

# Subcellular localization and quantification of cholesterol in cultured human fibroblasts exposed to human low density lipoprotein

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**Abstract** Subcellular localization of nonesterified cholesterol has been determined in normal human fibroblasts from cultures incubated with human low density lipoprotein (LDL). Nonesterified and esterified cholesterol content of fibroblasts, grown initially in the absence of cholesterol, increased significantly after a 1-hour incubation with LDL. Digitonin was used to localize nonesterified cholesterol that was accumulated within multivesicular and lamellar lysosomal inclusions. This was observed only in fibroblasts from cultures incubated with LDL. Accumulation of LDL-derived nonesterified cholesterol within lysosomes is consistent with the suggestion of other investigators that LDL is metabolized within lysosomes.—**Kruth, H. S., J. Blanchette-Mackie, J. Avigan, W. Gamble, and M. Vaughan.** Subcellular localization and quantification of cholesterol in cultured human fibroblasts exposed to human low density lipoprotein. *J. Lipid Res.* 1982. **23**: 1128–1135.

**Supplementary key words** digitonin • electron microscopy

Brown, Goldstein, and co-workers have shown that LDL taken up by cultured cells is degraded to nonesterified cholesterol and amino acids within lysosomes (reviewed in ref. 1). Digitonin complexes with  $3\beta$ -hydroxysterols, such as cholesterol, to form insoluble crystalline complexes that can be detected with the electron microscope (2). Using digitonin, we have demonstrated that nonesterified cholesterol accumulates within lysosomes during incubation of cultured human fibroblasts with human LDL. Chemical determinations of cellular cholesterol carried out on parallel cultures confirm cytochemical observations that significant intracellular cholesterol accumulation occurs during 1-hr incubation of cultured fibroblasts with LDL.

## MATERIALS AND METHODS

### Preparation of LDL

LDL (d 1.019–1.063 g/ml) was prepared from human plasma by standard techniques (3) of density flo-

tation in a preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, CA) at 215,000 *g* and 4–10°C for 18 hr using solid KBr for density adjustment (4). The LDL fraction was dialyzed 48 hr against four changes of 4 liters of 0.85% saline containing 0.01% EDTA at 4°C and filtered (sterile Millipore 0.45  $\mu$ m). Protein content of the LDL fraction was determined by the procedure of Lowry et al. (5).

### Cell culture

Normal human foreskin fibroblast cultures provided by Dr. Vincent C. Manganiello were maintained at 37°C in gassed (95% air, 5% CO<sub>2</sub>) and sealed 75-cm<sup>2</sup> tissue culture flasks (Corning Glass Works, Corning, NY) and fed weekly with Eagle's minimal essential medium (MEM) supplemented with 10% (v/v) fetal calf serum (FCS) (North American Biologicals, Inc., Miami, FL). After washing cultures twice with 10 ml of Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free Dulbecco's phosphate-buffered saline (DPBS), cultures were incubated with 1 ml of DPBS containing 0.125% trypsin for 5–10 min at 37°C. Trypsinization was terminated by addition of 10 ml of MEM containing 10% FCS to each flask. An aliquot of cell suspension was counted in a Neubauer chamber. Cells were diluted in MEM containing 10% FCS and plated at a nonconfluent density (6) of 10<sup>5</sup> cells per 60-mm culture dish (#3002 Falcon, Oxnard, CA) for electron microscopy or an equivalent density of 278,000 cells per 100-mm

Abbreviations: LDL, low density lipoprotein; MEM, minimal essential medium; FCS, fetal calf serum; DPBS, Dulbecco's phosphate-buffered saline; ACAT, fatty acyl-CoA:cholesterol acyltransferase.

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dish (#3003 Falcon, Oxnard, CA) for biochemical determinations. Cultures were incubated (37°C in 95% air, 5% CO<sub>2</sub>) for 24 hr, after which the medium was replaced with 5 ml of MEM without FCS. After incubation for 96 hr, medium was discarded and cells were incubated for 1 hr at 37°C in 5 ml of MEM with or without LDL (500 µg of protein).

#### Digitonin cytochemistry and biochemical analysis

Cultures were prepared for electron microscopy using measures to minimize lipid extraction (7–10). Parallel cultures were harvested by trypsinization for determination of cholesterol, protein, and cell count as described previously (11). Preparation of cells for electron microscopy was carried out at room temperature unless noted otherwise. Cultures were washed three times with 1 ml of DPBS then fixed for 30 min with 2 ml of 0.1 M Na-cacodylate/HCl buffer (pH 7.2) containing 2% paraformaldehyde, 2.5% glutaraldehyde, 0.05% anhydrous CaCl<sub>2</sub>, and 0.1 M sucrose (12), followed by fixation in the same solution (30 min) with or without 0.2% digitonin for an additional 30 min. Cells were then washed twice (30 min each) with 5 ml of 0.5 M Na-cacodylate/HCl buffer (pH 7.2) containing 0.05% anhydrous CaCl<sub>2</sub> and 0.1 M sucrose before post-fixation for 1 hr in 2 ml of 0.1 M Na-cacodylate/HCl buffer (pH 7.2) containing 0.05% anhydrous CaCl<sub>2</sub> and 2% osmium tetroxide (13). This was followed by dehydration in solutions of 70%, 80%, 90%, and 95% ethanol (5 ml per culture dish for 2 min each). Cultures were incubated twice for 10 min with 2 ml of Epon 812 (14) followed by final embedding with 4 ml Epon per dish. After 12 hr at room temperature to eliminate air bubbles and polymerization for 23 hr at 60°C, Epon-embedded cultures were released by partial immersion of dishes in liquid nitrogen. Cells were located in embedded monolayers using an inverted phase microscope and marked with a metal scribe. To facilitate mounting in the ultramicrotome, Epon capsule molds were glued to embedded cultures opposite previously located cells. The monolayer and attached mold were cut out as a unit with a jeweler's saw. Ultrathin *en face* sections were cut with a diamond knife and placed on bare 200 mesh copper grids before staining in aqueous uranyl acetate (15) and lead citrate (16) solutions. A thin carbon coat was applied for stability. Sections were viewed and photographed with a Phillips 400 electron microscope.

## RESULTS

Three types of cytoplasmic inclusions were present in fibroblasts from cultures incubated with LDL: 1) lipid-like inclusions of homogeneous electron density

lacking a boundary membrane; 2) membrane-enclosed multivesicular inclusions containing granular matrix of varying electron density; and 3) membrane-enclosed inclusions with lamellar and granular substructure. Fibroblasts from cultures not incubated with LDL contained fewer multivesicular inclusions and only rare lipid-like inclusions. Numerous lamellar inclusions were observed in fibroblasts from control and experimental cultures.

Multivesicular inclusions in cells incubated with LDL and reacted with digitonin contained digitonin-cholesterol complex in the form of stacks of short parallel electron dense lines (Figs. 1, 2, 3).<sup>4</sup> Those multivesicular inclusions with the most granular matrix also contained the greatest amount of digitonin-cholesterol complex (Fig. 2). Some inclusions of presumed multivesicular origin contained large amounts of digitonin-cholesterol complex and granular matrix without obvious intra-inclusion vesicles (Fig. 3). Multivesicular inclusions in cells from cultures incubated with MEM alone and reacted with digitonin or cultures incubated with MEM plus LDL but not reacted with digitonin did not contain digitonin-cholesterol complex (Fig. 4).

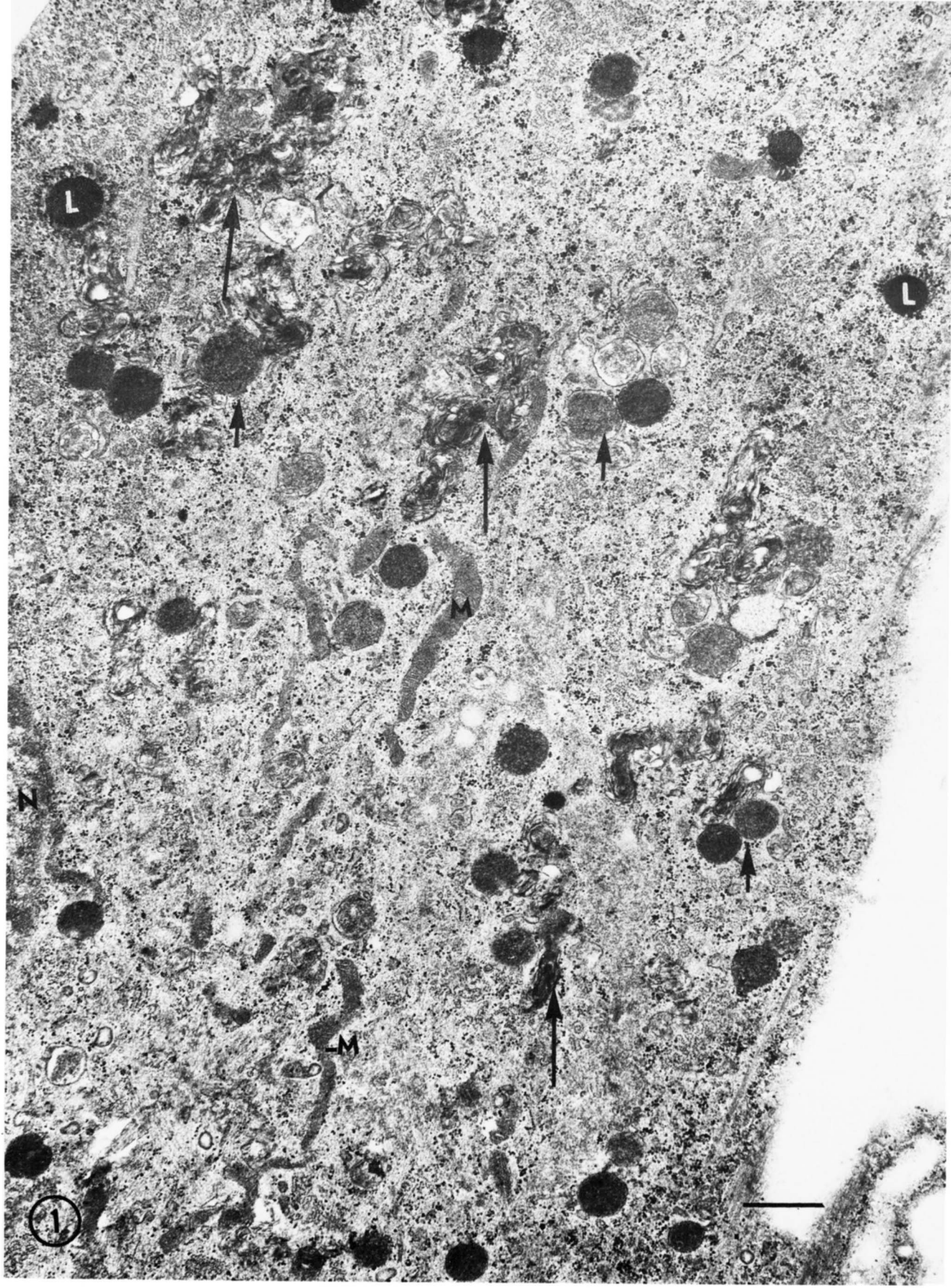
Membrane-enclosed inclusions containing concentric and curvilinear lamellae and granular structure were observed in cells from all cultures (Fig. 5a). Each lamella displayed a trilaminar "unit membrane" appearance which was more electron dense than the outermost limiting membrane or other cellular membranes. Digitonin-cholesterol complex was observed only in lamellar inclusions in cells incubated with LDL. In these cells, parallel electron dense curvilinear arrays replaced unit membrane lamella seen in lamellar inclusions in cells incubated with LDL but not treated with digitonin (Figs. 1, 5b and c).<sup>5</sup>

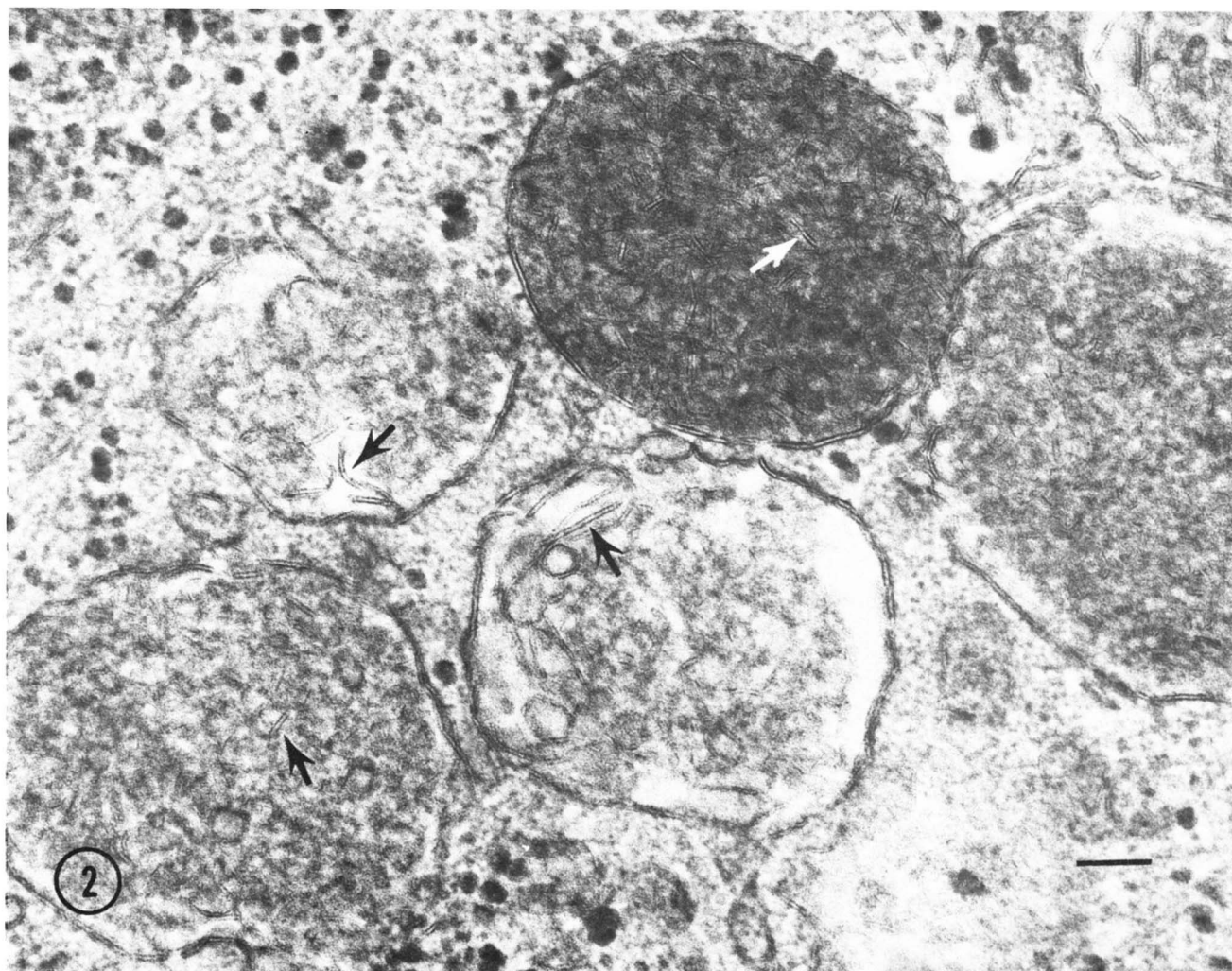
Lipid inclusions never contained digitonin-cholesterol complex and were often surrounded by dense glycogen deposits (Fig. 6). Digitonin-cholesterol complex was not observed within mitochondria and was rarely present within endoplasmic reticulum or coated vesicles in cells incubated with LDL.

Chemical analysis of parallel cultures revealed an average increase in both nonesterified (34%) and esterified cholesterol (133%) per cell in cultures incubated 1 hr with LDL (Table 1).

<sup>4</sup> Digitonin-induced structures of this type have been shown to depend on the presence of nonesterified cholesterol, whereas other digitonin-induced structures can result from complexes of digitonin and other lipids (10).

<sup>5</sup> It appears that digitonin reacts preferentially with accumulated nonesterified cholesterol rather than nonesterified cholesterol present within all cellular membranes. This could reflect the relatively low concentration of membrane-associated nonesterified cholesterol or result from an orientation of cholesterol within these membranes that precludes interaction with digitonin.





**Fig. 2.** Multivesicular inclusions in a fibroblast incubated with MEM plus LDL, 100  $\mu\text{g}$  of protein/ml, for 1 hr and fixed with paraformaldehyde-glutaraldehyde containing 0.2% digitonin. Digitonin-cholesterol complex (arrows) in the form of short parallel electron dense lines appears within the granular matrix of these inclusions. Note that inclusions with the greatest granular matrix also contain the most digitonin-cholesterol complex. Bar, 0.1  $\mu\text{m}$ ;  $\times 103,200$ .

## DISCUSSION

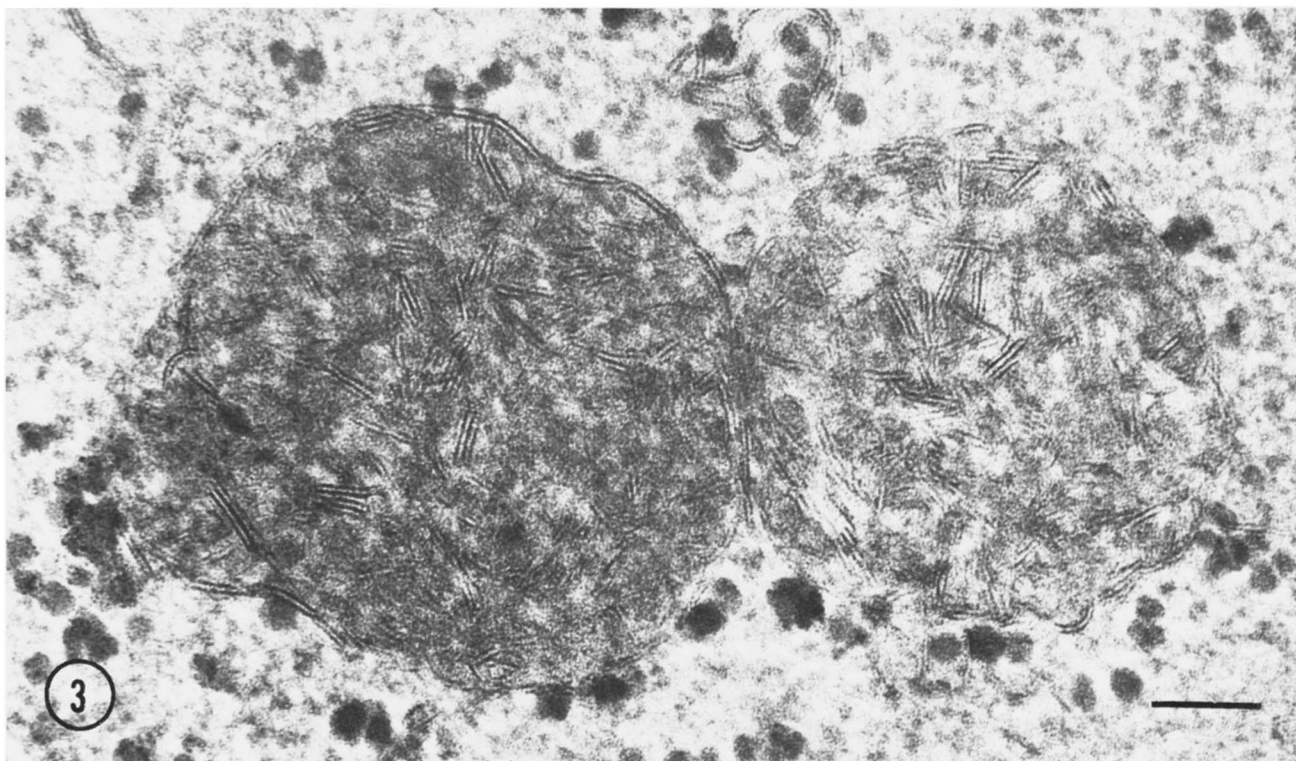
Although there was a greater percentage increase in cholesteryl ester, there was a greater molar increase in nonesterified cholesterol in human fibroblasts from nonconfluent cultures incubated with human LDL for 1 hr. Esterified cholesterol continued to increase during the subsequent 23 hr, whereas nonesterified cholesterol did not further increase after 2 hr.<sup>6</sup> Nonconfluent cul-

<sup>6</sup> Kruth, H. S., J. Blanchette-Mackie, J. Avigan, W. Gamble, and M. Vaughan. Unpublished observation.

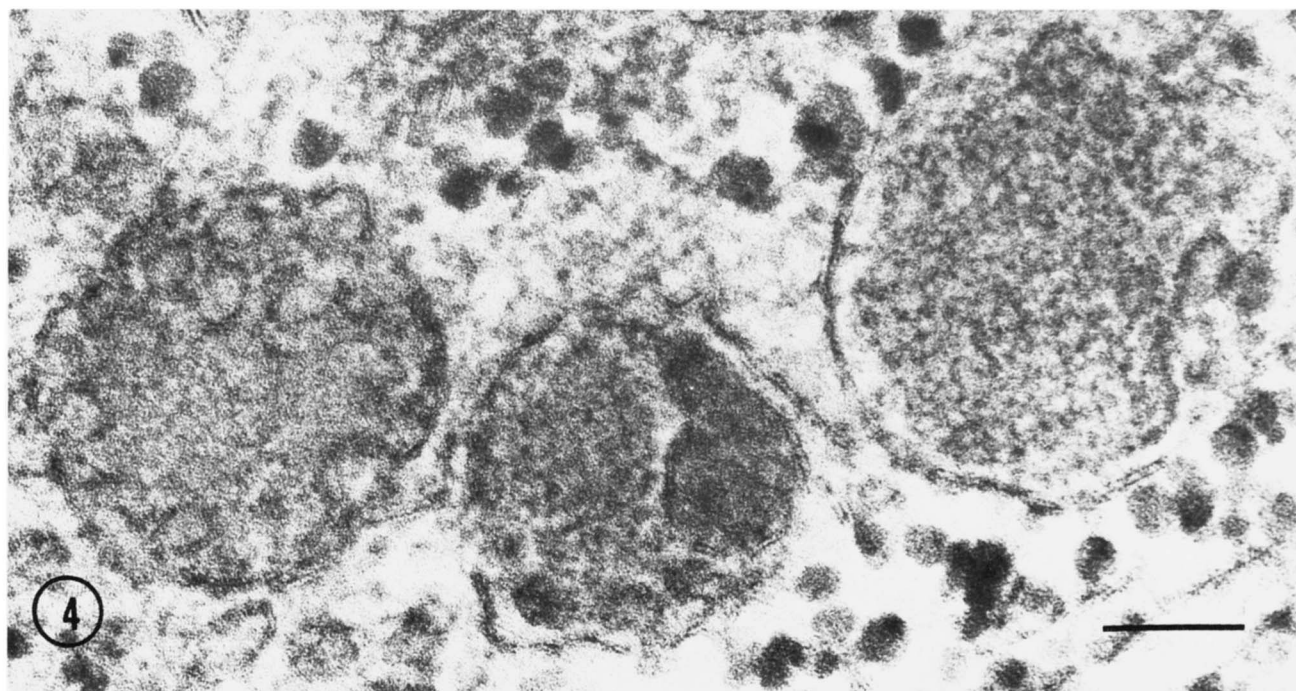
tures were used for study because cells at low density accumulate more LDL-derived cholesterol than cells grown at higher density (6). This is because, with increasing cell density, LDL receptor number decreases (6, 17, 18).

Ultrastructural analysis using digitonin cytochemistry revealed accumulation of nonesterified cholesterol within multivesicular and lamellar inclusions in cells from cultures incubated with LDL. Both multivesicular and lamellar inclusions have been previously characterized as lysosomes (19, 20). It is interesting that, in this study,

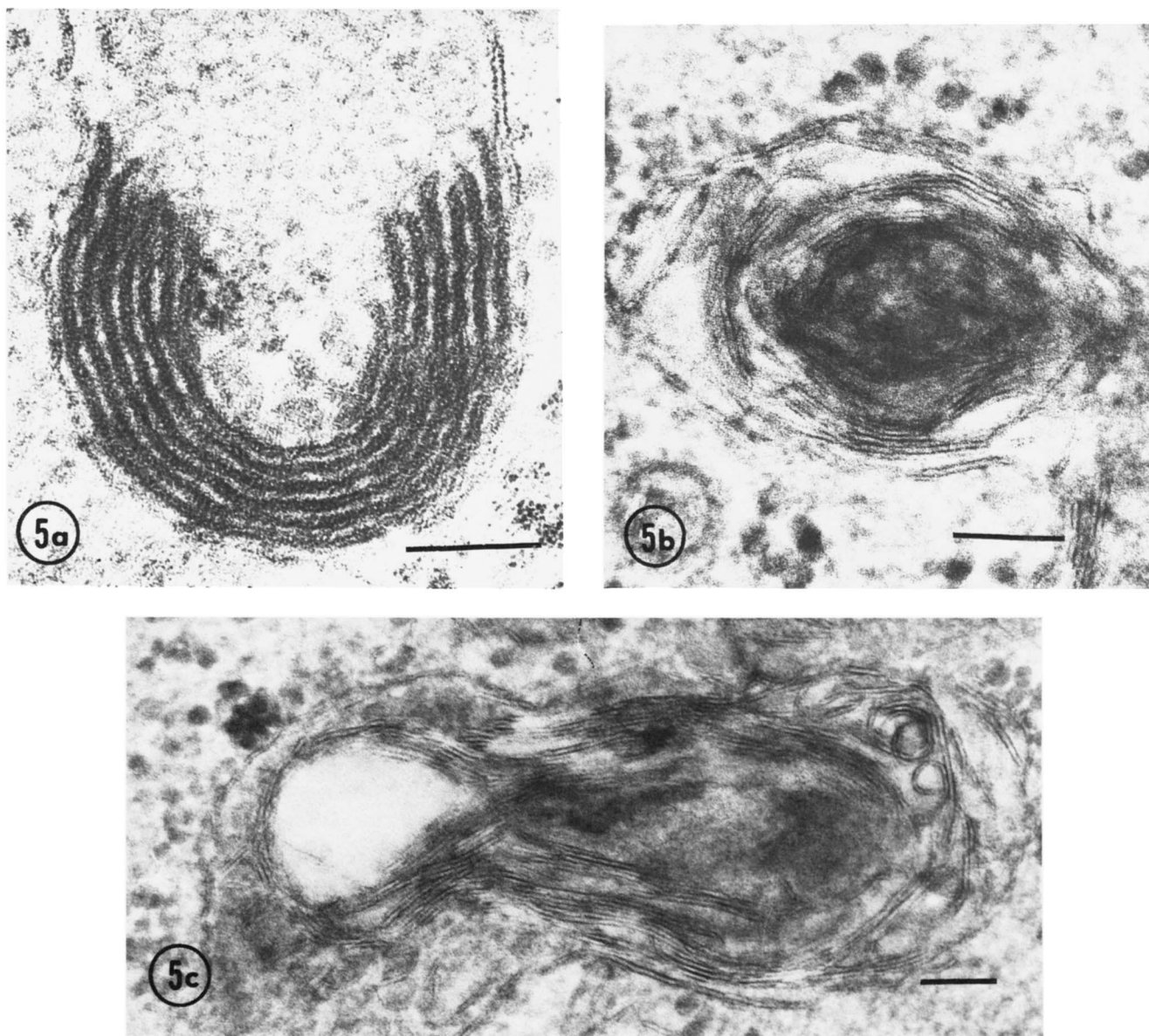
**Fig. 1.** Three types of inclusions are observed in *en face*-stained thin sections of fibroblasts incubated with MEM plus LDL, 100  $\mu\text{g}$  of protein/ml, for 1 hr and fixed with paraformaldehyde-glutaraldehyde containing 0.2% digitonin. Membrane-enclosed multivesicular inclusions (indicated by short arrows), membrane-enclosed lamellar inclusions (long arrows), and lipid-like inclusions (L) of homogeneous electron density lacking a boundary membrane are seen in addition to numerous mitochondria (M). The nucleus (N) is just apparent in the lower left-hand corner of the micrograph. Multivesicular inclusions indicated by upper right-hand arrow are shown at higher magnification in Fig. 2. Bar, 1.0  $\mu\text{m}$ ;  $\times 14,600$ .



**Fig. 3.** Inclusions in fibroblasts incubated with MEM plus LDL, 100  $\mu$ g of protein/ml, for 1 hr and fixed with paraformaldehyde-glutaraldehyde containing 0.2% digitonin. These inclusions contain abundant digitonin-cholesterol complex and granular matrix. Intra-inclusion vesicles are not seen. Bar, 0.1  $\mu$ m;  $\times$ 150,500.



**Fig. 4.** Multivesicular inclusions in fibroblasts incubated in MEM for 1 hr and fixed with paraformaldehyde-glutaraldehyde containing 0.2% digitonin. No digitonin-cholesterol complex is seen within these inclusions. Bar, 0.1  $\mu$ m;  $\times$ 181,500.

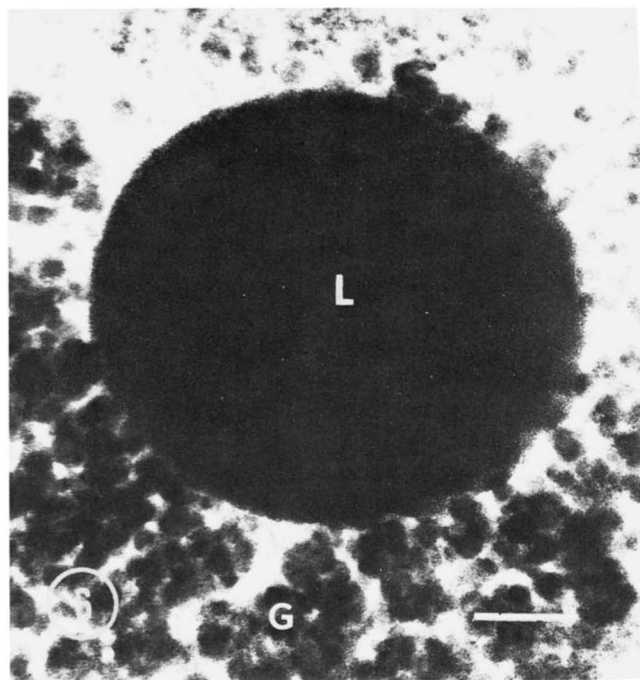


**Fig. 5.** Lamellar inclusions in fibroblasts incubated without (a) or with (b, c) LDL, 100  $\mu$ g of protein/ml, and fixed with paraformaldehyde-glutaraldehyde containing 0.2% digitonin. Inclusions are membrane-enclosed and contain trilaminar lamellae and granular matrix substructure (a). Lamellar inclusions observed in fibroblasts from cultures incubated with MEM plus LDL but not reacted with digitonin appear similar to those in a. Only in fibroblasts incubated with LDL and reacted with digitonin (b, c) do lamellar inclusions contain curvilinear electron dense lines that replace trilaminar lamellae seen in inclusions in control cells. Bar, 0.1  $\mu$ m; a,  $\times 193,500$ ; b,  $\times 187,000$ ; c,  $\times 116,100$ .

nonesterified cholesterol accumulated within two morphologically distinct types of lysosomes. While functional differences between these lysosomal subtypes are not well established, the possibility that they have different roles in cholesterol accumulation or excretion should be considered.

Lipid droplets observed in cells from cultures incubated with LDL did not show cytochemical evidence of nonesterified cholesterol, suggesting that these inclusions may contain predominantly esterified cholesterol.

This finding also suggests that compartmentalization of accumulated nonesterified cholesterol within lysosomes and esterified cholesterol within lipid droplets may occur within these cells. The above cytochemical findings using digitonin are consistent with recent fluorescence microscopic studies of cholesterol accumulation in human fibroblasts from cultures incubated with LDL (21). When these cultures were stained with filipin, a fluorescent probe that detects nonesterified cholesterol, both fluorescent and nonfluorescent phase refractile in-



**Fig. 6.** Lipid inclusion in fibroblast incubated with LDL, 100  $\mu\text{g}$  of protein/ml, for 1 hr and fixed in paraformaldehyde-glutaraldehyde containing 0.2% digitonin. Lipid inclusions show homogeneous electron density and lack a limiting membrane. No digitonin-cholesterol complex is observed. Abundant glycogen (G) surrounds this lipid inclusion. Bar, 0.1  $\mu\text{m}$ ;  $\times 132,000$ .

clusions were observed within cells. However, after enzymatic hydrolysis of cellular cholesteryl esters followed by filipin staining, all cellular inclusions were fluorescent, indicating the presence of separate nonesterified and esterified cholesterol-containing inclusions.

Previous studies by others suggest lysosomal degradation of endocytosed LDL. This is based on observations that LDL degradation is prevented by inhibitors of lysosomal function (22) and by ultrastructural local-

ization of gold, ferritin, or radiolabeled LDL within lysosomes (23–26). The latter investigations have examined subcellular localization of LDL-associated protein in contrast to this study which has examined subcellular localization of LDL-derived cholesterol. Our results are consistent with these studies and indicate that significant cholesterol accumulates within lysosomal inclusions during internalization and degradation of LDL. Brown and Goldstein (1) have proposed that nonesterified cholesterol from degraded LDL is released from lysosomes and re-esterified within the cytoplasm by fatty acyl-CoA:cholesteryl acyltransferase (ACAT). In our study, cytoplasmic lipid inclusions lacking boundary membranes observed in fibroblasts from cultures incubated with LDL may represent cytoplasmic sites of cholesterol esterification mediated by ACAT. Our results do not indicate how nonesterified cholesterol is transferred from sites of accumulation in multivesicular and lamellar lysosomes to cytoplasmic sites of cholesterol esterification. Possibly transfer is mediated by sterol carrier protein (27) or lateral membrane diffusion as described for fatty acids (28).

Previous biochemical and cytochemical investigations demonstrated the presence of cholesterol within lysosomes of cells in experimental atherosclerotic lesions (29, 30). This and the fact that multivesicular and lamellar lysosomal inclusions are sites of cholesterol accumulation in human fibroblasts suggest that lysosomes participate in both physiologic and pathologic accumulation of cellular cholesterol. ■

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**TABLE 1.** Accumulation of cholesterol by fibroblasts incubated with LDL

Culture Medium	Cells per Dish	Protein per Dish	Cholesterol Content	
			Nonesterified	Esterified
	$\times 10^{-3}$	$\mu\text{g}$	$\mu\text{g}/\text{mg protein}$	
MEM	169 $\pm$ 18	26.6 $\pm$ 3.2	66.7 $\pm$ 2.2	5.4 $\pm$ 1.6
MEM + LDL	169 $\pm$ 4	28.7 $\pm$ 4.9	83.0 $\pm$ 6.2	12.6 $\pm$ 1.5

Cultures were initiated in MEM plus 10% (v/v) FCS at a density of 278,000 cells per 100-mm culture dish and incubated 24 hr at 37°C. Culture medium was then changed to 5 ml of MEM without FCS for an additional 96 hr incubation at 37°C. Finally, cultures received new medium containing either 5 ml MEM or MEM plus LDL (500  $\mu\text{g}$  of protein) and incubated 1 hr at 37°C before cells were harvested for determination of protein and cholesterol contents as described elsewhere (11). Values are the means of data from triplicate cultures  $\pm$  SEM.

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