

Morphological Characterization of the Cholesteryl Ester Cycle in Cultured Mouse Macrophage Foam Cells

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ABSTRACT Mouse peritoneal macrophages can be induced to accumulate cholesteryl esters by incubating them in the presence of acetylated low density lipoprotein. The cholesteryl esters are sequestered in neutral lipid droplets that remain in the cell even when the acetylated low density lipoprotein is removed from the culture media. Previous biochemical studies have determined that the cholesterol component of cholesteryl ester droplets constantly turns over with a half time of 24 h by a cyclic process of de-esterification and re-esterification. We have used morphologic techniques to determine the spatial relationship of cholesteryl ester, free cholesterol, and lipase activity during normal turnover and when turnover is disrupted. Lipid droplets were surrounded by numerous 7.5–10.0-nm filaments; moreover, at focal sites on the margin of each droplet there were whorles of concentrically arranged membrane that penetrated the matrix. Histochemically detectable lipase activity was associated with these stacks of membrane. Using filipin as a light and electron microscopic probe for free cholesterol, we determined that a pool of free cholesterol was associated with each lipid droplet. Following incubation in the presence of the exogenous cholesterol acceptor, high density lipoprotein, the cholesteryl ester droplets disappeared and were replaced with lipid droplets of a different lipid composition. Inhibition of cholesterol esterification caused cholesteryl ester droplets to disappear and free cholesterol to accumulate in numerous myelin-like structures in the body of the cell.

Cholesterol homeostasis is important for normal cell function. The activities of the enzymes involved in the endogenous synthesis of cholesterol are in balance with cell surface receptors that mediate the internalization of exogenous cholesterol transport proteins such as low density lipoprotein (LDL¹) (1). Because of this tightly regulated process, cholesterol ordinarily does not accumulate in cells. However, when cells do accumulate more cholesterol than is required to meet metabolic needs, it is stored as cholesteryl ester lipid droplets, which are nonmembrane bound inclusions that are distributed in the cytoplasm of the cell (2). These lipid droplets are found in some steroid secreting cells (3, 4). Moreover, in certain disease processes, abnormal numbers of cholesteryl ester lipid droplets accumulate in cells, and as a result these cells have a foamy appearance (5). Heretofore, it has been difficult to study the metabolic activity of these lipid droplets in either normal or diseased cells of the body. However, cultured

peritoneal macrophages can be induced to accumulate large amounts of cholesteryl esters (2, 6) and this in vitro cell system can be used to study cholesterol metabolism in cells that have large amounts of stored cholesterol (7).

Previous biochemical and morphologic studies have established several important features about cholesteryl ester accumulation in cultured macrophages. (a) These cells accumulate large amounts of cholesteryl esters when incubated with chemically modified LDL (acetylated LDL [acetyl-LDL]) but not with normal LDL (6). (b) Cholesteryl ester-containing lipid droplets, which are birefringent under polarized light, appear by electron microscopy as nonmembrane bound inclusions that are free in the cytoplasm of the cell (2). (c) When macrophages that have accumulated large numbers of cholesteryl ester lipid droplets are incubated in the absence of acetyl-LDL, the cholesteryl ester level remains constant. However, when these cells are incubated in the presence of an extracellular cholesterol acceptor such as high density lipoprotein (HDL), there is a rapid loss of cholesteryl esters in response to the net egress of cholesterol from the cell (7).

Because the cholesteryl ester-laden macrophages resemble

¹ Abbreviations used in this paper: ACAT, acyl-CoA:cholesterol acyltransferase; acetyl-LDL, acetylated low density lipoprotein; DME, Dulbecco's modified Eagle medium; HDL, high density lipoprotein.

in many respects the foam cells found in atherosclerotic lesions (5, 8), this culture system has been used to study how cholesterol acceptors, such as HDL, can induce the removal of cholesterol. These studies have established the important concept that the cholesteryl ester lipid droplets are in a state of dynamic flux (7). They are continually being broken down by a nonlysosomal cholesteryl esterase into free cholesterol and fatty acids. When an extracellular cholesterol acceptor is not present, the free cholesterol generated by the esterase is re-esterified by the enzyme acyl-CoA: cholesterol acyltransferase (ACAT) in an energy-dependent reaction. These cholesteryl esters are sequestered into neutral lipid droplets. If, however, an extracellular cholesterol acceptor is present, the free cholesterol generated by the esterase leaves the cell and becomes associated with the cholesterol acceptor. Finally, if the ACAT enzyme is inhibited by pharmacological concentrations of progesterone, the de-esterification reaction continues and, in the absence of an extracellular cholesterol acceptor, the cell accumulates free cholesterol. This process of de-esterification and re-esterification of cholesterol has been termed the cholesteryl ester cycle (7).

In the present study we have utilized morphological and cytochemical techniques to study the cholesteryl ester cycle in cholesteryl ester-loaded macrophages. Using filipin as a specific cytochemical probe for free cholesterol, we have determined the location of the free cholesterol pool using both fluorescence and electron microscopy. In addition, this probe has been used to identify the cellular compartment that accumulates free cholesterol in the presence of progesterone. Using cytochemical methods, we have localized a lipase that has the properties of the cholesteryl esterase involved in the de-esterification reaction. Finally, we have studied the morphologic changes that take place when cholesterol leaves the cell in response to the presence of an exogenous cholesterol acceptor. These studies provide new and important information about the spatial arrangement of the enzymes and substrates involved in the cholesteryl ester cycle.

MATERIALS AND METHODS

Male Swiss Webster mice (25–30 g) were obtained from Simon Laboratories (Gilroy, CA). Dulbecco's modified Eagle medium (DME) and Dulbecco's PBS were purchased from Gibco Laboratories (Grand Island, NY). All other tissue culture supplies were obtained as previously reported (7). Glutaraldehyde was obtained from Electron Microscopy Sciences (Fort Washington, PA). Araldite was obtained from Ladd Research Industries, Inc. (Burlington, VT). Filipin was generously provided by Dr. Joseph Grady at Upjohn Co. (Kalamazoo, MI). Taurocholate was purchased from P.-L. Biochemicals, Inc. (Milwaukee, WI). Tween 80 and quinine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). Triolein was purchased from NuChek Prep (Elysian, MN).

Lipoproteins: Human LDL (d 1.019–1.063 g/ml) and HDL₃ (d 1.125–1.215 g/ml) were isolated from the plasma of individual healthy subjects by ultracentrifugation (9). LDL was acetylated with repeated additions of acetic anhydride as previously described (6). The concentrations of acetyl-LDL and HDL are given in terms of the protein content of the lipoproteins.

Reconstituted Acetyl-LDL: Acetyl-LDL was reconstituted with triglycerides by extracting the endogenous cholesteryl esters with heptane and reconstituting with triolein as previously described (10). The reconstituted lipoprotein was designated r[triolein] acetyl-LDL.

Preparation of Mouse Macrophage Monolayers: Resident peritoneal macrophages were harvested from mice in PBS by a previously described modification (2, 6) of the procedure developed by Edelson and Cohn (11). The peritoneal fluid of 5–30 mice was pooled (6–10 × 10⁶ cells/mouse) and the cells were collected by centrifugation (400 g, min, room temperature) and washed once with 30 ml of DME. The cells were resuspended in DME containing 20% (vol/vol) fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) at a final concentration of 2–4 × 10⁶ cells/ml. Aliquots of this suspension (0.5–1 ml) were dispensed into plastic Petri dishes (35 mm ×

10 mm) and incubated in a humidified CO₂ (5%) incubator at 37°C. After 2 h, each dish was washed three times with 2 ml of DME to remove nonadherent cells, and then used in the experimental protocol described.

Standard Protocol for Loading Macrophages with Cholesteryl Esters: On day 0, each dish of macrophages received 1 ml of DME containing 1 mg/ml of human albumin, 50 µg/ml of acetyl-LDL, penicillin (100 U/ml), and streptomycin (100 µg/ml) and was incubated at 37°C for 48 h with a change of media at 24 h. On day 2, each monolayer was washed twice with 2 ml of DME containing 5% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) and then washed once with 2 ml of DME alone. Each monolayer was then incubated at 37° for 24 h with 1 ml of DME containing 1 mg/ml of human albumin, penicillin (100 U/ml), and streptomycin (100 µg/ml). On day 3, the media was discarded and each dish was treated as described.

Polarized Light Microscopy: Monolayers of macrophages were grown on glass coverslips as described above. After the indicated incubation, the coverslips were removed and mounted on glass slides in a drop of media. To enhance birefringence, the slides were first warmed to 40–45°C for 10 min, then cooled to 10°C for 10 min before examination at 23°C (2). The cells were photographed with phase and polarized optics using a Zeiss Photomicroscope III (Carl Zeiss, Inc. NY.).

Localization of Filipin-binding by Fluorescence Microscopy: Monolayers of macrophages were grown on glass coverslips. After the indicated incubation, the cells were fixed for 30 min with 3% paraformaldehyde containing 50 µg/ml filipin (prepared from a fresh stock solution of 2.5 mg/ml filipin in dimethyl formamide). After washing three times with 2 ml of Dulbecco's PBS, the coverslips were mounted on glass slides with 90% glycerol in 0.1 M Tris-HCl (H 9.4) and viewed with a Zeiss Photomicroscope III using a filter package for ultraviolet light excitation (exciter filter, 365/12 nm; chromatic beam splitter, 395 nm; barrier filter, 420 nm). Photographs were taken either with Kodak Tri-X film (ASA 400) and developed in Microdol-X or Ektachrome 400 and printed commercially.

Electron Microscopy: Monolayers of macrophages were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3) for 20 min at room temperature. The monolayers were postfixed for 1 h at room temperature with 2% OsO₄ in 0.1 M sodium cacodylate (pH 7.3) and 4.5% sucrose. The cells were en bloc stained with 0.5% uranyl acetate in 0.03 M veronal acetate buffer pH 5.4 for 30 min at room temperature. The cells were then dehydrated in a graded series of ethanol. Following dehydration, the cells were transferred to propylene oxide and embedded in araldite.

To localize free cholesterol in cells by electron microscopy, macrophages were fixed overnight at room temperature in a fixative that contained 2% glutaraldehyde, 0.1 M sodium cacodylate (pH 7.3), and 50 µg/ml filipin (prepared from a fresh stock solution of 2.5 mg/ml filipin in dimethyl formamide). The cells were washed and processed as above or embedded directly in EPON after dehydration in ethanol.

Thin sections were prepared with a Sorvall MT 2-B ultramicrotome, stained, and viewed with a JEOL 100 CX electron microscope.

Microscopic Quantitation: Quantitative measurements of lipid droplet size and number were made directly on electron micrographs. 15 cells for each experiment were selected so that the plane of section was through the body of the cell (see Fig. 7 as an example). Micrographs were printed at a constant magnification and the relative area occupied by lipid droplets was measured using an Apple II computer with digitizer tablet (software provided by Dr. H. Hagler, University of Texas Health Science Center at Dallas).

Histochemical Localization of Lipase Activity: Monolayers of macrophages were fixed on ice with 2.5% glutaraldehyde in 0.1 M cacodylate, and then incubated in one of the following solutions for 4 h at 37°C. Solutions: (a) 0.2% (vol/vol) Tween 80, 36 mM CaCl₂, 20 mM Tris maleate buffer, pH 7.2; (b) 0.2% Tween 80, 36 mM CaCl₂, 2.5 mg/ml sodium taurocholate, 20 mM Tris maleate buffer, pH 7.2; (c) 0.2% Tween 80, 2.5 mg/ml taurocholate, 20 mM Tris maleate buffer, pH 7.2; (d) 0.2% Tween 80, 4 mg/ml quinine hydrochloride, 2.5 mg/ml taurocholate, 36 mM CaCl₂, 20 mM Tris maleate buffer, pH 7.2; (e) 0.2% Tween 80, .003% (wt/vol) lead nitrate, 2.5 mg/ml taurocholate, 20 mM Tris maleate pH 7.2. All water used in preparing the solutions was boiled 5 min before use. Tris maleate buffer was prepared as follows: 5.8 g maleic acid and 6 g Tris (hydroxymethyl) aminomethane were dissolved in 100 ml of water. After adding 1 g of activated charcoal, the solution was stirred for 10 min and filtered. To 40 ml of this solution, 20 ml of 1 N NaOH and 40 ml distilled water were added.

After incubating 4 h, the monolayers were washed with a buffer that contained 2 mg/ml EDTA and 0.1 M sodium cacodylate pH 7.2 for 3 min at room temperature, followed by a 3 min wash in 0.1 M cacodylate pH 7.2. The cells were then incubated in 0.15% (wt/vol) aqueous lead nitrate for 10 min at room temperature. The cells were washed with 0.1 M cacodylate pH 7.2,

postfixed with 2% OsO₄ in 0.1 M cacodylate pH 7.3 and processed for electron microscopy.

RESULTS

Sites of Free Cholesterol and Lipase Activity

The purpose of this series of experiments was to characterize by light and electron microscopy cholesteryl ester-loaded cultured macrophages during the cholesteryl ester cycle. For this reason, the conditions for the various experiments were chosen to match those employed in the previous biochemical studies that defined the cholesteryl ester cycle (7).

Morphology of Cholesteryl Ester-loaded Macrophages: Cultured macrophages were induced to accumulate cholesteryl ester lipid droplets by incubating them in the presence of 50 µg/ml of acetyl-LDL for 48 h. Following the initial incubation with acetyl-LDL, cells were cultured an additional 24 h in the absence of acetyl-LDL to permit the internalized acetyl-LDL to be degraded and the liberated free cholesterol to be converted to cholesteryl esters. Cells prepared in this way were examined with polarized light microscopy and 100% of the cells contained numerous birefringent droplets that were clustered in the cell body near the nucleus. When viewed by phase microscopy, each of the birefringent droplets, which displayed a formée cross that is characteristic for the cholesteryl ester droplet (12), appeared as a round, phase dense inclusion.

By electron microscopy, these cells contained numerous lipid droplets, which were not surrounded by a limiting membrane, that occupied 6–10% of the area of the cell body. Since these cells had been incubated in the absence of acetyl-LDL for 24 h, there was little indication of lipoprotein degradation taking place in lysosomes. This contrasts to cells examined during exposure to acetyl-LDL, which have numerous vacuoles present that often appear to contain lipoprotein particles (2).

The lipid droplets had several distinctive morphological features. Each droplet had a relatively electron-dense rim that surrounded an electron lucent core (best seen in Fig. 7A). This appearance suggested that the central portion of the droplet had been extracted during the embedding procedure. Many of the droplets were enmeshed in an organized arrangement of filaments that measured 7.5–10-nm diam (Fig. 1). These filaments, which frequently were arranged in sets of two, appeared to be wrapped around the lipid droplets.

A most remarkable feature of the droplets was that they frequently contained a marginally disposed, lamellar arrangement of membrane (large arrows, Fig. 2, A and B). Typically, these membranes were arranged in concentric whorles of three to four layers and they projected into the matrix of the lipid droplet (Fig. 2, C and D). In some lipid droplets, more membrane layers were seen and these membranes were arranged in stacks rather than whorles. (See arrows, Fig. 4.) Sometimes the membrane of these structures appeared to be in continuity with the endoplasmic reticulum juxtaposed to lipid droplets (Fig. 2, B–D). The endoplasmic reticulum was often studded with ribosomes (small arrows, Fig. 2B). Approximately 20% of the lipid droplets contained these specialized arrangements of membrane. Occasionally lipid droplets were found that had two sets of lamellar membrane.

Sites of Free Cholesterol: During the cholesteryl ester cycle, a pool of free cholesterol is generated that subsequently serves as a substrate for the ACAT enzyme. To understand

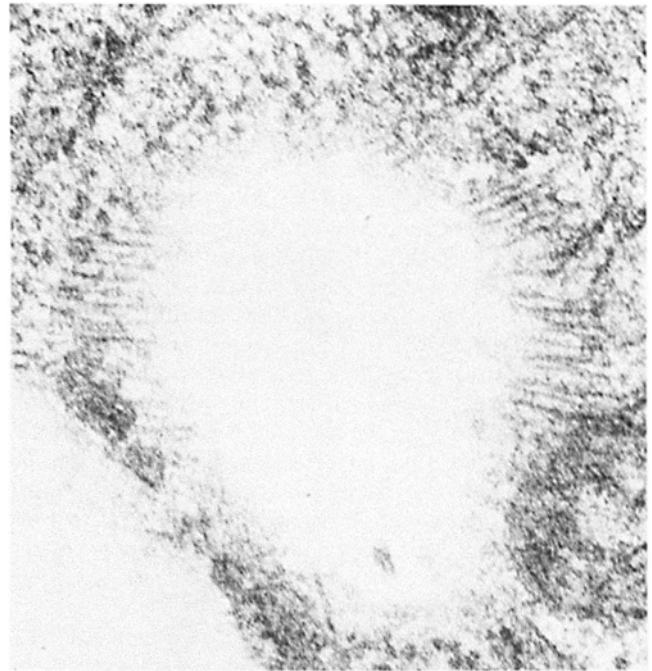


FIGURE 1 Electron micrograph of filaments associated with cholesteryl ester lipid droplets. Peritoneal macrophages (4×10^6) were plated in 35-mm culture dishes. Each dish received DME (supplemented with penicillin and streptomycin) plus 1 mg/ml human albumin and 50 µg/ml acetyl-LDL for 48 h followed by a 24-h incubation in the absence of acetyl-LDL. The cells were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.3 and processed for electron microscopy. $\times 70,000$.

the spatial relationship of this pool of cholesterol to the cholesteryl ester lipid droplet, cells were prepared for light and electron microscopy after fixation in the presence of filipin, a polyene antibiotic that forms complexes with 3 β -hydroxysterols. These complexes can be visualized by both fluorescence microscopy (13) and electron microscopy (14, 15).

To localize filipin-sterol complexes by fluorescence microscopy, we used a fluorescence filter package that is optimal for the spectral characteristics of filipin. Fig. 3 shows a fluorescence photomicrograph of a cholesteryl ester-loaded macrophage after fixation in the presence of filipin. This photograph was taken using a combination of phase contrast and epifluorescence light sources to visualize the relationship of filipin fluorescence to the phase dense lipid droplets. The centrosome region of each cell (C, Fig. 3) was fluorescent due to the presence of intracellular membranes that contained cholesterol. In addition to this site of filipin binding, each cell had small, punctate sites of fluorescence that were scattered throughout the cytoplasm of the cell. As can be seen in Fig. 3, each of these punctate sites of fluorescence was associated with the margin of a phase-dense lipid droplet (arrowheads). These fluorescent sites were not present in macrophages that had not been exposed to acetyl-LDL nor were they associated with triglyceride lipid droplets that formed in cells incubated with [triolein] acetyl-LDL (data not shown). Therefore, the margin of virtually every lipid droplet contained a focal region of filipin binding, an indication that free cholesterol was located at these sites.

The resolution of the fluorescence microscope was not sufficient to positively identify the location of the filipin-binding sites. However, filipin-cholesterol complexes are eas-

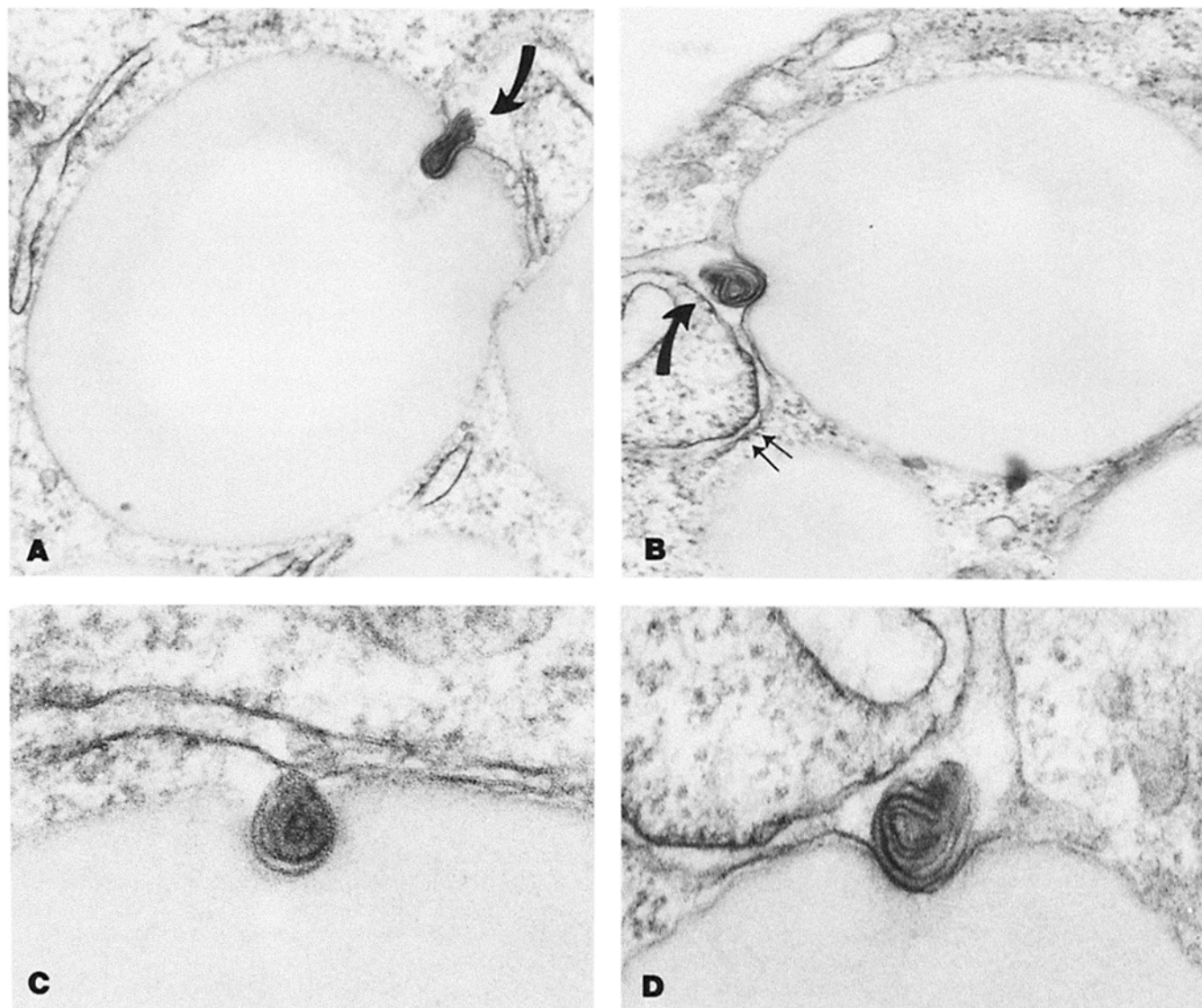


FIGURE 2 Low magnification (A and B) and high magnification (C and D) views of lamellar membrane associated with cholesteryl ester lipid droplets. Peritoneal macrophages were plated and cultured as described in Fig. 1. The cells were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.3 and processed for electron microscopy. Large arrows indicate lamellar membrane. Small arrows indicate ribosomes bound to the endoplasmic reticulum. $\times 57,000$ (A and B); $\times 140,000$ (C); $\times 117,000$ (D).

ily identified with the electron microscope because they have a characteristic globular morphology. Therefore, cholesteryl ester loaded-macrophages were processed for electron microscopy after fixation in the presence of filipin. Fig. 4 shows several lipid droplets in a filipin-treated cell. At the margin of three lipid droplets (*L*, Fig. 4) were dense accumulations of globular material (between brackets, Fig. 4). At higher magnification (Fig. 4 *inset*, between brackets) these regions appeared to be composed of numerous globules that measured ~ 200 Å in diameter and had the same dimensions as filipin-cholesterol complexes present in the plasma membrane (Fig. 4 *inset*, circles). Frequently, lamellar membrane was associated with these sites (arrows, Fig. 4). Based on quantitative measurements of electron micrographs, we estimated that 20% of the droplets contained accumulations of globular material.

The morphologic characteristics of the globules suggested that they were filipin-cholesterol complexes. Moreover, the location of the aggregates agreed with what was seen by

fluorescence microscopy. As further evidence that they corresponded to accumulations of filipin-sterol complexes, we did not see aggregates in cells that were not fixed in the presence of filipin, and they were not associated with triglyceride-containing lipid droplets in macrophages that had been incubated with r[triolein] acetyl-LDL (data not shown).

Cholesteryl ester-loaded macrophages were prepared for histochemical localization of lipase activity according to the method of Nagata (16). This method utilizes Tween 80 as a substrate for the lipase and Ca^{++} as a capturing agent. Once the Ca^{++} -fatty acid complex was formed, the cells were washed with EDTA to remove unbound Ca^{++} and incubated with lead, which replaces the bound Ca^{++} to form an electron-dense precipitate. Lipase can be distinguished from other esterases if the reaction is carried out in the presence of taurocholate, which preferentially activates the lipase (16). The histochemical reaction depends upon using gentle fixation conditions; therefore, all cells were fixed at 4°C .

When cells were processed for general localization of lipase,

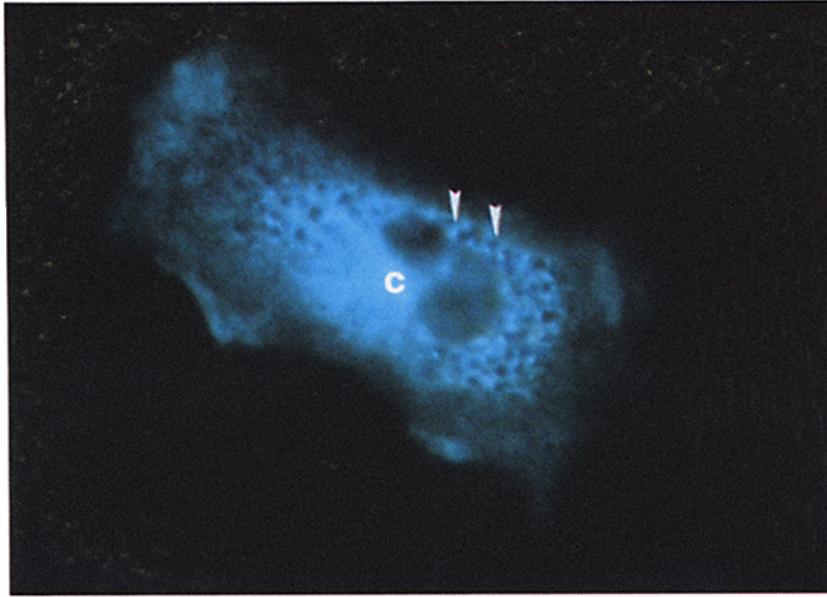


FIGURE 3 Combined phase contrast and fluorescence micrographs of cholesteryl ester lipid droplets after fixation in the presence of filipin. Macrophages (2×10^6 cells) were cultured in $50 \mu\text{g/ml}$ of acetyl-LDL as described in Fig. 1. The cells were further incubated for 24 h in the absence of acetyl-LDL, fixed with 3% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.3 plus $50 \mu\text{g/ml}$ filipin, and photographed. The photograph was made of a cell that was simultaneously illuminated with phase contrast and epifluorescence light. Arrowheads indicate, fluorescence associated with phase dense lipid droplets. C, centrosome, $\times 1,100$.

reaction product was found in the lumen of the endoplasmic reticulum, in lysosomes, and at focal sites on the margin of the lipid droplets (arrows, 5, *A* and *B*). The lipid droplet associated activity appeared in small indentations of the lipid material, and although poorly defined, the reaction product seemed to be associated with structures that resembled the lamellar membrane seen in Fig. 2. This relationship was more apparent in cells that had been incubated with taurocholate to activate the lipase (Fig. 5, *C* and *D*). A much more intense reaction product was found under these conditions and the precipitate was arranged in concentric whorles, as if following the contour of lamellar membrane. Moreover, the size of the focal site was larger than in cells reacted in the absence of taurocholate (Fig. 5, *A* and *B*). If Ca^{++} was omitted from the taurocholate-containing reaction mixture, an expanded lamellar membrane was seen, but reaction product was not detected (Fig. 5, *E* and *F*). Reaction product also was not seen when quinine, an inhibitor of lipase (15), was present in the reaction medium (data not shown.)

Interruption of the Cholesteryl Ester Cycle

Brown et al. (7) showed that in cholesteryl ester-loaded macrophages the cholesteryl ester cycle could be interrupted at two key sites. (*a*) If the macrophages were incubated in the presence of HDL the cholesterol left the cell and there was a corresponding loss of cholesteryl esters. (*b*) When macrophages were exposed to the ACAT inhibitor progesterone, there was a 40% increase in free cholesterol, which was derived from the hydrolysis of the cholesteryl esters. We found that both the loss of cholesteryl esters and the build-up of free cholesterol were accompanied by distinct morphological changes in these cells.

Lipid Droplets in Cholesteryl Ester-loaded Macrophages Exposed to HDL: Macrophages were loaded with cholesteryl esters and then incubated for 48 h in the presence or absence of $250 \mu\text{g/ml}$ HDL and fixed in the presence of filipin. Biochemical measurements have established that in the presence of HDL there is a substantial loss of cholesteryl esters from these cells in 48 h (2, 7). Fig. 6 shows the result of this experiment. *D-F* shows two cells that were incubated in the presence of HDL and *A-C* shows two cells that were incubated

in the absence of HDL. Whereas the number of phase dense droplets in HDL-treated cells did not change (compare Fig. 6*A* with 6*D*), many of these droplets were not birefringent (compare the left with the right cell in Fig. 6*E*), an indication that cholesteryl esters were not present. By quantitative analysis, we determined that only 40% of the HDL-treated cells contained birefringent droplets and that most of the positive cells contained both birefringent and nonbirefringent droplets. In contrast, 100% of the lipid droplets in untreated cells were birefringent (Fig. 6*B*). In both sets of cells, the phase dense inclusions stained with the fat specific dye Oil Red O (data not shown). Finally, regardless of the treatment, fluorescent filipin-sterol complexes (white dots, Fig. 6*C* and right cell, Fig. 6*F*) were associated only with the birefringent cholesteryl ester lipid droplets.

These light microscopic observations suggested that the cholesteryl ester lipid droplets were being replaced by another type of lipid droplet when cells were incubated in the presence of HDL. We also found that the ultrastructure of the lipid droplets was altered by HDL. Fig. 7 shows cells that were exposed to HDL for 0 h (*A*), and 48 h (*B*). Each cell contained numerous inclusions that had the morphological characteristics of lipid droplets. Although there was not any obvious change in the number of lipid droplets, the morphology of these inclusions changed in response to HDL. In untreated cells, the inclusions had the typical morphology of cholesteryl ester droplets (Fig. 7*A*): an electron-dense rim surrounding an electron-lucent core. After 12 h of exposure to HDL, the electron-dense area of the droplet increased with a concomitant decrease in the electron lucent portion (see Table I). By 48 h, most of the lipid droplets were completely electron dense (Fig. 7*B*).

These visual impressions were confirmed by measuring electron micrographs (see Materials and Methods). Whereas there was little change in the percent of the cell area occupied by lipid droplets during HDL exposure (Table I), there was a loss in the portion of the lipid droplet that was electron lucent (39% at 0 h vs. 0% at 48 h). Moreover, the area of the electron-lucent region was proportional to the time of exposure to HDL, which suggests that the change in electron density was correlated with the loss of cholesteryl esters.

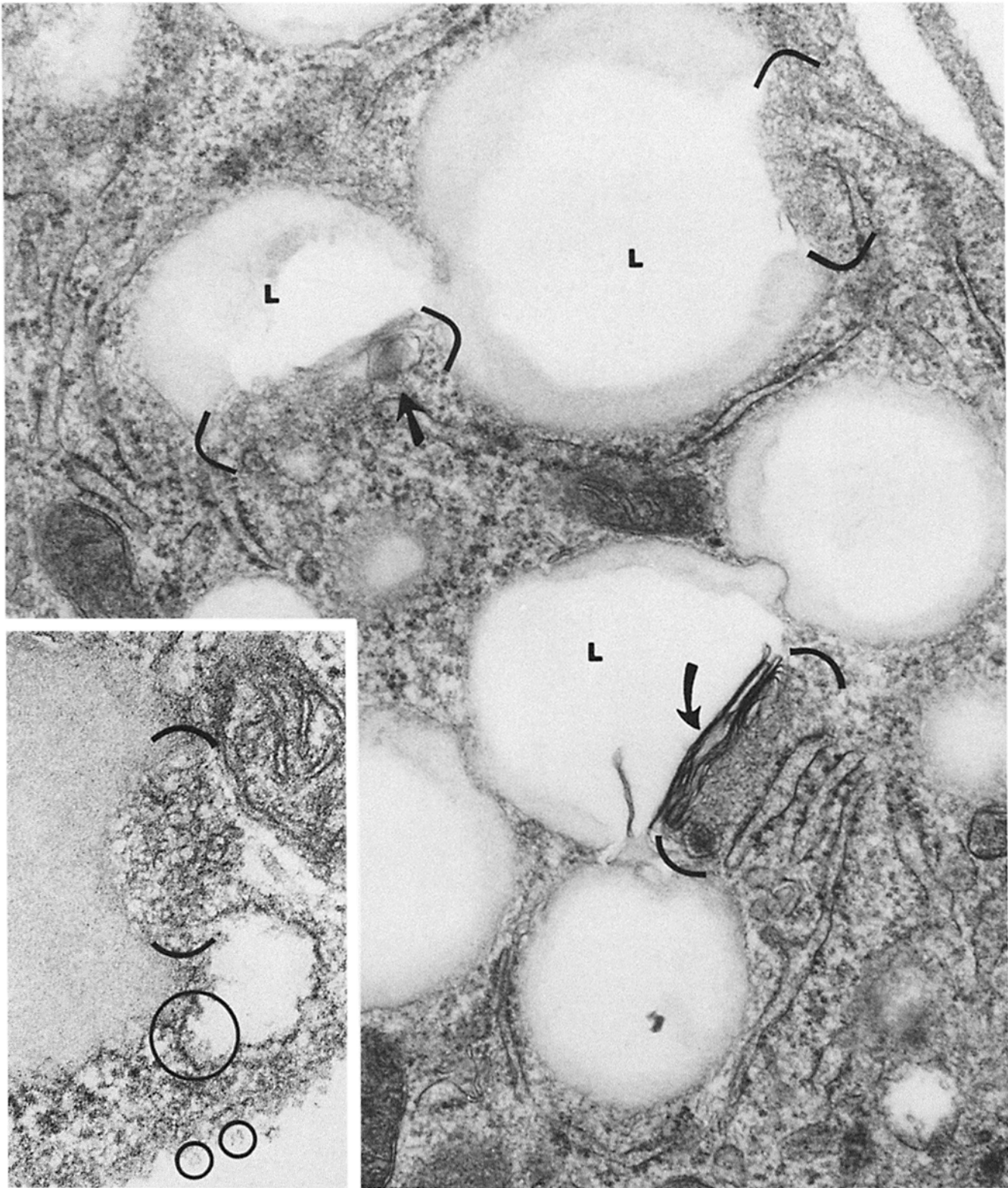
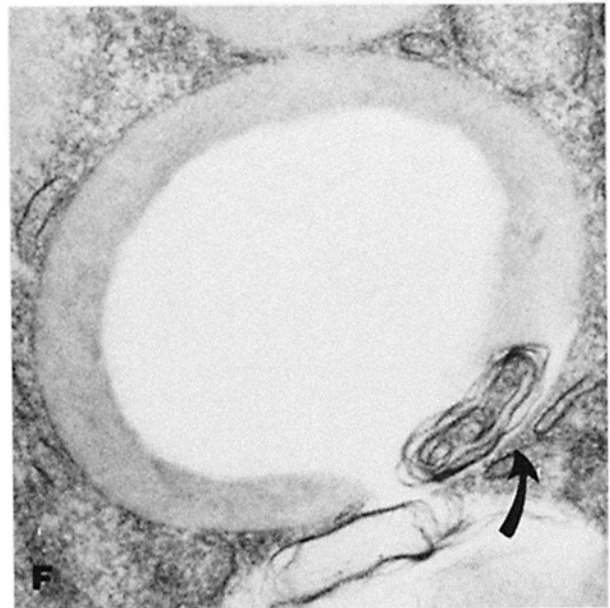
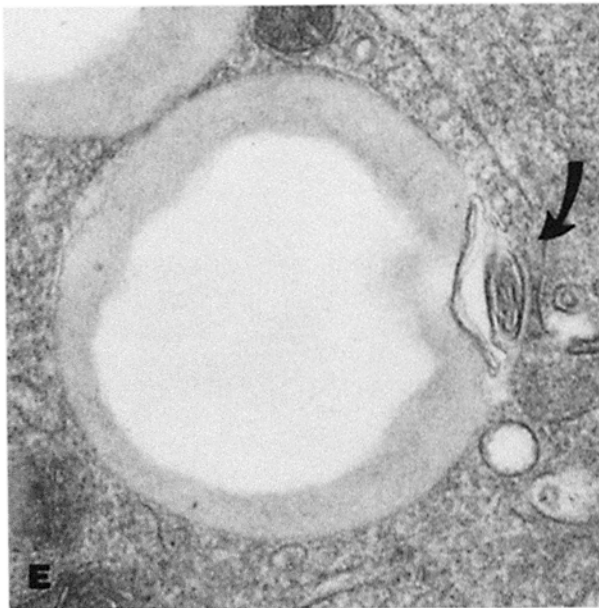
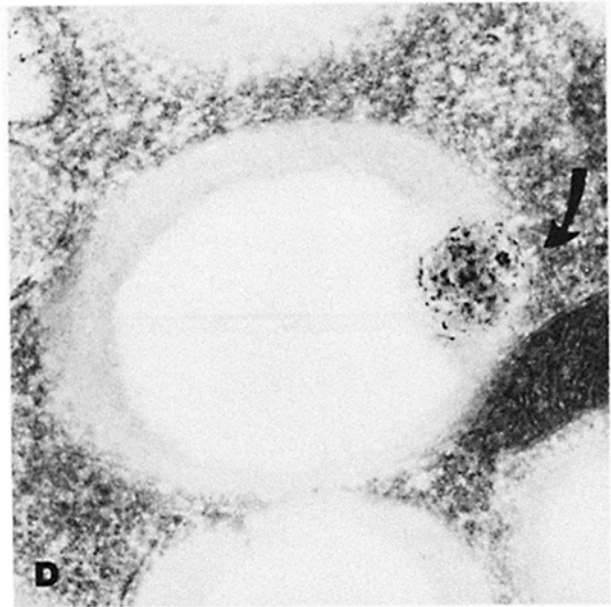
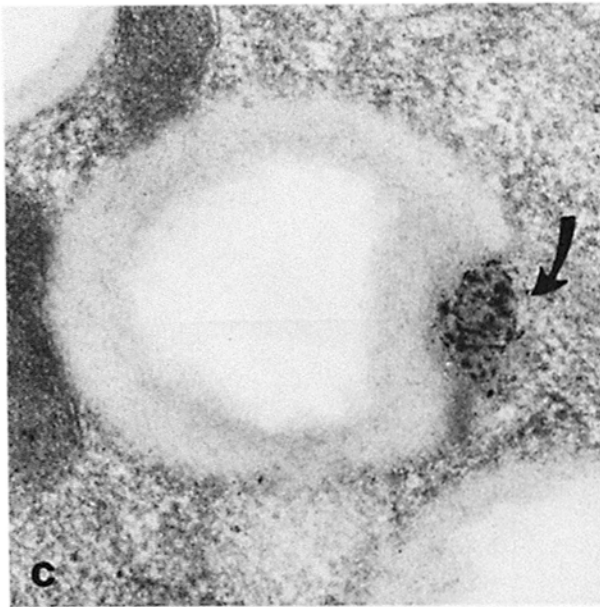
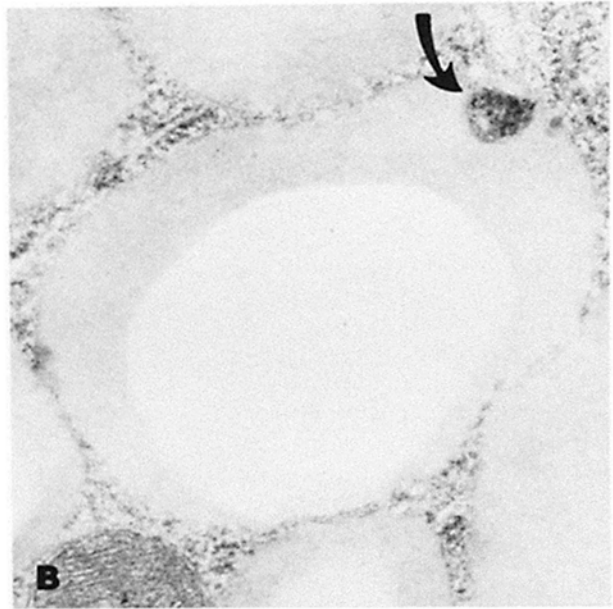
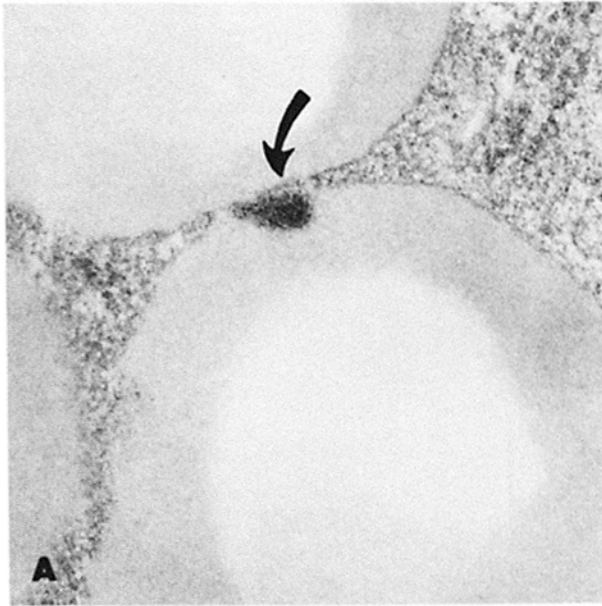


FIGURE 4 Electron microscopic view of cholesterol ester lipid droplets after fixation in the presence of filipin. Peritoneal macrophages (4×10^6 cells) were cultured as described in Fig. 1. At the end of the incubation, the cells were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.3 and incubated overnight at 23°C in 200 $\mu\text{g}/\text{ml}$ filipin in 0.1 M sodium cacodylate buffer pH 7.3. The cells were then postfixated with 2% OsO_4 in 0.1 M cacodylate buffer pH 7.3 plus 4.5% sucrose and en bloc stained with 0.5% uranyl acetate. The cells were embedded as described. Brackets delineate areas of globular deposits. Arrows indicate sites of lamellar membrane. Circles mark filipin-sterol complexes in membrane. L, lipid droplets with globular deposits. $\times 61,000$; $\times 104,000$ (inset).



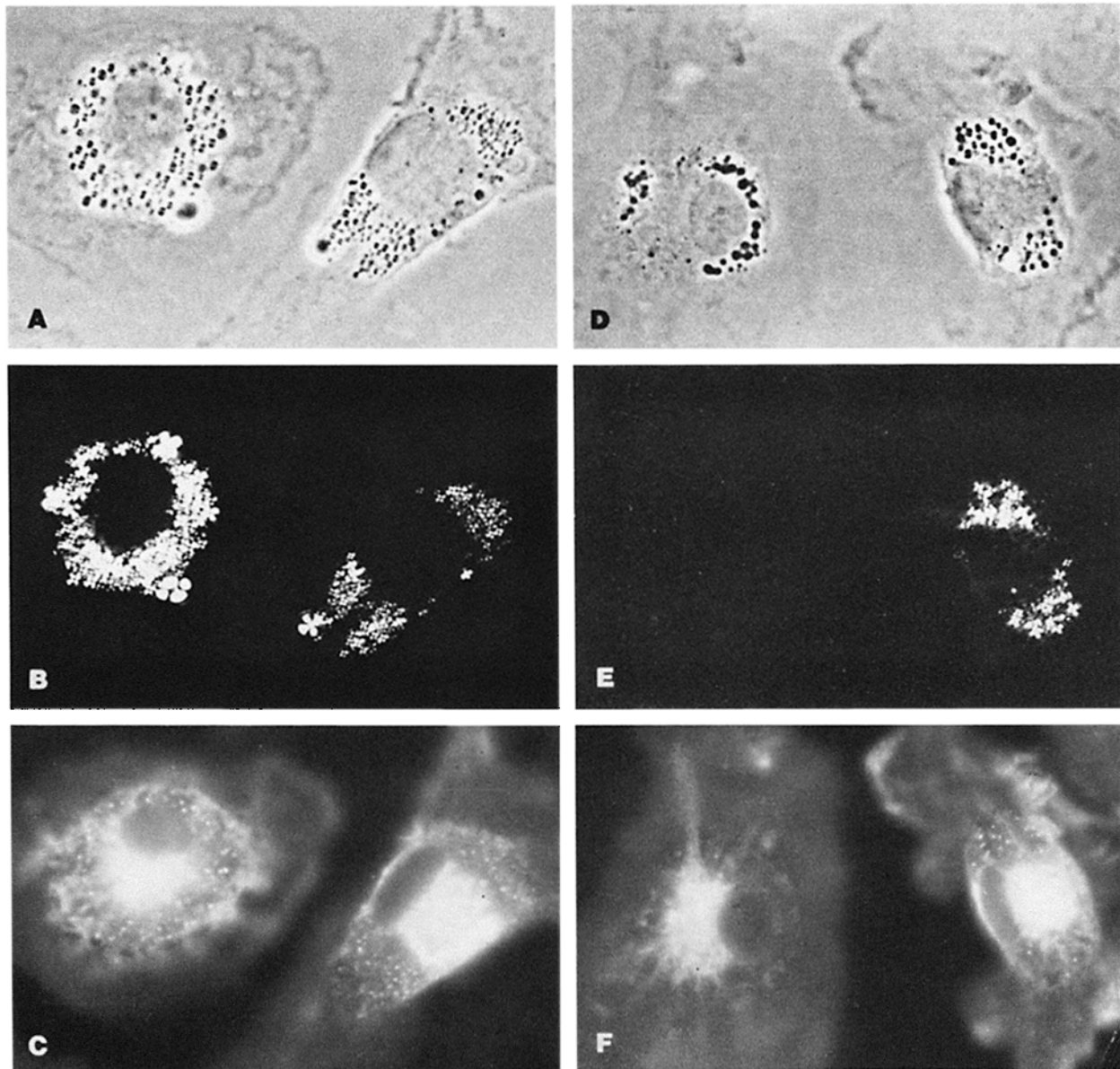


FIGURE 6 Phase contrast (A and D), polarized light (B and E), and fluorescence (C and F) micrographs of the same set of cholesterol ester-loaded macrophages incubated in the presence (D–F) and absence (A–C) of HDL. Peritoneal macrophages (2×10^6 cells) were cultured on glass coverslips for 48 h on DME (supplement with antibiotics) plus 1 mg/ml human albumin and 50 μ g/ml acetyl-LDL followed by a 24-h incubation in the absence of acetyl-LDL. One set of cells (A–C) was further incubated in this media for 48 h. A second set of cells (D–F) was incubated for 48 h in media plus 250 μ g/ml of HDL. All cells were fixed with 3% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.3 that contained 50 μ g/ml filipin. The coverslips were mounted in glycerol and the same cells within each set were photographed using phase contrast (A and D), polarized light (B and E), or fluorescence (C and F) microscopy. $\times 950$.

FIGURE 5 Electron microscopic visualization of lipase activity associated with cholesterol ester lipid droplets. Peritoneal macrophages (4×10^6 cells) were cultured as described in Fig. 1. Following this incubation, the cells were fixed for 0.5 h with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.3 at 4°C. The cells were then incubated in the indicated media for 4 h at 37°C, washed with EDTA buffer, and incubated with 0.15% aqueous lead nitrate for 10 min at 23°C. Cells were postfixed with 1% OsO₄ in 0.1 M sodium cacodylate buffer pH 7.3 and embedded in araldite. Arrows indicate sites of reaction product (A–D) or lamellar membrane (E and F). (A and B) Reaction mixture without taurocholate (medium A, Materials and Methods); (C and D) reaction mixture with taurocholate (medium B, Materials and Methods); (E and F) reaction mixture without calcium and with taurocholate (medium E, Materials and Methods). $\times 58,000$ (A and B); $\times 73,000$ (C); $\times 66,000$ (D); $\times 60,000$ (E and F).

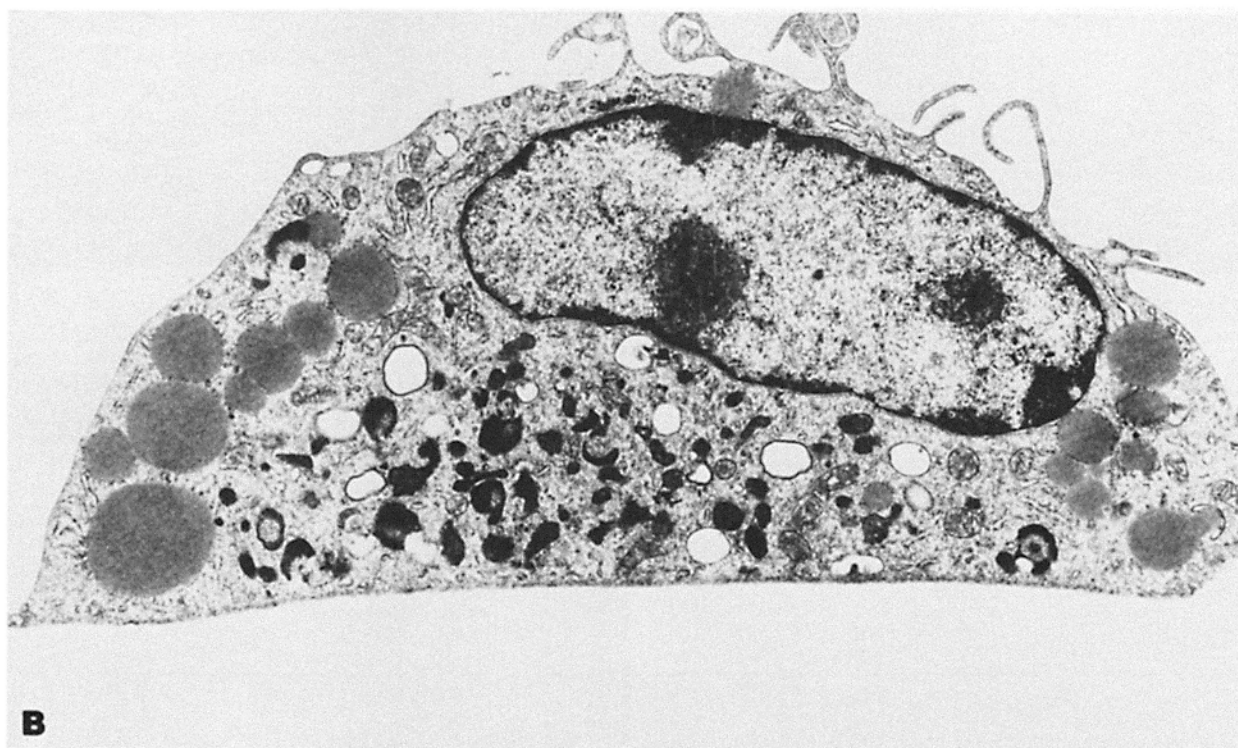
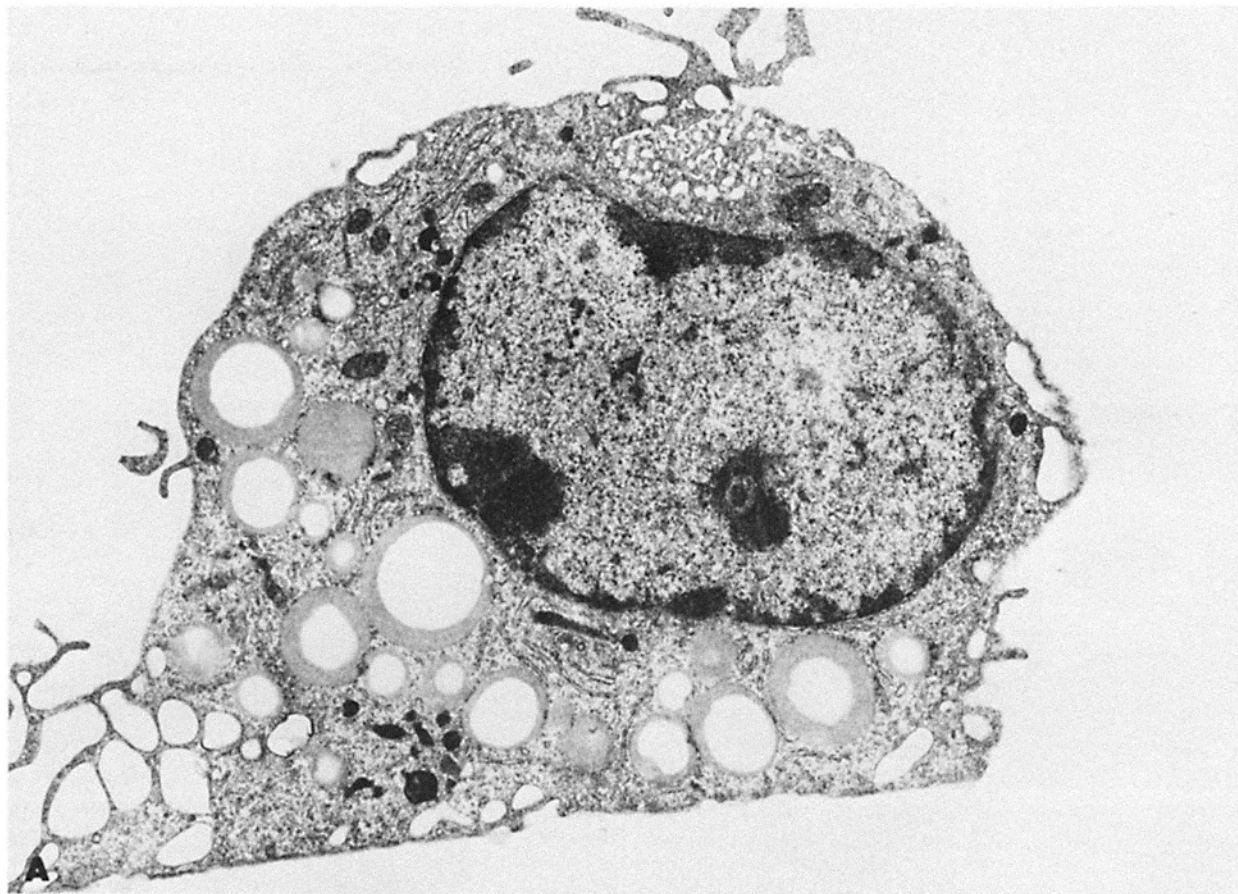


FIGURE 7 Electron microscopic view of cholesteryl ester-loaded macrophages after incubation with HDL for 0 h (A), or 48 h (B). Peritoneal macrophages (4×10^6 cells) were cultured in the presence of $50 \mu\text{g/ml}$ acetyl-LDL 48 h and then in the absence of acetyl-LDL for 24 h as described in Fig. 6. At this time, dishes were further incubated with $250 \mu\text{g/ml}$ HDL for 0 h (A) or 48 h (C) as described in Fig. 6. The cells were then fixed and processed for electron microscopy. $\times 13,000$.

TABLE I
Quantitation of Lipid Droplet Morphology

Time h	Cell area occupied by lipid droplets		Lipid droplet area that was electron lucent	
	+HDL	-HDL	+HDL	-HDL
	%		%	
0	10.5 ± 2.6*		39.3 ± 1.6	
8	7.7 ± 2.0	6.5 ± 1.4	27.0 ± 2.7	37.8 ± 2.8
24	7.6 ± 2.0	8.38 ± 2.47	10.3 ± 6.1	33.1 ± 3.5
48	6.7 ± 2.4	10.45 ± 1.4	0 ± 0	38.7 ± 3.2

Macrophages were loaded with cholesteryl esters as described and then exposed to 250 µg/ml HDL for various times. After the indicated times, the cells were fixed and processed for electron microscopy. Photomicrographs of cells were analyzed for the percent area of the cell occupied by lipid droplets and the percent of the lipid droplet area that was electron lucent. All measurements were made on electron micrographs where the plane of section was through the cell body.

* Mean ± SEM. Values are percentages.

Localization of the Free Cholesterol Pool in Progesterone-treated Macrophages: Cholesteryl ester-containing macrophages were exposed to progesterone for 48 h and processed for fluorescence visualization of filipin binding. Fig. 8A shows a phase contrast photomicrograph of two filipin-treated cells. Numerous phase dense lipid droplets were present in these cells; however, as seen in Fig. 8B, these inclusions were only weakly birefringent. When viewed by fluorescence microscopy (Fig. 8C), the same cells contained an array of fluorescent vacuoles that occupied the perinuclear area of the cell. These vacuoles were not seen in untreated cells (Fig. 6C). At this level of resolution, the cholesterol appeared to have accumulated in a vesicular compartment.

Fig. 9A shows an electron micrograph of a macrophage that had been induced to accumulate cholesteryl esters and then treated with progesterone for 48 h in the absence of acetyl-LDL (same protocol as Fig. 8). Lipid droplets in these cells were almost completely electron dense, an indication that they had lost cholesteryl esters. Most remarkably, within these cells there were numerous membrane-bound vacuoles that contained multiple layers of membrane arranged in concentric whorles (Fig. 9A inset). These structures, which had the appearance of myelin figures, were not found in progesterone-treated macrophages that had not been induced to accumulate cholesteryl esters (data not shown).

If the cholesteryl ester-loaded macrophages were exposed to progesterone for 48 h and fixed in the presence of filipin (Fig. 9B), the membrane-containing vesicles were completely disrupted (arrowheads, 9B). The organizational features of the tripartite membrane were difficult to resolve (9B inset) and the vesicles appeared to be filled with globular deposits that had the appearance of filipin-cholesterol complexes. These observations indicate that the cholesterol was sequestered in these membrane-containing vesicles.

DISCUSSION

Each lipid droplet was a phase-dense inclusion that characteristically appeared as a formée cross when viewed with polarized light. In the electron microscope, many of the nonmembrane bound droplets appeared to be surrounded by filaments that measured 7.5–10-nm diam. A similar basket-like arrangement of filaments has been seen associated with triglyceride lipid droplets in adipocytes (17, 18); however, in macrophages not every droplet was decorated by filaments and they were

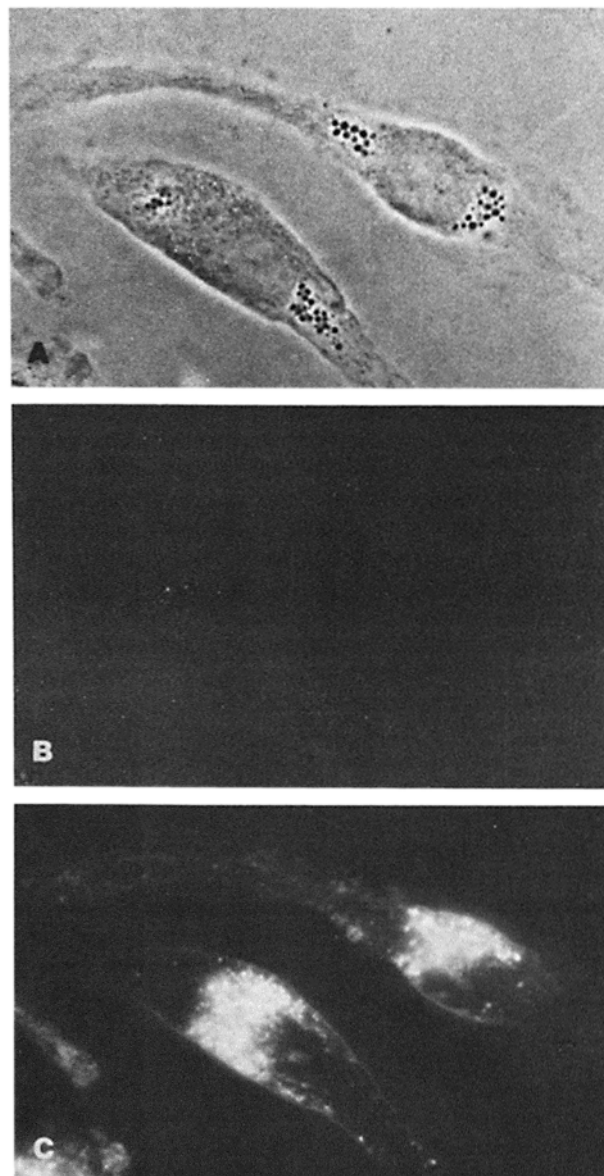
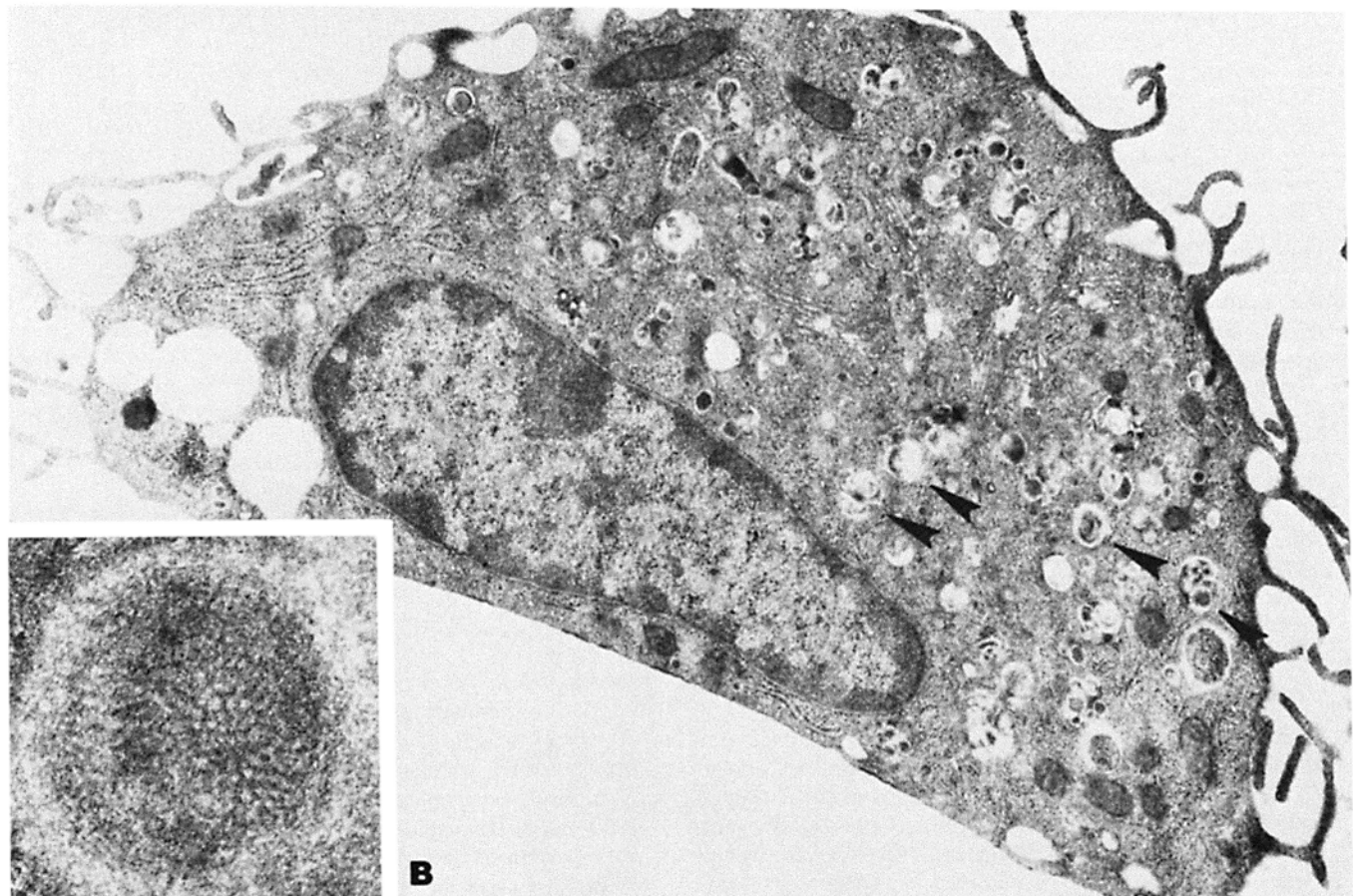
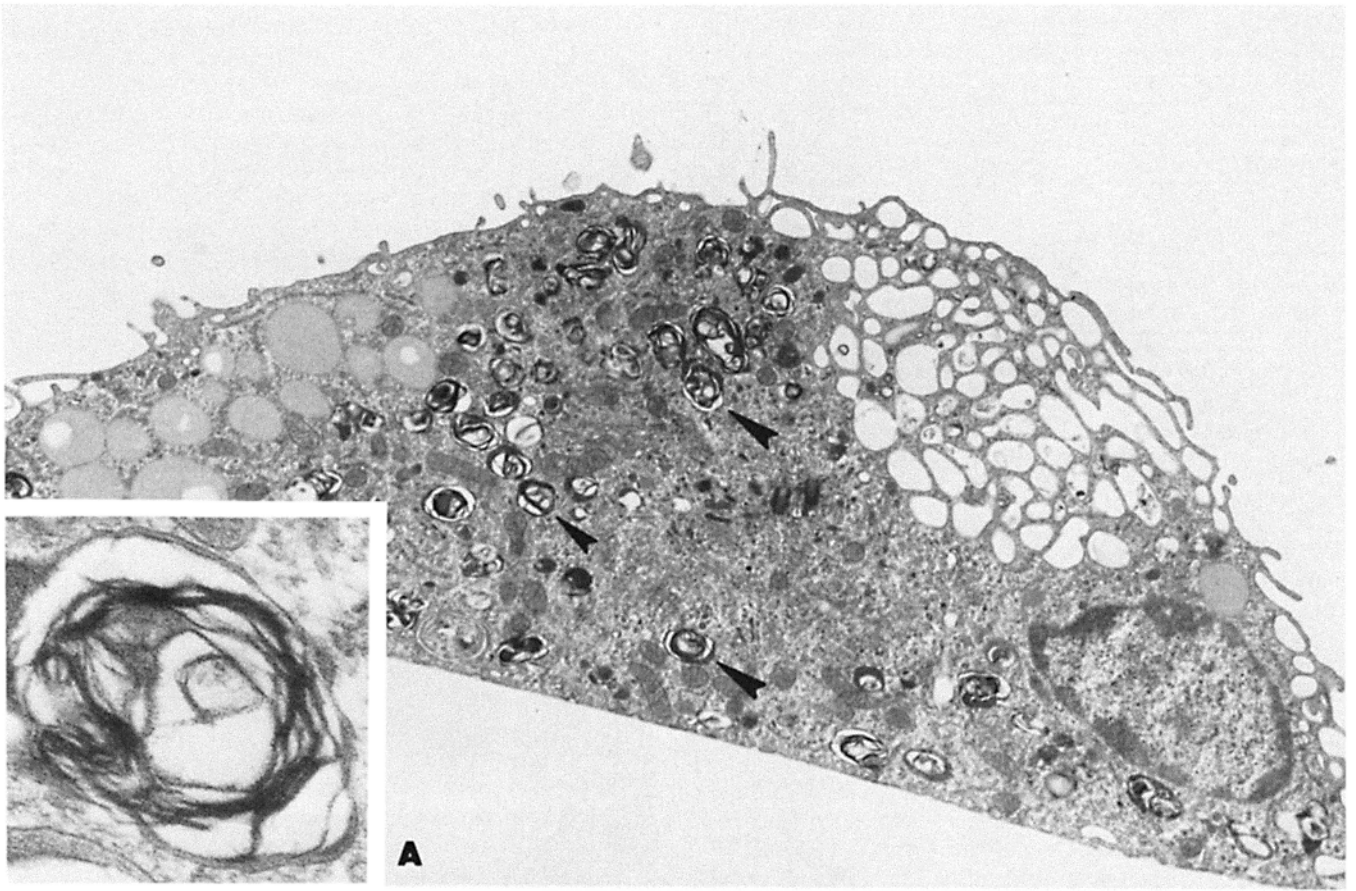


FIGURE 8 Phase contrast, polarized light, and fluorescence micrographs of the same cholesteryl ester-loaded macrophages after incubation with progesterone. Peritoneal macrophages (2×10^6 cells) were cultured on glass coverslips for 48 h in DME (supplemented with antibiotics) plus 1 mg/ml human albumin and 50 µg/ml acetyl-LDL. The cells were subsequently incubated for 24 h in the absence of acetyl-LDL before the addition of 10 µg/ml of progesterone. Cells were incubated in progesterone for 48 h, and fixed with 3% paraformaldehyde in 0.1 M sodium phosphate buffer and 50 µg/ml filipin. The same set of cells was photographed using phase contrast (A), polarized light (B), and fluorescence (C) microscopy. $\times 950$.

not as uniformly organized. Therefore, regardless of the lipid composition, lipid droplets tend to be enmeshed in filaments.

Another structural feature of the cholesteryl ester lipid droplet was the lamellar membrane. At the margin of ~20% of the droplets there was a circularly arranged stack of membrane that protruded into the lipid matrix. Given the thinness of the sections relative to the size of the lipid droplet, this quantitative data suggests that lamellar membrane was associated with every lipid droplet. We also found lamellar membrane associated with triglyceride containing lipid droplets in



macrophages that had been incubated in the presence of r[triolein] acetyl-LDL.

For the cholesteryl ester cycle to work, a pool of free cholesterol must be generated by the action of a nonlysosomal cholesteryl esterase (7). The filipin binding studies indicate that this free cholesterol is primarily associated with the cholesteryl ester droplets. Filipin is known to bind exclusively to 3, β -hydroxysterols (19), and cholesterol is the most likely sterol to be present in these cells. By both fluorescence and electron microscopy, we were able to detect sites of filipin binding at the margin of each lipid droplet. When cholesteryl ester-loaded macrophages were incubated in the presence of HDL, the number of lipid droplets that contained cholesteryl ester diminished and the filipin binding sites disappeared. Therefore, the presence of lipid droplet associated-free cholesterol was correlated with the existence of a functional cholesteryl ester cycle.

Sterol binding agents such as digitonin and filipin have been used to localize free cholesterol in many different cells (14, 15, 20). Most commonly these agents have been used to analyze the distribution of cholesterol in membranes. However, Shio et al. (21) used digitonin to identify sites of free cholesterol in isolated aortic foam cells. Although they did not report that digitonin-cholesterol complexes were associated with the lipid droplets, they did find a considerable number of these complexes in lysosomes. Recently Montesano et al. (22) have shown, by freeze fracture analysis, that filipin-cholesterol complexes are associated with lipid droplets, of unknown lipid composition, in mammary gland epithelial cells. Possibly free cholesterol is associated with all cholesteryl ester lipid droplets, regardless of the cell type.

The presence of a pool of free cholesterol, focally deposited at the margin of each lipid droplet, could be accounted for if the de-esterification limb of the cholesteryl ester cycle took place at this site. The enzyme activity localized to lamellar membrane in the lipid droplets had the characteristics of this lipase. The enzyme utilized Tween 80 as a substrate and appeared to be activated by taurocholate because considerably more reaction product was present when the histochemical incubation contained this trihydroxy bile salt. No reaction product was seen if the capturing agent, Ca^{++} , was omitted from the reaction mixture or when quinine hydrochloride, a lipase inhibitor, was present. Finally, lamellar membrane structures were often associated with the pool of free cholesterol that formed filipin-cholesterol complexes.

We cannot completely rule out the possibility that the reaction product was an artifact of the histochemical procedure. Some investigators have noted that myelin-like membrane whorles have a nonspecific affinity for lead precipitates (E. Holtzman, personal communication). However, the presence of reaction product appeared to be dependent on the conditions established by several different histochemists (16) for localizing lipase activity. Moreover, our results agree with

the observations of both Wigglesworth (23, 24), who found by light microscopic histochemistry that esterase activity was associated with the lipid droplets in insect tissue, and Madreiter and Deimling (25), who used ultrastructural histochemistry to demonstrate esterase activity on the margins of lipid droplets in mouse liver and kidney.

Khoo et al. (26) have reported that one-third of the triglyceride lipase in rat adipocytes is associated with the fat-cake in fractionated adipocytes. More recently Hajjar et al. (27) reported that a substantial portion of the neutral cholesteryl esterase fractionated with the lipid droplets of foam cells. The co-migration on density gradients of lipid and lipase suggests that the two might be tightly associated within the cell. Nishikawa et al. (4) found that most of the cholesteryl esterase in rat adrenal was in the microsome fraction. These biochemical findings could be explained by our observation that lipase is part of a membrane system that is preferentially associated with the lipid droplet.

We anticipated that in response to HDL, the cholesteryl ester-loaded macrophage would lose lipid droplets as the cellular content of cholesterol diminished. However, we found that under the conditions of these experiments, the number of lipid droplets remained constant but changed in lipid composition. Possibly the hydrolysis of cholesteryl esters liberated fatty acids that were used to synthesize triglycerides, which were then incorporated into lipid droplets.

Pharmacological amounts of progesterone will inhibit the ACAT enzyme and disrupt the cholesteryl ester cycle. After 24-h exposure to this steroid, on the average each cell contains almost twice as much free cholesterol as an untreated cholesteryl ester-loaded macrophage (7). The present study indicates that much of this cholesterol was located in the membrane of myelin-like structures that had accumulated in the cell. The appearance of this new membrane system must be related to the inhibition of ACAT in cells that are generating large amounts of free cholesterol. These structures might represent the accumulation sites of specialized membrane that normally functions to shuttle cholesterol from the lipid droplet to the cell surface.

Taken together, the morphologic information about the cholesteryl ester cycle emphasizes the dynamic nature of the lipid droplet. An important next step in these studies is to isolate the lipid droplets and characterize the lipid composition as well as the enzymes that might be associated with this inclusion. Possibly lamellar membrane is a specialized membrane system that houses all of the cellular enzymes responsible for the cholesteryl ester cycle. If this is the case, then various exogenous factors, such as cyclic nucleotides, may be able to modulate either the activity of these enzymes (28, 29) or their spatial arrangement (26).

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FIGURE 9 Cholesteryl ester-loaded macrophages fixed in the presence (B) and absence (A) of filipin after treatment with progesterone. Peritoneal macrophages (4×10^6 cells per dish) were incubated with 10 $\mu\text{g}/\text{ml}$ of progesterone as described in Fig. 8. The cells in (A) were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed with 2% OsO_4 , and embedded in araldite. The cells in (B) were fixed in glutaraldehyde and then incubated overnight in the presence of 200 $\mu\text{g}/\text{ml}$ filipin in 0.1 M cacodylate pH 7.3. These cells were then embedded in EPON as described in Materials and Methods. Arrowheads indicate membrane containing myelin-like structures. $\times 16,600$, inset $\times 69,000$ (A); $\times 14,000$, inset $\times 78,000$ (B).

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