Purification and partial characterization of extracellular liposomes isolated from the hyperlipidemic rabbit aorta

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Abstract Extracellular liposomes (EL) that accumulated in the aortic intima of rabbits on 2 weeks (prelesional stage) and 16 weeks (lesional stage) of diet-induced hyperlipidemia were isolated and purified by gel filtration, ultracentrifugation, and affinity chromatography on anti-apoB and anti-albumin Sepharose. The material obtained after each step was examined by negative staining electron microscopy, by protein analysis (SDS-PAGE, immunoblotting, autoradiography, uronic acid), and by lipid analysis for unesterified cholesterol (UC), cholesteryl esters (CE), phospholipids (PL), triglycerides (TG), thiobarbituric acid reactants (TBAR). EL represented the major constituent of intimal lipid deposits; their predominance on particulate beta-lipoproteins (LP) increased with the duration of hyperlipoproteinemia. As compared with serum low density lipoproteins (LDL) and beta-very low density lipoproteins (beta-VLDL), the crude EL fraction obtained after gel filtration and ultracentrifugation had a decrease in CE and TG, with augmentation of UC, PL, and apoB. After removal of apoB and some albumin by immunoadsorption, the purified EL fraction consisted only of UC, PL, and albumin. The albumin was resistant to proteolytic digestion with pronase, and reacted with anti-albumin antibody only after delipidation of EL. This indicated that albumin was trapped in the aqueous core of vesicles, presumably acting as a scavenger of oxygen-free radicals. TBAR was highly associated with intact or degraded beta-LP. The EL that accumulate in the aortic intima of hyperlipidemic rabbits represent the predominant form of lipid deposits, resulting from the transcytosed excess beta-LP, which is degraded and reassembled upon interaction with the extracellular matrix components. —**Mora, R., M. Simionescu, and N. Simionescu.** Purification and partial characterization of extracellular liposomes isolated from the hyperlipidemic rabbit aorta. *J. Lipid Res.* 1990. **31:** 1793–1807.

Supplementary key words albumin • LDL • beta-VLDL

In experimental diet-induced atherogenesis in the rabbit and hamster, the earliest ultrastrauctural modification so far detected has been the progressive accumulation within the aortic and valvular intima of extracellular liposome-like phospholipid vesicles (100–300 nm) rich in unesterified cholesterol (UC) (1–3). The extracellular liposomes (EL) colocalize with immunoreactive apoprotein B (4) which suggests that their origin is from transcytosed serum beta lipoproteins (beta-LP) whose components are partially degraded and reassembled upon interaction with the extracellular matrix components (1, 5). The progressive EL deposition and the beta-LP distribution within the rabbit aortic wall vary characteristically during successive stages of hyperlipoproteinemic atherogenesis (6). Because of their high content of unesterified cholesterol, these EL were assumed to represent the ultrastructural equivalent of the filipin-positive cholesterol-rich particles revealed by light microscopy in human and animal atherosclerotic lesions (7). Structures similar to the EL have been mentioned in early accounts on the plaque ultrastructure (see ref. 1) as well as in more recent reports (8–10). Of particular relevance was the detection of such structures in the intima of WHHL and cholesterol-fed rabbit aortas prepared by quick-freezing and freeze-etching (10).

Isolation and partial characterization of EL have been recently reported for the early atherogenesis of the aortic valve of the hyperlipidemic hamster (1–2 weeks of diet) (11) and for the advanced atherosclerotic lesions of rabbit (6–9 months of diet) and humans (autopsy material) (12).

In the present study we have developed a procedure for the isolation and purification of EL extracted from the hyperlipidemic rabbit aortas after 2 weeks (prelesional stage, i.e., prior to monocyte adhesion and diapedesis) and 16 weeks (lesional stage) of a cholesterol-rich diet. The purified EL fractions were examined for their structural and chemical composition as compared to serum beta-LP. An unexpected finding was that the EL trap serum albumin within their core.

Abbreviations: UC, unesterified cholesterol; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; H-diet, hypercholesterolemic diet; EM, electron microscopy; LP, lipoproteins; LDL, low density lipoproteins; R-LDL, rabbit LDL; RSA, rabbit serum albumin; VLDL, very low density lipoproteins.

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METHODS

Preparation of special materials

Rabbit low density lipoprotein (R-LDL). Serum LDL fraction was isolated by a single-step density gradient ultracentrifugation using a modification of the procedure of Redgrave, Roberts, and West (13). The discontinuous gradient was constructed from bottom to top with 7 ml serum (brought to d 1.3 g/ml by addition of solid KBr), 4 ml of d 1.006 g/ml KBr solution, and 1 ml water. The centrifugation in a Beckman SW-41 rotor in an L5-75 Beckman ultracentrifuge was carried out for 24 h at 272,000 *g*.

Rabbit apolipoprotein B (R-apoB) was obtained from rabbit serum LDL of normolipidemic animals, according to Helenius and Simons (14).

Rabbit serum albumin (RSA) was prepared from pooled rabbit sera subjected to affinity chromatography on Blue Sepharose CL-6B (Pharmacia-LKB Biotechnology, Uppsala, Sweden) using a 1.2 × 40 cm column (15). The albumin fraction thus obtained was then gel-filtered on a Sephadex G-200 column (2 × 90 cm) (Pharmacia-LKB Biotechnology, Uppsala, Sweden) using PBS as eluant at a flow rate of 8 ml/h.

Radioiodinated albumin and radioiodinated immunoglobulin. Rabbit serum albumin (RSA) and affinity-purified rat or goat immunoglobulin G (IgG) were iodinated with carrier-free $Na^{125}I$ (from the Institute of Atomic Energy, Otwock, Swierk, Poland) using the iodogen procedure (10 μg/100 μg protein) according to Fraker and Speck (16). Unbound iodine was removed by chromatocentrifugation (17) and dialysis against PBS. The specific radioactivities were 0.893 μCi/μg RSA and 0.222–0.802 μCi/μg IgG. In the trichloroacetic acid-precipitated protein, the radioactivity represented ~95% for RSA and ~93% for IgG.

Anti-rabbit apoB antiserum was raised in 50 female rats (R strain). The antigen (0.50 and 0.25 mg of R-apoB, in Freund's complete and incomplete adjuvants, respectively) was administered subcutaneously 1 week apart. Two weeks after the last injection, blood was collected.

Anti-rabbit albumin antiserum was prepared by immunizing goats, at 1-week intervals, with three subcutaneous injections of 40, 60, and 40 mg RSA; the first dose was in complete and the boosters in incomplete Freund's adjuvants. The blood was collected 2 weeks after the last injection.

Anti-apoB IgG and immunoadsorbent column. Normolipidemic rabbit LDL was attached to CNBr-activated Sepharose CL-4B (Pharmacia-LKB Biotechnology) (18). After coupling, the Sepharose was inactivated with 1 M ethanolamine, pH 8.5. A 40-ml column (1 mg R-LDL protein/ml gel) was equilibrated with PBS and 80 ml of rat anti-rabbit apoB antiserum was passed through it. After extensive washing with buffer, the anti-apoB IgG was eluted with 0.2 M glycine–HCl, pH 2.8. After dialysis against water, the IgG fraction was lyophilized. The anti-apoB IgG was then attached to CNBr-activated Sepharose CL-4B and 10-ml columns (2 mg IgG/ml gel), with a retaining capacity of 10 mg apoB, were prepared.

Anti-albumin IgG and immunoadsorbent column. Forty mg of rabbit albumin was coupled to CNBr-activated Sepharose CL-4B at a concentration of 2 mg protein/ml gel. The anti-albumin IgG (obtained by affinity chromatography from 100 ml goat anti-albumin antiserum) was attached to CNBr-activated Sepharose CL-4B and 10-ml columns (2.2 mg IgG/ml gel), with a retaining capacity of 12 mg albumin, were prepared.

Animal model

Chinchilla adult male rabbits, 2.3–2.7 kg body weight, housed separately in single cages, were divided into two groups. One set (80 animals) was fed a standard pelleted chow diet supplemented with 0.5% cholesterol and 5% butter. The control group (28 animals) was fed the same diet without supplement. At 2 weeks and 16 weeks of the hypercholesterolemic diet, the rabbits were killed under general anesthesia with an intraperitoneal injection of 10% chloral hydrate (0.3 g/kg body weight). Blood samples were collected by cardiac puncture.

The total serum cholesterol before the induction of the diet was 40–60 mg/dl (normolipidemic rabbits); the values increased to 300–900 mg/dl after 2 weeks, and to 2,000–2,800 mg/dl after 16 weeks of the fat-rich diet (hyperlipidemic rabbits).

Isolation and purification of extracellular liposomes

The protocol to be described was established by several preliminary experiments from which the most efficient steps for extraction and purification of a highly enriched EL fraction were selected.

Preparation of aortic extracts. The thoracic aorta, including the aortic arch, was excised, briefly rinsed in cold Tris-buffered saline (10 mM Tris-HCl in 0.14 M NaCl, pH 7.4) to completely remove the blood. The vessel was then immersed in ice-cooled TBS containing 0.01% ethylenediamine tetraacetate, sodium salt (Na_2EDTA), 0.01% sodium azide, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.008% gentamycin, and 0.001% butylated hydroxytoluene (BHT), and the adventitia was excised under a stereomicroscope. At 2 weeks of diet, the aortic intima appear grossly normal; in these animals the intima-media tunics were removed together. At 16 weeks of hypercholesterolemic (H)-diet, the aortic intima displayed frequent, often confluent, fatty streaks which under a stereomicroscope, were stripped off from the underlying media. The aortic tissue thus collected from 10–20 aortas (2 weeks of H-diet) or 1 or 2 aortas (16 weeks of H-diet) were minced and suspended in cold TBS (3 mg/g tissue) of same composition as mentioned above, and centrifuged at 8,700 *g* for 10 min at 4°C. The very thin creamy top layer (in which the presence of lipid

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droplets was detected by phase contrast microscopy) that separated from samples of aortas with atherosclerotic lesions was discarded. The supernatant fractions were then subjected to the following procedure to obtain a purified fraction of extracellular liposomes. The flow diagram in **Fig. 1** gives the main steps of this protocol.

Gel filtration chromatography. The low-speed supernatants from the aortic saline extracts were brought to 2 M NaCl and fractionated by gel filtration on a 75 × 2 cm column of Sepharose CL-6B (Pharmacia-LKB Biotechnology) equilibrated with the elution buffer (2 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.05% Na_2EDTA, 0.02% NaN_3, and 0.001% BHT). The run was performed at a flow rate of 8 ml/h and 2-ml fractions were collected. The elution profiles are illustrated in **Fig. 2**. Peak I collected in the void volumes (at 40 to 60 ml) was shown, by negative staining (19), to contain a large population of extracellular liposomes, particulate lipoproteins, and fibrillar material, probably originating from the extracellular aggregated matrix (**Fig. 3**).

Density gradient ultracentrifugation. The material eluted in the void volume of the gel filtration column under peak I was subjected to density gradient ultracentrifugation for 24 h at 272,000 *g* using the procedure outlined above for the isolation of serum lipoproteins. The refractive indices of the fractions were measured with an Abbe refractometer (Carl Zeiss, Jena, GDR) and their corresponding densities were determined with a conversion table. As determined by negative staining of corresponding aliquots, the fractions of densities 1.02 to 1.08 g/ml appeared to contain the bulk of EL. These fractions were dialyzed against 0.14 M NaCl containing 0.01% Na_2EDTA, pH 7.4, and the dialysates were subjected to affinity chromatography.

Affinity chromatography on anti-apoB-Sepharose. To separate the EL from the particular beta-lipoproteins known to occur in the rabbit aortic intima at this time of H-diet (4, 6, 20, 21), the dialyzed EL-enriched fraction was applied to an IgG anti-apoB–Sepharose CL-6B column equilibrated with the elution buffer (0.14 M NaCl and 0.01% Na_2EDTA, pH 7.4). The sample remained in contact with the immunoadsorbent for 3 h at 4°C, then was eluted

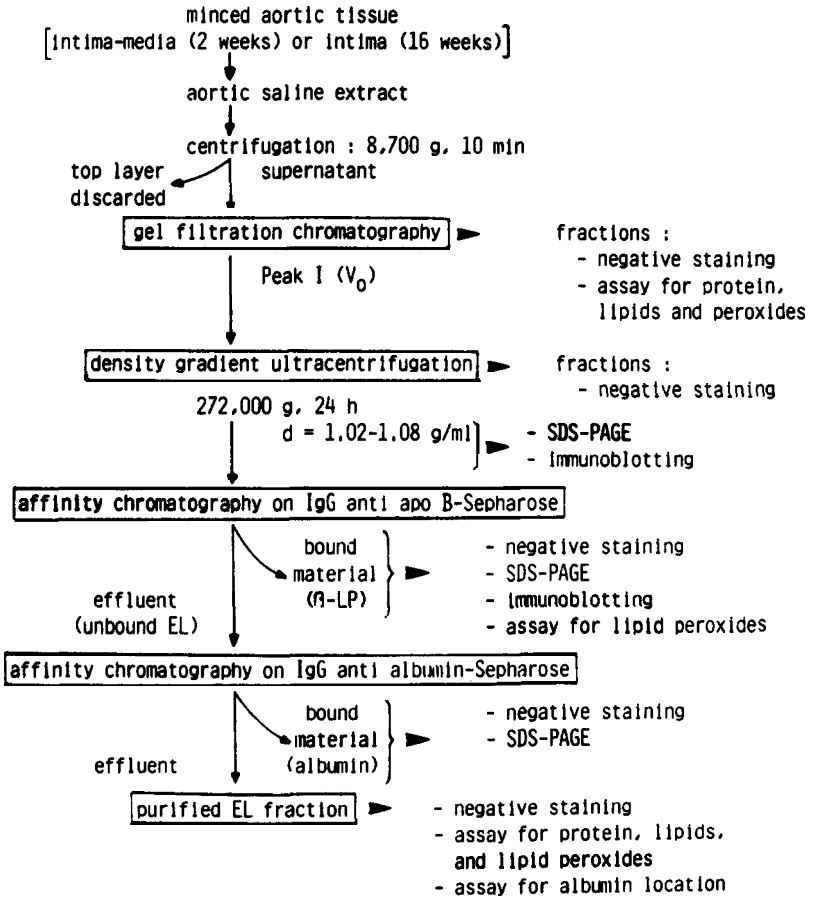

Fig. 1. Flow diagram of the extraction, isolation, and purification procedures used to obtain extracellular liposomes (EL) from the aorta of normal hyperlipidemic rabbits, after 2 or 16 weeks of cholesterol-rich diet. On the right side of the panel are indicated the main analysis performed for the characterization of the EL fraction obtained after each step of the procedure.

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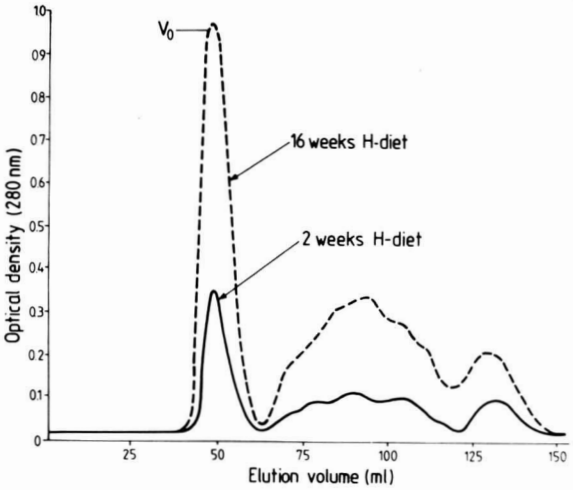

Fig. 2. Gel filtration chromatography on a Sepharose CL-6B column of aortic saline extracts of rabbits fed a hyperlipidemic diet for 2 weeks (——) or 16 weeks (----). As shown by the negative staining preparations, the great majority of the extracellular liposome fraction (including particulate lipoproteins) eluted in the void volume (V_o). Aortic extracts from normal animals showed a very small peak in the void volume fraction (not illustrated).

with the same buffer at a flow rate of 10 ml/h. The recovered effluent was examined by negative staining and used for the second affinity chromatography. The bound material was eluted with 0.2 M NaCl, 0.05 M $NaHCO_3$-NaOH, pH 11.0, and analyzed by negative staining and assayed by SDS-PAGE for its content of apoB and albumin.

Affinity chromatography on anti-albumin–Sepharose. To further purify the EL fraction from its possible contaminant albumin (known to be present in the intima of these hyperlipidemic animals even at 2 weeks of H-diet [Mora, R., M. Simionescu, and N. Simionescu, unpublished observations]), the EL fraction recovered in the unbound material of the previous affinity chromatography was applied to an IgG anti-albumin–Sepharose CL-6B column. Both the unadsorbed and the adsorbed materials were eluted as described above and examined by negative staining and SDS-PAGE assays for apoB and albumin. The unbound effluent of this column was considered the purified EL fraction which was used for chemical characterization.

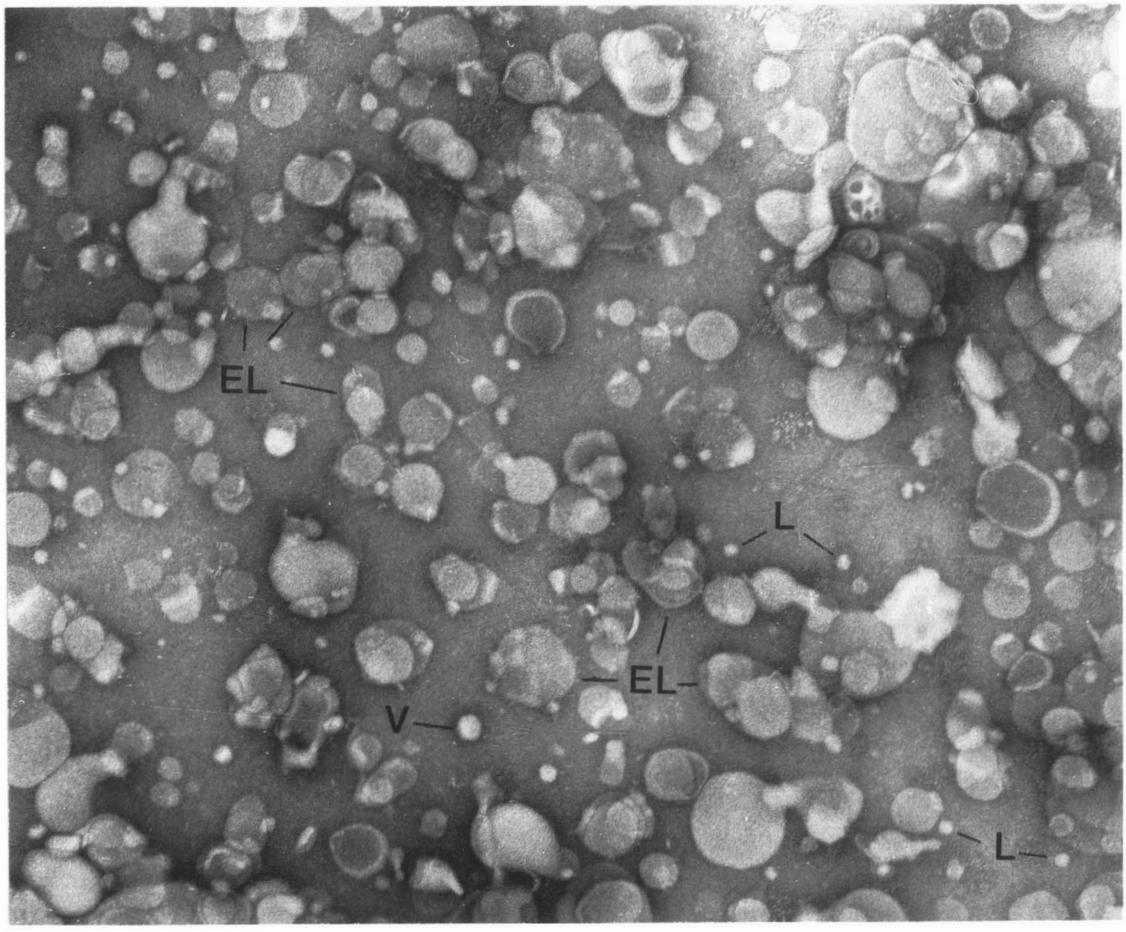

Fig. 3. Electron micrograph of a negatively stained aliquot of the aortic EL fraction (lesional stage) obtained by gel-filtration chromatography. The fraction contains a heterogeneous population of vesicle (EL) and lipoprotein-like particles in the size range of serum LDL (L) and beta-VLDL (V); × 77,000.

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Analysis of EL fractions as compared with serum LDL and beta-VLDL

Negative staining. The efficiency of each step used for EL purification was monitored by negative staining of aliquots of EL fractions using 1% sodium phosphotungstate adjusted to pH 8,0 (19). The grids were examined with a Philips 201C and 400 HM electron microscopes.

Thin-section electron microscopy of purified EL fractions. For thin-section EM analysis of purified EL fractions, the procedure using sequential tannic acid and *p*-phenylenediamine treatments of osmicated tissue was used, as recently described by Guyton and Klemp (22). Briefly, EL fractions were included in 0.8% agarose (previously melted and then cooled to about 40°C). The samples were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 18–24 h at 4°C, followed by rinsing in buffer. All subsequent steps were at 25°C. Postfixation was in 1% OsO_4 in the same buffer for 120–150 min. This was followed by three quick changes of buffer over a total of 5 min, treatment with 1% tannic acid in 0.05 M Na cacodylate, pH 7.4, for 30 min, and a wash in 1% Na_2SO_4 in 0.05 M Na cacodylate, pH 7.4, for 5 min. Samples were rinsed in 70% ethanol for three changes of 5 min each, then placed in fresh 1% *p*-phenylenediamine in 70% ethanol for 30 min, and rinsed again with three changes of 70% ethanol of 5 min each. The dehydration and embedding schedule was as follows: 95% ethanol, 15 min; 100% ethanol, 15 min; 1:1 Epon-resin mixture: ethanol, 1 h, resin overnight, resin 2 h, and embedding. Thin sections were cut using a Reichert OmV3 Ultratome. Sections were viewed after brief staining with 7% aqueous uranyl acetate and with alkaline lead citrate.

Protein analysis

Protein concentration was determined by Peterson's modification (23) of the Lowry method using BSA as standard.

Gel electrophoresis. Samples of EL fraction, concentrated by precipitation with 7% trichloracetic acid and 0.015% sodium deoxycholate (final concentration) and delipidated with hexane–isopropanol 3:2 were denatured at 100°C for 3 min in 0.06 M Tris-HCl buffer, pH 6.8, containing 2% sodium dodecylsulfate (SDS) and 5% 2-beta-mercaptoethanol. The aliquots were applied on SDS-polyacrylamide 5–15% gradient gels (24) and electrophoresed at a constant current of 10 mA for 14 h. After fixation in 30% ethanol and 10% acetic acid, the proteins were stained with 0.1% Coomassie Brilliant Blue G-250 in 50% methanol-10% glacial acetic acid.

Immunoelectroblotting and autoradiography. The proteins separated by the above SDS-PAGE were electrophoretically transferred on nitrocellulose membranes (NC) (Schleicher and Schuell Inc., Keene, NH) in 0.04 M Tris-boric acid, pH 8.3, (25), except that methanol was omitted for the transfer buffer. The electrotransfer was conducted for 7 h at 60 V, at 10–12°C using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Labs., Richmond, CA). The NC membranes were then removed and quenched with 1% hemoglobin in PBS, overnight at 22°C. The strips were then incubated overnight at room temperature with the radioiodinated affinity-purified rat anti-rabbit apoB IgG or goat anti-rabbit albumin IgG at a concentration of 3.6–4.0 μg IgG/ml in PBS containing 0.1% hemoglobin (PBS/Hb). Strips were rinsed for 2 h × 2 in PBS containing 0.05% Triton X-100, then thoroughly washed in PBS. The strips mounted on a piece of paper were radioautographed overnight at −70°C on X-ray films from Eastman Kodak (Rochester, NY) and Azo-Mures (Tg. Mures, Romania). The gels and electroblots were calibrated with high molecular weight protein standards (Pharmacia-LKB Biotechnology or Merck, Darmstadt, FRG).

Quantitation of uronic acid content. After lyophilization and delipidation with chloroform–methanol 2:1 followed by ether, the EL were digested with papain (28 U/mg) for 24 h at 60°C to obtain the glycosaminoglycan (GAG) moieties of proteoglycans (26). The hexuronic acid content of GAG content was evaluated by using the borax–H_2SO_4–carbazole procedure (27). The GAG content was evaluated by using a factor of 3.3 to convert the uronic acid values to GAG (26).

Lipid analysis

Total cholesterol was measured either enzymatically using a Sigma kit, or chemically by the method of Zak (28).

Unesterified cholesterol precipitated with digitonin was determined according to Zak as described by Fukuda, Azain, and Ontko (29). The difference between the total and unesterified cholesterol content was multiplied by a factor of 1.7 to calculate the cholesteryl ester content (29).

Lipid phosphorus was determined according to Ames and Dubin (30) and phospholipids were calculated using a multiplication factor of 25.

Triglyceride was determined chemically using a Chemapol reagent kit or enzymatically with a Sigma enzymatic kit (31).

Alternatively, the main lipid classes were measured after solvent extraction (32) and subsequent separation by thin-layer chromatography (TLC). Silica gel TLC was performed on Sigma precoated plates developed in petroleum ether–diethyl ether–acetic acid 90:10:1. The plates were exposed to I_2 vapors and spots corresponding to the lipid standards run on the same plates were then scraped from the plates. The lipids were eluted from the gel (33) and measured using the methods outlined above. Solvent blanks and blank areas from each TLC plate were used as controls.

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Determining the location of EL-associated albumin

Pilot experiments indicated that the final purified EL fraction contained detectable amounts of albumin although passed through the anti-albumin affinity column. To reveal whether this albumin is attached on the EL contour or is located inside EL, we designed experiments in which the purified EL fraction was subjected to one of the following treatments: *a*) peptic digestion (to remove albumin if adsorbed on EL surface); *b*) delipidation to release the EL content; and *c*) incubation of aortic fragments with radiolabeled albumin before mincing, to check whether albumin is artifactually trapped within EL during the isolation manipulations.

Pronase digestion. Samples of purified EL fraction and RSA aliquots (5–15 μg protein) dialyzed against 0.07 M phosphate buffer or 0.1 M Tris-HCl, pH 7.4, were digested with 100 or 200 μg Pronase E (70,000 PVK/g, Merck-Darmstadt) for 2–7 h at 37°C. Samples were then precipitated with 7% trichloroacetic acid (final concentration). After centrifugation for 5 min at 8,700 *g*, the pellet was resuspended in solubilization buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 5% 2-beta-mercaptoethanol), boiled at 100°C for 3 min, and analyzed by SDS-PAGE as described above. Gels were silver-stained (34).

Delipidation followed by dot-blot immunolabeling and autoradiography. Aliquots (3–10 μg protein) of purified EL fraction were applied to nitrocellulose, membranes and the sheets were used either wet or dried for 15 min at 22°C. The undried sheets were immediately immersed in PBS. The dried membranes were delipidated with chloroform–methanol 2:1 for 15 min, then kept for 15 min at 22°C evaporate the solvent. Both membranes (undelipidated and delipidated) were incubated overnight with 1% Hb in PBS to quench the unreacted sites. Sheets were then overlayered with (3.6–4.0 μg/ml) ^{125}I-labeled goat anti-rabbit albumin IgG or rat anti-rabbit apoB IgG in PBS/Hb. After washing with PBS, the dot blots were exposed overnight to X-ray films at −70°C.

Delipidation followed by agarose gel electorphoresis. Aliquots of affinity-purified EL fraction, before and after delipidation with hexane–isopropanol 3:2 (v/v), were electrophoresed in 0.8% agarose in 0.075 M sodium barbiturate–barbituric acid, pH 8.6, for 4 h at 100 V. The plates were stained for protein with 0.1% Coomassie Brilliant Blue G-250 in 50% methanol and 10% glacial acetic acid.

Control experiments with albumin. To assess whether free albumin can be artifactually trapped within EL during the extraction procedure and subsequent manipulations, we added 125 μg of ^{125}I-labeled RSA (111.6 μCi) to the aortas (at 2 weeks H-diet) before mincing. The protein moiety (~80 μg) or EL fraction thus prepared was precipitated with trichloroacetic acid, delipidated with chloroform–methanol, and radioactivity was measured.

Assay for lipid peroxides

The malondialdehyde (MDA) adducts for serum LDL, VLDL, and EL fractions were determined spectrophotometrically upon reaction with thiobarbituric acid (35–37) and expressed as nmoles of MDA equivalents per mole liquid.

RESULTS

As previously reported, the aortic intima in normal rabbit is virtually devoid of macrophages and smooth muscle cells (1). Material gently extracted with saline from the aortic intima of normolipidemic rabbits when subjected to gel filtration chromatography under the conditions mentioned above, yielded a very small lipid peak which, upon density gradient ultracentrifugation, remained included in the bottom fraction (d = >1.1 g/ml). By phase contrast microscopy, this material contained cell membrane debris originating probably from the overlying endothelium.

Gel filtration chromatography

After 2 weeks of H-diet, the saline extracts subjected to gel filtration yielded, in the void volume (Fig. 2), a major fraction of a nonuniform population of vesicular structures (extracellular liposomes) of 40–300 nm in diameter, as well as beta-lipoprotein-like particles (20–55 nm) (Fig. 3). The retarded fraction also contained a certain amount of beta-lipoprotein-like particles 20–30 nm in diameter. After 16 weeks of H-diet the elution pattern had a similar feature with a markedly higher content in EL (Fig. 2).

In each of these two groups of experiments, the void volume fractions were pooled, examined for their chemical composition, and further concentrated by density gradient ultracentrifugation.

Chemical composition

The chemical composition, expressed on molar basis, of the EL fractions extracted from rabbit aortas in prelesional and lesional stages of atherogenesis, and subjected to gel filtration chromatography, is shown in **Table 1**. As compared with the serum LDL and beta-VLDL (which represented 25% of plasma beta-lipoproteins [LP]) collected from the same animals, at 2 weeks of H-diet, the EL fraction was characterized by a decreased relative concentration of cholesteryl ester (CE) and triglycerides (TG) with increased concentration of unesterified cholesterol (UC), phospholipids (PL), and protein (P). The percentage UC/total cholesterol (TC) ratio was about double the value found in plasma LP. In the EL fraction from lesional aortas (16 weeks on H-diet) when beta-VLDL accounted for 50% of serum LP, the percentage of cholesterol in unesterified form, as well as PL, further augmented, while TG continued to decline.

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TABLE 1. Composition of the extracellular liposome fraction (EL) subjected to gel filtration chromatography; comparison with serum β-VLDL and LDL

Component	2 Weeks on H-Diet (Prelesional Stage)			16 Weeks on H-Diet (Lesional Stage)		
	β-VLDL	LDL	EL	β-VLDL	LDL	EL
Cholesteryl ester (CE)[a]	44.9 ± 4.4	53.2 ± 1.7	33.1 ± 1.7	55.8 ± 3.1	54.8 ± 2.2	19.8 ± 1.3
Unesterified cholesterol (UC)[a]	15.4 ± 1.1	20.2 ± 0.7	31.0 ± 0.9	18.0 ± 0.8	20.4 ± 1.0	49.5 ± 1.4
Phospholipid (PL)[a]	22.2 ± 1.8	17.3 ± 0.7	32.7 ± 1.7	18.2 ± 1.1	17.5 ± 1.0	29.0 ± 0.9
Triglyceride (TG)[a]	17.5 ± 2.5	9.3 ± 1.6	3.2 ± 0.3	8.0 ± 1.1	7.3 ± 1.3	1.7 ± 0.2
Lipid % of total weight	95.1 ± 0.5	82.8 ± 2.0	70.1 ± 2.5	96.2 ± 0.3	86.3 ± 1.5	81.5 ± 1.1
Protein % of total weight	4.9 ± 0.5	17.2 ± 2.0	29.9 ± 2.5	3.8 ± 0.3	13.7 ± 1.5	18.5 ± 1.1
UC/PL (molar ratio)	0.7 ± 0.1	1.2 ± 0.1	0.95 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.7 ± 0.1
% UC/TC[b]	25.5 ± 4.2	27.5 ± 1.9	48.4 ± 3.4	24.4 ± 2.4	27.1 ± 2.5	71.4 ± 4.8

[a]Values for lipids are mole %, means ± SEM of three to five preparations. The molecular weights of CE, UC, PL, and TG were taken as 666, 387, 800, and 885 Da, respectively.
[b]TC, total cholesterol.

Density gradient ultracentrifugation

In the range density of 1.02–1.08 g/ml, a broad opaque band was found in which about 85–90% of the total cholesterol of the gradient was recovered. By electron microscopy of negatively stained aliquots, this band contained EL of 40–300 nm and lipoprotein-like particles of 20–55 nm (Fig. 3). The relative fraction represented by polymorphic EL was largely predominant in advanced (16 weeks of H-diet) stages of aortic lesions. As a general pattern, at prelesional stages of atherogenesis (2 weeks of H-diet), the EL concentration was maximum around d 1.06 g/ml, whereas at 16 weeks of diet the EL were centered around d 1.06 g/ml, whereas at 16 weeks of diet the EL were centered around d 1.04 g/ml. Both in prelesional and lesional stages of hyperlipidemia, trace amounts of EL and LDL-like particles were found in the 1.2 g/ml density fraction which contained 7–10% of the total cholesterol of the gradient. In lesioned aortas, about 3–5% of total cholesterol was recovered in the d < 1.02 g/ml fraction.

The d 1.02–1.08 g/ml fraction containing the bulk amount of EL was analyzed for its proteins by SDS-PAGE and subsequent immunoblotting; representative results are shown in **Fig. 4**. Among the electrophoresed proteins of this fraction, several bands located at positions below the level of the seric LDL-apoB-100 (lane a) immunostained positively for apoB, suggesting that these peptides represented immunoreactive fragments of apolipoprotein B-100 (Fig. 4, lanes b and c). This was an indication that the lipoprotein-like particles seen in negatively stained preparations of this fraction were largely represented by degraded beta-lipoproteins. In the same ultracentrifugal isolates a band of 66 kDa was immunologically identified as albumin (Fig. 4, lane d). This protein could not be removed by repeated ultracentrifugations, suggesting its tight association with EL. In the electrophoresed serum beta-lipoproteins, no detectable bands comigrating with rabbit serum albumin were observed (Fig. 4, lane a).

Affinity chromatography

At the completion of the affinity chromatography on IgG anti-apoB-Sepharose, both the unbound effluent and the immunoadsorbed material that was subsequently eluted were examined by electron microscopy (negative staining), SDS-PAGE, and immunoblotting. The nonadsorbed effluent contained a high concentration of EL and was practically devoid of reactive apoB, but expressed an albumin band. The bound material consisted mostly of lipoprotein-like particles and few small EL; immunoblots were positively stained by anti-apoB antibody and to a very little extent by anti-albumin antibody.

After passing the above unbound effluent through the second affinity column (IgG anti-albumin-Sepharose) the subsequent unadsorbed effluent as well as the bound (and then eluted) material were examined by negative staining and analyzed for lipid and protein composition. The unbound effluent contained almost exclusively EL, which in gel electrophoresis expressed a sole protein band corresponding to albumin (Fig. 4, lane e). The final purified EL fraction of rabbit aortas at either prelesional or lesional stages of atherogenesis displayed a similar electrophoretic pattern with a single protein, albumin (Fig. 4, lane e).

Characterization of affinity-purified EL fraction

When prepared for negative staining, some EL upon drying, appeared as collapsed vesicles but the majority maintained their sac-like shape with a slightly flattened electron-lucent appearance (**Fig. 5**). Some of them had surface protrusion which may suggest either coalescence of lipoprotein-derived vesicles or locally condensed lipid material (Fig. 5). The size range of EL isolated at both prelesional and lesional stages of atherogenesis was between 40 and 300 nm. Approximately 92% of the EL at prelesional stage and 85% of the EL at lesional stage measured between 45 and 160 nm. About 78% of the

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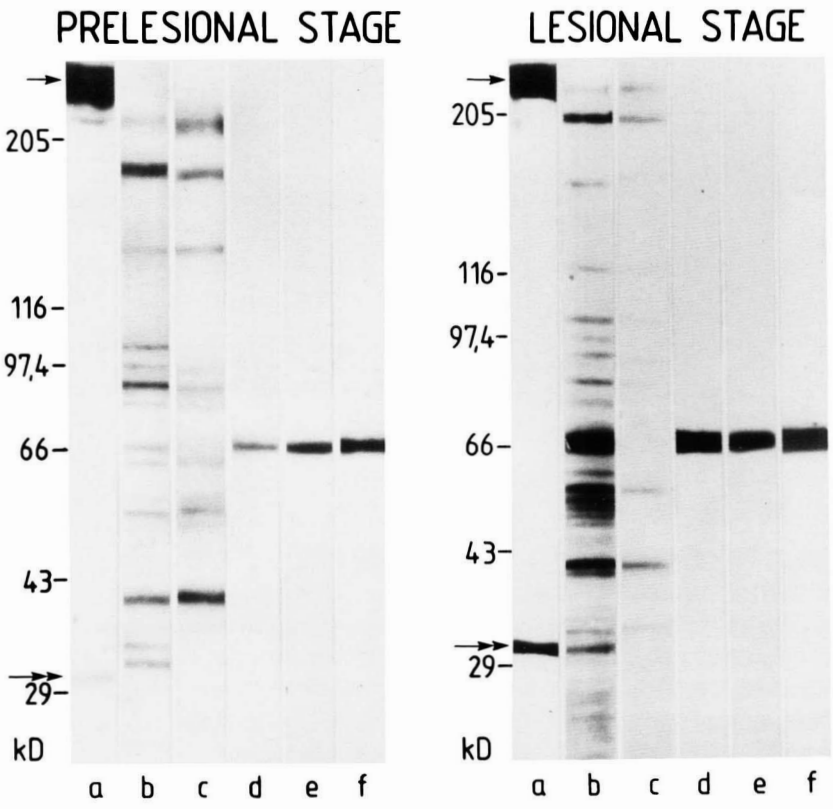

Fig. 4. SDS-polyacrylamide gel-electrophoresis at different steps of purification of the EL fraction obtained from extracts of rabbit aorta at prelesional (2 weeks) and lesional (16 weeks on H-diet) stages of hyperlipidemia; (a) rabbit serum LDL; (b) EL fraction obtained after gel filtration chromatography followed by density gradient ultracentrifugation; (c) electroimmunoblotting of EL fraction in b, showing that the partially purified EL react with anti-apoB, revealing several immunoreactive bands; (d) immunoblotting of the EL fraction in b revealing its reactivity with anti-albumin; (e) the EL fraction purified by affinity chromatography (on anti-apoB and anti-albumin-Sepharose columns) displays albumin as the sole protein component; (f) rabbit serum albumin; arrow: position of apoB; double-head arrow: position of apoE.

vesicles at prelesional stage had a diameter between 45 and 125 nm with a peak diameter of 85 nm. At lesional stage, 66% of the vesicles had a diameter between 60 and 140 nm with a peak diameter of about 100 nm. The lesional EL were more heterogeneous in size and shape than prelesional EL. As shown by both the negative staining as well as by the tannic acid–phenylenediamine preparations (**Fig. 6**), the prelesional EL were commonly vesicles formed by one or two phospholipid lamellae (about 5 nm thick) whereas the lesional EL were frequently oligolamellar. At the completion of the four-step purification procedure, the EL fraction extracted from prelesional aortas (2 weeks of H-diet) contained cholesterol in a predominantly unesterified form, bringing the UC/TC ratio to about 98% (**Table 2**). The relative high proportion of protein (~19%) was represented by trapped albumin exclusively. PL accounted for about 50% of the total EL fraction mass. The EL fraction extracted from lesional aortas (16 weeks of H-diet) was largely represented by PL, UC, and albumin (Table 2). The yield of EL-cholesterol obtained after four-step purification accounted for 12 ± 0.6% and 59 ± 3% of total cholesterol of the initial aortic saline extract from prelesional and lesional stages of hyperlipidemia, respectively (**Table 3**). The EL-trapped albumin accounted for about 1.7% and 17.5% of total albumin isolated from saline aortic extracts at prelesional and lesional stages of atherogenesis, respectively (Table 3).

The thiobarbituric acid-reactants (TBAR) were determined in serum lipoproteins and in the EL fraction extracted from aortas in the lesional stage (16 weeks of H-diet). TBAR were highly concentrated in the affinity-adsorbed beta-lipoproteins (**Table 4**).

Under the preparation conditions used in our experiments, the proteoglycan content was largely variable in the crude EL fraction obtained by gel filtration chromatography (4 to 8 μg/100 μg protein). In the affinity-purified EL fraction, proteoglycans were virtually undetectable.

The affinity-purified aortic beta-LP had a decreased lipid and an increased apoB concentration as well as a

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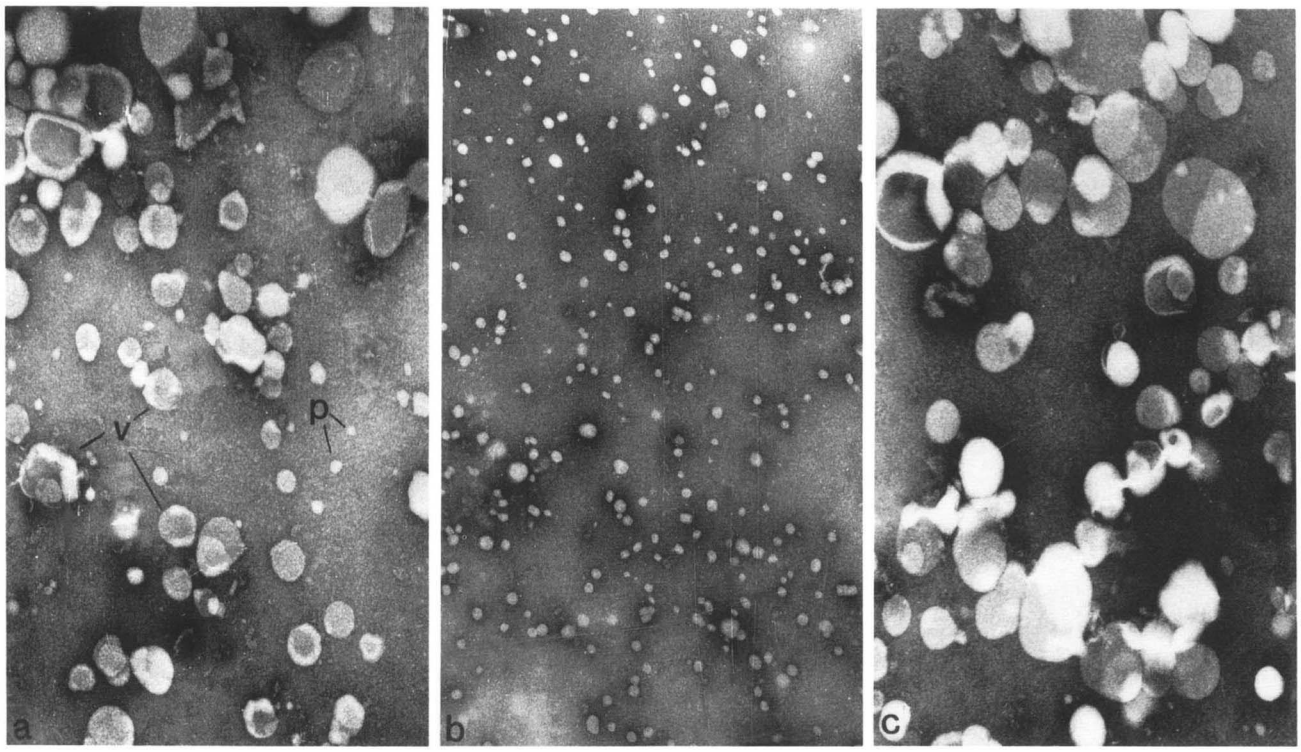

Fig. 5. Electron micrographs of negatively stained aliquots of the aortic EL fractions (lesional stage) at different stages of purification. (a) The crude EL fraction obtained after gel filtration and density gradient ultracentrifugation consisted of a mixture of vesicles (v) and lipoprotein particles (p) in the size range of LDL and beta-VLDL. (b) Affinity chromatography on anti-apoB Sepharose retained the beta-lipoproteins; after elution, the aliquots showed particles of various sizes. (c) The nonretained fraction virtually consisted of heterogeneous EL vesicles only; (a) × 47,500; (b) × 33,250; (c) × 56,050.

lower CE and an augmented UC content as compared to serum beta-LP (**Table 5** and Table 1).

EL-trapped albumin

The failure of the anti-albumin immunoadsorbent to remove the albumin present in the post-ultracentrifugation EL fraction suggested that either the antigenic sites for immunorecognition were masked, or that the albumin was not located on the EL surface, but rather trapped inside these vesicles.

To exclude the possible albumin association with the EL surface, the affinity-purified fraction was subjected to

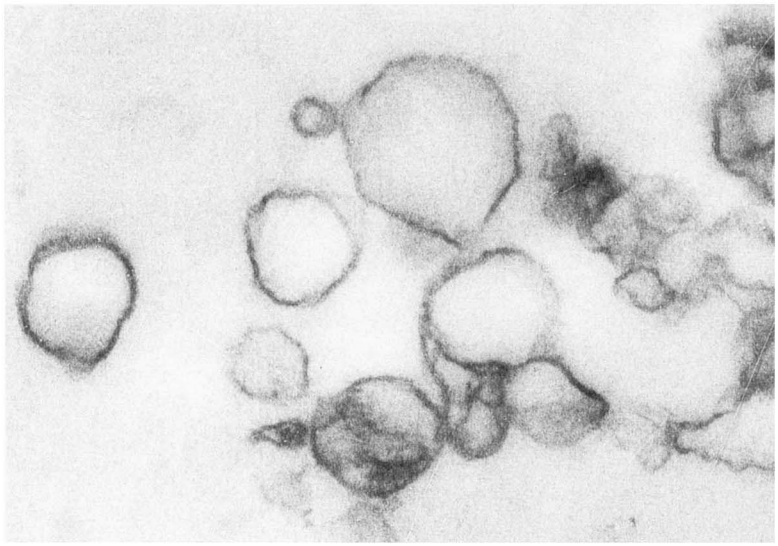

Fig. 6. Electron micrograph of a thin section through an aliquot of extracellular liposomes embedded in agarose and processed by tannic acid-*p*-phenylenediamine technique (22); × 87,600.

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TABLE 2. Composition of the extracellular liposome fraction after the four-step purification procedure

Component	2 Weeks on H-Diet (Prelesional Stage)	16 Weeks on H-Diet (Lesional Stage)
Cholesteryl ester (CE)[a]	1.1 ± 0.5	5.6 ± 1.4
Unesterified cholesterol (UC)[a]	54.3 ± 4.6	61.2 ± 6.6
Phospholipid (PL)[a]	44.3 ± 2.3	32.2 ± 2.4
Triglyceride (TG)[a]	0.3 ± 0.2	1.0 ± 0.5
Lipid % of total weight	80.4 ± 1.0	87.0 ± 0.5
Protein % of total weight	19.6 ± 1.0	13.0 ± 0.5
UC/PL (molar ratio)	1.2 ± 0.2	1.9 ± 0.3
% UC/TC[b]	98.0 ± 1.4	91.6 ± 2.0

The values for serum β-VLDL and LDL from the corresponding experimental conditions are given in Table 1.

[a]Values for lipids are mole %, means ± SEM of five preparations. The molecular weights for CE, UC, PL, and TG were taken as 666, 387, 800, and 885 Da, respectively.

[b]TC, total cholesterol.

proteolytic digestion with pronase E; the albumin moiety of EL was not affected (**Fig. 7**).

To expose the EL core by breaking their phospholipid lamellae, affinity-purified EL preparations were delipidated with chloroform-methanol and aliquots were applied on nitrocellulose membranes. By dot-blot immunolabeling with antibodies to RSA, the delipidated EL stained positively for albumin. The intact nondelipidated EL subjected to the same procedure gave a negative result (**Fig. 8**). Agarose gel electrophoresis of affinity-purified EL fraction before and after delipidation with hexane-isopropanol, showed that while undelipidated EL remained at the site of sample application, upon delipidation one protein band migrated at the same position as the rabbit serum albumin used as control (not illustrated). In contrast, the aortic beta-lipoprotein fraction (isolated by affinity chromatography) displayed an increased electrophoretic mobility in the agarose gels, as compared with serum LDL and beta-VLDL (not illustrated).

Albumin appeared not to be artifactually trapped within EL during the isolation procedure since incubation of aortic tissue minces with radioiodinated albumin failed to show any trace of radioactivity in the isolated EL fraction.

These findings indicated that albumin is a constituent of the EL formed in vivo, being trapped in the aqueous core of these vesicles.

DISCUSSION

Under normal conditions, plasma lipoproteins (LP) are transported through arterial endothelium by a dual mechanism: endocytosis by which LP are internalized to the endothelial lysosomal compartment, and transcytosis which delivers LP to the interstitial fluid of the vessel wall (38, 39) without significant accumulation within the latter (1, 4, 6, 38-40). In diet-induced hyperlipidemia, there is an increased transport of LDL (41) and beta-VLDL (39) with deposition of LP and their components in the subendothelial extracellular matrix (1-12). In the prelesional stages of atherogenesis in fat-fed rabbits and hamsters, the earliest ultrastructural change so far detected in the intima of the aorta and cardiac valves is the progressive accumulation of LP-derived extracellular liposomes rich in unesterified cholesterol (1-5) and closely associated with

TABLE 3. Distribution of cholesterol, beta-lipoprotein (beta-LP), and albumin in the different fractions isolated from aortic saline extracts

Fraction	Stage	Cholesterol	β-LP (Protein)	Albumin
			μg/g aortic tissue[a]	
Saline extract[b]	pL[c]	550 ± 50[d]	220 ± 20	2600 ± 100
	L	4800 ± 800	530 ± 50	4200 ± 200
After gel filtration (V_o)	pL	160 ± 12	ND	ND
	L	3650 ± 150	ND	ND
Retained by anti-apoB	pL	75 ± 4	107 ± 8[e]	
	L	300 ± 50	210 ± 20	
Retained by anti-albumin	pL	3.3 ± 0.5		2.2 ± 0.3
	L	30 ± 10	9.4 ± 3.2	
Purified EL[b]	pL	66 ± 3	44 ± 2	
	L	2850 ± 150		890 ± 50

[a]Wet weight (intima + media).

[b]In some experiments the saline aortic extracts were divided into two parts: one part was subjected directly to gel filtration (Fig. 1) to isolate EL; the other part was applied first on an anti-apoB column (to remove the aortic beta-LP) and the fraction unretained by the immunoadsorbent was further subjected to affinity chromatography on Blue Sepharose CL-6B to isolate the aortic albumin. Beta-LP were eluted with 0.2 M NaCl in 0.05 M $NaHCO_3$-NaOH, pH 11.0, and albumin with 1.5 M KCl in 10 mM KH_2PO_4-NaOH, pH 7.4.

[c]pL, prelesional stage; L, lesional stage.

[d]Values represent the mean ± SEM of three preparations; ND, not determined.

[e]The amount of beta-LP coeluting with EL in the void volume fraction (V_o); the rest of beta-LP eluted in the included volume (66-75 ml) by gel filtration.

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TABLE 4. Thiobarbituric acid-reactants (TBAR) detected in serum beta-lipoproteins and in the EL fractions of rabbit aortas in the lesional stage of hyperlipidemia

Sample	TBAR (Malondialdehyde Equivalents as nmol/mol Lipid × 10^4)
Serum β-VLDL	1.0 ± 0.1[a]
Serum LDL	0.9 ± 0.2
EL fraction	
After gel filtration	3.1 ± 0.4
After affinity chromatography	
Adsorbed β-lipoproteins[b]	14.3 ± 2.2
Nonadsorbed EL fractions	0.9 ± 0.1

[a]Values are means ± SEM of three-five preparations.
[b]Aortic beta-lipoproteins retained by the immunoadsorbent anti-apoB.

apoprotein B (4, 6). Such structures [presumably the equivalent of the filipin-positive particles seen in light microscopy (7)] have been recently identified also by other investigators using either conventional thin-section electron microscopy (8), cytochemistry (42, 43), quick-freezing (9), or ultra-rapid freezing and freeze-etching (10). There is a substantial body of evidence that the source of the augmented aortic lipids in swine, rabbits, and humans is the plasma lipoproteins (1, 18, 20–22, 43–46). Arterial lipid deposits have been extracted and partially characterized only from relatively advanced human lesions or after extended duration of hypercholesterolemia (from 1 to 12 months of diet) (12, 20–22, 43–47). Data on the lipid composition of normal aortic intima in humans and experimental animals emerged from the control experiments reported in various studies (12, 44–46) or inquiries specifically addressed to normal specimens (48). In the present investigation, the isolation and purification procedure we have developed was applied to the cell-free aortic intima in the prelesional stage of atherogenesis, i.e., before monocyte migration (2 weeks on H-diet) or in a lesional stage (16 weeks on H-diet) (1).

TABLE 5. Composition of beta-lipoproteins isolated by affinity chromatography from saline extracts of rabbit aortas at prelesional (2 weeks) and lesional (16 weeks) stages of hyperlipidemia

Component	2 Weeks on H-Diet (Prelesional Stage)	16 Weeks on H-Diet (Lesional Stage)
Cholesteryl ester (CE)[a]	39.0 ± 2.7[b]	35.0 ± 2.5
Unesterified cholesterol (UC)	23.1 ± 1.0	32.6 ± 1.7
Phospholipid (PL)	27.5 ± 2.9	23.4 ± 1.2
Triglyceride (TG)	10.4 ± 1.3	9.0 ± 0.8
Lipid % of total weight	65.7 ± 2.5	75.5 ± 2.2
Protein % of total weight	34.3 ± 2.0	24.5 ± 2.0
UC/PL	0.8 ± 0.1	1.4 ± 0.1
% UC/TC[c]	37.2 ± 2.2	48.2 ± 2.8

[a]The molecular weights of CE, UC, PL, and TG were taken as 666, 387, 800, and 885 Da, respectively.
[b]Values for lipids are mole %, means ± SEM of three preparations.
[c]TC, total cholesterol.

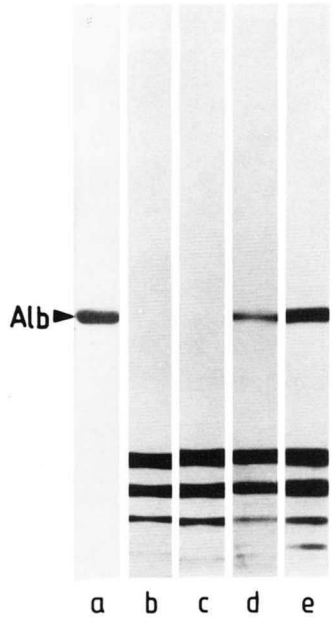

Fig. 7. SDS-PAGE analysis of purified EL after treatment with 100 μg pronase E for 7 h at 37°C; (a) rabbit serum albumin (RSA); (b) pronase E; (c) RSA after proteolysis with pronase E: note that the protein is completely digested by the enzymatic proteolysis, the only protein bands visualized being those of the enzyme; (d) EL fraction obtained at prelesional (2 weeks) and (e) at lesional stages (16 weeks) of hyperlipidemia after proteolytic digestion with pronase E: note that albumin associated with EL fractions (lanes d and e) was not affected by the proteolytic enzyme; Alb, albumin.

By comparison with the method used by Chao et al. (12), our procedure used two additional affinity chromatography steps that allowed a further purification of the extracellular liposome fraction to yield solely the phospholipid vesicles and their content. As such, through our technique two EL fractions could be sequentially isolated: *i*) a crude EL fraction obtained after gel filtration and ultracentrifugation, and *ii*) a purified EL fraction separated from the latter by two affinity chromatography steps (on anti-apoB, and anti-albumin) to remove the beta-LP particles and albumin.

The amount of aortic EL fraction thus isolated was proportional to the duration of the H-diet. At 2 weeks (prelesional stage), the expected fraction consisted of an almost one-to-one molar mixture of unesterified cholesterol (UC) and phospholipid. This ratio was increased to about 2:1 in the fraction isolated from the lesional stage (16 weeks).

As compared to plasma LDL and beta-VLDL from the same animals, the crude EL fraction was characterized by a relative decrease in cholesteryl esters (CE) and triglycerides (TG) with a marked augmentation in the UC and PL content, especially in the lesional stage (Tables 1 and 2). Negatively stained aliquots reveled the heterogeneous composition in which, in addition to the prevalent EL, relatively frequent LP particles of LDL and VLDL size could be observed (Fig. 3). The LP particle size (20–55

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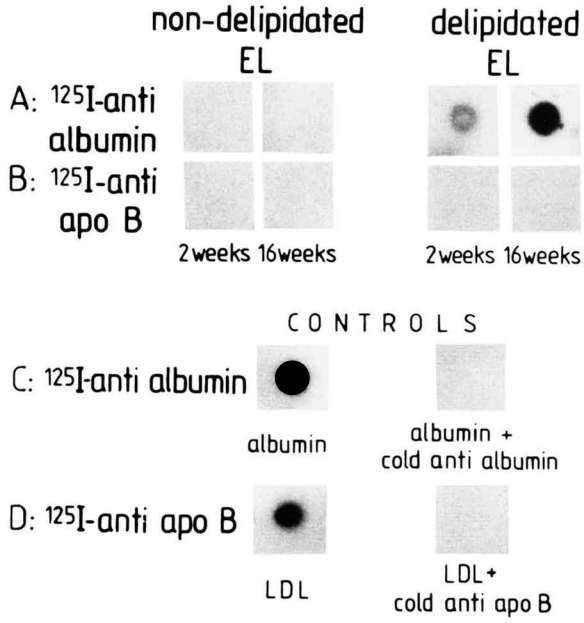

Fig. 8. Autoradiography of the dot-blot immunoanalysis of purified extracellular liposomes (EL) isolated from extracts of aortic intima at 2 weeks (prelesional stage) and 16 weeks (lesional stage) of diet-induced hyperlipidemia, in rabbits. A: Upon incubation with radioiodinated anti-rabbit serum albumin, there is a lack of immunoreactivity in the dots of the nondelipidated EL and a positive reaction of the EL blots after delipidation with chloroform–methanol. B: Incubation with radioiodinated anti-apoB does not reveal any immunoreactivity of the either nondelipidated or delipidated EL. C and D: Controls for the specificity of the immunoreaction, in which dots of the albumin alone, or LDL alone, or each of them overlayered with the corresponding cold antibody, were incubated with the specific radioiodinated antibody. Note the negative results obtained when the ligand was previously reacted with its nonlabeled antibody.

nm, with peak diameter of 36 nm) was generally comparable with the dimensions of similar structures found in other studies: 20–30 nm in rabbit and human aortas (12), 23–45 nm in rabbit intima (10), 25–35 nm in human atheromas (49). The thiobarbituric acid reactants (TBAR) detected in the crude EL fraction were about threefold higher than in the serum LDL or beta-VLDL (Table 4). Our data for compositional and morphological characteristics of crude EL fraction obtained from rabbit aorta at lesional stage of hyperlipidemia by gel filtration and ultracentrifugation are in agreement with those reported by Chao et al. (12) for UC-rich lipid particles from atherosclerotic lesions of rabbit aorta isolated in a similar manner. Since we used additional purification steps (affinity chromatography), further comparisons of the data are not available. In addition, we have isolated EL from aortas at the prelesional stage of atherogenesis, for which the UC/PL molar ratio is different than that for EL obtained at the lesional stage. The presence of large lipid particles (corresponding to our crude EL fraction) has also been demonstrated in human atherosclerotic plaques by several investigators (12, 20, 50–53).

After immunoadsorption removal of apoB and albumin, the purified EL fraction remained represented by UC, PL, and still a significant amount of protein. The retention of apoB and albumin by their respective antibodies linked to Sepharose indicated that these proteins were relatively loosely associated with the external contour of EL vesicles, as otherwise suggested by the immunocytochemical studies on the aorta of hyperlipidemic rabbits (4, 6). A striking finding was that almost all the TBAR associated with the EL was adsorbed together with the beta-LP by the anti-apoB immunoadsorbent column (Table 4); only an insignificant quantity was found in the eluted nonadsorbed EL fraction. This was a suggestion that the TBAR, irrespective of the site of generation, were eventually associated with the intact or degraded apoB. The breakdown of apoB-100 of the EL fraction was demonstrated by SDS-PAGE (Fig. 4). Such breakdown accompanies the oxidation of beta-LP (54). The presence of oxidatively modified LP was convincingly demonstrated in atheroma of WHHL rabbits (35, 55). However, the occurrence of oxidized LP in these lesions does not reveal whether the LP oxidation is a cause or a consequence (part of a defense reaction) of the atherogenic process.

As revealed by the analyses performed on the purified EL fraction, the protein contained in the core of these phospholipid vesicles was albumin. This protein could not be removed by either gel filtration with high ionic strength buffer, by high concentrations of KBr during repetitive ultracentrifugations, or by anti-albumin immunoadsorbent. The EL-associated albumin was resistant to proteolytic digestion with pronase and reacted with anti-albumin antibody only after EL delipidation. From these observations it was inferred that albumin is a structual constituent of EL, being trapped in the aqueous core of the vesicle. Since complexing of serum albumin with phospholipid liposomes has been extensively documented in vitro (56, 57), the physicochemical interaction between EL and its trapped albumin remains to be elucidated, as well as the physiopathological significance of the albumin tight association and trapping within the core of EL. At this stage, one can speculate that albumin occurrence in these degraded and reassembled beta-LP may reflect its participation in the local defense reaction as a natural potent extracellular antioxidant (58), bearing on its activity as scavenger of oxygen-free radicals (59, 60).

In vitro formation of UC-PL-albumin liposomes (vesicular LP) from VLDL (during triglyceride lipolysis) (61) raises the possibility that aortic EL might be assembled extracellularly from degraded LP. The altered protein pattern of aortic beta-LP indicates modifications in the transcytosed serum beta-LP that are accompanied by changes in their compositional characteristics (decreased lipid and increased apoB content). The compositional

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alteration of aortic beta-LP could be explained by a partial delipidation of transcytosed serum beta-LP in the intima, a process contributing to the formation of vesicular lipid structures (EL) in the subendothelial space. The decreased CE content (increased UC/TC ratio) of the aortic beta-LP could be attributed to the hydrolysis of CE of beta-LP (21) in the intima, an additional process that might also contribute to the appearance of UC-rich phospholipid vesicles (EL).

The albumin-containing EL have many morphological and compositional similarities to the abnormal vesicular lipoproteins that appear in the plasma of patients with cholestatic jaundice (62–65) or in rabbit plasma after phospholipid infusion (65).

The isolation and characterization of the chemical composition of the EL accumulated in the aortic intima at early phases of hyperlipoproteinemic atherogenesis set the stage for further work on the elucidation of the molecular events occurring at the onset of plaque formation.

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