Development of unesterified cholesterol-rich lipid particles in atherosclerotic lesions of WHHL and cholesterol-fed NZW rabbits

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Abstract Previously, we isolated and characterized unesterified cholesterol-rich lipid particles (UCLP) that accumulate in extracellular spaces of atherosclerotic lesions of humans and cholesterol-fed rabbits. In the present study, we examined early developing atherosclerotic lesions to determine when UCLP appear and when they become enriched in cholesterol and sphingomyelin. Cholesterol-fed NZW rabbits, which rapidly develop atherosclerotic lesions, and genetically hyperlipidemic WHHL rabbits, which develop lesions over a longer period of time, were studied. UCLP of peak density 1.04 g/ml appear as early as 4 weeks after the onset of cholesterol feeding and progressively accumulate during atherosclerotic lesion development. Beginning with their appearance and afterwards, UCLP contain a saturating level (2:1 molar ratio) of cholesterol relative to phospholipid. Whereas, early UCLP are enriched in phosphatidylcholine, with time UCLP become enriched with sphingoymelin. Another UCLP population having a peak density of 1.09 g/ml was present in control aortas and increased in amount more slowly than the d 1.04 g/ml UCLP during cholesterol feeding. The d 1.09 g/ml particles were predominantly unilamellar vesicles, the majority between 100 and 200 nm in diameter. They contained > 90% of their cholesterol in unesterified form and their ratio of unesterified cholesterol to phospholipid progressively increased from 0.6 to 1.7 during cholesterol feeding. Liposome resistance to solubilization by high density lipoproteins is known to be increased by enrichment with unesterified cholesterol and sphingomyelin. Sphingomyelin enrichment of unesterified cholesterol-rich lipid particles (UCLP) could stabilize cholesterol in a form that does not readily crystallize. However, at the same time, the early and progressive accumulation of UCLP in developing atherosclerotic lesions may limit reverse cholesterol transport and accelerate disease progression.—**Chao, F-F., E. J. Blanchette-Mackie, B. F. Dickens, W. Gamble, and H. S. Kruth.** Development of unesterified cholesterol-rich lipid particles in atherosclerotic lesions of WHHL and cholesterol-fed NZW rabbits. *J. Lipid Res.* 1994. **35:** 71–83.

Supplementary key words lipoproteins • phospholipids • liposomes • sphingomyelin • cardiovascular disease

Accumulation of cholesterol in arterial vessels is a prominent feature of atherosclerosis (1–4). Much of this cholesterol is unesterified. We previously reported the accumulation of unesterified cholesterol-rich lipid particles (UCLP) in the extracellular spaces of human and animal atherosclerotic lesions (5, 6). Filipin, which binds to unesterified cholesterol, was used to detect these particles in lesions. The particles accumulate at the onset of atherosclerosis (7, 8) and co-exist with lipid droplet-filled foam cells in advanced lesions (5, 6). Isolated UCLP are uni- and multilamellar liposomes approximately 110 nm in diameter. They have a high molar ratio (> 2:1) of unesterified cholesterol to phospholipid (UC/PL), a high molar % (> 75%) of cholesterol in unesterified form, and sphingomyelin as their major phospholipid (9, 10).

High density lipoprotein is the major serum factor considered to mediate reverse cholesterol transport from the blood vessel wall. Many previous investigations have shown that unesterified cholesterol or sphingomyelin (SPH) enrichment promotes liposome stability in the presence of high density lipoprotein or apolipoprotein A-I, the major protein constituent of HDL (11–17). Thus, the lipid composition of UCLP would make these particles difficult to remove from atherosclerotic lesions. Additionally, because UCLP are cholesterol-rich, they potentially

Abbreviations: UCLP, unesterified cholesterol-rich lipid particles; UC, unesterified cholesterol; TC, total cholesterol; PL, phospholipid; SPH, sphingomyelin; HDL, high density lipoprotein; WHHL, Watanabe Heritable Hyperlipidemic; NZW, New Zealand White; DPBS, Dulbecco's phosphate-buffered saline; LDL, low density lipoprotein.

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may activate complement (18, 19) promoting the inflammatory events accompanying lesion development.

For these reasons, we examined early developing atherosclerotic lesions to determine when UCLP appear and when they become enriched in cholesterol and sphingomyelin. Cholesterol-fed New Zealand White (NZW) rabbits, which rapidly develop atherosclerotic lesions, and hyperlipidemic Watanabe Heritable Hyperlipidemic (WHHL) rabbits, which genetically develop lesions over a longer period of time, were used to study UCLP development in atherosclerotic lesions.

MATERIALS AND METHODS

Aortic tissue specimens

Male NZW rabbits (3-month-old, Hazleton Dutchland, Denver, PA) were fed NIH-09 standard rabbit chow (NIH Dietary Specifications, NIH-11-136f) supplemented with 1% (wt/wt) cholesterol obtained from Ziegler Bros. (Gardners, PA) for 0–30 weeks. Groups of ten rabbits were fed for 0, 1, 2, and 3 weeks; seven rabbits were fed for 4 weeks; and groups of three rabbits were fed for 6, 8, 11, 16, 20, and 30 weeks. Male and female WHHL rabbits (up to 4 yr of age) were obtained from the NIH breeding colony and were fed NIH-09 standard rabbit chow (essentially cholesterol-free) their entire lives.

Rabbits were killed by intravenous injection of ketamine, and their aortas (from arch to bifurcation) were immediately removed. The aortic tissues were rinsed several times in cold (4°C) Dulbecco's phosphate-buffered saline (DPBS) and dissected free of adventitia and the outer portion of the media. The dissected aortas were rinsed in DPBS, blotted dry, and weighed. Lipid particles were isolated from aortas pooled from NZW rabbits and individual aortas of WHHL rabbits. Serum was prepared from blood specimens collected by cardiac puncture at the time of killing.

Isolation and purification of aortic lipid particles

Rabbit aortic tissues were minced for 40 min with scissors in extraction buffer (pH 7.4) containing 0.15 M NaCl, 0.1% EDTA, 0.05% glutathione, 0.02% NaN_3, and 0.13% ϵ-aminocaproic acid (1 g of tissue per 8 ml of extraction buffer). Minced tissues were gently stirred for 2 h. These and all subsequent procedures were carried out at 4°C.

The isolation and purification procedure was the same as we described previously (9, 10). Aortic lipid particles were purified by centrifuging samples at 1500 *g* for 15 min, followed by microfiltration of the supernatant through 0.45 μm (pore-size) filters (Acrodisc 4184, Gelman Sciences, Ann Arbor, MI). Microfiltration removed lipid droplets and crystalline lipid from the extracts of minced aortas. The filtrate was dialyzed overnight against 2 l of 1.5 M NaCl (pH 7.4) containing 0.1% EDTA, 0.05% glutathione, 0.02% NaN_3, and 0.13% ϵ-aminocaproic acid. The dialyzed filtrate was subjected to gel-filtration column chromatography (Bio-Gel A-50m, Bio-Rad, Richmond, CA) using a 1.6 × 50 cm column with a flow rate of 7 ml/h per sq cm. Sample was eluted with the same high salt buffer that was used for dialysis. This step separated large lipid particles from any smaller lipoproteins and soluble protein. Most of the recovered cholesterol eluted in the void volume, whereas less than 10% of cholesterol eluted with the internal volume.

Density gradient centrifugation of the pooled void volume fractions was carried out in a 14 × 89 mm Beckman Ultraclear centrifuge tube. A discontinuous gradient was constructed from bottom to top with 3 ml of 1.21 g/ml (density adjusted with NaCl and KBr solutions), 3 ml of void volume fraction (d 1.061 g/ml), 3 ml of 1.019 g/ml NaCl solution, and 2.5 ml of 1.006 g/ml NaCl solution (all salt solutions contained 0.1% EDTA and 0.01% thimerosal, pH 7.0). The gradient was centrifuged for 22 h and 170,000 *g* at 4°C in an SW-41 rotor (Beckman, Palo Alto, CA) to produce a continuous gradient as described by Redgrave, Roberts, and West (20). This step separated lipid particles according to their different hydrated densities.

After centrifugation, 15 0.8-ml fractions were collected from the top of the gradient with pump-driven aspiration (Auto Densi-Flow II, Haake Buchler, Saddle Brook, NJ). The refractive indices of the fractions were determined with an Abbe Refractometer (Reichert, Buffalo, NY) and their corresponding densities were determined with a conversion table (21). Because KBr and NaCl were present in the higher density fractions (fractions 12 to 15), density was determined in these fractions using a hydrometer.

Chemical analysis of each fraction was carried out. All values for isolated UCLP were adjusted for cholesterol recovery at each step of the isolation procedure: 10–20% of cholesterol was released from the tissue during the mincing procedure, 95–100% of cholesterol was recovered from high salt dialysis, 80–90% from gel-filtration column chromatography, and 75–85% from density gradient ultracentrifugation. The d 1.04 g/ml UCLP were considered to be the low density peak fraction, number 7. The d 1.09 g/ml UCLP were considered to be the high density peak fraction, number 11.

Chemical analysis

Lipids of serum, tissues, and isolated aortic particles were extracted with chloroform methanol 2:1 (v/v) according to Folch, Lees, and Sloane Stanley (22). Unesterified and esterified cholesterol were determined enzymatically with the fluorometric method described by Gamble et al. (23). Total phospholipid phosphorus was determined by the Fiske-SubbaRow method (24) modified by Bartlett (25) with Fiske-SubbaRow reagent obtained from Fisher Scientific Co. (Fair Lawn, NJ).

Phospholipid classes were separated by high-performance liquid chromatography using a modified procedure of Kaduce, Norton, and Spector (26). The isolated phospholipid classes were then quantified by measuring total phospholipid phosphorus. The detailed procedure was described in our previous report (10).

Electron microscopy

Samples of isolated UCLP were negatively stained with phosphotungstic acid. A drop of sample was applied to carbon-stabilized, formvar-coated copper grids and allowed to remain 15 sec. The adhered lipid particles were then washed with DPBS and stained with 4 drops of 2% potassium phosphotungstic acid (adjusted to pH 6 with KOH) for 30 sec. Excess fluid was drawn off with filter paper. Grids were examined with a Phillips EM 300 electron microscope operated at 80 Kv (Phillips Electronics, Mahwah, NJ). Sizes of the lipid particles were measured from photomicrographs of negatively stained particles.

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). The Student's *t*-test was used to establish statistical significance. Probability values of 0.05 or less were considered significant (27).

RESULTS

Serum lipid levels and lesion development

Serum cholesterol of NZW rabbits increased during the first 6 weeks of cholesterol feeding, remained level between 2000 and 3000 mg/dl from 6 to 11 weeks, and then slowly decreased to ≅ 1000 mg/dl (**Fig. 1**). Serum phospholipid also increased during the first 6 weeks of cholesterol feeding to a level of 800 mg/dl, remained at this level until 16 weeks, and then declined (data not shown).

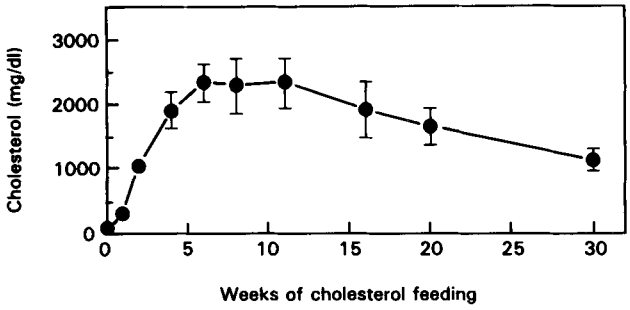

Fig. 1. Serum total cholesterol levels of NZW rabbits fed 1% cholesterol. Each point indicates an average cholesterol level of sera from 10 rabbits (0, 1, 2, and 3 weeks of cholesterol feeding), from 7 rabbits (4 weeks of cholesterol feeding), and from 3 rabbits for the remaining weeks of cholesterol feeding. The bars indicate the SEM and are not shown when smaller than the symbol.

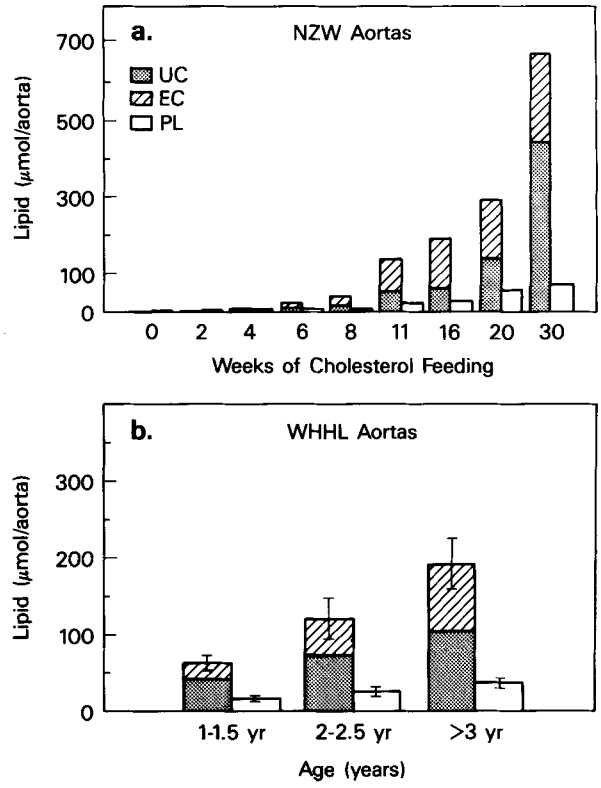

Fig. 2. The amount of cholesterol and phospholipid in aortas of NZW and WHHL rabbits. The number of rabbit aortas combined for each NZW preparation (a) is the same as the number listed for serum samples in Fig. 1. The number of individual WHHL rabbit aortas (b) analyzed were four for 1 to 1.5-yr and > 3-yr groups, and five for the 2 to 2.5-yr group. A portion of minced aortic tissue was weighed, extracted, and analyzed for each constituent as described in the Materials and Methods section. The bars indicate SEM for total cholesterol and PL. Total cholesterol, UC, EC, and PL for the > 3-yr group were significantly different than the 1 to 1.5-yr group. UC, unesterified cholesterol; EC, esterified cholesterol; PL, phospholipid.

The development of disease lagged behind the rapid increase in serum cholesterol and phospholipid levels. Grossly visible raised and flat lesions, which included white streaks, dots, and patches, appeared in the thoracic arch region of rabbit aortas at 4 weeks of cholesterol feeding. White flat and raised lesions were observed throughout the entire aorta by 11 weeks. As lesions appeared during cholesterol feeding, the average weights of the intima progressively increased from 0.3 g at 0–2 weeks, 0.5 g at 4–8 weeks, 0.9 g at 11 weeks, 1.8 g at 20 weeks, and 2.6 g at 30 weeks.

WHHL rabbits developed atherosclerosis more gradually than cholesterol-fed NZW rabbits. In a preliminary experiment with aortas of 3-month-old WHHL rabbits, we were unable to isolate lipid particles or detect grossly visible disease. Lesions were present in aortas of all WHHL rabbits older than 1 year. Most aortic lesions in WHHL rabbits were distributed in the thoracic arch region and also appeared as flat or raised white streaks,

dots, and patches. The lower serum cholesterol in WHHL rabbits compared with NZW rabbits fed cholesterol accounts for slower development of disease in WHHL rabbits. Serum cholesterol in WHHL rabbits ranged between 400 and 730 mg/dl in 12 out of 13 rabbits (ages ranged between 1 and 4 years old). One 2-year-old female rabbit had a very high serum cholesterol level of 1330 mg/dl. The aortic tissue weight of this rabbit was 2.3 g, higher than that of similar aged, as well as older WHHL rabbits. Excluding this rabbit, WHHL aortic tissue weights averaged 0.6 g in 1- to 1.5-year-old rabbits, 1.4 g in 2- to 2.5-year-old rabbits, and 1.5 g in rabbits greater than 3 years old.

Accumulation of cholesterol and phospholipid in aortic tissues of cholesterol-fed NZW and normal-fed WHHL rabbits

The amounts of unesterified cholesterol, esterified cholesterol, and phospholipid in the aortic tissues of NZW rabbits increased during cholesterol feeding (**Fig. 2a**). These increases were first detected after 4 weeks of cholesterol feeding. The increase in esterified cholesterol compared with 0-week controls exceeded that of unesterified cholesterol through the initial 20 weeks of cholesterol feeding. However, by 30 weeks of feeding, the

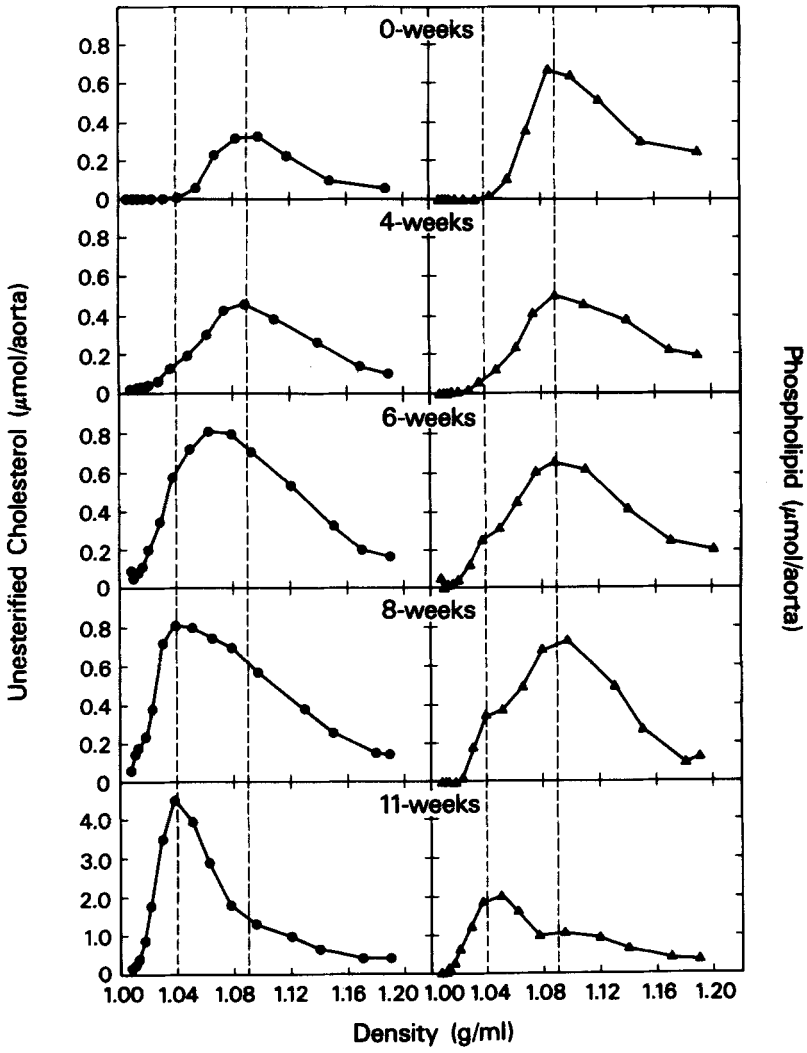

Fig. 3. The density gradient distribution of unesterified cholesterol and phospholipid of lipid particles isolated from cholesterol-fed NZW rabbits. Large lipid particles that eluted in the void volume of a Bio-Gel A-50m column were subjected to density gradient centrifugation. Vertical dashed lines indicate densities of the major UCLP populations, which occurred at d 1.04 g/ml and 1.09 g/ml. Note the change in scale of the y-axis for the 11-week density gradient distribution. The number of rabbit aortas combined for each preparation was the same as that indicated in Fig. 1.

increase of unesterified cholesterol was greater than that of esterified cholesterol.

The amounts of unesterified cholesterol, esterified cholesterol, and phospholipid in WHHL rabbit aortic tissues progressively increased with age (Fig. 2b). A comparison of cholesterol accumulation in aortas of cholesterol-fed NZW rabbits and genetically hypercholesterolemic WHHL rabbits showed the more rapid progression of disease in the cholesterol-fed NZW rabbits. The aortic cholesterol accumulation in the 1- to 1.5-year-old WHHL rabbits was intermediate between that of cholesterol accumulation in aortas of NZW rabbits fed cholesterol for 8–11 weeks. Aortic cholesterol accumulation in WHHL rabbits greater than 3 years old and in NZW rabbits fed cholesterol for 16 weeks were comparable. Also, the amount of cholesterol in the aorta of a 3-year-old, normal-fed NZW rabbit was comparable to that of the 3-month-old NZW rabbits prior to cholesterol feeding (both were approximately 2 μmol/aorta). This showed that no cholesterol accumulated in aortas of NZW rabbits in the absence of cholesterol feeding.

Accumulation of UCLP in developing atherosclerotic lesions

During cholesterol feeding, two UCLP populations were apparent from the distribution of unesterified cholesterol after density gradient centrifugation of aortic lipid particles. The first UCLP population, which was the only one in aortas of control NZW rabbits (**Fig. 3**, 0-weeks) or rabbits fed cholesterol for 1 to 3 weeks (data not shown), had a distribution of unesterified cholesterol that peaked at a density of about 1.09 g/ml. The second UCLP population, which appeared by 4 weeks and became the predominant form by 8 weeks of cholesterol feeding, showed a distribution of unesterified cholesterol that peaked at d 1.04 g/ml. The distribution of unesterified cholesterol peaked transiently at d 1.06 g/ml at 6 weeks reflecting overlapping d 1.04 g/ml and 1.09 g/ml UCLP populations.

We observed two distinct phospholipid peaks corresponding to the predominant unesterified cholesterol peaks at densities 1.09 and 1.04 g/ml (Fig. 3). Between 0 and 3 weeks of cholesterol feeding, there was one phospholipid peak at d 1.09 g/ml. An increase in phospholipid appeared at d 1.04 g/ml by 4 weeks of cholesterol feeding similar to the appearance of the unesterified cholesterol peak at this density at 4 weeks. This peak gradually enlarged and became the predominant phospholipid peak by 11 weeks of cholesterol feeding.

The amount of esterified cholesterol distributed in the density gradient was low and consistently occurred at different densities compared with unesterified cholesterol and phospholipid. This suggested that esterified cholesterol was not associated with the UCLP. However, esterified cholesterol in the gradient also progressively increased between 4 and 30 weeks of cholesterol feeding and accounted for 16–32% (molar basis) of the total cholesterol in the density gradient. A major peak of esterified cholesterol was present at d 1.03 g/ml after 11 weeks of cholesterol feeding (data not shown). By 20 weeks, a second peak occurred at d < 1.01 g/ml.

The pattern of density gradient distributions of unesterified cholesterol, phospholipid, and esterified cholesterol in particles from 1- to 4-year-old WHHL rabbits was similar to those lipid distributions for particles from NZW rabbits fed cholesterol for 11 weeks or longer (**Fig. 4**). Specifically, unesterified cholesterol and phospholipid peaked at d 1.04 g/ml, esterified cholesterol peaked at d 1.03 g/ml. As in NZW rabbits fed cholesterol for 20 weeks or longer, an additional esterified cholesterol peak occurred at d < 1.01 g/ml in WHHL rabbits older than 2 years (Fig. 4, b and c). The molar percentage of cholesterol in the gradient that was esterified averaged between 26 and 38% for the three WHHL age groups.

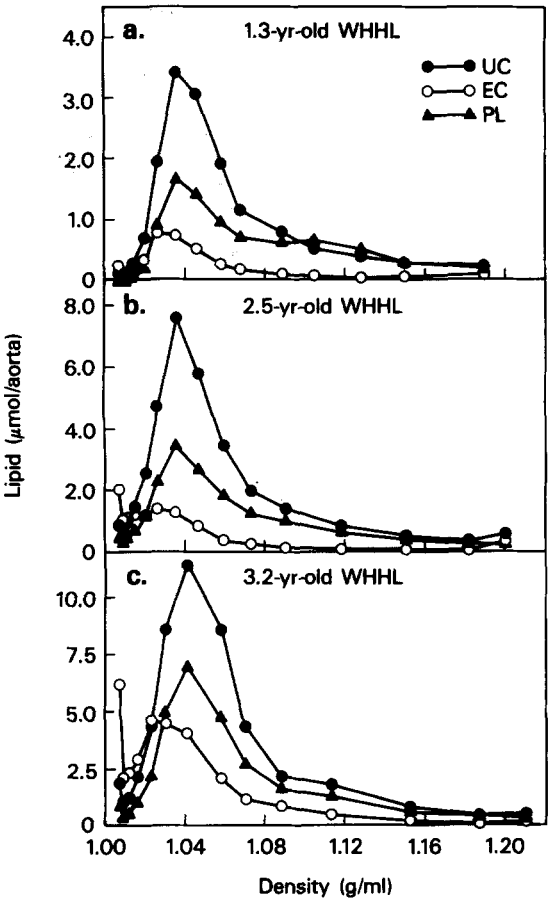

Fig. 4. The density gradient distribution of unesterified cholesterol, esterified cholesterol, and phospholipid of lipid particles isolated from aortas of WHHL rabbits. Large lipid particles that eluted in the void volume of a Bio-Gel A-50m column were subjected to density gradient centrifugation. (a) a 1.3-yr-old male WHHL rabbit with serum cholesterol level of 413 mg/dl; (b) a 2.5-yr-old female WHHL rabbit with serum cholesterol level of 558 mg/dl; and (c) a 3.2-yr-old male WHHL rabbit with serum cholesterol level of 379 mg/dl.

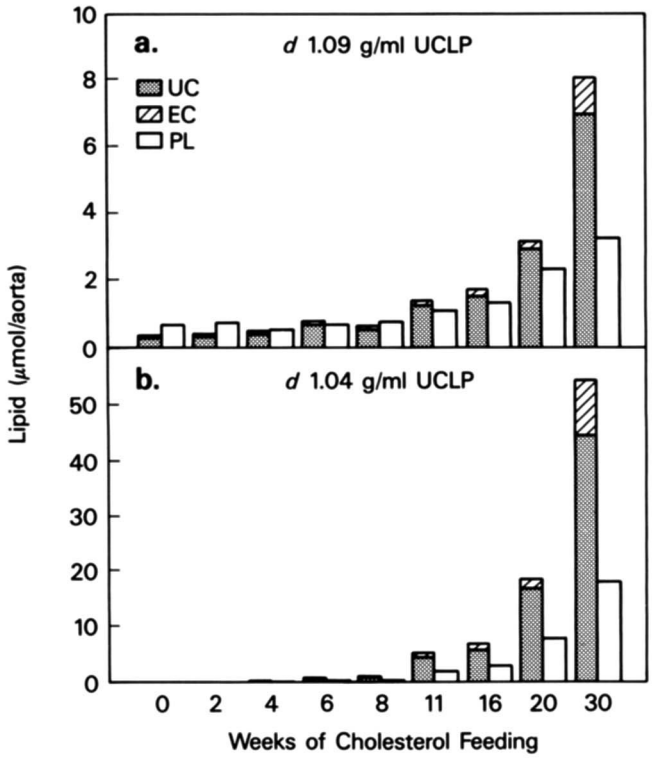

Fig. 5. The amount of unesterified cholesterol, esterified cholesterol, and phospholipid in UCLP isolated from aortas of cholesterol-fed NZW rabbits. (a) is d 1.09 g/ml UCLP and (b) is d 1.04 g/ml UCLP. The number of rabbit aortas analyzed at each time point was the same as indicated in Fig. 1. The values shown were determined from only the peak density fraction of the corresponding UCLP population and thus are an underestimate of the total amount of each UCLP population.

The density gradient distribution of phospholipid in young WHHL rabbits (Fig. 4a) showed a major phospholipid peak at d 1.04 g/ml and a small broad distribution of phospholipid with a peak density of about 1.10 g/ml. This was similar to the pattern observed for NZW rabbits fed cholesterol for 11 weeks. The phospholipid peak at d 1.09 g/ml was less apparent in older WHHL rabbits (Fig. 4, b and c) and in NZW rabbits fed cholesterol for longer than 11 weeks (data not shown). This was because the larger amount of phospholipid comprising the d 1.04 g/ml peak obscured the smaller amount of phospholipid in the d 1.09 g/ml peak.

Both cholesterol and phospholipid at d 1.09 g/ml and d 1.04 g/ml progressively increased during cholesterol feeding reflecting progressive lesion development (**Fig. 5**). Whereas, d 1.09 UCLP were present prior to the initiation of cholesterol feeding, d 1.04 g/ml UCLP appeared by 4 weeks or more of cholesterol feeding (Figs. 3 and 5). In both UCLP populations, the increment of total cholesterol was greater than that of phospholipid during cholesterol feeding. Similar to NZW rabbits, the amount of cholesterol and phospholipid in the UCLP populations at d 1.09 and d 1.04 g/ml for WHHL rabbits (**Fig. 6**) increased with age, also reflecting progressive lesion development in these rabbits. Density 1.04 g/ml UCLP were predominant in all WHHL age groups. For each UCLP population, the group of oldest WHHL rabbits (>3 yr) had significantly higher amounts of UCLP cholesterol and phospholipid than the youngest group of WHHL rabbits (1–1.5 yr).

Lipid composition of serum, aortic tissue, and UCLP

The d 1.04 g/ml UCLP had a UC/PL molar ratio of 2:1 or greater from the time of appearance of these particles at 4 weeks throughout the duration of cholesterol feeding (**Table 1**). The UC/PL ratio of d 1.04 g/ml UCLP in WHHL rabbits was also about 2:1 (**Table 2**). This high ratio was maintained although the serum UC/PL ratio of WHHL rabbits remained below 1:1, and that of cholesterol-fed NZW rabbits remained below 2:1 through

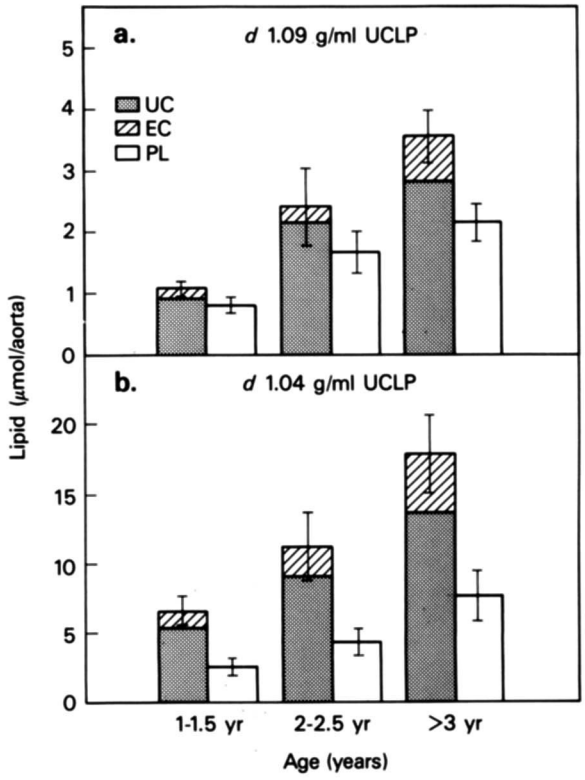

Fig. 6. The amount of unesterified cholesterol, esterified cholesterol, and phospholipid in UCLP isolated from aortas of WHHL rabbits. (a) is d 1.09 g/ml UCLP and (b) is d 1.04 g/ml UCLP. Four rabbits were analyzed in the 1 to 1.5-yr and > 3-yr groups and five rabbits were analyzed in the 2 to 2.5-yr group. The values are averages of the amounts of UCLP constituent for rabbits in each group. The bars indicate SEM for total cholesterol and phospholipid. The values shown were determined from only the peak density fraction of the corresponding UCLP population and thus are an underestimate of the total amount of each UCLP population. There were significant differences for total cholesterol, UC, EC, and PL between > 3-yr and 1 to 1.5-yr groups. PL was also significantly different between the 2 to 2.5-yr and > 1 to 1.5-yr group. EC was significantly different between the 2 to 2.5-yr and > 3-yr groups. UC, unesterified cholesterol; EC, esterified cholesterol; PL, phospholipid.

TABLE 1. Effect of cholesterol feeding on lipid composition of NZW rabbit serum, aortic tissues, and UCLP

Lipid Composition	Weeks of Cholesterol Feeding								
	0	2	4	6	8	11	16	20	30
	mole%								
Serum									
Total cholesterol	53	85	85	85	85	86	86	85	91
Unesterified	18	18	21	27	21	23	24	24	25
Esterified	35	68	64	59	64	62	62	61	66
Phospholipid	47	15	15	15	15	15	14	15	9
% UC/TC	35	20	25	31	25	27	28	29	28
UC/PL	0.4	1.2	1.4	1.8	1.4	1.6	1.6	1.6	2.8
Aortic tissues									
Total cholesterol	34	37	62	78	86	86	88	84	91
Unesterified	34	35	30	35	28	30	25	38	59
Esterified	0	1	32	43	58	56	63	46	32
Phospholipid	66	63	38	22	14	14	12	16	9
% UC/TC	98	94	48	44	33	35	28	46	65
UC/PL	0.5	0.6	0.8	1.6	2.0	2.2	2.1	2.4	6.2
d 1.09 g/ml UCLP									
Total cholesterol	34	34	47	51	45	54	56	58	68
Unesterified	33	33	46	48	42	52	52	55	56
Esterified	1	1	1	3	3	2	4	3	12
Phospholipid	66	66	53	49	55	46	44	42	32
% UC/TC	98	98	97	95	94	96	92	94	82
UC/PL	0.5	0.5	0.9	1.0	0.8	1.1	1.2	1.3	1.7
d 1.04 g/ml UCLP									
Total cholesterol	–	–	72	73	73	73	71	70	75
Unesterified	–	–	56	62	64	65	59	64	62
Esterified	–	–	16	11	9	8	12	16	13
Phospholipid	–	–	28	27	27	27	29	30	25
% UC/TC	–	–	78	85	88	89	83	92	82
UC/PL	–	–	2.0	2.3	2.3	2.4	2.0	2.2	2.5

The number of serum lipid values that were averaged and the number of aortas that were pooled for each time point are the same as those listed in Fig. 1. Dash indicates no lipid particles were present at these time points. % UC/TC, % of cholesterol unesterified; UC/PL, molar ratio of unesterified cholesterol to phospholipid.

20 weeks. Thus, the UC/PL ratio of d 1.04 g/ml UCLP was usually higher than the UC/PL ratio of serum in both rabbit models. By contrast, although the UC/PL molar ratio of the d 1.09 g/ml UCLP showed a progressive increase during cholesterol feeding, this ratio remained below 2:1 in both cholesterol-fed NZW and WHHL rabbits.

As d 1.04 g/ml UCLP accumulated in aortic tissues of cholesterol-fed rabbits, the UC/PL ratio of aortic tissue progressively increased from 0.5:1 to a level of about 2:1, a ratio similar to that of d 1.04 g/ml UCLP. WHHL aortic tissue showed a similar UC/PL ratio that ranged between 2.3:1 and 2.8:1. The UC/PL ratio of aortic tissue from a 3-year-old NZW rabbit never fed cholesterol remained at 0.5:1.

By 30 weeks of cholesterol feeding, the UC/PL ratio of aortic tissue from cholesterol-fed rabbits increased substantially to a value of 6.2:1, suggesting the presence of cholesterol crystal formation at this advanced stage of the disease process. We confirmed this finding by feeding an additional rabbit the same cholesterol diet for another 30 weeks. The UC/PL ratio of that rabbit tissue was 6.3:1.

The large amount of cholesterol that was in unesterified form (% UC/TC) in UCLP appeared to be independent of the serum and aortic tissue lipid composition (Tables 1 and 2). Both d 1.04 and 1.09 g/ml UCLP consistently contained >75% of their cholesterol in unesterified form in WHHL and cholesterol-fed NZW rabbits. This was substantially higher than the % UC/TC of serum which was about 30% in both rabbit models. The % UC/TC of UCLP was also generally higher than the % UC/TC (<50% between 4 and 20 weeks) of aortic tissue from cholesterol-fed NZW rabbits and higher than the % UC/TC (<65%) of aortic tissue from WHHL rabbits.

Phospholipid classes of serum, aortic tissue, and UCLP

Phosphatidylcholine (PC) and sphingomyelin (SPH) were the two predominant phospholipids in d 1.04 g/ml

TABLE 2. Effect of age on lipid composition of WHHL rabbit serum, aortic tissues, and UCLP

Lipid Composition	Age (years) 1–1.5 (4)	2–2.5 (5)	> 3 (4)
		mole%	
Serum			
Total Cholesterol	70 ± 2.2	77 ± 1.4	72 ± 2.2
Unesterified	21 ± 1.5	22 ± 0.5	19 ± 0.5
Esterified	49 ± 4.8	55 ± 1.5	53 ± 1.8
Phospholipid	30 ± 4.0	24 ± 1.4	29 ± 2.2
% UC/TC	31 ± 3.7	28 ± 0.9	27 ± 0.5
UC/PL	0.9 ± 0.1	0.7 ± 0.2	0.7 ± 0.1
Aortic tissues			
Total cholesterol	79 ± 1.6	82 ± 1.2	84 ± 1.3
Unesterified	51 ± 1.8	48 ± 2.7	46 ± 0.9
Esterified	28 ± 2.2	34 ± 3.1	38 ± 1.3
Phospholipid	21 ± 1.6	18 ± 1.2	17 ± 1.3
% UC/TC	65 ± 2.3	59 ± 3.6	55 ± 1.2
UC/PL	2.3 ± 0.4	2.7 ± 0.2	2.8 ± 0.3
d 1.09 g/ml UCLP			
Total Cholesterol	51 ± 1.5	54 ± 2.5	63 ± 1.9
Unesterified	45 ± 1.5	48 ± 2.0	50 ± 0.9
Esterified	6 ± 1.2	6 ± 0.6	12 ± 2.3
Phospholipid	49 ± 1.5	46 ± 3.5	38 ± 1.9
% UC/TC	88 ± 1.8	89 ± 0.7	80 ± 3.2
UC/PL	0.9 ± 0.1	1.0 ± 0.1	1.4 ± 0.1
d 1.04 g/ml UCLP			
Total Cholesterol	72 ± 1.0	72 ± 1.6	71 ± 2.5
Unesterified	58 ± 1.8	58 ± 1.2	54 ± 1.2
Esterified	14 ± 2.6	14 ± 1.5	17 ± 2.2
Phospholipid	29 ± 1.0	28 ± 1.6	29 ± 2.5
% UC/TC	81 ± 3.4	81 ± 1.8	77 ± 2.4
UC/PL	2.1 ± 0.1	2.2 ± 0.1	1.9 ± 0.2

Values are averages ± SEM of determinations made on the number of rabbit aortas indicated in parentheses. Total cholesterol, PL, and UC/PL were significantly different between 1- to 1.5- and > 3-year-old d 1.09 g/ml UCLP groups. % UC/TC, % of cholesterol unesterified; UC/PL, molar ratio of unesterified cholesterol to phospholipid.

UCLP (**Table 3**). The proportion of SPH in d 1.04 g/ml UCLP of NZW rabbits increased during cholesterol feeding. The PC/SPH molar ratio decreased from 1.8:1 at 11 weeks of cholesterol feeding to 0.9:1 by 30 weeks. The d 1.04 g/ml UCLP PC/SPH ratio of WHHL rabbits 1 to 1.5 years old was the same as NZW rabbits fed cholesterol for 30 weeks. The PC/SPH ratio of d 1.04 g/ml UCLP isolated from aortas of the oldest WHHL rabbits (>3 years old) reached an even lower level of 0.5:1, similar to what we have previously reported for d 1.04 g/ml UCLP isolated from human aortas (10). Serum PC/SPH ratios were >2.5:1 and were always higher than their corresponding tissue ratios, which ranged between 0.5:1 and 2.0:1. The tissue ratios were generally more comparable to the ratios observed for d 1.04 g/ml UCLP which ranged between 0.9:1 and 1.8:1. The phospholipid classes of the d 1.09 g/ml UCLP were not analyzed due to insufficient material.

Electron microscopic analysis of UCLP

UCLP of d 1.09 g/ml isolated from aortas of WHHL and cholesterol-fed NZW rabbits were comprised of both spherical and irregularly shaped vesicles, most of which were unilamellar (**Fig. 7**). The longest diameter of the vesicles ranged between 100 and 200 nm. Some of the vesicles had a granular content (Fig. 7a) and some had knob-like projections covering their surfaces (Fig. 7c).

Most of the UCLP isolated from aortas of cholesterol-fed rabbits at d 1.04 g/ml were multilamellar vesicles containing regular tightly apposed concentric membranes that were 35-nm thick (**Fig. 8b**). Most of the d 1.04 g/ml UCLP isolated from WHHL rabbit aortas (Fig. 8a) were multilamellar vesicles with fewer lamella as compared to d 1.04 g/ml UCLP from cholesterol-fed rabbits. Some d 1.04 g/ml UCLP from both rabbit models demonstrated

TABLE 3. Phospholipid classes of d 1.04 g/ml UCLP isolated from aortas of cholesterol-fed NZW and normal-fed WHHL rabbits

Phospholipid	NZW			WHHL	
	Weeks of Cholesterol Feeding			Age (years)	
Class	11 (3)	16 (3)	30 (2)	1–1.5 (3)	> 3 (2)
			mole%		
CL	4.6	3.1	1.5	3.0 ± 0.9	0.2
PI	1.0	0.3	0.2	0.6 ± 0.4	1.3
PS	1.0	2.2	0.2	1.2 ± 0.6	1.1
PE	2.6	1.5	1.8	0.5 ± 0.5	0.1
PC	53.1	51.5	41.2	38.5 ± 2.5	27.3
LPC	8.4	5.3	10.9	11.1 ± 0.8	8.8
SPH	29.3	36.1	44.3	45.0 ± 2.5	61.4
PC/SPH	1.8	1.4	0.9	0.9 ± 0.1	0.5 (0.4, 0.6)

Aortas were pooled for isolation of UCLP from NZW rabbits fed cholesterol. UCLP isolated from individual WHHL aortas were analyzed and the values for each preparation were averaged. The number of aortas pooled for NZW and individual aortas analyzed for WHHL rabbits is shown in parentheses. CL, cardiolipin; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; SPH, sphingomyelin; PC/SPH, phosphatidylcholine/sphingomyelin molar ratio.

a content of loose irregular membranes (65-nm thick) (Fig. 8c) rather than the tightly apposed regular concentric membranes that characterized the majority of d 1.04 g/ml UCLP. Also, some d 1.04 g/ml UCLP were unilamellar vesicles.

The sizes of the d 1.04 g/ml UCLP isolated from aortas of WHHL and NZW rabbits ranged between 60 and 300 nm. An additional population of spherical solid particles was present in the d 1.04 g/ml UCLP density fraction isolated from aortas of WHHL rabbits. These solid spheres (indicated by arrow in Fig. 8a) ranged in size between 30 and 100 nm.

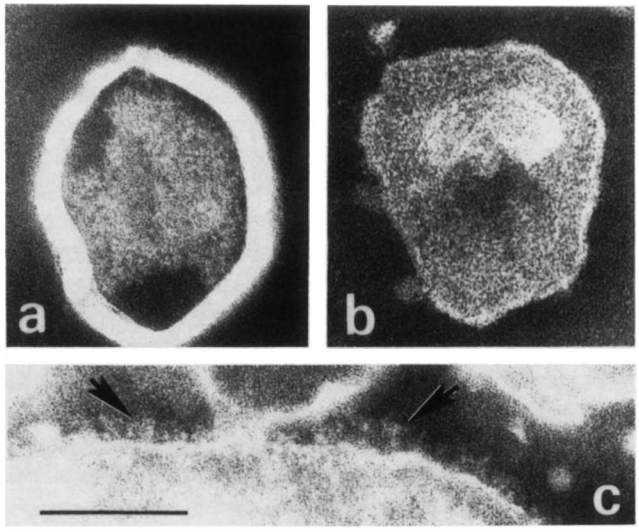

Fig. 7. Electron micrographs of negatively stained d 1.09 g/ml UCLP. UCLP at d 1.09 g/ml were comprised of vesicular structures (a and b). Those shown were isolated from cholesterol-fed NZW rabbits. Those isolated from WHHL rabbits were similar. The d 1.09 g/ml UCLP often showed a granular content (a). Note the knob-like projections (indicated with arrows) that surrounded the surface of some d 1.09 g/ml UCLP particles (c). Bar = 0.1 μm.

The unesterified cholesterol peak that occurred transiently at d 1.06 g/ml at 6 weeks of cholesterol feeding appeared to be a consequence of overlapping d 1.04 g/ml and 1.09 g/ml UCLP populations. The d 1.06 g/ml peak fraction contained a mixture of particles similar to d 1.04 g/ml UCLP (characterized by their multilamellar structure) and d 1.09 g/ml UCLP (characterized by their internal granular content) (data not shown).

No cholesterol crystals were present in any fraction, confirming their removal by the microfiltration step during isolation of the UCLP.

DISCUSSION

Two predominant populations of UCLP with peak densities of d 1.04 and 1.09 g/ml accumulated in aortas of cholesterol-fed NZW and normal-fed WHHL rabbits. Density 1.09 g/ml UCLP were present in control aortas and increased moderately in amount with progressive atherosclerosis. Density 1.04 g/ml UCLP first appeared at 4 weeks of cholesterol feeding and increased greatly with developing atherosclerosis.

Even though both UCLP contained most of their cholesterol in unesterified form (>75%), they differed in other chemical components. Density 1.09 g/ml UCLP tended to contain approximately equal amounts of

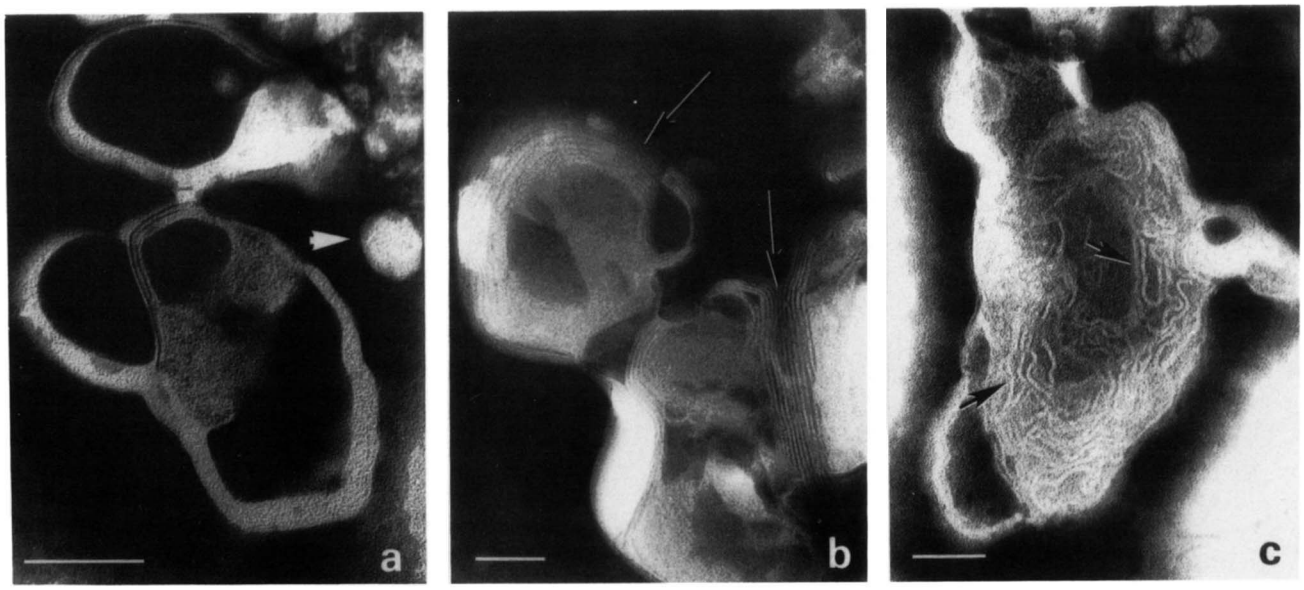

Fig. 8. Electron micrographs of negatively stained d 1.04 g/ml UCLP. UCLP at d 1.04 g/ml were of two types; those that contained regular multilamellar concentric tightly apposed 35-nm wide membranes (a, WHHL and b, NZW), and those that contained irregular loosely arranged 65-nm wide membranes (c). WHHL UCLP had less lamella than cholesterol-fed NZW UCLP (compare a and b). Some spherical, solid-appearing lipid particles (one indicated with arrowhead in a) were present in the d 1.04 g/ml UCLP isolated from aortas of WHHL rabbits. All bars = 0.1 μm.

cholesterol and phospholipid. On the other hand, d 1.04 g/ml UCLP contained about twice as much cholesterol as phospholipid. A 2:1 UC/PL molar ratio has been shown to be the maximum amount of cholesterol that can associate with phospholipid (28, 29). Thus, the d 1.04 g/ml UCLP appear to be fully saturated with unesterified cholesterol when these lipid particles were first detected at 4 weeks of cholesterol feeding.

The two UCLP also were different in their structures. The d 1.09 g/ml UCLP were comprised primarily of unilamellar vesicular particles. The surfaces of some of these particles were covered by small knob-like projections. Such projections are associated with membranes of cells (30, 31) and suggests these particles were derived from cellular membranes. Similar appearing membranous particles are released from several cell types including intestinal goblet cells (32), thyroid epithelial cells (33), red blood cells (34), lymphocytes (35), and platelets (36). These particles provide a mechanism for the selective externalization of membrane components. Although the d 1.04 g/ml UCLP and d 1.09 g/ml UCLP showed differences in composition and structure, it remains possible that the higher density UCLP are precursors of the lower density UCLP.

Some d 1.04 g/ml UCLP are possibly derived from plasma low density lipoprotein (LDL). We have recently shown that in vitro hydrolysis of the esterified cholesterol core of LDL (37) (normally a 25-nm solid spherical particle) converts LDL to larger unesterified cholesterol-rich lipid liposomal particles similar to the d 1.04 g/ml UCLP. Mora, Simionescu, and Simionescu (38) have reported that most unesterified cholesterol-rich lipid particles isolated from thoracic aortas of cholesterol-fed rabbits are not retained by an anti-apolipoprotein B affinity column. Thus, if d 1.04 g/ml UCLP are derived from degradation of LDL, apolipoprotein B (the major protein constituent of LDL) also would appear to be degraded. Indeed, in our in vitro study, we found that hydrolysis of the cholesteryl ester core of LDL did not occur unless apolipoprotein B was first degraded.

In vivo conversion of LDL to liposomes could occur as a result of uptake and re-secretion of LDL-derived cholesterol in the form of UCLP. Schmitz et al. (39) and Robenek and Schmitz (40) showed that mouse peritoneal macrophages take up acetylated LDL and, when treated with nifedipine, secrete multilamellar liposomes enriched in unesterified cholesterol, similar in size to the d 1.04 g/ml UCLP. Their findings and the fact that UCLP accumulate in the extracellular spaces of atherosclerotic lesions (5–8) suggest that many UCLP particles may be constituents of a reverse cholesterol transport pathway.

The cholesteryl ester that was isolated with the d 1.04 g/ml UCLP is probably derived from plasma lipoproteins that had entered the vessel wall. We have previously shown that the fatty acid composition of this cholesteryl ester is more similar to plasma lipoproteins than to cellular lipid droplets (10). This cholesteryl ester does not appear to be an intrinsic constituent of the liposomal UCLP. This was suggested by the fact that the peak densities of cholesteryl ester were d 1.03 g/ml and d <1.01 g/ml as

compared to d 1.04 g/ml for UCLP. Some solid appearing lipoprotein-like spherical particles were present in the d 1.04 g/ml UCLP fraction. These particles most likely contained the cholesteryl ester found with the UCLP.

SPH enrichment of d 1.04 g/ml UCLP occurred during cholesterol feeding in NZW rabbits and with prolonged genetically induced hypercholesterolemia in WHHL rabbits. Whereas the d 1.04 g/ml UCLP isolated from early lesions in cholesterol-fed rabbits were enriched in PC, UCLP from more advanced lesions showed progressive enrichment in SPH. The UC/PL ratio of the early and late d 1.04 g/ml UCLP was approximately 2:1 in both instances. Thus, it appears that UCLP are initially enriched with PC but SPH progressively replaces the PC in UCLP. Consistent with this conclusion is the fact that multilamellar liposomes released from mouse peritoneal macrophages are enriched with PC rather than SPH (39, 40).

The proportion of SPH in aortic tissue phospholipids increases with increasing age and severity of atherosclerosis (41–44). SPH eventually becomes the most abundant aortic phospholipid. Our results show that d 1.04 g/ml UCLP is one location of sphingomyelin accumulation in atherosclerotic lesions. In WHHL rabbits greater than 3 years old, the PC/SPH ratio of UCLP showed the greatest enrichment with SPH. The PC/SPH ratio of 0.5:1 in these older rabbits is the same ratio that we previously reported for UCLP isolated from human aortas (10).

The source of the SPH that accumulates in atherosclerotic lesions is not known. It has been suggested that the accumulated SPH is derived from the plasma (45) or by synthesis in the vessel wall (46). Regarding the latter possibility, Blumenfeld, Schwartz, and Adamany (47) showed that cultured vascular smooth muscle cells synthesize and secrete significant amounts of SPH and PC. Whatever the source of SPH, our data indicate that SPH is enriched in d 1.04 g/ml UCLP relative to serum. This is most evident from a comparison of the PC/SPH ratios of serum and UCLP from WHHL rabbits. The PC/SPH ratio of serum from WHHL rabbits was 3:1 or greater, whereas the PC/SPH ratio of their UCLP was about 1:1 or less.

The accumulation of liposomal UCLP enriched with unesterified cholesterol and SPH during development of atherosclerotic lesions has important implications for the disease process. It has been previously shown that a 2:1 cholesterol-enrichment of phospholipid liposomes can result in spontaneous activation of complement in vitro (18, 19). However, addition of SPH to cholesterol-enriched PC liposomes inhibits this spontaneous activation of complement. Recently, Seifert et al. (48) reported that a cholesterol-rich liposomal particle fraction isolated from human aortas does activate complement. It remains to be determined whether the particles isolated by Seifert et al. are the same UCLP we have isolated from human aortas previously (9, 10) and from rabbit aortas in this study.

The accumulation of lipid particles enriched with unesterified cholesterol and SPH may limit cholesterol removal from lesions. HDL is thought to mediate reverse cholesterol transport from the vessel wall. A number of previous studies (11–17) have shown that artificially produced liposomes, when enriched with either cholesterol or SPH, become resistant to solubilization by HDL. Our studies indicate that the cholesterol- and SPH-enriched UCLP we isolated from atherosclerotic lesions are poorly solubilized by HDL (49).

SPH enrichment may stabilize UCLP and prevent cholesterol from crystallizing in lesions. This possibility is consistent with our experience with both human- and rabbit-derived d 1.04 g/ml UCLP; isolated particles remain stable for months and do not demonstrate cholesterol crystallization. Such stability may reflect the stronger interaction of cholesterol with SPH as compared with other phospholipids (50). This strong interaction of cholesterol and SPH may also explain the progressive enrichment of UCLP with SPH.

The present results demonstrate that UCLP accumulate early in lesion development. They exhibit unusual characteristics possessing a saturated level of cholesterol relative to phospholipid (UC/PL of 2:1) and showing progressive enrichment with SPH. Whereas a high unesterified cholesterol concentration in these particles should predispose to activation of complement and crystallization of cholesterol, progressive enrichment with sphingomyelin should minimize both of these pathologic processes. At the same time, SPH enrichment may limit removal of cholesterol contained within these lipid particles. Because of the unique properties of these lipid particles we believe it is important to further investigate their formation, removal, and role in the pathogenesis of atherosclerosis.

The authors acknowledge Yung-Sing Li, Ya-Jun Chen, Rani Rao, Rufus Seabron, and Nancy Dwyer for their assistance in this study; Carol Kosh for help in preparation of the manuscript; the Veterinary Resources Branch, National Institutes of Health, and the Section on Laboratory Animal Medicine and Surgery, National Heart, Lung, and Blood Institute, for help in care and feeding of the animals.

Manuscript received 26 April 1993 and in revised form 21 July 1993.

REFERENCES

1. Buck, R. C., and R. J. Rossiter. 1951. Lipids of normal and atherosclerotic aortas. *Arch. Pathol.* **51:** 224–237.
2. Böttcher, C. J. F., and F. P. Woodford. 1962. Chemical changes in the arterial wall associated with atherosclerosis. *Fed. Proc.* **21:** (Suppl. 11) 15–19.
3. Insull, W., Jr., and G. E. Bartoch. 1966. Cholesterol, triglyceride, and phospholipid content of intima, media and atherosclerotic fatty streak in human thoracic aorta. *J. Clin. Invest.* **45:** 513–523.
4. Katz, S. S., G. G. Shipley, and D. M. Small. 1976. Physical

chemistry of the lipids of human atherosclerotic lesions: demonstration of a lesion intermediate between fatty streaks and advanced plaques. *J. Clin. Invest.* **58:** 200–211.
5. Kruth, H. S. 1983. Filipin-positive, oil red O-negative particles in atherosclerotic lesions induced by cholesterol feeding. *Lab. Invest.* **50:** 87–93.
6. Kruth, H. S. 1984. Localization of unesterified cholesterol in human atherosclerotic lesions. Demonstration of filipin-positive, oil-red-O-negative particles. *Am. J. Pathol.* **114:** 201–208.
7. Kruth, H. S. 1985. Subendothelial accumulation of unesterified cholesterol: an early event in atherosclerotic lesion development. *Atherosclerosis.* **57:** 337–341.
8. Simionescu, N., E. Vasile, F. Lupu, G. Popescu, and M. Simionescu. 1986. Prelesion events in atherogenesis: accumulation of extracellular cholesterol-rich liposomes in the arterial intima and cardiac valves of the hyperlipidemic rabbit. *Am. J. Pathol.* **123:** 109–125.
9. Chao, F. F., L. M. Amende, E. J. Blanchette-Mackie, S. I. Skarlatos, W. Gamble, J. H. Resau, W. T. Mergner, and H. S. Kruth. 1988. Unesterified cholesterol-rich lipid particles in atherosclerotic lesions of human and rabbit aortas. *Am. J. Pathol.* **131:** 73–83.
10. Chao, F. F., E. J. Blanchette-Mackie, Y. J. Chen, B. F. Dickens, E. Berlin, L. M. Amende, S. I. Skarlatos, W. Gamble, J. H. Resau, W. T. Mergner, and H. S. Kruth. 1990. Characterization of two unique cholesterol-rich lipid particles isolated from human atherosclerotic lesions. *Am. J. Pathol.* **136:** 169–179.
11. Jonas, A., and D. J. Krajnovich. 1978. Effect of cholesterol on the formation of micellar complexes between bovine A-I apolipoprotein and L-α-dimyristoylphosphatidylcholine. *J. Biol. Chem.* **253:** 5758–5763.
12. Tall, A. R., and Y. Lange. 1978. Incorporation of cholesterol into high density lipoprotein recombinants. *Biochem. Biophys. Res. Commun.* **80:** 206–212.
13. Pownall, H. J., J. B. Massey, S. K. Kusserow, and A. M. Gotto, Jr. 1979. Kinetics of lipid-protein interactions: effects of cholesterol on the association of human plasma high density apolipoprotein A-I with L-α-dimyristoylphosphatidylcholine. *Biochemistry.* **18:** 574–579.
14. Guo, L. S. S., R. L. Hamilton, J. Goerke, J. N. Weinstein, and R. J. Havel. 1980. Interaction of unilamellar liposomes with serum lipoproteins and apolipoproteins. *J. Lipid Res.* **21:** 993–1003.
15. Tall, A. R. 1980. Studies on the transfer of phosphatidylcholine from unilamellar vesicles into plasma high density lipoproteins in the rat. *J. Lipid Res.* **21:** 354–363.
16. Tall, A. R., I. Tabas, and K. J. Williams. 1986. Lipoprotein–liposome interactions. *Methods Enzymol.* **128:** 647–668.
17. Swaney, J. B. 1983. Reconstitution of apolipoprotein A-I from human high density lipoprotein with bovine brain sphingomyelin. *J. Biol. Chem.* **258:** 1254–1259.
18. Cunningham, C. M., M. Kingzette, R. L. Richards, C. R. Alving, T. F. Lint, and H. Gewurz. 1975. Activation of human complement by liposomes: a model for membrane activation of the alternative pathway. *J. Immunol.* **122:** 1237–1242.
19. Alving, C. R., R. L. Richards, and A. A. Guirguis. 1977. Cholesterol-dependent human complement activation resulting in damage to liposomal model membranes. *J. Immunol.* **118:** 342–347.
20. Redgrave, T. G., D. C. K. Roberts, and C. E. West. 1975. Separation of plasma lipoproteins by density gradient ultracentrifugation. *Anal. Biochem.* **65:** 42–49.
21. Wolf, A. V., and M. S. Brown. 1979. Concentrative properties of aqueous solutions: conversion tables. *In* Handbook of Chemistry and Physics. CRC Press, Boca Raton, Florida. 227–276.
22. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
23. Gamble, W., M. Vaughan, H. S. Kruth, and J. Avigan. 1978. Procedure for determination of free and total cholesterol in micro- or nanogram amounts suitable for studies with cultured cells. *J. Lipid Res.* **19:** 1068–1070.
24. Fiske, C. H., and Y. SubbaRow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66:** 375–400.
25. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
26. Kaduce, T. L., K. C. Norton, and A. A. Spector. 1983. A rapid, isocratic method for phospholipid separation by high-performance liquid chromatography. *J. Lipid Res.* **24:** 1398–1403.
27. SAS User's Guide. 1979. SAS Institute, Raleigh, NC.
28. Reiber, H. 1978. Cholesterol-lipid interactions in membranes—the saturation concentration of cholesterol in bilayers of various lipids. *Biochim. Biophys. Acta.* **512:** 72–83.
29. Collins, J. J., and M. C. Phillips. 1982. The stability and structure of cholesterol-rich codispersions of cholesterol and phosphatidylcholine. *J. Lipid Res.* **23:** 291–298.
30. Cunningham, W. P., and F. L. Crane. 1966. Variation in membrane structure as revealed by negative staining technique. *Exp. Cell Res.* **44:** 31–45.
31. Benedetti, E. L., and P. Emmelot. 1965. Electron microscopic observations on negatively stained plasma membranes isolated from rat liver. *J. Cell Biol.* **26:** 299–305.
32. Specian, R. D., and M. R. Neutra. 1980. Mechanism of rapid mucous secretion in goblet cells stimulated by acetylcholine. *J. Cell Biol.* **85:** 626–640.
33. Taichiwaki, O., and S. H. Wollman. 1982. Shedding of dense cell fragments into the follicular lumen early in involution of the hyperplastic thyroid gland. *Lab. Invest.* **47:** 91–98.
34. Allan, D., M. M. Billah, J. B. Finean, and R. H. Michell. 1976. Release of diacylglycerol-enriched vesicles from erythrocytes with increased intracellular [Ca^{2+}]. *Nature.* **261:** 58–60.
35. Sachs, D. H., P. Kiszkiss, and K. J. Kim. 1980. Release of Ia antigens by a cultured B cell line. *J. Immunol.* **124:** 2130–2136.
36. Skarlatos, S. I., L. M. Amende, F. F. Chao, E. J. Blanchette-Mackie, W. Gamble, and H. S. Kruth. 1988. Biochemical characterization of isolated cholesterol-phospholipid particles continuously released from rat and human platelets after activation. *Lab. Invest.* **59:** 344–352.
37. Chao, F. F., E. J. Blanchette-Mackie, V. V. Tertov, S. I. Skarlatos, Y-J. Chen, and H. S. Kruth. 1992. Hydrolysis of cholesteryl ester in low density lipoprotein converts this lipoprotein to a liposome. *J. Biol. Chem.* **267:** 4992–4998.
38. Mora, R., M. Simionescu, and N. Simionescu. 1990. Purification and partial characterization of extracellular liposomes isolated from the hyperlipidemic rabbit aorta. *J. Lipid Res.* **31:** 1793–1807.
39. Schmitz, G., H. Robenek, M. Beuck, R. Krause, A. Schurek, and R. Niemann. 1988. Ca^{++} antagonists and ACAT inhibitors promote cholesterol efflux from macrophages by different mechanisms. I. Characterization of cellular lipid metabolism. *Arteriosclerosis.* **8:** 46–56.
40. Robenek, H., and G. Schmitz. 1988. Ca^{++} anatogonists and ACAT inhibitors promote cholesterol efflux from macro-

phages by different mechanisms. II. Characterization of intracellular morphologic changes. *Arteriosclerosis.* **8:** 57–67.
41. Böttcher, C. J. F., and C. M. Van Gent. 1961. Changes in the composition of phospholipids and of phospholipid fatty acids associated with atherosclerosis in the human aortic wall. *J. Atheroscler. Res.* **1:** 36–46.
42. Smith, E. B. 1965. The influence of age and atherosclerosis on the chemistry of aortic intima. Part 1. The lipids. *J. Atheroscler. Res.* **5:** 224–240.
43. Rouser, G., and R. D. Solomon. 1969. Changes in phospholipid composition of human aorta with age. *Lipids.* **4:** 232–234.
44. Eisenberg, S., Y. Stein, and O. Stein. 1969. Phospholipases in arterial tissues. IV. The role of phosphatide acyl hydrolase, lysophosphatide acyl hydrolase, and sphingomyelin choline phosphohydrolase in the regulation of phospholipid composition in the normal human aorta with age. *J. Clin. Invest.* **48:** 2320–2329.
45. Seth, S. K., and H. A. I. Newman. 1975. Sphingomyelin and other phospholipid metabolism in the rabbit atheromatous and normal aorta. *Circ. Res.* **36:** 294–299.
46. McCandless, E. L., and D. B. Zilversmit. 1956. The effect of cholesterol on the turnover of lecithin, cephalin and sphingomyelin in the rabbit. *Arch. Biochem. Biophys.* **62:** 402–410.
47. Blumenfeld, O. O., E. Schwartz, and A. M. Adamany. 1979. Efflux of phospholipids from cultured aortic smooth muscle cells. *J. Biol. Chem.* **254:** 7183–7190.
48. Seifert, P. S., F. Hugo, J. Tranum-Jensen, U. Zâhringer, M. Muhly, and S. Bhakdi. 1990. Isolation and characterization of a complement-activating lipid extracted from human atherosclerotic lesions. *J. Exp. Med.* **172:** 547–557.
49. Chao, F-F., N. Rifai, E. J. Blanchette-Mackie, J. H. Resau, L. M. Amende, and H. S. Kruth. 1991. In vitro solubilization of aortic unesterified cholesterol-rich lipid particles by high density lipoproteins. *Clin. Chem.* **37:** 921 (abstract).
50. Lund-Katz, S., H. M. Laboda, L. R. McLean, and M. C. Phillips. 1988. Influence of molecular packing and phospholipid type on rates of cholesterol exchange. *Biochemistry.* **27:** 3416–3423.