A low density lipoprotein-sized particle isolated from human atherosclerotic lesions is internalized by macrophages via a non-scavenger-receptor mechanism

Richard E. Morton, Gail A. West, and Henry F. Hoff[1]

Atherosclerosis Section, Department of Brain and Vascular Research, Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44106

Abstract A lipoprotein particle designated A-LDL, which contains apolipoprotein B (apoB) and which is the size of plasma low density lipoproteins (LDL), was isolated from homogenates of human aortic atherosclerotic plaques by a combination of affinity chromatography and gel-filtration. Compared to plasma LDL, A-LDL was more electronegative, its hydrated density was lower and more heterogeneous, and its protein-to-lipid ratio was lower. In addition, apoB in A-LDL was highly degraded, and A-LDL was recognized by mouse peritoneal macrophages (MPM) as indicated by its ability to stimulate cholesterol esterification. Cholesterol esterification was saturable with an apparent K_m of 100 μg of A-LDL cholesterol/ml. Stimulation of cholesterol esterification was linear with time, leading to extensive accumulation of cholesteryl ester in MPM over a 48-hr time interval. The uptake or degradation of acetyl-LDL (radiolabeled either in the protein with ^{125}I or hydrophobic core with [^{3}H]cholesteryl ether) was markedly decreased by excess unlabeled acetyl-LDL but not by A-LDL, and excess acetyl-LDL did not inhibit the uptake or degradation of labeled A-LDL. However, a 10-fold excess of A-LDL also failed to inhibit the uptake of labeled A-LDL. This finding was consistent with the observation that, unlike the saturable stimulation of cholesterol esterification in MPM induced by A-LDL, the uptake of cholesteryl ether-labeled A-LDL was almost linear over a 0–400 μg cholesterol/ml range. This discrepancy between dose response curves for A-LDL, which did not occur for acetyl-LDL, could be eliminated by a 24-hr postincubation period in the absence of lipoprotein, suggesting that A-LDL is catabolized less efficiently than acetyl-LDL following internalization. In summary, we conclude that A-LDL uptake by MPM occurs via a low affinity–high capacity process. Although the uptake of A-LDL is not readily saturated, it is of sufficient affinity to lead to lipid loading of macrophages even when A-LDL is present at relatively low concentrations. If these mechanisms are operative in vivo, they could explain how foam cells in human fatty streak lesions develop.—**Morton, R. E., G. A. West, and H. F. Hoff.** A low density lipoprotein-sized particle isolated from human atherosclerotic lesions is internalized by macrophages via a non-scavenger-receptor mechanism. *J. Lipid Res.* 1986. **27:** 1124–1134.

Supplementary key words cholesterol esterification • acetyl-LDL • foam cells

The earliest atherosclerotic lesion is considered by many to be the fatty streak (1, 2). These lesions are characterized by the presence of numerous cholesteryl ester-laden cells (3), called foam cells, that appear to be derived, in large part, from macrophages (3–5). The mechanism leading to the accumulation of cholesteryl ester in macrophages in vivo is unknown, but may result from the uptake of modified or abnormal lipoproteins that enter from the blood or are modified within the arterial wall. This suggestion is based on the in vitro observation that mouse peritoneal macrophages (MPM) take up plasma LDL only after it has been modified to become more electronegative (6–8). Since the scavenger-receptor, which mediates the uptake of these modified lipoproteins (6), is not down-regulated by cellular cholesterol levels, uptake continues unabated until the macrophage morphologically resembles the foam cells of the fatty streak (6).

We have purified and characterized lipoproteins extracted from advanced human atherosclerotic lesions (9–11) to test the hypothesis that plasma lipoproteins that have accumulated within the arterial wall are responsible for the formation of arterial foam cells. Recently, we reported the isolation of lipoproteins containing apoB from the human aorta designated A-LP (11). These lipoproteins were more electronegative and more heterogeneous in size than plasma LDL. In addition, they were readily internalized by mouse peritoneal macrophages (MPM), in contrast to

Abbreviations: LDL, low density lipoprotein; A-LDL, arterial LDL; apoB, apolipoprotein B; SDS, sodium dodecyl sulfate; MPM, mouse peritoneal macrophages; DMEM, Dulbecco's modified Eagle's medium; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; β-VLDL, the β-migrating d <1.006 g/ml fraction from cholesterol-fed dogs.

[1]To whom reprint requests should be addressed.

plasma LDL (6). This uptake resulted in intracellular accumulation of cholesteryl esters, thus forming cells morphologically similar to foam cells (11).

In light of the previously reported size heterogeneity of A-LP, in this study we have further purified these arterial lipoproteins by isolating a fraction similar in size to plasma LDL, designated A-LDL. We report the structural and functional properties of this lipoprotein and partially characterize the uptake mechanism of A-LDL by MPM.

EXPERIMENTAL PROCEDURES

Materials

Male Swiss Webster mice were purchased from Charles River Laboratories (Wilmington, MA). [1-^{14}C]Oleic acid (48 mCi/nmol) was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA), and [1,2,6,7-^{3}H(N)]cholesteryl oleate (82.7 Ci/nmol), [1,2-^{3}H(N)]cholesterol (55 Ci/nmol), and sodium [^{125}I]iodide were from New England Nuclear (Boston, MA). [^{3}H]Cholesteryl oleyl ether was synthesized from [1,2-^{3}H(N)]cholesterol and oleyl alcohol by the method of Halperin and Gatt (12). Oleic acid, cholesteryl oleate, bovine serum albumin (fatty acid free), penicillin G, streptomycin, leupeptin, pepstatin, cholesterol oxidase, cholesteryl esterase, horseradish peroxidase, and bovine pancreatic trypsin (T-8642) were purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture plates were from Costar (Cambridge, MA). Dulbecco's modified Eagle's medium (DMEM, Cat. #430-1600), penicillin-streptomycin solution, and fetal calf serum were obtained from Gibco Laboratories (Grand Island, NY). Canine β-VLDL was a gift from Dr. Thomas Innerarity, Gladstone Foundation, San Francisco, CA.

Methods

Isolation of lipoproteins from aortic tissue. Human aortas (arch to bifurcation) with numerous raised fatty fibrous atherosclerotic lesions were obtained at autopsy within 24 hr of death from individuals usually over 40 years of age (Pathology Service, Cleveland Clinic Foundation). The tunica intima was dissected away from the underlying tunica media, rinsed briefly in 0.9% NaCl, and finely minced. The tissue was dispersed in a Polytron homogenizer (Brinkman Instruments, Westbury, NY) at full speed for 30 sec at 4°C in 130 mM Tris–HCl, 150 mM NaCl, 0.05% Na_2 EDTA, pH 7.4, at a ratio of 1 g wet weight of minced tissue to 5 ml of buffer. The homogenization buffer also contained 10 mM ϵ-amino caproic acid, 500 U/ml penicillin G, 500 μg/ml streptomycin, 0.05% Na_2 EDTA, 0.002% chloramphenicol, 5 μM leupeptin, and 35 μM pepstatin. The supernatant fractions obtained after centrifugation (35,300 *g*, 60 min) were stored for up to 2 weeks at 4°C. Typically, three such supernatants (i.e., from three separated aortas) were pooled and applied to an anti-LDL Sepharose affinity column (11) equilibrated in 50 mM Tris–HCl, 150 mM NaCl, 0.05% Na_2 EDTA, pH 7.4 (Tris–NaCl buffer) at a flow rate of 25 ml/hr. After application of the pooled samples, the column was extensively rinsed with Tris–NaCl buffer until the eluant absorbance at 280 nm was $\leq$ 0.05. Bound material (apoB-containing lipoproteins) was eluted at a flow rate of 25 ml/hr with 150 mM NaCl, 0.05% Na_2 EDTA adjusted to pH 11 with NH_4OH, and fractions (4.5 ml) were collected into tubes containing 0.5 ml of 0.5 M Tris–HCl, pH 7.4. Eluted fractions having absorbance (280 nm) values of $\geq$ 0.1 were pooled, dialyzed against 2.5 mM Tris–HCl, 0.005% Na_2 EDTA, pH 8.8, and then concentrated by electrophoresis for 3 hr in a sample concentrator (Isco 1750, Lincoln, NE) containing 25 mM Tris–HCl, 30 mM NaCl, 0.05% Na_2 EDTA, pH 8.8, as electrode buffer. The concentrated lipoproteins, derived from plaques and containing apoB (A-LP), were either used as is or further purified by gel filtration. Concentrated A-LP (5–8 ml) was applied to a Bio-Gel A-15m column (Bio-Rad, Richmond, CA) (2 $\times$ 60 cm) equilibrated in Tris–NaCl buffer, and chromatographed at a flow rate of 10 ml/hr. Two cholesterol-containing peaks were resolved, one eluting at the column void volume, and a second peak coeluting with plasma LDL, designated arterial LDL, and abbreviated A-LDL. The peak fractions were pooled and electrophoretically concentrated as described above. A-LP, or subsequent gel filtration fractions, were stored in phosphate buffer containing 0.05% EDTA. Nine such preparations were used in the studies reported here. All procedures were carried out at 4°C.

Lipoprotein isolation and chemical modification. LDL was isolated from fresh (< 24 hr old) plasma, obtained from the Red Cross Blood Bank, by differential ultracentrifugation as the 1.019 < d < 1.063 g/ml fraction as described by Hatch and Lees (13). LDL was acetylated by repetitive additions of acetic anhydride (14) and then extensively dialyzed against 150 mM NaCl, 0.05% Na_2 EDTA, pH 7.4. The extent of lysyl modification was measured by changes in 2,4,6-trinitrobenzene sulfonic acid reactivity (15). Routinely, > 45% of the lysyl residues of LDL were modified. Acetyl-LDL and A-LDL were labeled with ^{125}I following the iodine monochloride procedure of McFarlane (16) as modified by Bilheimer, Eisenberg, and Levy (17); the specific activity of labeled preparations was generally 200–300 cpm/ng protein.

Lipoproteins were labeled with [^{3}H]cholesteryl oleyl ether by first incorporating the [^{3}H]cholesteryl ether into high density lipoproteins following the lipid dispersion technique of Morton and Zilversmit (18). [^{3}]Cholesteryl ether-labeled high density lipoprotein (100 μg cholesterol) was then in-

cubated with the lipoprotein of interest (2 mg of cholesterol) plus partially purified lipid transfer protein (500 μl of CM-cellulose fraction) (18). After incubation at 37°C for 6 hr, the radiolabeled lipoproteins were re-isolated by ultracentrifugation as the d < 1.063 g/ml fraction.

Characterization of lipoproteins. The chemical and physical properties of isolated plasma and aorta-derived lipoproteins were evaluated as follows. Transmission electron microscopy was performed utilizing phosphotungstic acid as a negative stain (19). Agarose electrophoresis was performed on pre-made agarose gels (Corning, Palo Alto, CA); samples were electrophoresed according to the manufacturer's instructions at 90 V for 55 min. Lipoproteins were detected by staining with 0.3% oil red O in 60% ethanol. Lipoprotein total cholesterol was determined enzymatically with Reagent Set (Boehringer, Mannheim, Indianapolis, IN), a modification of the method of Roeschlau, Bernt, and Gruber (20). Protein was determined by the method of Lowry et al. (21) as modified by Peterson (22) with human serum albumin as standard. To evaluate the protein components, lipoproteins were delipidated as previously described (11) and applied to SDS-polyacrylamide gradient gels (3–12%, Separation Science, Inc., Attleboro, MA) and electrophoresed at 40 mA at 15°C. Proteins were visualized by the silver staining procedure of Wray et al. (23). For amino acid analysis, lipoproteins were delipidated with ethanol–ether (24), and after derivatization with phenylisothiocyanate the amino acid composition of hydrolysates was determined on the PICO-TAG system of Waters Associates, Milford, MA (25).

The density of lipoproteins was measured by density gradient ultracentrifugation. Lipoproteins labeled with either [^{3}H]cholesterol (26) or ^{125}I (17, 18) were applied to the top of 60 Ti centrifuge tubes containing layers (7.5 ml each) at densities of 1.080, 1.065, 1.050, 1.035, and 1.019 g/ml, and ultracentrifuged for 41 hr at 60,000 rpm at 4°C. Samples were removed from the top of the tube by displacement with d = 1.48 g/ml NaBr solution pumped into the bottom of the tube at 40 ml/hr. Aliquots (2 ml) were taken for the determination of solvent density (gravimetrically) and radioactivity.

Cellular metabolism of lipoproteins. Resident peritoneal macrophages from unstimulated adult male mice were collected and cultured as previously described (11) except that 2 × 10^6 cells were plated per 16 mm diameter well, and cells were allowed to adhere overnight in DMEM containing 10% fetal calf serum. For cholesterol esterification studies, cells were rinsed with DMEM, and then to each well was added: [^{14}C]oleate–albumin (final oleate concentration of 0.27 mM (9)), the test lipoprotein in DMEM at the indicated concentration, and medium for a final volume of 0.35 ml. At the indicated time, the cells were rinsed four times with phosphate-buffered saline and the cellular lipid was extracted three times with hexane–isopropanol 3:2 (v/v). During the first extraction, [^{3}H]cholesteryl oleate was added as an internal standard. Organic solvents were removed under a stream of N_2 and the lipids were separated by thin-layer chromatography on SG-60 plates (Merck, Darmstadt, Germany) in a developing solvent of heptane–diethyl ether–acetic acid 85:15:2 (v/v). Cholesteryl esters were identified by comigration with a cholesteryl oleate standard. Radioactivity was quantitated by liquid scintillation counting in a toluene-based scintillant containing 0.4% PPO, 0.01% POPOP, and 2% absolute ethanol. The protein content of solvent-extracted cells was quantitated by a modification (22) of the protein assay of Lowry et al. (21). In experiments in which the accumulation of cholesterol mass in the cells was studied, the total and unesterified cholesterol in lipid extracts of cells was determined by a fluorometric, enzymatic method (27). Esterified cholesterol levels were calculated by the difference between these values.

In studies measuring the cellular accumulation of [^{3}H]cholesteryl oleyl ether from [^{3}H]ether-labeled lipoproteins, and in some esterification studies, cells were cultured as described above, but at the end of the experiment the cell layer was washed four times with phosphate-buffered saline, after which the cells were scraped and washed twice by centrifugation. Cell pellets were resuspended in 1 ml of phosphate-buffered saline by brief sonication with a probe (Heat Systems Co., Plainview, NY). The radioactivity in an aliquot was either determined directly, or the lipids were extracted (28) and chromatographed as described above. Cellular protein was calculated from measurements of DNA (29) and an experimentally determined conversion factor of 90 μg of DNA/mg of protein.

The degradation of ^{125}I-labeled acetyl-LDL and A-LDL by macrophages was measured essentially as described by Goldstein and Brown (30). Wells contained ^{125}I-labeled lipoproteins ± unlabeled lipoproteins, 10% lipoprotein-deficient human plasma, and DMEM medium in a total volume of 0.35 ml. At the indicated time, the medium was removed, the cells were briefly rinsed with phosphate-buffered saline, and the combined medium plus rinse was subjected to trichloroacetic acid precipitation. The extent of degradation was calculated from the acid-soluble non-iodide radioactivity (30).

RESULTS

When A-LP, the lipoprotein fraction isolated by immunoaffinity chromatography on anti-LDL-Sepharose, was further purified by gel filtration chromatography, two distinct, well-separated absorption peaks were routinely observed. The first eluted at the column void volume, whereas the second peak coeluted with radiolabeled plasma LDL. In several preparations the applied lipoprotein cholesterol

was approximately equally distributed between these two fractions, whereas most (> 80%) of the applied apoB, as assessed by electroimmunoassay, was recovered in the LDL-sized lipoprotein fraction. In a typical preparation, ≃ 50 μg (cholesterol) of the LDL-sized lipoprotein was obtained from a gram of intima (wet wt), although this yield was variable, ranging between 15 and 91 μg/g. Usually, the intima from three aortas yielded ≃ 2 mg of lipoprotein cholesterol.

A-LDL, the fraction of A-LP coeluting with plasma LDL, was composed of discrete, spherical particles which were approximately the same size as plasma LDL (220 nm diameter) (**Fig. 1**). By contrast, the particles present in the void volume fraction were much larger and more heterogeneous in size than plasma LDL (Fig. 1 insert). Aggregates of LDL-sized particles were rarely seen in the void volume fraction.

Although similar in size, A-LDL differed from plasma LDL in several respects. A-LDL migrated 75–100% faster than plasma LDL during agarose electrophoresis, having an electrophoretic mobility similar to that of very low density lipoproteins. The protein component of A-LDL, as evaluated by SDS-polyacrylamide gel electrophoresis combined with immunoblotting for apoB, was very similar to that previously published for A-LP (11). That is, A-LDL demonstrated numerous protein bands, most of which were positive for apoB immunoreactivity (data not shown). We have previously shown that the presence of highly degraded apoB in arterial lipoproteins is not a postmortem or preparation artifact (11). A-LDL demonstrated a lower protein-to-total cholesterol ratio than plasma LDL (0.37 ± 0.05 vs. 0.56 ± 0.02, respectively, mean ± SD, n = 3), suggesting that the extensive degradation of apoB may have resulted in the loss of part of the protein component

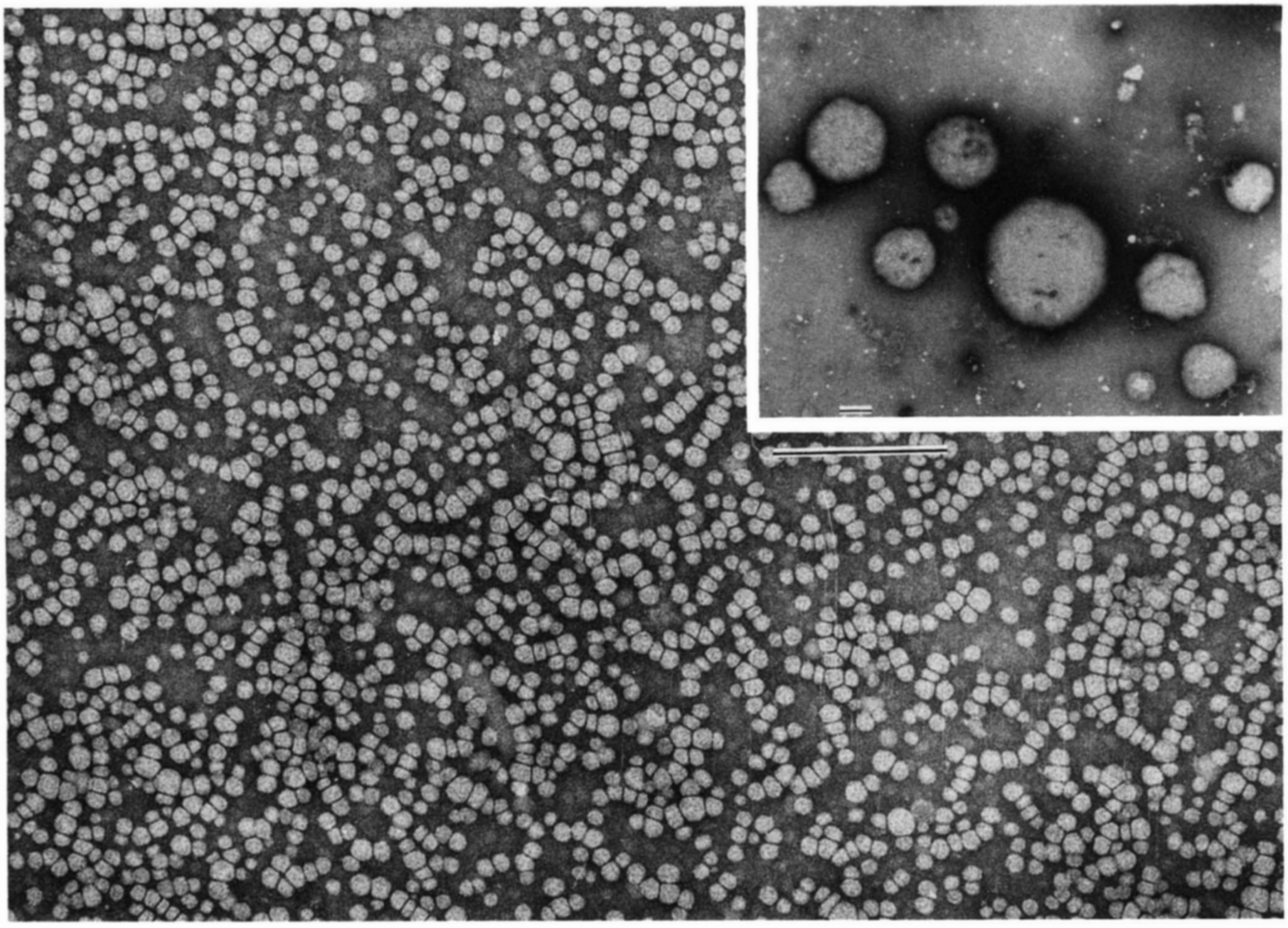

Fig. 1. Electron micrographs of negatively-stained preparations. Fig. 1 illustrates a preparation of A-LDL, demonstrating particles similar in size to those of plasma LDL (size range in diameters = 200 to 300 Å). Fig. 1 (insert) shows a similarly treated preparation of the void volume fraction from the Bio-Gel A-15m column chromatography of affinity-purified lipoproteins isolated from human aortic plaques illustrating large-sized particles of variable diameters. Magnifications are indicated by bars that signify 2500 Å (magnification of Fig. 1 is about five times greater than that of Fig. 1 insert). Desalted samples of lipoproteins were mixed 1:1 (v:v) with 2% phosphotungstic acid (PTA), pH 7.4, as previously described (19). Samples of the mixture were dried on collodion-carbon-coated grids and viewed by electron microscopy. Bovine serum albumin, 0.1 mg/ml was added to samples of the void volume fraction prior to mixing with PTA to facilitate spreading on the coated grids by lowering the surface tension.

TABLE 1. Amino acid analysis

Amino Acid	Mole %[a] A-LDL	Plasma LDL
Asp	10.6 ± 0.4	9.7 ± 0.9
Glu	12.0 ± 0.9	11.8 ± 0.4
Ser	9.4 ± 2.4	8.7 ± 1.4
Gly	5.4 ± 0.2	4.9 ± 0.1
His	3.0 ± 1.0	2.1 ± 0.1
Arg	4.3 ± 0.2	3.7 ± 0.1
Thr	6.6 ± 0.1	6.7 ± 0.1
Ala	6.5 ± 0.2	6.5 ± 0.2
Pro	4.4 ± 0.2	4.4 ± 0.5
Try	2.5 ± 0.2	2.9 ± 0.0
Val	5.2 ± 0.1	5.4 ± 0.1
Met	1.5 ± 0.2	1.7 ± 0.1
Cys	0.2 ± 0.2	0.2 ± 0.2
Ile	5.5 ± 0.1	5.9 ± 0.0
Leu	11.6 ± 0.8	12.4 ± 0.5
Phe	4.6 ± 0.8	4.9 ± 0.6
Lys	7.2 ± 1.4	8.5 ± 0.8

Lipoproteins were delipidated and then the protein portion was hydrolyzed and derivatized and the amino acids were quantitated by high pressure liquid chromatography as described in Methods.

[a] Average of values contained for two separate lipoprotein preparations ± SEM.

of A-LDL. Amino acid analysis, however, showed that A-LDL and plasma LDL were nearly identical in the mole fraction of all amino acids measured, indicating that if such a protein loss has occurred, there was no selective loss of amino acids (**Table 1**). Consistent with the lower protein/total cholesterol ratio of A-LDL, the mean particle density of A-LDL (labeled with [^{3}H]cholesterol) was significantly lower than that of labeled plasma LDL (**Fig. 2**), with A-LDL also demonstrating greater heterogeneity in particle density. Very similar density profiles were obtained for A-LDL and plasma LDL labeled in their protein moiety with ^{125}I (data not shown).

As demonstrated earlier (11), lipoproteins isolated from human aortic plaques by affinity chromatography alone (A-LP) stimulated cholesterol esterification in MPM in a dose-dependent fashion, yet failed to completely saturate this process at lipoprotein concentrations of up to 150 μg of cholesterol/ml (**Fig. 3A**), suggesting that A-LP contained lipoproteins that stimulate esterification by saturable and nonsaturable mechanisms. When A-LP was fractionated by gel filtration, the void volume lipoproteins were found to saturate cholesterol esterification at relatively low concentrations, yielding an apparent K_m of 20 μg of cholesterol/ml. On the other hand, A-LDL did not saturate cholesteryl ester synthesis at concentrations up to 150 μg of cholesterol/ml (11), suggesting that the apparently nonsaturable component in A-LP was A-LDL (Fig. 3A). However, the response of MPM to A-LDL was saturable at higher lipoprotein concentrations (Fig. 3B), demonstrating an apparent K_m of 100 μg of cholesterol/ml. By comparison, the response of macrophages to acetyl-LDL was saturated at much lower lipoprotein levels with an apparent K_m of ~ 15 μg of cholesterol/ml.

The extent of cholesterol esterification stimulated by A-LDL was time-dependent. Both A-LDL and acetyl-LDL

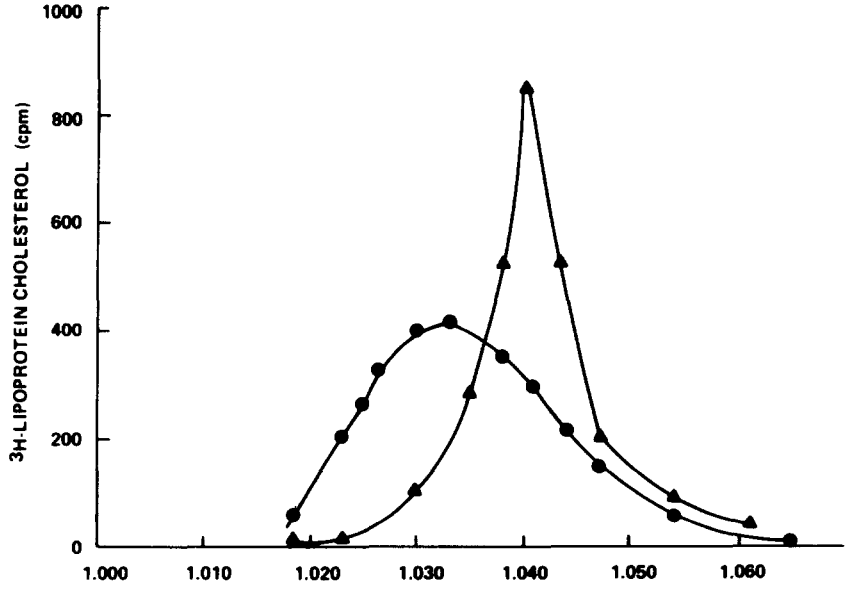

Fig. 2. Density gradient ultracentrifugation of A-LDL. A-LDL (●) and plasma LDL (▲) (100 μg of total cholesterol each) were labeled with [^{3}H]cholesterol by incubating the lipoprotein with filter paper (impregnated with [^{3}H]cholesterol) and 1 ml of 2 mg/ml BSA for 2 hr at 37°C. Labeled lipoproteins were loaded onto the top of a discontinuous NaBr gradient and centrifuged as outlined in Methods. The results shown are the average of duplicate determinations on the same sample. The profiles were essentially the same when samples were centrifuged for 64 hr instead of 41 hr. These results are representative of three experiments carried out on two different preparations of A-LDL.

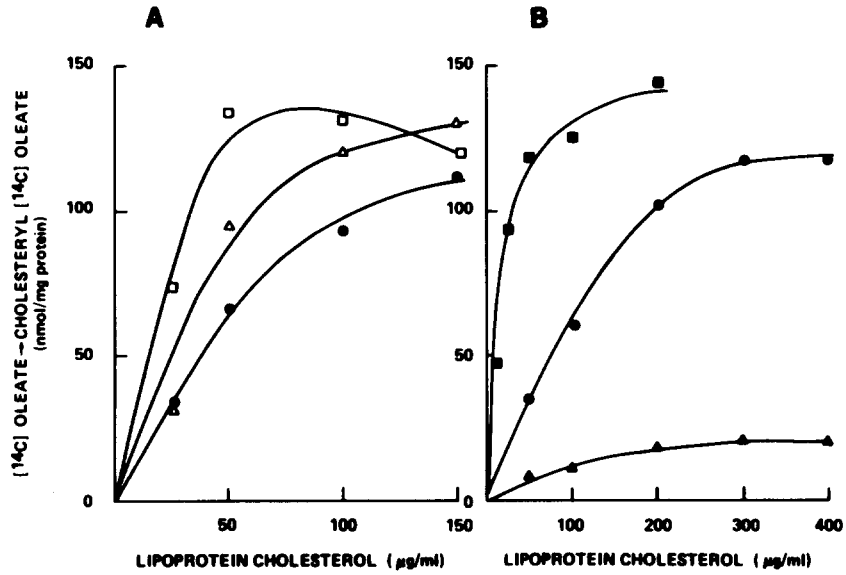

Fig. 3. Dose response of cholesterol esterification to various lipoprotein fractions. Mouse peritoneal macrophages were incubated as described in Methods with various concentrations of A-LP (△), gel-filtration void fractions (□), A-LDL (●), acetyl-LDL (■), or plasma LDL (▲) for 16 hr. Data are presented as two graphs, panels A and B, for clarity. The esterification values are the average of duplicate wells. These results are representative of at least two experiments for each lipoprotein fraction.

induced a linear increase in cholesteryl ester formation over the 48-hr period studied (**Fig. 4**). Thus, the binding site recognizing A-LDL is not down-regulated over this period, similar to the scavenger receptor recognizing acetyl-LDL. The elevated incorporation of [^{14}C]oleic acid into cholesteryl ester correlated with an increase in cellular cholesteryl ester mass (**Table 2**). A-LDL caused an 18-fold increase in cholesteryl ester mass relative to cells incubated with plasma LDL. By comparison, acetyl-LDL increased cholesteryl ester mass 50-fold over the 48-hr period. These levels of cholesteryl ester accumulation are similar to those previously reported by us for A-LP (11), in which oil red O-stainable lipid inclusions were noted to accumulate within the macrophage, giving the cells the appearance of foam cells. Both A-LDL and acetyl-LDL caused a substantial (2- to 3-fold) increase in cellular free cholesterol.

We considered the possibility that A-LDL might be recognized by the acetyl-LDL or scavenger receptor on MPM, since both A-LDL and acetyl-LDL have increased net negative surface charges. To test this hypothesis, we assessed the ability of A-LDL to inhibit the catabolism of ^{125}I-labeled acetyl-LDL in direct competition studies. As shown in **Table 3**, an apparently saturating level of A-LDL (refer to Fig. 3B) did not inhibit ^{125}I-labeled acetyl-LDL degradation. By contrast, excess unlabeled acetyl-LDL effectively inhibited the degradation of ^{125}I-labeled acetyl-LDL. In the reverse experiment, excess unlabeled acetyl-LDL (450 μg of cholesterol/ml) had no effect on the degradation of 45 μg/ml cholesterol of ^{125}I-labeled A-LDL by MPM (Table 3). Unexpectedly, however, excess A-LDL (450 μg of cholesterol/ml) was also unable to inhibit the degradation of ^{125}I-labeled A-LDL to a significant extent. This suggests that the uptake of A-LDL by MPM is not saturable. Since it is possible that the iodination procedure might have altered A-LDL, and hence its recognition by MPM, we further tested this observation with lipoproteins labeled with [^{3}H]cholesteryl ether. When the uptake of cholesteryl ether-labeled acetyl-LDL was compared in the presence and absence of a 10-fold excess amount (200 μg of cholesterol/ml) of unlabeled acetyl-LDL, A-LDL, plasma LDL, and β-VLDL, only acetyl-LDL caused a significant reduction (≃ 75%) in labeled acetyl-LDL uptake (**Fig. 5a**). However, when the uptake of cholesteryl ether-labeled A-LDL was compared in the presence or absence of a 10-fold excess of the same lipoproteins, none of the lipoproteins including excess A-LDL, markedly inhibited the uptake of labeled A-LDL (Fig. 5b), although all four test lipoproteins decreased A-LDL uptake by 25–30%. The reason for this partial inhibition by all lipoproteins is unclear; however, it is unlikely that these data suggest A-LDL recognition by multiple receptors since plasma LDL, which is taken up by non-receptor-mediated pathways in MPM, also caused the same extent of inhibition of A-LDL uptake. Thus, the results with [^{3}H]cholesteryl ether-labeled lipoproteins confirmed the observations made using ^{125}I-labeled lipoproteins. Collectively, these data indicate that the uptake mechanisms for acetyl-LDL and A-LDL are different. This conclusion was further substantiated in a separate experiment in which maleyl-albumin (400 μg/ml) inhibited labeled acetyl-LDL uptake by 87% but had no effect on labeled A-LDL uptake by MPM (data not shown).

The data thus far suggest conflicting mechanisms of A-

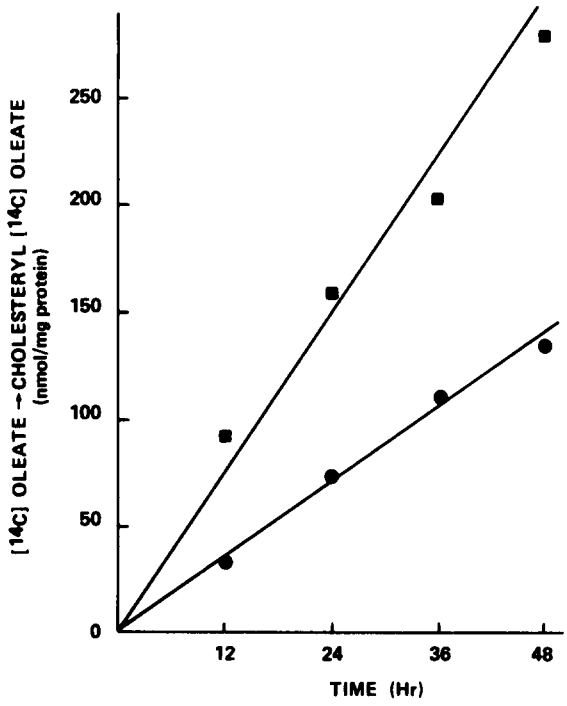

Fig. 4. Time course of cholesterol esterification in response to A-LDL. Mouse peritoneal macrophages were incubated as described in Methods with 100 μg of lipoprotein cholesterol/ml of A-LDL (●) or acetyl-LDL (■) for the indicated times. The medium was replenished at the 24-hr point. The esterification values shown are the average of duplicate wells.

LDL recognition by MPM. Dose–response studies measuring stimulation of cholesterol esterification indicate a saturable uptake mechanism for A-LDL, whereas competition studies with radiolabeled A-LDL suggest that uptake is not saturable over a large concentration range. To address this discrepancy, the dependence of lipoprotein uptake (as measured by [^{3}H]cholesteryl ether accumulation) and the subsequent stimulation of cholesterol esterification on A-LDL concentration were studied concurrently. As seen in **Fig. 6**, the uptake of [^{3}H]cholesteryl ether-labeled A-LDL increased almost linearly over the concentration range studied, whereas cholesterol esterification showed a saturable response. Treatment of cell layers with 0.5 ml of 500 μg/ml trypsin for 5 min at 37°C did not alter the cell-associated cholesteryl ether radioactivity. These results contrast with acetyl-LDL where uptake and cholesterol esterification followed similar, saturable kinetics (data not shown). Thus, unlike acetyl-LDL, the kinetics of cholesterol esterification do not mirror the uptake of A-LDL. It is notable that the kinetics of cholesterol esterification promoted by unlabeled (Fig. 3B) and [^{3}H]cholesteryl ether-labeled A-LDL (Fig. 6) were the same, demonstrating that this labeling procedure did not alter the interaction of A-LDL with MPM.

The data in Fig. 6 indicate that an impairment exists in the processing of A-LDL by MPM which causes cholesterol esterification to diverge from lipoprotein uptake. This blockage was temporary, however, since cells that were allowed to process internalized A-LDL for a 24-hr equilibration period after their initial incubation with the lipoprotein (i.e., postincubation in the absence of A-LDL) could convert all lipoprotein cholesterol to cellular cholesteryl oleate. As seen in **Table 4**, this complete processing was evidenced by a change in the ratio of lipoprotein uptake to cholesterol esterification from an initial value of ≥ 2 to 1 after the equilibration period.

DISCUSSION

In this study we have described characteristics of a lipoprotein (A-LDL) isolated from homogenates of human aortic atherosclerotic lesions, which possesses apoB immunoreactivity and is LDL-sized. When compared to plasma LDL, A-LDL was found to be less dense and more electronegative, and its apoB component was highly degraded. Furthermore, when incubated with mouse peritoneal macrophages (MPM), A-LDL caused a concentration-dependent increase in cellular cholesterol esterification and cholesteryl ester mass. A-LDL, isolated by affinity chromatography and gel filtration techniques, does not appear to be identical to the LDL-sized lipoproteins previously isolated by us (10) and others (31) through a combination of ultracentrifugation and gel filtration. Although the lipoproteins isolated by either approach demonstrated an increased electrophoretic mobility relative to plasma LDL, these lipoprotein preparations were markedly different in their ratios of protein/cholesterol (10, 31) and in amino acid composition (31). These apparent discrepancies might be due, in part, to the fact that A-LDL isolated from tissue homogenates by ultracentrifugation and gel

TABLE 2. Cholesterol accumulation in mouse peritoneal macrophages

Lipoprotein	Cellular Cholesterol Content	
	FC	CE
	nmol/mg protein	
None	88.2	ND
Plasma LDL	76.8	9.3
A-LDL	182.1	164.0
Acetyl-LDL	215.4	490.8

Mouse macrophages were incubated with the indicated lipoprotein (100 μg of lipoprotein cholesterol/ml) as described in Methods. After 24 hr, the media were replaced with fresh media containing the same lipoprotein at the same concentration and incubated for an additional 24 hr (48 hr total incubation time). Cellular total and free cholesterol (FC) contents were determined; cholesteryl ester (CE) content was calculated from the difference between the values, times 1.7 to correct for the fatty acid content of the ester. Data are the average of triplicate determinations and are representative of two experiments. ND, none detected.

TABLE 3. Effect of unlabeled lipoproteins on the degradation of ^{125}I-labeled acetyl-LDL and ^{125}I-labeled A-LDL

Radiolabeled Lipoprotein	Unlabeled Lipoprotein Added	^{125}I-Labeled Lipoprotein Protein Degraded	% of Control
		μg/mg protein per 4.5 hr	
^{125}I-Labeled acetyl LDL		9.1	100
^{125}I-Labeled acetyl LDL	plasma LDL	10.4	114
^{125}I-Labeled acetyl LDL	acetyl LDL	2.6	29
^{125}I-Labeled acetyl LDL	A-LDL	9.1	100
^{125}I-Labeled A-LDL		2.8	100
^{125}I-Labeled A-LDL	plasma LDL	2.4	86
^{125}I-Labeled A-LDL	acetyl LDL	2.7	96
^{125}I-Labeled A-LDL	A-LDL	2.6	90

Mouse peritoneal macrophages were incubated as described in Methods with the indicated radiolabeled lipoprotein in the presence or absence of unlabeled acetyl-LDL, plasma LDL, or A-LDL, and 10% lipoprotein-deficient human plasma. In the experiment shown, cells were incubated with either ^{125}I-labeled acetyl LDL (20 μg of cholesterol/ml) and 200 μg of cholesterol/ml of the unlabeled lipoprotein indicated, or with ^{125}I-labeled A-LDL (45 μg of cholesterol/ml) and 450 μg of cholesterol/ml unlabeled lipoprotein. After 4.5 hr, cells were rinsed and the trichloroacetic acid-soluble, non-iodide radioactivity of the combined medium plus rinse was determined. Values are the average of two experiments, each performed in duplicate.

filtration may contain lipid–protein complexes that have the same size and hydrated density of LDL but are devoid of immunoreactive apoB. Thus, the isolation procedure reported here most likely results in a more homogeneous preparation of the LDL-like lipoproteins that accumulate in atherosclerotic lesions.

In these studies, cholesterol esterification has been used as a measure of lipoprotein–cell surface interactions. Although cholesterol esterification is several steps removed from the interaction of a lipoprotein with its receptor, studies of the LDL- (30), scavenger- (32, 33), and β-VLDL-receptors (34) have shown that the kinetics of intracellular processing events (i.e., degradation and cholesterol esterification) mirror the kinetics of ligand binding to the receptor. In our earlier studies on the interaction of MPM with lipoproteins isolated by anti-LDL affinity chromatography alone (A-LP), we demonstrated that A-LP stimulated cholesterol esterification in MPM to a far greater extent than did plasma LDL, suggesting an enhanced interaction of A-LP with the MPM surface. We additionally demonstrated that this enhanced uptake of A-LP was not an artifact due to the isolation procedure or the postmortem interval (11). Over the concentration range tested, A-LP-induced cholesterol esterification did not reach saturation. In the present study, when the two major subfractions of A-LP, the gel filtration void-volume fraction and A-LDL, were assayed over the same dose range, it appeared that the failure of A-LP to saturate cellular cholesterol esterification was due to A-LDL. At higher A-LDL concentrations, however, cholesterol esterification did plateau, suggesting that A-LDL is taken up by a saturable process. In light of the increased electronegativity of A-LDL relative to plasma LDL, it seemed that this apparently saturable uptake might be mediated by the macrophage scavenger-receptor. However, excess unlabeled A-LDL was unable to inhibit uptake or degradation of radiolabeled acetyl-LDL, and conversely, excess unlabeled acetyl-LDL had little effect on the uptake or degradation of radiolabeled A-LDL. Although only a 10-fold excess of unlabeled lipoprotein was used in these competition studies, this level of competing lipoprotein was clearly sufficient to markedly inhibit the uptake and degradation of acetyl-LDL (Table 3 and Fig. 5). Likewise, the recognition of A-LDL by the β-VLDL receptor on MPM seems unlikely since the uptake of labeled A-LDL was not significantly affected by an amount of β-VLDL competitor which is sufficient to saturate its receptor (35).

An unexpected finding of the present study was the observation that excess A-LDL also failed to inhibit the uptake of A-LDL radiolabeled in either the protein or lipid moiety. This is in contrast to conclusions based on cholesterol esterification, which predicted that this level of unlabeled A-LDL would have caused a 55–60% inhibition of labeled A-LDL uptake. The reason for this descrepancy was apparent when cholesterol esterification and lipoprotein uptake were studied simultaneously; unlike LDL (30), acetyl-LDL (32, 33), and β-VLDL (34), A-LDL-induced cholesterol esterification did not reflect lipoprotein uptake. The almost linear uptake of [^{3}H]cholesteryl ether-labeled A-LDL observed over the 0–400 μg/ml concentration range is consistent with A-LDL uptake by either a non-receptor-mediated mechanism or one of very low affinity requiring concentrations of ligand far higher than those used here to saturate uptake. The concept of non-receptor-mediated uptake mechanisms for lipoproteins has been discussed in the literature (36–38). Those mechanisms have been proposed to rectify the apparent discrepancy that plasma LDL is internalized by cells even in the absence of LDL receptors, as in patients with homozygous familial hypercholesterolemia (36, 37). It should be stressed here that the

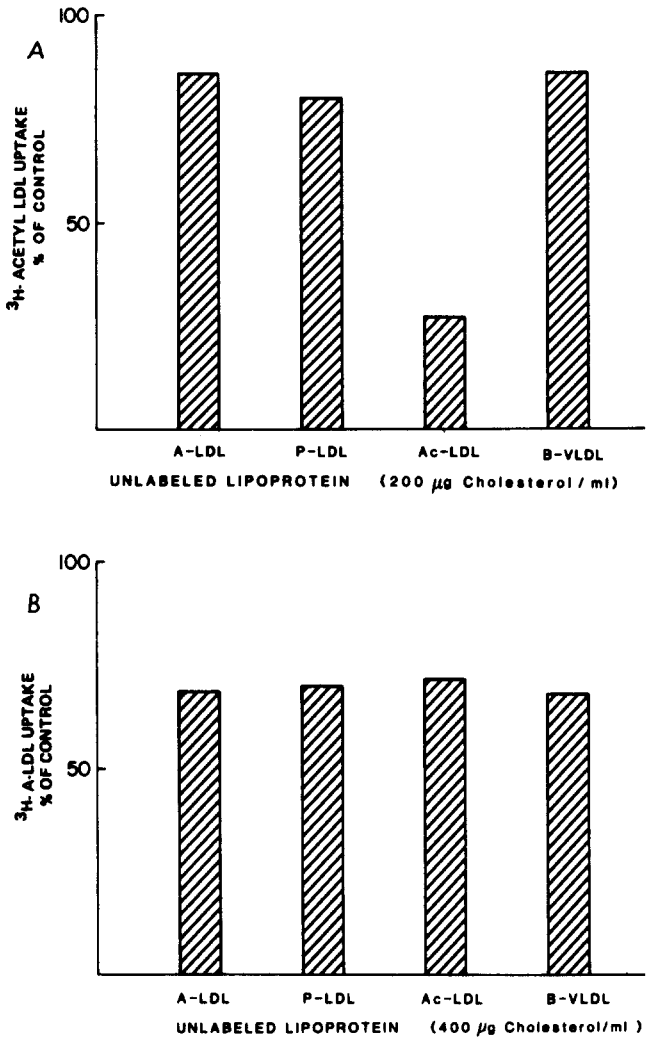

Fig. 5. Effect of unlabeled lipoproteins on uptake of cholesteryl ether-labeled lipoproteins. Macrophages were incubated with: panel A) [^{3}H]cholesteryl oleyl ether-labeled acetyl-LDL (20 μg of cholesterol/ml, 4,575 cpm/nmol of cholesterol), or panel B [^{3}H]cholesteryl oleyl ether-labeled A-LDL (40 μg of cholesterol/ml, 6,816 cpm/nmol of cholesterol) in the presence or absence of a 10-fold amount of unlabeled lipoprotein. After 16 hr, cells were washed and harvested as described in Methods and the cellular content of [^{3}H]cholesteryl ether was determined. In the absence of unlabeled lipoproteins, the uptake of [^{3}H]cholesteryl ether, expressed as lipoprotein cholesterol uptake (i.e., assuming intact particle uptake) was 75.4 and 111.0 nmol/mg of protein for acetyl-LDL and A-LDL, respectively. The results are the average of duplicate wells. Abbreviations: P-LDL, plasma LDL; Ac-LDL, acetyl LDL.

uptake of A-LDL by MPM is significantly higher than that of plasma LDL, which presumably enters via a "nonspecific" uptake mechanism since MPM have no measurable LDL receptor activity under these culture conditions (33). Alternative mechanisms of uptake that have been proposed to explain the accumulation of cholesteryl ester in cells involve internalization of the lipid portion of lipoproteins independent of the protein portion (36, 38), possibly via lipid transfer proteins. However, in this study, both the protein and lipid portions appear to enter into the cell as evidenced from the internalization and degradation of labeled components. To our knowledge, this is the first example of an in vivo-derived lipoprotein being internalized by a high capacity, low affinity mechanism that is sufficient to cause cholesteryl ester loading at moderate lipoprotein concentrations. Further studies are needed to better characterize this uptake mechanism and to examine the possible role of increased electronegative surface charge in this mechanism.

The kinetics of A-LDL uptake (as measured with [^{3}H]cholesteryl ether-labeled lipoproteins) and stimulation of cholesterol esterification were clearly different; the former was linear with increasing concentration, whereas the latter was saturable. Thus, in contrast to acetyl-LDL, A-LDL apparently takes longer to be processed, so that a delay occurs between lipoprotein uptake and cholesterol esterification. It is not clear whether this slower processing is a result of physical differences in A-LDL relative to acetyl-LDL, such as a higher melting temperature of lipid components of A-LDL. Moreover, it is also uncertain whether the ac-

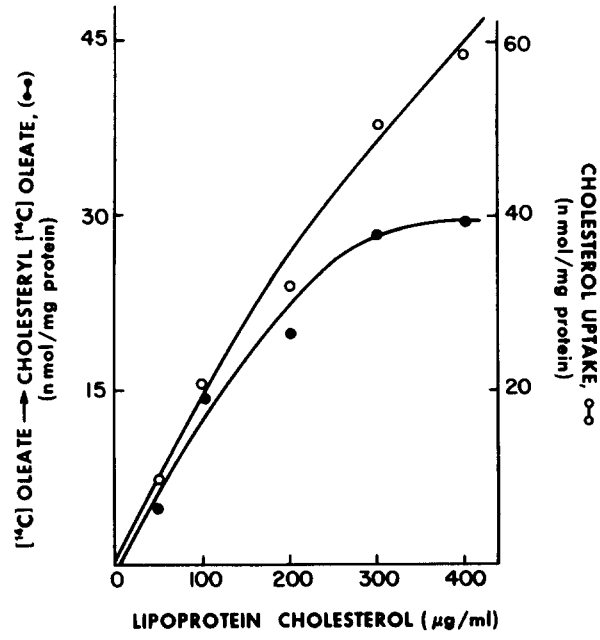

Fig. 6. Cholesterol esterification and cholesteryl ether accumulation in macrophages. Mouse peritoneal macrophages were incubated with the indicated amount of A-LDL (3,621 cpm/nmol cholesterol) for 16 hr at 37°C in the presence of [^{14}C]oleate-albumin. After incubation, the medium was removed, and the cell layer was processed as described in Methods to quantitate the [^{3}H]cholesteryl ether label accumulated (○), expressed in terms of lipoprotein cholesterol, and the amount of [^{14}C]oleate incorporated into cholesterol ester (●). Esterification and ether accumulation due to plasma LDL, which reflects receptor-independent uptake, were subtracted from the values presented. For plasma LDL, esterification was relatively constant and averaged 2.8 nmol/mg of protein over the range of 50 to 400 μg cholesterol, whereas ether accumulation increased almost linearly and reached a value of 27.8 nmol/mg of protein at the highest plasma LDL concentration. The values shown are the average of duplicate wells.

TABLE 4. Effect of postincubation on extent of cholesterol esterification

Amount of Labeled A-LDL Added Initially	Time of Postincubation	Lipoprotein Uptake	Cholesterol Esterification	Ratio Uptake/Esterification
μg cholesterol / ml	*hr*	*nmol/mg protein*		
100	0	38.6	19.0	2.0
300	0	80.9	34.0	2.4
100	24	34.9	37.0	0.9
300	24	87.7	91.7	1.0

Macrophages were incubated with the indicated amount of [^{3}H]cholesteryl ether-labeled A-LDL in the presence of [^{14}C]oleate–BSA. After 24 hr, cells were either processed as described in Methods (t = 0) or incubated an additional 24 hr with [^{14}C]oleate–BSA but without lipoprotein, and then processed (t = 24 hr). Data are the average of duplicate wells.

tual impairment in processing of the internalized lipoprotein occurs at the level of lipid hydrolysis in the lysosome, or in the re-esterification of liberated unesterified cholesterol via the ACAT reaction. If the defect is at the lysosomal level, one might expect secondary lysosomes to become lipid-filled. In fact, in foam cells of rabbit lesions, about half of the lipid inclusions are within secondary lysosomes (4).

In summary, we have described some of the structural and functional characteristics of a lipoprotein particle, designated A-LDL, isolated from human aortic plaques that possesses apoB immunoreactivity and is LDL-sized. A-LDL was capable of causing massive cholesteryl ester accumulation in MPM. Although A-LDL is more electronegative than plasma LDL, its uptake was not mediated by the scavenger-receptor which recognizes negatively charged ligands. The uptake of A-LDL by MPM was linear over a high concentration range, suggesting a high-capacity, low affinity uptake mechanism. Unlike other lipoproteins, cholesterol esterification induced by A-LDL did not reflect the uptake of A-LDL by MPM. This observation suggests a slower cellular processing of internalized A-LDL.

The authors wish to thank Dr. William Opsahl for performing the amino acid analysis of A-LDL, Ms. Muriel Daly for her excellent editorial assistance, and Mr. Endre Ritly for photographic services. This work was supported by Public Health Service Research Grant HL-29582 from the National Heart, Lung, and Blood Institute of the United States Public Health Service and by the Bleeksma Fund.

Manuscript received 16 December 1985.

REFERENCES

1. McGill, H. C. Jr. 1968. Fatty streaks in the coronary arteries and aorta. *Lab. Invest.* **18:** 560–564.
2. Katz, S. S., G. G. Shipley, and D. M. Small. 1976. Physical density of the lipids of human atherosclerotic lesion. Demonstration of a lesion intermediate between fatty streaks and advanced plaques. *J. Clin. Invest.* **58:** 200–211.
3. Lang, P. D., and W. Insull, Jr. 1970. Lipid droplets in atherosclerotic fatty streaks of human aorta. *J. Clin. Invest.* **49:** 1479–1488.
4. Fowler, S., I. I. Shio, and N. J. Haley. 1979. Characterization of lipid-laden aortic cells from cholesterol-fed rabbits. IV. Investigation of macrophage-like properties of aortic cell populations. *Lab. Invest.* **41:** 372–378.
5. Schaffner, T., K. Taylor, E. J. Bartucci, K. Fischer-Dzoga, J. H. Beeson, S. Glagov, and R. W. Wissler. 1980. Arterial foam cells with distinctive immunomorphologic and histochemical features of macrophages. *Am. J. Pathol.* **100:** 57–80.
6. Brown, M. S., S. K. Basu, J. R. Falck, Y. K. Ho, and J. L. Goldstein. 1980. The scavenger cell pathway for lipoprotein degradation: specificity of the binding site that mediates the uptake of negative-charged LDL by macrophages. *J. Supramol. Struct.* **13:** 67–81.
7. Mahley, R. W., T. C. Innerarity, K. H. Weisgraber, and S. U. Oh. 1979. Altered metabolism (in vivo and in vitro) of plasma lipoproteins after selective chemical modification of lysine residues of the apoproteins. *J. Clin. Invest.* **64:** 743–750.
8. Fogelman, A. M., I. Shechter, J. Seager, M. Hokom, J. S. Child and P. A. Edwards. 1980. Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. *Proc. Natl. Acad. Sci. USA.* **77:** 2214–2218.
9. Goldstein, J. L., H. F. Hoff, Y. K. Ho, S. K. Basu, and M. S. Brown. 1981. Stimulation of cholesteryl ester synthesis in macrophages by extracts of atherosclerotic human aortas and complexes of albumin/cholesterol esters. *Arteriosclerosis.* **1:** 210–226.
10. Hoff, H. F., and J. W. Gaubatz. 1982. Isolation, purification, and characterization of a lipoprotein containing apoB from the human aorta. *Atherosclerosis.* **42:** 273–297.
11. Clevidence, B. A., R. E. Morton, G. West, D. M. Dusek, and H. F. Hoff. 1984. Cholesterol esterification in macrophages: stimulation by lipoproteins containing apoB isolated from human aortas. *Arteriosclerosis.* **4:** 196–207.
12. Halperin, G., and S. Gatt. 1980. The synthesis of cholesteryl alkyl ethers. *Steroids.* **35:** 39–42.
13. Hatch, F. T., and R. S. Lees. 1968. Practical method for plasma lipoprotein analysis. *Adv. Lipid Res.* **6:** 2–63.

14. Fraenkal-Conrat, H. 1957. Methods for investigating the essential groups for enzyme activity. *Methods Enzymol.* **4:** 247–269.
15. Habeeb, A. F. S. A. 1966. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem.* **14:** 328–336.
16. McFarlane, A. S. 1958. Efficient trace-labelling of proteins with iodine. *Nature.* **182:** 53.
17. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.
18. Morton, R. E., and D. B. Zilversmit. 1981. A plasma inhibitor of triglyceride and cholesteryl ester transfer activities. *J. Biol. Chem.* **256:** 11992–11995.
19. Hoff, H. F., J. D. Morrisett, and A. M. Gotto. 1973. Interaction of phosphatidylcholine and apolipoprotein alanine: electron microscopic studies. *Biochim. Biophys. Acta.* **296:** 653–660.
20. Roeschlau, P., E. Bernt, and W. Gruber. 1974. Enzymatische Bestimmung des Gesamt-Cholesterins im Serum. *J. Clin. Chem. Clin. Biochem.* **12:** 403–407.
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193.** 265–275.
22. Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83:** 346–356.
23. Wray, W., T. Boulikas, V. P. Wray, and R. Hancock. 1981. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118:** 197–203.
24. Kane, J. P., D. A. Hardman, and H. E. Paulus. 1980. Heterogeneity of apolipoprotein B: isolation of a new species from human chylomicrons. *Proc. Natl. Acad. Sci. USA.* **77:** 2465–2468.
25. Cohen, S. A., T. L. Tarvin, and B. A. Bidlingmeyer. 1984. Analysis of amino acids: using precolumn derivatization with phenylisothiocyanate. *Am. Lab.* **16:** 48–59.
26. Nilsson, A., and D. B. Zilversmit. 1972. Fate of intravenously administered particulate and lipoprotein cholesterol in the rat. *J. Lipid Res.* **13:** 32–38.
27. 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.
28. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
29. Labarca, C., and K. Paigen. 1980. A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* **102:** 344–352.
30. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts: comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* **249:** 5153–5162.
31. Hollander, W., J. Paddock, and M. Colombo. 1979. Lipoproteins in human atherosclerotic vessels. I. Biochemical properties of arterial low density lipoproteins, very low density lipoproteins, and high density lipoproteins. *Exp. Mol. Pathol.* **30:** 144–171.
32. Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophage: implications for cholsterol deposition in atherosclerosis. *Annu. Rev. Biochem.* **52:** 223–261.
33. Goldstein, J. L., Y. K. Ho, S. K. Basu, and M. S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* **76:** 333–337.
34. Goldstein, J. L., Y. K. Ho, M. S. Brown, T. L. Innerarity, and R. W. Mahley. 1980. Cholesteryl ester accumulation in macrophages resulting from receptor-mediated uptake and degradation of hypercholesterolemic canine-β-very low density lipoproteins. *J. Biol. Chem.* **255:** 1839–1848.
35. Innerarity, T. L., K. S. Arnold, K. H. Weisgraber, and R. W. Mahley. 1986. Apolipoprotein E is the determinant that mediates the receptor uptake of β-very low density lipoproteins by mouse macrophages. *Arteriosclerosis.* **6:** 114–122.
36. Pittman, R. C., and D. Steinberg. 1984. Sites and mechanisms of uptake and degradation of high density and low density lipoproteins. *J. Lipid Res.* **25:** 1577–1585.
37. Simons, L. A., D. Reichl, N. B. Myant, and M. Mancini. 1975. The metabolism of the apoprotein of plasma low density lipoprotein in familial hyperbetalipoproteinemia in the homozygous form. *Atherosclerosis.* **21:** 283–298.
38. Steinbrecher, U. P., J. L. Witzum, Y. A. Kesaniemi, and R. Elam. 1983. Comparison of glucosylated low density lipoprotein with methylated or cyclohexanedione-treated low density lipoprotein in the measurement of receptor-independent low density lipoprotein catabolism. *J. Clin. Invest.* **71:** 960–964.