous work has shown that considerable remodeling occurs in the tissue fluids of animals (1–5, 15, 22–24). However, much remains to be learned about the mechanisms involved and the extent to which those results apply to humans.

Reichl and co-workers (6–9, 25) studied human foot lymph lipoproteins, but their studies were usually limited to a few subjects, who often had hyperlipidemia, lymphedema, or postphlebitis syndrome. As the last two conditions raise both endothelial permeability and the extravascular residence time of macromolecules (10), lymph lipoproteins in such patients are likely to differ from normal lipoproteins. Apart from one study in lymphedema patients (9), the work of Reichl's group on the composition of size subclasses was limited to gradient gel electrophoresis of prestained lymph, or to measurements of apoA-I with or without apoA-II or cholesterol in gel-filtration fractions (6–8, 25, 26). No data were reported on the size distributions of apoA-IV, apoB, apoC-III, apoE, TG, or PL in normal lymph, and no ultrastructural studies of normal lymph lipoproteins were reported.

Using ultracentrifugation, Hong, Pflug, and Reichl (27) showed that human lymph lipoproteins are predominantly non-apoB-containing species, presumably owing to the effect of size on transendothelial transport (10, 17, 18). We have also found that the apoA-I/apoB ratio is greater in lymph than in plasma (11), and in the present study have confirmed that VLDL are present only in low concentration. As the size distribution of LDL apoB in lymph did not differ consistently from that in plasma, we found no evidence that the smallest LDL are preferentially transferred across endothelium. However, our Superose 6 HP-SEC procedure is unlikely to have had sufficient resolving power to detect a small difference in LDL size distribution.

The most significant findings of our study relate to the distributions of lipids and apolipoproteins in particles smaller than LDL. As in plasma, most apoA-I was in sphe-

roidal HDL. Within this size range, in lymph compared with plasma, proportionately more apoA-I eluted in the fractions that contained the largest particles. This was particularly evident in the case of apoA-I in LpA-I without A-II particles, and was also apparent for apoA-II and apoE. Shifts in distribution toward larger particles were also seen for UC, CE, and PL in that rank order, giving the largest HDL much greater UC/CE ratios than in plasma. Our experiments, in which lymph was collected at ambient temperature and at 0–4°C, showed that these differences between plasma and lymph were unlikely to have been attributable to remodeling of particles ex vivo. Reichl and co-workers (6–8, 25, 26) also found that the distributions of cholesterol, apoA-I, and apoA-II in normal lymph were shifted toward larger HDL but did not quantify UC, CE, PL, or apoE. In lymphedema fluid Reichl et al. (9) found that all apoE and more than 25% of apoA-I was in particles larger than plasma HDL.

On electron microscopy of the d 1.019- to 1.085-g/ml fraction, Reichl et al. (9) found that lymphedema fluid contained square-packing particles, a feature indicative of enrichment with surface polar lipids (28). Occasional discoidal particles, presumably devoid of core lipids, were also seen in addition to spheroidal HDL. When we fractionated the d < 1.21-g/ml fraction of lymph through Superose 6 and examined pooled fractions by electron microscopy, most particles were spheroidal. No square-packing particles were seen, but all pools except that containing the medium-sized and small HDL were seen to contain discs. Although these were relatively few in number, their presence is significant as similar particles were never seen in any fraction of plasma, and their numbers are underestimated as fewer of them are likely to stand on edge compared with en face. This is the first demonstration of discoidal lipoproteins in normal human peripheral tissue fluid.

Dory et al. (15) reported that, of two HDL fractions isolated by SEC from peripheral lymph of cholesterol-fed dogs, the larger particles were mostly discs. These contained large amounts of apoE and UC, in addition to apoA-I and apoA-IV. Sloop et al. (23, 29) had earlier demonstrated the presence of discoidal HDL in the 1.063–1.21 g/ml density range of canine lymph, and had shown that these increased in number when the animals were fed cholesterol. These discoidal particles were converted to CE-rich spheroidal HDL when incubated in vitro with LCAT (30), confirming the prediction of Hamilton et al. (31) that nascent discoidal HDL represent a preferred substrate for LCAT that converts them into spheroidal particles. Forte et al. (24) have shown that sheep lung lymph contains both square-packing particles and discoidal HDL rich in UC and apoE.

Thus, our results are consistent with the notion that in normal human tissue fluids particles are produced that are similar in size to the large HDL of plasma and are rich in cell-derived cholesterol. LpA-I without A-II particles, apoA-II-containing particles, and apoE-containing particles all appear to participate in this process. At least some of these particles have high UC/CE ratios, and at least some are discoidal. On the basis of previous work (32–34), it is likely that at least a proportion of the discs are produced from lipid-free apoA-I and small lipid-poor pre-β_1 HDL that have acquired cell-derived PL and UC in tissue fluid, which is an environment of low LCAT activity (30, 35) (M. N. Nanjee, J. C. Cooke, W. L. Olszewski, and N. E. Miller, unpublished observations). Other discs may contain apoE derived from local synthesis in peripheral tissues (22, 36). Further work, using immunoaffinity chromatography and other separation procedures, will be needed to identify the lipoprotein species involved.

We found that lymph also contained abundant apoA-I-containing particles of 40–60 kDa, resolved by Superdex HP-SEC but not by Superose 6. These were essentially identical in size to their counterparts in plasma, where they are a mixture of lipid-poor pre-β_1 HDL and lipid-free apoA-I (14). Reichl et al. (7) described similar particles in human foot lymph, but on other occasions detected none (8, 25). Lefevre, Sloop, and Roheim (34) have demonstrated lipid-free apoA-I in canine lymph, and Asztalos et al. (37) showed that canine lymph contains multiple subclasses of partially lipidated apoA-I-containing species. Castro and Fielding (33) have demonstrated that similar particles function as the primary acceptors of UC when cultured fibroblasts are exposed to plasma, but no similar experiments with tissue fluid have been reported.

There are no published data on the size distribution of apoA-IV in human peripheral lymph. Using immunoaffinity chromatography, Duverger et al. (38) identified two populations of apoA-IV-containing particles in both suction blister fluid and plasma from humans: a major population of LpA-IV and a minor population of LpA-I:A-IV particles. No differences between the two matrices were observed in the relative concentrations of the two types of particle. However, the composition of suction blister fluid is unlikely to be representative of normal tissue fluid, as it is the product of an unphysiologic increase in the transport of water and solutes across capillary endothelium (39). Duverger et al. found that the LpA-IV particles of plasma were composed of three populations in the VLDL, LDL, and HDL size ranges. Those in the HDL range contained two overlapping subpopulations, one the size of and the other smaller than HDL_3. No similar data were presented for blister fluid. In the present study we found that in both lymph and plasma apoA-IV was confined to two size subfractions: a minor population within the HDL size range, and a major population of smaller particles. The size of the latter was consistent with their being lipid-free and/or lipid-poor apoA-IV. Lymph contained proportionately more of the smaller species, possibly attributable to the sieving effect of endothelium. In contrast, canine peripheral lymph HDL have been shown to be enriched in apoA-IV relative to plasma HDL, particularly during cholesterol feeding, when much of the apoA-IV was in discs (15, 23, 29).

By combining the data on total protein and cholesterol concentrations in Superdex fractions, we found that lymph HDL and, more specifically, the larger particles carried more cholesterol than could be explained by filtration of plasma HDL across endothelium. Our calculation

of the mass of new cholesterol carried by lymph HDL was based on four assumptions. One was that plasma HDL of different sizes cross endothelium at the same rates as plasma proteins of the same size. Although no studies of the transendothelial transport of lipoproteins have been described, the rates of appearance in lymph of macromolecules after their intravenous infusion into animals have shown that molecular size is a major determinant (40–43). This underlies the inverse association that exists between the L/P ratios of endogenous protein concentrations and their molecular weights (10, 17, 18). We have shown that this relation holds over the size range of HDL in humans (11).

Another assumption was that all HDL return to plasma from tissue fluids via lymph, not via venous capillaries. This accords with our current understanding of the general behavior of macromolecules (39, 44). Thus, when lactate dehydrogenase (140 kDa) was released from skeletal muscle by electrical stimulation in dogs, no arteriovenous difference in enzyme concentration developed, even though the concentrations in leg lymph were 7-fold those in plasma (45).

A third assumption was that peripheral cells do not catabolize apoA-I-containing particles. Tissue culture studies have shown that HDL without apoE are taken up by peripheral cells only via the relatively slow processes of nonspecific fluid and adsorptive endocytosis (46). Although studies in vivo, using HDL tagged with nonbiodegradable markers, have documented the accumulation of some radioactivity in the peripheral tissues of rats (47) and monkeys (48), the amounts were small compared with those in liver and kidney. Furthermore, at least some of the tissue radioactivity in those experiments is likely to have been that of undegraded HDL trapped in the extravascular space.

Our fourth assumption was that the chromatographic fractions used for the calculation included all the HDL that carried additional cholesterol. This was probably not always absolutely true, as traces of apoA-I were sometimes found in the adjacent fractions that contained small LDL. Thus, our calculations may have slightly underestimated the true net uptake of cholesterol by HDL in tissue fluid.

When we incubated lymph for 24 h at 37°C in vitro with LCAT inhibited by iodoacetate, HDL cholesterol concentration did not change (M. N. Nanjee and N. E. Miller, unpublished observations).Thus, it seems likely that there is little or no mass transfer of UC or CE between HDL and other lipoproteins in tissue fluid, and that the extra cholesterol in lymph HDL is derived from cells. Published evidence that human lymph HDL transport cholesterol from tissues is limited to a report that the specific radioactivity of lymph HDL cholesterol exceeded that of lymph LDL cholesterol in four subjects given radiolabeled cholesterol several weeks earlier (49). However, that result might merely have reflected preferential exchange of UC between HDL and cells. Therefore, our data provide the first direct confirmation that HDL mediate reverse cholesterol transport in human tissue fluid, although they do not identify the extent to which native plasma-derived HDL and HDL species that are produced in tissue fluid contribute to the process.

If the total flow rate of prenodal peripheral lymph in our subjects is assumed to have been about 8 l/d (5, 18, 43), our data correspond to a mean value for whole body reverse cholesterol transport by HDL via lymph of 0.89 mmol (344 mg)/day, which corresponds to about 40% of cholesterol turnover in humans. Taking into account the probability that there are regional and diurnal variations in cholesterol homeostasis in peripheral tissues, this is close to indirect estimates of whole body reverse cholesterol transport in humans (50, 51).

This work was supported by grants from the British Heart Foundation, the Joint Research Board of St. Bartholomew's Hospital, and the Royal Society to N.E.M., and from the National Institutes of Health to J.S.W. and R.L.H. (NIH R01 HL60844). The authors thank Professor J-C. Fruchart (Lille) for providing antiserum to human apoA-IV, and Drs. M. Rosseneu (Brugge) and N. Duverger (Paris) for providing pure human apoA-IV.

Manuscript received 8 August 2000 and in revised form 18 December 2000.

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