Human lymphedema fluid lipoproteins: particle size, cholesterol and apolipoprotein distributions, and electron microscopic structure[1]

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Abstract The concentration of cholesterol, apolipoproteins A-I, B, and E has been determined in lymphedema fluid from nine patients with chronic primary lymphedema. The concentrations were: 38.14 ± 21.06 mg/dl for cholesterol, 15.6 ± 6.17 mg/dl for apolipoprotein A-I, 7.5 ± 2.8 mg/dl for apolipoprotein B, and 1.87 ± 0.50 mg/dl for apolipoprotein E. These values represent 23%, 12%, 6%, and 38% of plasma concentrations, respectively. The ratio of esterified to unesterified cholesterol in lymphedema fluid was 1.46 ± 0.45. Lipoproteins of lymphedema fluid were fractionated according to particle size by gradient gel electrophoresis and by exclusion chromatography. Gradient gel electrophoresis showed that a majority of high density lipoproteins (HDL) of lymphedema fluid were larger than ferritin (mol wt 440,000) and smaller than low density lipoproteins (LDL); several discrete subpopulations could be seen with the large HDL region. Fractionation by exclusion chromatography showed that more than 25% of apolipoprotein A-I and all of apolipoprotein E in lymphedema fluid was associated with particles larger than plasma HDL_2. Apolipoprotein A-I also eluted in fractions that contained particles the size of or smaller than albumin. Isolation of lipoproteins by sequential ultracentrifugation showed that less than 25% of lymphedema fluid cholesterol was associated with apolipoprotein B. The majority of apolipoprotein A-containing lipoproteins of lymphedema fluid were less dense than those in plasma. Ultracentrifugally separated fractions of lipoproteins were examined by electron microscopy. The fraction d < 1.019 g/ml contained little material, while fraction d 1.019–1.063 g/ml contained two types of particles: round particles 17–26 nm in diameter and square-packing particles 13–17 nm on a side. Fractions d 1.063–1.085 g/ml had extensive arrays of square-packing particles 13–14 nm in size. Fractions d 1.085–1.11 g/ml and fractions d 1.11–1.21 g/ml contained round HDL, 12–13 nm diameter and 10 nm diameter, respectively. Discoidal particles were observed infrequently.—**Reichl, D., T. M. Forte, J-L. Hong, D. N. Rudra, and J. Pflug.** Human lymphedema fluid lipoproteins: particle size, cholesterol and apolipoprotein distributions, and electron microscopic structure. *J. Lipid Res.* 1985. **26:** 1399–1411.

Supplementary key words reverse cholesterol transport

Transport of cholesterol from cells of peripheral tissues to the liver is usually referred to as "reverse cholesterol transport" (1). The first steps of reverse transport take place at or near cell surfaces in the interstitium of tissues. Hence, it is difficult to study them directly in vivo. However, studies in vitro have shown that sustained efflux of cholesterol mass from cells of mammalian peripheral tissues takes place only in the presence of suitable acceptors of cholesterol in the medium (2). Plasma high density lipoprotein (HDL) (1) and complexes of phospholipids with apolipoproteins of HDL have proven to be the most efficient acceptors in vitro (3–6).

Several lines of evidence show that in vivo HDL are intimately linked with reverse cholesterol transport in man. Firstly, Nestel and Miller (7) have shown that in subjects undergoing extensive weight loss by caloric restriction, HDL is the predominant carrier of tissue-derived cholesterol in plasma. Secondly, patients deficient in HDL or HDL metabolism (i.e., familial lecithin:cholesterol acyltransferase deficiency; familial apolipoprotein (apo) A-I and C-III deficiency; and apoA-I, C-III, and D deficiency) are prone to premature atherosclerosis related to defective removal of cholesterol from cells (1, 8, 9). Thirdly, we have previously shown that HDL is involved with early steps of reverse transport that take place in tissues by analyzing peripheral prenodal lymph, a fluid whose composition reflects that of interstitial fluids (10). In normal subjects whose tissue cholesterol was prelabeled, we could show that radioactive cholesterol is transferred from tissues of the foot preferentially to HDL of lymph draining those tissues (11.) Subsequent studies have shown that lymph HDL differ from their plasma counterparts in that size distribution was shifted towards larger diameters when compared to plasma HDL (12, 13).

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

[1]This work was presented in part at the 38th Annual Meeting of the American Heart Association, 1984.

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The nature and function of these large HDL (2) of human peripheral lymph are not known. It has recently been demonstrated that dog peripheral lymph contains numerous large discoidal HDL particles (14). Sheep lung lymph has been shown to contain a unique, large apoA-I and apoE-containing particle that forms square-packing arrays when examined by electron microscopy (15). The physico-chemical properties of human lymph lipoproteins, however, are not well understood and such information could provide insights into the metabolism of lipoproteins in the extravascular compartment. Sample size of human peripheral lymph is usually less than 1 ml and thus does not lend itself to extensive analysis; however, lymphedema fluid, which in many respects is comparable with that of lymph, can easily be obtained from patients with primary lymphedema, which is a form of chronic edema with no known cause outside the lymphatic system (16). The latter source of extravascular fluid was used in the present study in order to determine lipoprotein size distribution and morphology as well as cholesterol and apolipoprotein distribution among particles of different sizes. The findings are discussed in terms of their possible bearing on the initial steps of reverse cholesterol transport.

METHODS

Samples of lymphedema fluid and plasma

Lymphedema fluid was obtained from eight women and one man in the course of staged operations on lymphedematous legs (16). Dynamic venography and measurements of ambulatory venous pressure before the operation showed normal values in all patients of this study. Apart from their chronic primary lymphedema, all subjects were apparently healthy at the time of the operation. The procedure was the same as described previously (17). Preoperatively the leg was elevated and an Esmarch bandage was applied under maximal pressure in order to empty the vascular bed as much as possible; the bloodless field was maintained by a Kiddes tourniquet, insufflated 50–80 mm above arterial pressure. The excised tissue of the patients showed no signs of inflammation as judged by light microscopy performed by Prof. N. Huth (Rosenheim, Germany).

Only the clear fluid that accumulated spontaneously in the incision was aspirated with a syringe and transferred into centrifuge tubes that contained 1 mg disodium EDTA and 1 mg of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma Chemical Co., St. Louis, MO) for each ml of the fluid. Cell counting revealed less than 10,000 erythrocytes/μl. The samples were passed through Millipore (2μ pore size) filters and stored in stoppered plastic vials at 4°C until used for analyses. On average, 15 ml of lymphedema fluid was obtained during each operation. Blood was obtained from the cubital vein in the course of the operation; 1 mg of disodium EDTA/ml was added and samples were centrifuged at 5000 *g* and 4°C for 15 min. Plasma was separated and 1 mg of DTNB was added for each ml.

Molecular sieve chromatography

Lymphedema fluid and plasma were fractionated by gel chromatography on a 90 × 1.8 cm column of 10% agarose (Bio-Gel A-0.5 m, Bio-Rad Laboratories, Richmond, CA) using 0.15 M NaCl-0.01 M phosphate, pH 7.2, as equilibrating and running buffer. The column was operated at 4°C and 8 ml/hr flow rate. Fractions of 1.8 ml were taken. Two ml of lymphedema fluid or of plasma that had been diluted fivefold with the equilibrating buffer was applied. The column was calibrated with human plasma low density lipoprotein (LDL) and high density lipoproteins 2 and 3 (HDL_2 and HDL_3) isolated by ultracentrifugation and with crystallized bovine serum albumin (Miles Laboratories Ltd., Elkhart, IN) (**Fig. 1**). Relevant constituents were assayed either in each column fraction or in a pool of two consecutive fractions.

Ultracentrifugation procedures

Sequential ultracentrifugation of lymphedema fluid and of plasma that had been diluted fivefold with the equilibrating buffer was carried out at 10°C at 43,000 rpm in a Beckman L65 ultracentrifuge using an SW51 rotor essentially according to Glomset, Norum, and King (18). Prior to ultracentrifugation, both of the fluids were adjusted to a background density of 1.019 g/ml by addi-

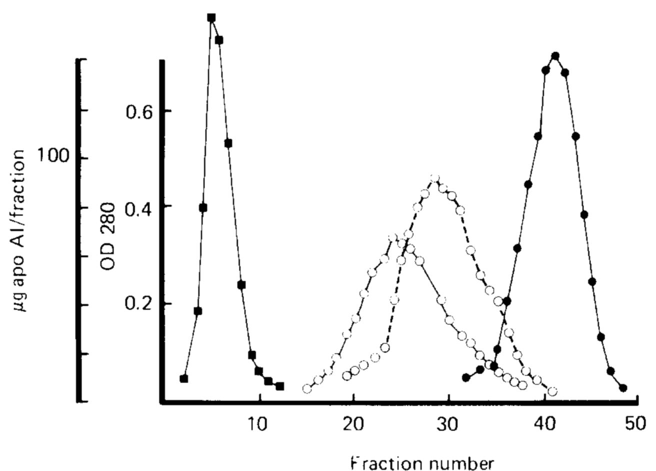

Fig. 1. Exclusion chromatography of plasma and serum albumin in a Bio-Gel A-0.5m column. Isolated human plasma LDL (d 1.019–1.063 g/ml, 5 mg of protein) ■—■; HDL_2 (d 1.085–1.10 g/dl, 1 mg of apoA-I) ○—○; HDL_3 (d 1.10–1.21 g/dl, 1 mg of apoA-I) ○- - -○; and crystallized bovine serum albumin (10 mg) ●—● were applied in separated runs. Elution patterns of LDL and albumin were monitored in terms of absorbance at 280 nm, and HDL_2 and HDL_3 in terms of apoA-I concentration. Elution buffer: 0.15 M NaCl-0.01 M phosphate, pH 7.2.

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tion of KBr. The following density fractions were obtained by sequential flotation: <1.019 g/ml, 1.063 g/ml, 1.085 g/ml, 1.10 g/ml, and 1.21 g/ml. The first two density fractions were obtained after 18 hr centrifugation and the last two after 40 hr. The fractions were washed as previously described (18). They were used for analyses directly or after dialysis against the equilibrating buffer.

Cholesterol and apolipoprotein analyses

Cholesterol was determined enzymatically (19), using commercial reagents: Human (Taunusstein-Neuhof, GFR) for the determination of total cholesterol and Boehringer Mannheim (Mannheim, GFR) for unesterified cholesterol. The concentrations of total and unesterified cholesterol in body fluids were determined directly as recommended by the manufacturers. Column fractions were first extracted with chloroform–ethanol 1:2 (v/v) according to Folch, Lees, and Sloane Stanley (20), taken to dryness, and dissolved in a measured volume of isopropanol. In this way, samples were usually concentrated tenfold. Aliquots of lipids in isopropanol were added to commercial reagents; crystalline cholesterol (Sigma Chemical Co.) dissolved in isopropanol was used as standard for this colorimetric determination. Apolipoprotein B (apoB) was quantified immunoelectrophoretically in the biological fluids and column fractions as described previously (21). Albumin was quantified immunoelectrophoretically using commercial antiserum (Behringwerke A.G. Marburg, GFR). For the determination of apolipoprotein A-I (apoA-I) and apolipoprotein E (apoE), samples were first delipidated. This served two purposes. Firstly, most samples of fractionated body fluids had to be concentrated before apoE could be determined. Preliminary experiments have shown that best results were obtained by precipitating apoproteins with ethanol–ether. Secondly, following delipidation, apoA-I of body fluids becomes comparable in its immunochemical properties to those of the standard (12). Therefore, all samples were delipidated by adding to them a tenfold volume of ethanol–diethylether 3:1 (v/v). One mg of crystallized bovine serum albumin (Miles, USA) was added to each sample as carrier for the precipitating constituents. The resulting precipitates were centrifuged at 4°C and 3000 *g* for 10 min, the supernate was decanted, the sediments were resuspended in diethylether and centrifuged under the above conditions, and the supernate was decanted. The precipitates were dried under a stream of nitrogen and dissolved in a known volume of 0.15 M NaCl that was made to 2% (w/v) in respect to Nonidet P40 (BDH, Poole, England). In this way, samples were concentrated approximately tenfold. The respective apolipoproteins were determined immunoelectrophoretically: apoA-I as previously described (12); apoE according to Curry et al. (22) using antibodies that were a gift from Dr. Patsy Wang-Iverson, Mount Sinai Medical Center, New York, NY; and as a standard, apoE that was a gift from Dr. A. Sutar, London. Concentrations were determined by measuring rocket height. The lowest part of the linear concentration-peak height relation was at 0.2 mg/dl for apoA-I and at 0.8 mg/dl for apoE. Recoveries of standard added to samples before delipidation were 86% ± 5 (n = 4) for apoA-I and 83% ± 7 (n = 4) for apoE.

Polyacrylamide gradient gel electrophoresis

Electrophoresis of unfractionated plasma and lymphedema fluid was carried out in precast polyacrylamide gels of linear concentrations (2–16% and 4–30% polyacrylamide, Pharmacia, Uppsala, Sweden) essentially as previously described (13). Samples of lymphedema fluid and plasma were prestained with a 1/10 volume of saturated Sudan black in 1,2-propanediol before application to the gels.

Polyacrylamide gradient gel electrophoresis of isolated fractions of lymphedema fluid and plasma was carried out in Berkeley. Particles of d < 1.063 g/ml were electrophoresed on 2–16% Pharmacia precast gradient gels, while particles with d > 1.063 g/ml were electrophoresed on 4–30% precast gels (Pharmacia, Piscataway, NJ). Gels were stained for protein with Coomassie blue G-250; following destaining, the gels were scanned with the RFT Scanning Densitometer (Transidyne Corporation, Ann Arbor, MI) at 603 nm. Electrophoretic methods used were those described by Krauss and Burke (23) for 2–16% gels and by Nichols, Blanche, and Gong (24) for 4–30% gels.

Electron microscopy

Samples of ultracentrifugally isolated plasma and lymphedema fluid lipoproteins were packed in ice and air-shipped to Berkeley, CA; less than 24 hr intervened between shipping and receiving of samples. Isolated fractions of plasma and lymphedema fluid were immediately dialyzed to 0.134 M ammonium acetate buffer containing 345 μM EDTA. Negative staining and particle sizing procedures were the same as previously described (25).

RESULTS

The concentration of cholesterol and apolipoproteins in lymphedema fluid and blood plasma

The present results show that the composition of lymphedema fluid obtained from patients with chronic lymphedema differs substantially from that of plasma (**Table 1**). When the concentrations in interstitial fluid are expressed as percentages of respective concentrations in patients' own plasma, then the average of apoB is only

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TABLE 1. Concentration of cholesterol, apoB, apoA-I, and apoE in lymphedema fluid

Patient	Age	Sex	Cholesterol				ApoB		ApoA-I		ApoE	
			Total mg/dl	% of Plasma	UC mg/dl	CE/UC[a]	mg/dl	% of Plasma	mg/dl	% of Plasma	mg/dl	% of Plasma
	yr											
1	43	F	18.2	9.3	8.1	1.24	5.9	5	7.4	6.2		
2	23	F	64.3	47.5	25.4	1.53	5.8	6.6	26.2	19.1		
3	36	F	69.1	42.1	22.1	2.11	6.7	5.7	22.1	19.4	1.5	37.5
4	44	F	15.9	7.2	6.9	1.30	5.5	5.0	7.1	6.0	1.1	24.4
5	34	F	35.7	15.7	10.9	2.27	7.8	6.8	17.3	14.3	1.8	42.5
6	29	F	23.2	13.2	12.4	0.87	5.7	4.7	12.9	10.0	2.6	43.8
7	54	M	58.6	39.3	27.2	1.15	8.9	6.5	15.1	13.6	2.4	41.0
8	32	F	19.0	11.1	8.7	1.37	12.1	9.2	15.8	12.0	1.9	39.6
9	30	F	39.3	27.1	17.1	1.29	9.8	8.6	16.2	12.5	1.8	40.0
Mean ± SD			38.14 ± 21.06	23.6 ± 15.7	15.38 ± 7.83	1.46 ± 0.45	7.5 ± 2.28	6.4 ± 1.58	15.6 ± 6.17	12.5 ± 4.79	1.87 ± 0.50	38.4 ± 6.5

[a]UC, unesterified cholesterol; CE, cholesteryl ester.

6.4% ± 1.58 (Table 1). This is similar to that previously found for the concentration of apoB in peripheral lymph of apparently healthy subjects [7.6% ± 1.89 (21)]. For apoA-I, the average concentration in lymphedema fluid relative to its concentration in plasma was 12.5% ± 4.79 (Table 1), while the concentration of apoE was 38.4% ± 6.5 (Table 1). Relative to plasma concentrations, apoE is the most abundant apolipoprotein in lymphedema fluid. The concentration of albumin in lymphedema fluid relative to plasma, determined in five patients, was 42.2% ± 8.7.

The present results also show that the concentration of cholesterol in lymphedema fluid is much lower than that in plasma (Table 1). It can be seen that there is a considerable variation from patient to patient. We have observed that in patients in whom the excised edematous tissue was to a large extent fibrous, the concentration of cholesterol in the lymphedema fluid was low (7-16%) relative to plasma values (patients 1, 4, 5, 6, and 8). The higher levels of cholesterol in lymphedema fluid of the remaining patients (2, 3, 7, and 9) may have been due to a difference in the histological composition of the edematous tissue, duration of lymphedema, or other as yet unknown processes. Tissue in the latter four patients was mainly subcutaneous adipose tissue with minor signs of fibrosis. The mean ratio of cholesteryl ester to unesterified cholesterol in lymphedema fluid was much lower than in plasma (1.46 ± 0.45 vs 2.77 ± 0.2).

Comparison of native lipoproteins of lymphedema fluid and plasma by gradient gel electrophoresis

The concentrations of apoB and apoA-I in lymphedema fluid in the present study are similar to their concentrations in human peripheral lymph (12, 21). In order to determine whether particle size distributions in lymphedema fluid are similar to those in lymph, Sudan black-prestained lymphedema fluid was electrophoresed on polyacrylamide gradient gels. **Fig. 2** shows representative patterns of prestained lymphedema fluid and corresponding plasma. It can be seen that lipoproteins of lymphedema fluid differ considerably from those of plasma in particle size distribution (Fig. 2A and B). On 4-30% gradient gels (Fig. 2A), lymphedema fluid has several discrete bands in the region between HDL_2 and LDL; these bands are not visible in plasma. Based on the position of the molecular weight marker, ferritin, the intermediate-sized particles are greater than 440,000 molecular weight. A discrete band can also be seen in the small pore region of the gel that migrates above albumin. Plasma lipoproteins stain most dominantly in the HDL_2 region, which is consistent with the greater lipid content of this fraction as compared with HDL_3. Particle size dis-

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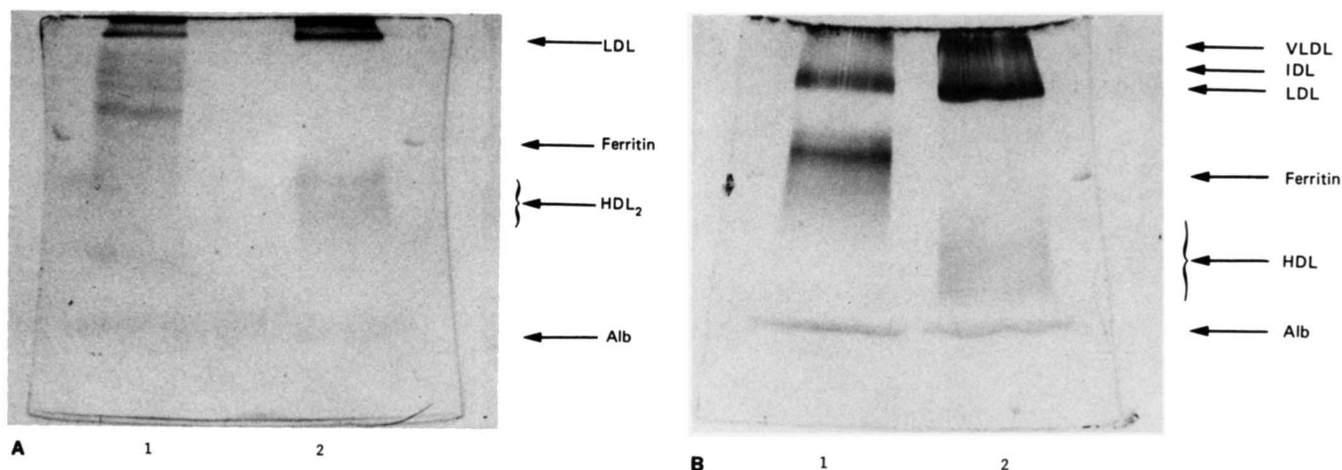

Fig. 2. Representative electrophoretograms of lymphedema fluid and plasma prestained with Sudan black. Note that the dye binds to albumin which functions as an internal size marker (66,000 molecular weight). Lane 1, lymphedema fluid; lane 2, plasma; the marker on both sides of polyacrylamide gels is ferritin. A, Electrophoresis in a 4–30% gel; B, electrophoresis in a 2–16% gel.

tribution on 2–16% gels (Fig. 2B) indicates that considerably less VLDL and IDL is present in lymphedema fluid than in plasma. In lymphedema fluid a major new species of particles is present in the region between LDL and HDL, a finding consistent with that observed in the 4–30% gel. The differences between lymphedema fluid and plasma observed in the present study are similar to those we reported previously for peripheral lymph (11).

Distribution of cholesterol and apolipoproteins among lymphedema fluid and plasma lipoproteins separated by exclusion chromatography

Gradient gel electrophoretograms show a marked difference between lymphedema fluid and plasma lipoproteins in terms of particle size distribution. In order to obtain insight into the distribution of cholesterol and apolipoproteins among these particles, lymphedema fluid and plasma samples from two patients were chromatographed on agarose gel. Recoveries of apolipoproteins and of cholesterol approached 100%. The results of these analyses are shown in **Fig. 3** (A: lymphedema fluid plasma from patient 5 and B: lymphedema fluid and plasma from patient 7). Inspection of Figs. 3A and B shows marked differences between lipoproteins of lymphedema fluid and plasma. ApoA-I of lymphedema fluid elutes from the column predominantly in fractions that contain particles larger than plasma HDL$_2$. This is in good agreement with our previous findings in human peripheral lymph. ApoA-I of lymphedema fluid is also present in fractions that comprise the descending limb of the albumin peak; corresponding fractions of plasma contain only traces of apoA-I. ApoA-I of these small particles can only be demonstrated after delipidation, which suggests that this small molecular weight apoA-I-containing structure is not merely an aggregate of free apoA-I but is probably complexed with lipids that shield it from recognition from antibodies.

The distribution of apoE among lipoproteins of lymphedema fluid is also different from that in plasma. ApoE elutes as a broad peak between the void volume and HDL$_2$ in one patient (Fig. 3A); in the other patient, apoE has a defined peak in the void volume and forms a subsequent broad band to the HDL$_2$ region (Fig. 3B). The pronounced void volume apoE peak in the latter patient may be related to the elevated plasma cholesterol level in this patient. In contrast, plasmas of both patients show two peaks, one in the void volume and the other in the HDL$_2$ region.

The difference between the distribution of cholesterol in lymphedema fluid and plasma is conspicuous; a large proportion of cholesterol in lymphedema fluid elutes from the column with particles that are larger than plasma HDL$_2$ and smaller than LDL (Fig. 3A and B). When the weight ratio of cholesterol to apoA-I is considered in lymphedema fluid lipoproteins, it is apparent that the ratio decreases as particle size decreases. A similar observation was made in peripheral lymph (13). In patient 7, the cholesterol:apoA-I ratio in the largest of apoA-I-containing particles (fraction 14) was 1.1, which is twice that in plasma HDL$_2$ from the same patient (i.e., fraction 20). In patient 5 with elevated lymphedema fluid choles-

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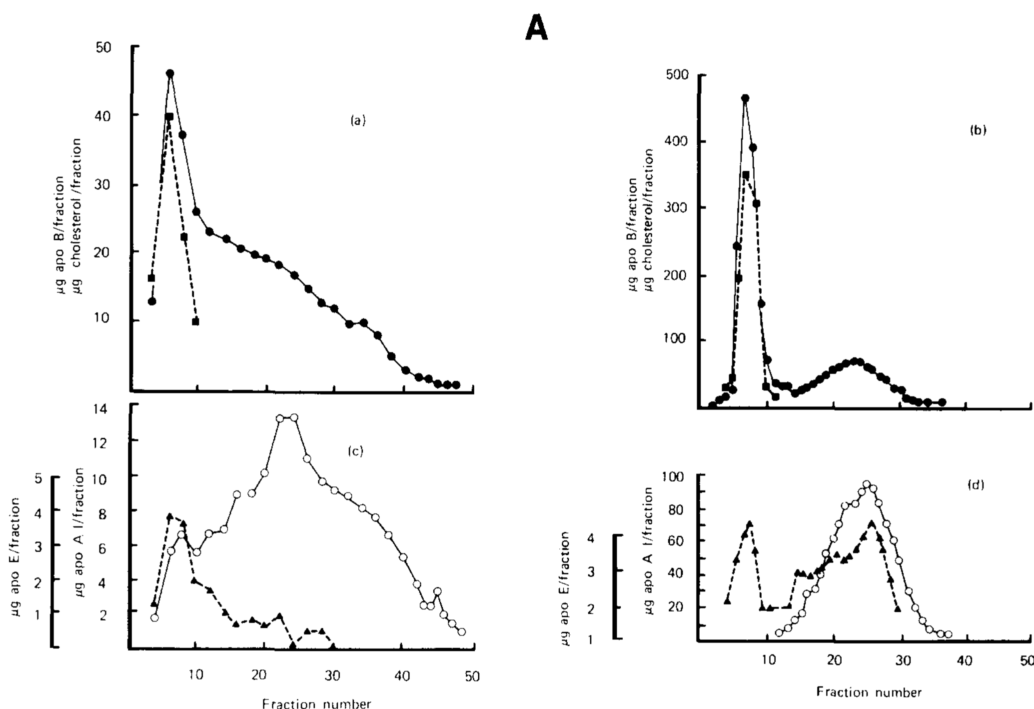

Fig. 3. Exclusion chromatography of plasma and lymphedema fluid in a Bio-Gel A-0.5m column. A: Elution pattern of lymphedema fluid (a and c) and plasma (b and d) from patient 5 (plasma cholesterol 326 mg/dl). Lymphedema fluid was applied directly; plasma was first diluted with elution buffer (as in Fig. 1). Concentration of cholesterol (● — ●), apoB (■- - -■), apoA-I (○ — ○), and apoE (▲- - -▲) is normalized to 1 ml of the respective body fluid.

terol, the cholesterol:apoA-I ratio in fraction 14 was 3.1, indicating that lymphedema fluid particles of comparable size can differ considerably in their load of cholesterol in relation to apoA-I content.

Comparison of lipoproteins of lymphedema fluid and plasma separated by sequential ultracentrifugation

Plasma and lymphedema fluid samples of five patients were subjected to sequential ultracentrifugation and the cholesterol and apolipoprotein distributions are shown in **Table 2**. Recoveries of cholesterol and of apoB in the fractionated lymphedema fluid and plasma were approximately 90%. However, the recovery of apoA-I and apoE was 70% and 80%, respectively, in lymphedema fluid, and 69% and 71%, respectively, in plasma. Low recoveries of apolipoproteins following sequential ultracentrifugation of plasma have been reported by others (26, 27). It can be seen that the distribution of lipoproteins among the density fractions of lymphedema fluid differs from that of plasma in several respects (Table 2). *1*) Only traces of lipoproteins are found in the lymphedema fluid fraction of d < 1.019 g/ml. *2*) A considerable proportion of apoA-I and apoE in lymphedema fluid is present in the density fraction d 1.020–1.063 g/ml; in plasma this fraction contains apoB. *3*) Twenty-three percent of cholesterol of the lymphedema fluid fraction of d > 1.063 g/ml is associated with particles corresponding in density to plasma HDL_3 as opposed to 33% in plasma. It can be concluded that in lymphedema fluid, most apoA-I-containing lipoproteins are less dense than their counterparts in plasma.

Electron microscopic characteristics of lymphedema fluid and plasma lipoprotein fractions

The electron microscopic structures of lipoprotein fractions from four patients were examined and are typified by the electron micrographs from one of the patients, shown in **Fig. 4**. Additionally, in two patients, plasma fractions were compared with those of lymphedema fluid. In all patients, the d < 1.019 g/ml fraction of lymphedema fluid contained few particles; however, the particles in this fraction were very heterogeneous in size and ranged from 16–60 nm in diameter (data not shown). This heterogeneity of size was also present in the plasma fraction where particles ranged from 18–90 nm. The d 1.019–1.063 g/ml fraction from lymphedema fluid (Fig. 4A) shows great variation in both size and morphology from patient to patient. In three of the patients, this fraction consisted of two morphologically distinct particles, one round and the

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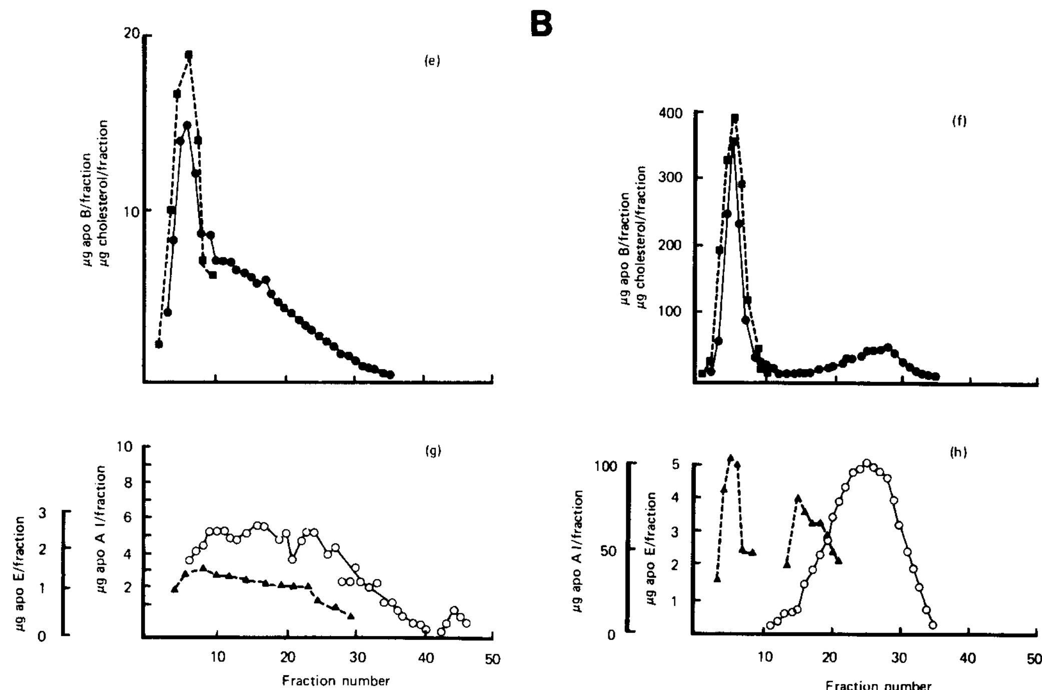

B. Elution pattern of lymphedema fluid (e and g) and plasma (f and h) from patient 7 (plasma cholesterol 144 mg/dl). Experimental conditions and symbols as Fig. 3A.

other square-packing. In the remaining patient, only round particles were noted. The round structures probably represent LDL structures and, for the four patients, the mean diameter of these particles was 21.9 ± 5.5 nm. The square-packing particles seen in three of the patients had a mean particle size of 14.9 ± 2.2 nm. The proportion of round to square-packing particles varied from patient to patient so that square particles formed the majority of particles in one case (see Fig. 4A) but were absent in this fraction in another patient. Unlike lymphedema fluid, plasma fractions contained typical LDL (Fig. 4F) with diameters of 23.0 ± 2.6 and 23.6 ± 2.8 nm for the two patients examined. The major constituent of the lymphedema fluid d 1.063–1.085 g/ml fraction was a particle with a strong tendency to form extensive square-packing arrays as shown in Fig. 4C; the mean particle size was 13.1 ± 1.8 nm on a side. A fraction of similar density from plasma of one of the two patients revealed the presence of large round particles, 19.8 ± 4.9 nm, and a few small round ones (8–11 nm diameter) (data not shown). The former particles probably represented small LDL particles while the latter represented HDL_2 particles.

The HDL density class of both lymphedema fluid and plasma was subfractionated into HDL_2 (d 1.085–1.11 g/ml) and HDL_3 (d 1.11–1.21 g/ml). The d 1.085–1.11 g/ml fraction from lymphedema fluid contained mainly round particles (Fig. 4D) with a mean diameter of 12.3 ± 2.0 nm for the four patients. As indicated in Fig. 4D, occasional discoidal structures were present but they were the exception and not the rule. In contrast, plasma HDL of d 1.085–1.110 g/ml possessed only round particles (12.1 ± 1.8 nm and 10.5 ± 1.9 nm for the two patients examined) which had a tendency to pack hexagonally (Fig. 4G). The more dense HDL fraction (d 1.110–1.21 g/ml) of lymphedema fluid contained little material; we could identify particles in only one of the four samples (Fig. 4E). In this case, the majority of particles were round with a mean diameter of 10.2 ± 1.7 nm; occasional discoidal structures were also present. Plasma HDL d 1.110–1.21 g/ml, on the other hand, were considerably smaller in diameter (8.7 ± 1.4 and 8.2 ± 1.2 nm) than lymphedema fluid HDL particles (Fig. 4H).

One of the two patients in whom plasma and lymphedema fluid were examined in parallel by electron microscopy had sufficient material to permit examination of the subfractions by gradient polyacrylamide gel electrophoresis. Electrophoretograms of lymphedema fluid and plasma fractions are shown in **Fig. 5A–D**. The distribu-

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TABLE 2. Distribution of cholesterol, apoA-I, apoB, and apoE in ultracentrifugally isolated fractions of lymphedema fluid and plasma

		% Recovery		Density (g/ml)									
				<1.019		1.019–1.063		1.063–1.085		1.085–1.11		1.11–1.21	
Patient	Constituent	Lymphedema Fluid	Plasma	L.F.[a] mg/dl	Plasma mg/dl	L.F. mg/dl	Plasma mg/dl	L.F. mg/dl	Plasma mg/dl	L.F. mg/dl	Plasma mg/dl	L.F. mg/dl	Plasma mg/dl
1	Cholesterol	71	ND[b]	0.8	ND	3.9	ND	4.8	ND	3.1	ND	0.8	ND
	ApoA-I	64	ND	0.2	ND	0.85	ND	0.98	ND	1.76	ND	0.9	ND
	ApoB	96	ND		ND	4.8	ND	0.85	ND		ND		ND
	ApoE	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	Cholesterol	96	ND	0.3	ND	34.5	ND	6.45	ND	18.7	ND	2.2	ND
	ApoA-I	88	ND		ND	15.7	ND	2.1	ND	3.2	ND	2.0	ND
	ApoB	84	ND		ND	3.1	ND	1.8	ND		ND		ND
	ApoE	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3	Cholesterol	89	119.0	3.0	21.8	23.6	92.2	10.1	15.2	9.2	22.8	16.0	39.2
	ApoA-I	54	64			4.5	0.4	2.6	3.3	3.6	27.3	1.2	42.5
	ApoB	92	111		27.2	5.8	106.0	0.4					
	ApoE	72	60		1.7	0.7		0.3	0.6				
4	Cholesterol	89	94	0.2	35.1	4.1	96.0	4.7	22.2	2.8	35.0	2.2	18.0
	ApoA-I	68	74	0.2		0.7		0.8	2.5	1.9	39.8	1.1	45.4
	ApoB	96	93		24.0	4.6	78.0	0.7	8.0				
	ApoE	88	77		1.4	0.5		0.4	1.3				
6	Cholesterol	91	94	0.3	23.3	7.6	98.7	3.9	16.2	8.2	28.3	0.9	10.7
	ApoA-I	79	71			5.5	2.1	2.3	8.4	1.4	35.4	1.1	45.8
	ApoB	104	90		34.1	5.3	78.8	0.6					
	ApoE	76	77		1.5	1.5	0.4	0.4	0.3	0.16			
Mean ± SD[c]	Cholesterol	87.2 ± 9.5	102 ± 14.4	0.9 ± 1.2	26.7 ± 7.3	17.9 ± 13.4	95.6 ± 3.2	6.0 ± 2.4	17.8 ± 3.8	8.4 ± 6.4	28.7 ± 6.1	4.4 ± 6.5	22.6 ± 17.8
	ApoA-I	70.6 ± 13.2	69.6 ± 5.1	0.08 ± 0.1		5.4 ± 6.1	0.8 ± 1.1	1.7 ± 0.8	4.7 ± 3.2	2.3 ± 0.9	34.3 ± 6.0	1.2 ± 0.4	44.5 ± 1.8
	ApoB	94.4 ± 7.2	98.0 ± 11.3		28.4 ± 5.1	4.7 ± 1.0	87.6 ± 15.9	0.8 ± 0.5	2.6 ± 4.6				
	ApoE	78.6 ± 8.3	71.3 ± 9.8		1.5 ± 0.1	0.9 ± 0.5	0.1 ± 0.2	0.3 ± 0.05	0.7 ± 0.05				

[a] L.F., lymphedema fluid.
[b] ND, not determined.
[c] SD σ n − 1.

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tion patterns on gradient gel electrophoresis are in good agreement with electron microscopic information. Gradient gel electrophoresis of d 1.019-1.063 g/ml fractions was carried out on a 2-16% gel (Fig. 5A) and shows that plasma particles are heterogeneous in size (24-27 nm) but confined to the large pore region of the gel. In contrast, lymphedema fluid d 1.019-1.063 g/ml Coomassie blue-staining material contains a small fraction of material in the LDL region (27 nm) and a major component in the smaller pore region of the gel (broad peak between 16-18 nm). The latter particles probably account for the square-packing structures seen by electron microscopy. Lymphedema fluid d 1.063-1.085 g/ml lipoproteins form a discrete band (peak at 14.3 nm) in the large pore region of the 4-30% gel (Fig. 5B); according to the nomenclature of Nichols et al. (24), these particles are larger in size than $(HDL_{2b})_{gge}$. The large-sized particles correspond in gradient gel position to the bands found between LDL and HDL_2 in electrophoretograms of native lymphedema fluid (e.g., Fig. 2A). The major peak on 4-30% gels of d 1.063-1.085 g/ml lymphedema fluid particles is similar in size to the dimensions (13-14 nm) of square-packing particles visualized by electron microscopy. The parallel plasma fraction contains a major component that barely enters the gel, the typical position for LDL, and corroborates the electron microscopic observation. A very minor component is also noted in the $(HDL_{2b})_{gge}$ region; this probably corresponds to the 8-11-nm size particles identified by electron microscopy. Lymphedema fluid lipoproteins of d 1.085-1.110 g/ml are the size of $(HDL_{2b})_{gge}$ or larger (Fig. 5C); this is consistent with larger particle diameter noted in electron microscopy. The d 1.110-1.21 g/ml fraction of lymphedema fluid has little or no identifiable material on the 4-30% gel, whereas the corresponding fraction from plasma contains particles primarily in the $(HDL_{2a})_{gge}$ and $(HDL_{3a})_{gge}$ regions (Fig. 5D).

DISCUSSION

The present results were obtained by analyzing lymphedema fluid from patients in whom, at the time of operation, inadequate drainage of lymph was the only detectable cause of edema. Although the sol-gel interstitial phases in tissues of normal and lymphedematous legs may differ, the present results are in many respects similar to those obtained previously by analyzing human prenodal peripheral lymph from apparently healthy subjects. The concentration of apoA-I, apoB, and cholesterol in the two fluids is similar (Table 1) (12, 21) as is that of apoA-II, apoA-IV, and apoE (Reichl, D., J. Pflug, and G. Uttermann, unpublished observations). More importantly, the distribution of Sudanophilic material on gradient gels (Fig. 2) (13) and that of apoA-I and of cholesterol among particles of different sizes isolated by gel filtration (Figs. 1 and 3) (13) is very similar in lymphedema fluid and peripheral lymph. In both fluids, apoA-I can be detected in lipoproteins that float at d 1.019-1.063 g/ml (Table 2) (13), while, in both instances, VLDL are virtually absent (Table 2) (21) and apoB-containing lipoproteins migrate with prebeta mobility when electrophoresed (17). The composition of the two extravascular fluids probably represents a weight average of the composition of fluids in subcompartments of the interstitium that have different exclusion characterstics (28, 29). Lymphedema fluid HDL differ considerably from plasma HDL in size, density, and apolipoprotein composition. Particles that are larger than plasma HDL_2 and smaller than LDL are the predominant apoA-I-containing lipoproteins; they also contain the majority of the lymphedema fluid apoE and a considerable proportion of lymphedema fluid cholesterol. Particles analagous to plasma HDL_3 are low in lymphedema fluid; however, the results from gel filtration suggest that particles the size of, or smaller than, albumin may be present in lymphedema fluid.

We have previously observed the presence of large HDL in lymph, and have postulated that they are the consequence of extravascular processing (12, 13). Potentially smaller HDL can increase in size, either by increasing their nonpolar core or by increasing polar components of their surface. An example of the former mechanism is the conversion of HDL_3 to HDL_2 during esterification of cholesterol to cholesteryl esters by lecithin:cholesterol acyltransferase (LCAT) (30); cholesteryl esters are incorporated into the core of the particles and expand its volume. A similar mechanism has been recently proposed for the conversion of smaller HDL particles into larger ones, including large apoE-rich HDL-E, when dog plasma or serum is incubated with cholesterol-coated Celite or with cholesterol-laden macrophages (31). It is tempting to speculate that a mechanism involving macrophages, and perhaps other cells of peripheral tissues that synthesize apoE (32), could also account for the presence of apoE in the large HDL particles of lymphedema fluid.

Increase in HDL surface components has been observed in human plasma in vivo after a fat meal; in this case, the content of phospholipids and of total protein increases (33). Similarly, HDL increase in size when incubated in the presence of VLDL and lipoprotein lipase (34). In functionally hepatectomized rats, it could be shown that phospholipids and apolipoproteins were transferred intravascularly from chylomicrons to HDL, thereby increasing their size and the surface/volume ratio (35). HDL incubated with phospholipid liposomes in vitro also take up phospholipids, increase their size and surface/volume ratio, and concomitantly displace free apoA-I from the lipoprotein (36, 37). The latter mechanism could account for the increase in HDL size and also for the

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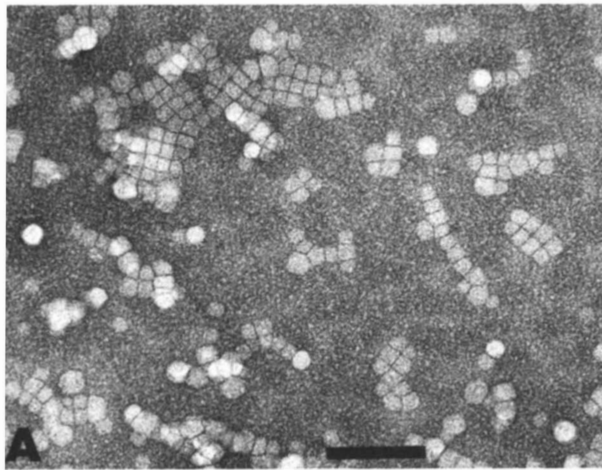

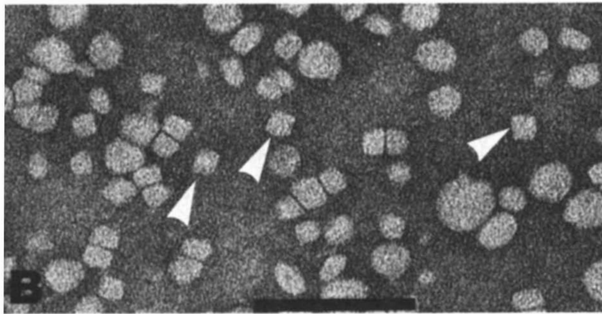

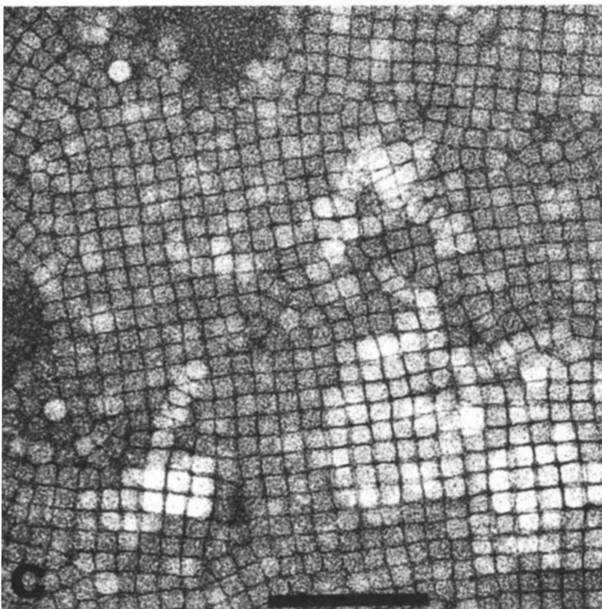

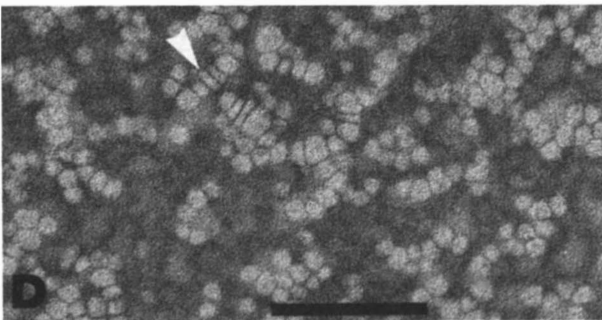

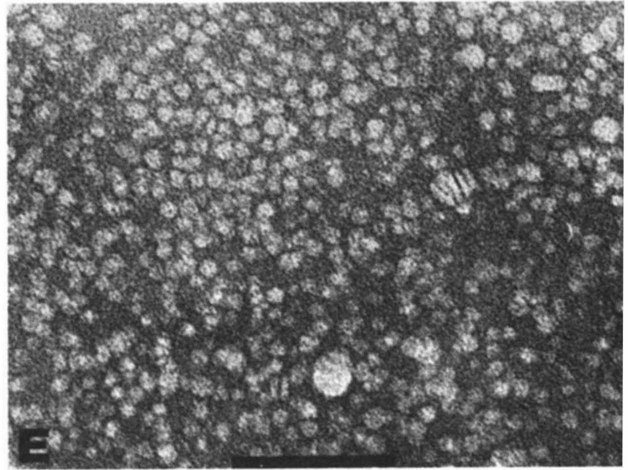

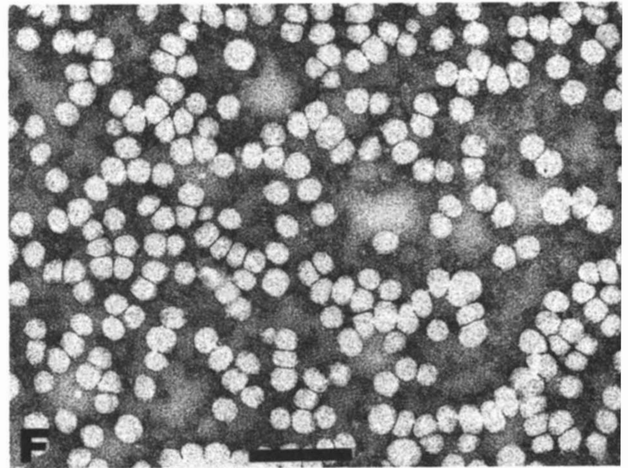

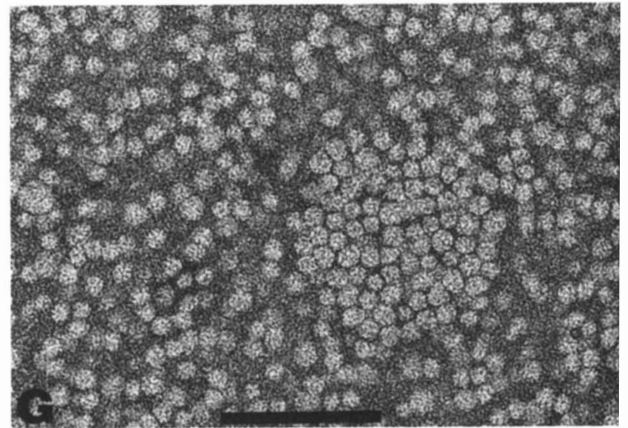

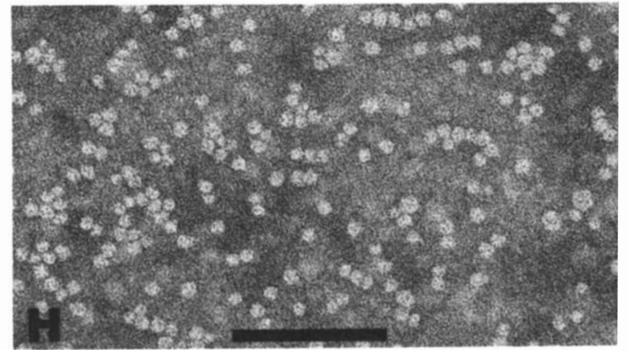

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generation of very small apoA-I-containing particles found in lymphedema fluid. The potential source within the interstitium of phospholipids and other compounds that expand the lipoprotein surface may be from products of degradation of chylomicrons and VLDL that gain access into extravascular compartments. Alternatively, they may be secreted by cells. Recent studies by Basu et al. (38) have shown that macrophages derived from circulating human monocytes secrete in vitro discoidal apoE-phospholipid complexes. It has been suggested that the particles with an increased surface/volume ratio could remain stable if apolipoproteins underwent conformational changes reducing their surface occupancy (39).

The morphology of human lymphedema fluid HDL particles was unlike that reported for peripheral lymph from cholesterol-fed dogs (14). In the dog, large numbers of discoidal structures were present, whereas in human lymphedema fluid discoidal particles were rarely encountered. The numerous discoidal particles seen in the dog may in part be related to the hypercholesterolemic condition of the animal.

The morphology of lymphedema fluid lipoproteins is clearly distinct from that of plasma. Most notably, the less dense HDL particles (apoA-I- and apoE-associated particles) of lymphedema fluid, which migrate on gradient gels between LDL and HDL, form square-packing arrays during negative staining. Although some square-packing particles were seen in the d 1.019–1.063 g/ml and the d 1.085–1.11 g/ml fractions, the bulk of the particles was in the d 1.063–1.085 g/ml fraction. Similar square-packing structures have also been found in sheep lung lymph (15), and in that study, particles were also associated with apoA-I and apoE. In sheep lung lymph, the fraction containing square-packing particles was also enriched in phospholipid and unesterified cholesterol. Square-packing particles have also been isolated by several laboratories from the plasma of patients with abetalipoproteinemia (40–42); these particles have a density less than that of classical HDL but are associated with HDL apolipoproteins. Compositional studies by Kostner et al. (43) indicate that the square-packing HDL fraction from abeta-

Fig. 4. Electron micrographs of negatively stained fractions of lymphedema fluid (A–E) and plasma (F–H) lipoproteins. A, Low magnification micrograph of lymphedema fluid d 1.019–1.063 g/ml fraction. Particles show a tendency to organize into square arrays. B, High magnification of lymphedema fluid 1.019–1.063 g/ml sample which shows that some freestanding particles (arrows) have a square profile. C, Lymphedema fluid d 1.085–1.110 g/ml fraction. This fraction forms extensive square-packing arrays upon negative staining. D, Lymphedema fluid d 1.085–1.110 g/ml fraction. Particles are heterogeneous round structures, but occasional discoidal structures (arrows) are also visible. E, Lymphedema fluid d 1.110–1.21 g/ml fraction. Particles are generally round, although an occasional disc is present. F, Plasma d 1.019–1.063 g/ml fraction consists of heterogeneous round particles. G, Plasma d 1.085–1.110 g/ml fraction. The particles are round in profile and tend to organize in hexagonal arrays when close-packed. H, Plasma d 1.110–1.21 g/ml fraction. Bar markers represent 100 nm.

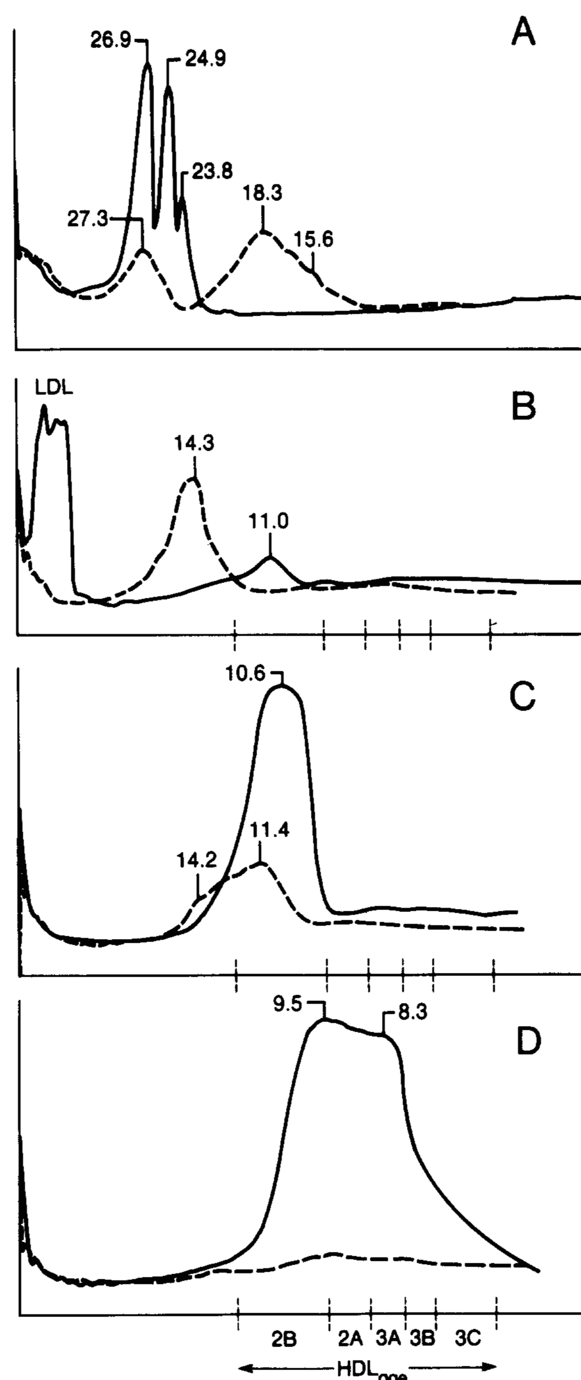

Fig. 5. Gradient polyacrylamide gel electrophoresis of lymphedema fluid and corresponding plasma fractions. Solid line indicates plasma, while broken line indicates lymphedema fluid; numbers over peaks and shoulders indicate particle size in nm. A, Fractions of d 1.019–1.063 g/ml electrophoresed on 2–16% gels. Note the appearance of a new peak at approximately 18.3 nm in lymphedema fluid. B, Fractions of d 1.063–1.085 g/ml on 4–30% gel. In plasma this fraction contains mainly contaminating LDL and a small amount of HDL_{2b} material. Lymphedema fluid in this density range contains particles that are intermediate to LDL and HDL in size. C, Fractions of d 1.085–1.110 g/ml on 4–30% gel. Lymphedema fluid lipoproteins are somewhat larger in size than corresponding HDL particles. D, Fractions of d 1.110–1.21 g/ml on 4–30% gel. Little material is present in the lymphedema fluid fraction, while in plasma this fraction contains predominantly HDL_{2a} and HDL_3 particles. The hatched lines on the X-axis of B, C, and D indicate the major gradient gel HDL fractions (HDL_{gge}) as defined by Nichols et al. (24) for human plasma. Gels were stained with Coomassie blue G250.

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lipoproteinemic patients is enriched in polar lipids. The unusual electron microscopic structure of less dense HDL from lymphedema fluid, sheep lung lymph, and abetalipoproteinemia may be related to increased surface components, principally phospholipid and unesterified cholesterol. Recent studies by Forte et al. (44) in an in vitro model system using bovine HDL_2 and dimyristoylphosphatidylcholine (DMPC) showed that spherical HDL particles could be converted into square-packing ones by incorporation of excess phospholipid into the particle. The product particles were larger than native HDL and had a gradient gel pattern similar to the d 1.063–1.085 g/ml fraction of lymphedema fluid. As noted above, in the case of lymphedema fluid, the excess phospholipid and/or cholesterol presumed to be incorporated into interstitial HDL particles could be derived from either chylomicron and VLDL degradation products or from cell-derived lipid. The bovine model system also provides additional insights into the generation of a small apoA-I particle in the lymphedema fluid. Saturation of the HDL particle with DMPC led to the displacement of one molecule of apoA-I from the native particle. Similar reactions may take place in the interstitial compartment wherein incorporation of excess phospholipid and/or unesterified cholesterol into HDL particles that crossed the vascular wall may destabilize and dissociate apoA-I from their surface. The dissociated apoA-I could react with other lipids, particularly phospholipid, to generate a particle of small molecular weight. Due to exclusion characteristics of the interstitium, such small particles have the highest probability of reaching cell surfaces.

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