REVERSIBLE ACCUMULATION OF
CHOLESTERYL ESTERS IN MACROPHAGES INCUBATED
WITH ACETYLATED LIPOPROTEINS

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ABSTRACT

Mouse peritoneal macrophages accumulate large amounts of cholesteryl ester
when incubated with human low-density lipoprotein that has been modified by
chemical acetylation (acetyl-LDL). This accumulation is related to a high-affinity
cell surface binding site that mediates the uptake of acetyl-LDL by adsorptive
endocytosis and its delivery to lysosomes. The current studies demonstrate that
the cholesteryl ester accumulation can be considered in terms of a two-compartment
model: (a) the incoming cholesteryl esters of acetyl-LDL are hydrolyzed in
lysosomes, and (b) the resultant free cholesterol is re-esterified in the cytosol where
the newly formed esters are stored as lipid droplets.

The following biochemical and morphologic evidence supports the hydrolysis-
re-esterification mechanism: (a) Incubation of macrophages with acetyl-LDL
markedly increased the rate of cholesteryl ester synthesis from \(^{14}\text{C}\)oleate, and
this was accompanied by an increase in the acyl-CoA:cholesteryl acyltransferase
activity of cell-free extracts. (b) When macrophages were incubated with reconstituted
acetyl-LDL in which the endogenous cholesterol was replaced with \(^{3}\text{H}\)cholesteryl linoleate, the \(^{3}\text{H}\)cholesteryl linoleate was hydrolyzed, and at least
one-half of the resultant \(^{3}\text{H}\)cholesterol was re-esterified to form \(^{3}\text{H}\)cholesteryl
oleate, which accumulated within the cell. The lysosomal enzyme inhibitor chlo-
roquine inhibited the hydrolysis of the \(^{3}\text{H}\)cholesteryl linoleate, thus preventing
the formation of \(^{3}\text{H}\)cholesteryl oleate and leading to the accumulation of unhy-
drolyzed \(^{3}\text{H}\)cholesteryl linoleate within the cells. (c) In the electron microscope,
macrophages incubated with acetyl-LDL had numerous cytoplasmic lipid droplets
that were not surrounded by a limiting membrane. The time course of droplet
accumulation was similar to the time course of cholesteryl ester accumulation as
measured biochemically. (d) When acetyl-LDL was removed from the incubation
medium, biochemical and morphological studies showed that cytoplasmic chole-
steryl esters were rapidly hydrolyzed and that the resultant free cholesterol was
excreted from the cell.
component of the acetyl-LDL was degraded to and degradation, of \textsuperscript{14}C-acetyl-LDL was blocked to recognize the enhanced negative charge of the acetyl-

cell surface site that bound the lipoprotein with native LDL\textsuperscript{(26)}. The uptake of \textsuperscript{14}C-LDL, heptane-extracted acetyl-LDL reconstituted

KEY WORDS macrophages • cholesteryl esters • lipoproteins • lysosomes • cell surface receptors

When the concentration of circulating lipoproteins rises to high levels in the plasma of man or experimental animals, lipid is deposited in macrophages throughout the body \textsuperscript{(16, 20, 27, 34, 39)}. This deposition is particularly striking in patients with familial hypercholesterolemia, a genetic disorder in which the cholesterol-transport protein low density lipoprotein (LDL)\textsuperscript{1} builds up to high levels in plasma and interstitial fluid \textsuperscript{(16, 21)}. As a result, macrophages in tissues throughout the body of these patients accumulate large quantities of cholesteryl ester, most of which is contained in cytoplasmic lipid droplets that are not surrounded by a membrane \textsuperscript{(12, 21)}. Prior attempts to reproduce this cholesteryl ester accumulation by incubating macrophages with large amounts of LDL in vitro have failed. This failure results from the fact that macrophages take up LDL in vitro at relatively low rates that are insufficient to reproduce the massive cholesteryl ester deposition that occurs in vivo \textsuperscript{(26, 30, 39)}.

We recently reported that mouse peritoneal macrophages and other macrophages take up and degrade LDL that has been modified by chemical acetylation (acetyl-LDL) at rates that are 20-fold greater than those for the uptake and degradation of native LDL \textsuperscript{(26)}. The uptake of \textsuperscript{125}I-acetyl-LDL was shown to be mediated by a trypsin-sensitive cell surface site that bound the lipoprotein with high affinity. This binding site appeared to recognize the enhanced negative charge of the acetyl-LDL. The binding, and consequently the uptake and degradation, of \textsuperscript{125}I-acetyl-LDL was blocked by certain negatively charged sulfated polysaccharides, such as dextran sulfate and fucoidan \textsuperscript{(26)}. After its uptake by the cells, the \textsuperscript{125}I-labeled protein component of the acetyl-LDL was degraded to monoo\textsubscript{[125]I]iodotyrosine, which was excreted into the culture medium \textsuperscript{(26)}. In the presence of the lysosomal enzyme inhibitor chloroquine \textsuperscript{(14, 22)}, the uptake of \textsuperscript{125}I-acetyl-LDL continued but the proteolytic degradation was blocked, suggesting that this degradation was occurring in lysosomes \textsuperscript{(26)}. In contrast to the protein component of acetyl-LDL whose hydrolysis products were released into the culture medium, the cholesterol component of acetyl-LDL accumulated progressively within the macrophages, producing a 38-fold increase in the cellular cholesterol content. Approx. 80\% of this accumulated cholesterol was in the form of cholesteryl esters \textsuperscript{(26)}.

Inasmuch as 75\% of the cholesterol of acetyl-LDL is in an esterified form, the question arose as to whether the macrophages accumulated these esters intact owing to a failure of lysosomal hydrolysis, or whether they hydrolyzed the esters and re-esterified the cholesterol for storage within the cytoplasm. This hydrolysis-re-esterification mechanism has been shown to account for much of the cholesteryl ester deposition in cultured human fibroblasts and arterial smooth muscle cells that take up large amounts of cholesteryl ester when incubated with polycationic LDL \textsuperscript{(2, 3, 17)}. Although previous studies have been carried out on the metabolism of cholesteryl esters in macrophages \textsuperscript{(30, 37, 39)}, the question of a hydrolysis-re-esterification mechanism as a major route for processing internalized cholesteryl esters in this cell type has not hitherto been addressed. The identification of a high affinity binding and uptake process for acetyl-LDL in the macrophage has now allowed a systematic biochemical and morphological study of one mechanism underlying the deposition of cholesteryl esters in these cells.

**MATERIALS AND METHODS**

**Materials**

Female NCS mice (25-30 g) were obtained from The Rockefeller University, New York. \textsuperscript{5, 11, 12}C-cholesterol (56 mCi/mmol) and \textsuperscript{1, 2-3}H-cholesterol (43 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, Ill.). \textsuperscript{1,2-3}H-cholesteryl linoleate was synthesized from \textsuperscript{1,2,3}H-cholesterol and linoleyl chloride as previously described \textsuperscript{(31)}. Fucoidin was obtained from ICN Nutritional Biochemicals (Cleveland, Ohio). Bovine serum albumin (cat. No. A4378) and chloroquine diphosphate were purchased from Sigma Chemical Co. (St. Louis, Mo.). Oil Red \textsubscript{O} was obtained from Chroma-Gesellschaft Schmid and Co. Glutaraldehyde was obtained from Electron Microscopy Sciences (Fort Washington, Pa.). Araldite was obtained from Ladd Research Industries, Inc. (Burlington, Vt.). Gill's hematoxylin was purchased from Polysciences, Inc. (Warrington, Pa.). Fetal calf serum, obtained from Flow Laboratories, Inc.
mobility on electrophoresis in agarose gel at pH 8.6 (see compared to native LDL, acetyl-LDL had an enhanced acetic anhydride as previously described (3). As com-

(d > 1.215 g/ml) were isolated by the reconstitution method of Krieger et al. in which the endogenous neutral lipids of acetyl-LDL were removed by heptane extraction and replaced with [3H]cholesterol linoleate (31). The reconsti-
tuted lipoprotein, which is designated r-[3H-cholesterol linoleate]acetyl-LDL, was shown to comigrate with ace-

in agarose gel electrophoresis at pH 8.6. 3°H-

acetyl-LDL was prepared as previously described (3, 9). The concentrations of native LDL, acetyl-LDL, and r-

[3H-cholesterol linoleate]acetyl-LDL are given in terms of the protein content of the lipoprotein.

Macrophage Monolayers

Monolayers were incubated at 37°C in medium A or B supplemented with 0.1 mM [1-14C]oleate bound to albumin (24). After the indicated interval, the cells from each monolayer were washed (18), scraped with a rubber policeman into 1 ml of phosphate-buffered saline, and centrifuged (400 g, 10 min, 4°C). The resulting cell pellet was extracted with chloroform/methanol (2:1), and the choles-
teryl [14C]oleate was isolated by thin-layer chromatography (24). Esterification activity is expressed as the nanomoles of choleseryl [14C]oleate formed per mil-

gram of total cell protein. Correction for procedural losses was made by utilizing [3H]cholesterol oleate as an internal standard added before the chloroform/methanol extraction (24).

Measurement of Cellular Content of Free and Esterified Cholesterol

Macrophage monolayers were harvested by the following procedure: Each monolayer (60-mm dish) was

washed three times at 4°C with 2 ml of buffer containing 0.15 M NaCl, 50 mM Tris-chloride (pH 7.4), and 2 mg/

ml of bovine serum albumin, followed by three addi-
tional washes with 2 ml of phosphate-buffered saline. Each monolayer was then incubated with 1.2 ml of 0.2 N NaOH for 15 min at room temperature. The cell solution was transferred with a Pasteur pipette to a glass tube containing 40 μl of 6 N HCl, and an aliquot was removed for protein determination. The steroids were extracted with chloroform/methanol (2:1), the free and esterified cholesterol fractions were separated on silicic acid/Celite columns (8), and the cholesterol content of each fraction was measured by gas-liquid chromatogra-

phy (after alkaline hydrolysis of the choleseryl ester fraction) as previously described (8). Correction for procedural losses was made using [3H]cholesterol, choles-
teryl [14C]oleate, and stigmasteryl as internal standards (8).

The relative composition of the fatty acyl components of choleseryl esters was determined by gas-liquid chro-

nyl acetate (3, 26). [3H]cholesterol linoleate-labeled ace-

tyld-LDL was prepared by the reconstitution method of Krieger et al. in which the endogenous neutral lipids of acetyl-LDL were removed by heptane extraction and replaced with [3H]cholesterol linoleate (31). The reconsti-
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r-

[3H-cholesterol linoleate]acetyl-LDL are given in terms of the protein content of the lipoprotein.

Incorporation of [1-14C]oleate into Cholesteryl Esters by Macrophage Monolayers

Peritoneal cells were harvested from unstimulated mice in phosphate-buffered saline as described by Edel-
son and Cohn (15). The fluid from 20–40 mice (6–10 × 106 cells/mouse) was pooled, and the cells were collected by

centrifugation (400 g, 10 min, room temperature) and washed once with 30 ml of DMEM. The cells were then

r-esuspended in DMEM containing 20% (vol/vol) fetal calf serum and adjusted to a final concentration of 2.5–

× 106 cells/ml. Aliquots of this cell suspension (0.5–2 ml) were dispensed into 35- or 60-mm plastic Petri dishes as indicated in the legends. The dishes were incubated in a humidified CO2 (5%) incubator at 37°C. After 2 h, each dish was washed three times with 2 ml of DMEM without serum to remove nonadherent cells. Each mono-
layer of macrophages then received 1 or 2 ml of DMEM containing penicillin (100 U/ml), streptomycin (100 μg/

ml), and either 10% fetal calf serum (medium A), 20% fetal calf lipoprotein-deficient serum (medium B), or

10% human lipoprotein-deficient serum (medium C) as indicated in the legends. Each dish of adherent macro-

phages contained ~50% of the total number of cells originally plated, and each 10th adherent cells contained ~60–75 μg of protein. Throughout these experiments, we observed that the rate of uptake and degradation of acetyl-LDL varied by as much as threefold from experiment to experiment (26). This variation was minimized by incubating the macrophages for 24 h in medium A before the addition of acetyl-LDL.

Lipoproteins

Human LDL (d = 1.019–1.063 g/ml) and human lipoprotein-deficient serum (d > 1.215) were isolated by ultracentrifugation (8). Fetal calf lipoprotein-deficient serum (d = 1.215 g/ml) was prepared by differential ultracentrifugation as previously described (10). LDL was acetylated with repeated additions of acetic anhydride as previously described (3). As com-
pared to native LDL, acetyl-LDL had an enhanced mobility on electrophoresis in agarose gel at pH 8.6 (see Fig. 1, reference 3) and a normal particle size as judged by electron microscopy after negative staining with ura-
matography of the fatty acid methyl esters as previously described (method 2, reference 25).

Hydrolysis of r-[3H]-Cholesteryl Linoleate-Acetyl-LDL and Re-esterification of the Liberated [3H]-Cholesterol by Macrophage Monolayers

Monolayers were incubated at 37°C with the indicated concentrations of r-[3H]-cholesteryl linoleate-acetyl-LDL and [3H]oleate-albumin in medium C. After the indicated interval, the medium was removed and saved for analysis, and each monolayer was washed (18) and harvested as described in the preceding section. The medium (1 ml/dish) was extracted with chloroform/methanol according to the method of Bligh and Dyer (5) after the addition of an internal standard containing [14C]cholesterol (30 μg, 1,500 cpm) and unlabelled cholesteryl oleate (10 μg). The cells were extracted with chloroform/methanol (2:1) after addition of the internal standard of [14C]cholesterol (31). The free and esterified [3H]cholesterol fractions were separated by thin-layer chromatography on silica gel sheets using benzene/ethyl acetate (2:1) for the cells or heptane/diethyl ether/acetic acid (90:30:1) for the medium (31). In some experiments, [3H]cholesteryl linoleate and [3H]cholesteryl oleate were resolved from each other by chromatography on silica gel sheets impregnated with silver nitrate as previously described (method 1, reference 25). Each isolated fraction was cut from the chromatogram and its content of [3H] and [14C] radioactivity was determined by double-label scintillation counting in Aquasol (New England Nuclear, Pilot Chemicals Div., Watertown, Mass.).

Oil Red O Staining

Monolayers of macrophages were prepared on glass coverslips contained in Petri dishes. After the indicated incubation, the coverslips were removed, fixed with 6% paraformaldehyde in 0.1 M sodium phosphate (pH 7.3) for 30 min at room temperature, and then fixed for 60 min in 2.5% potassium dichromate and 1% osmium tetroxide in water. The coverslips were then stained with Oil Red O (2) and counterstained with Gill's double-strength hematoxylin for 10 min.

Polarized Light Microscopy

Monolayers of macrophages were prepared on glass coverslips in Petri dishes. After the indicated incubation, the coverslips were removed and mounted on glass slides. To enhance birefringence, the slides were first warmed to 40°-45°C for 10 min, then cooled to 10°C for 10 min and examined immediately thereafter (17). The cells were photographed under either polarizing optics or differential interference contrast optics with a Zeiss Photomicroscope III (Carl Zeiss, Inc., New York).

Electron Microscopy

Monolayers of macrophages were fixed in situ with 2% glutaraldehyde in 0.1 M sodium phosphate (pH 7.3) for 30 min at room temperature, scraped from the dish, and centrifuged (5 min, 4°C, 12,000 rpm) in a microfuge tube (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The cell pellets were postfixed with 2% osmium tetroxide and 4.5% sucrose in 0.1 M sodium phosphate (pH 7.3) for 60 min at room temperature, dehydrated, and embedded in Araldite. Material for electron microscopy was sectioned on a Sorvall MT 2-B ultramicrotome (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.), stained with uranyl acetate and lead citrate, and viewed with a JEOL 100 CX electron microscope.

Assay of Acyl-CoA:Cholesterol Acyltransferase (ACAT) Activity of Cell-Free Extracts

Enzyme activity was assayed by a modification (7) of the method of Goodman et al. (28). Cell-free extracts were prepared by suspending pooled pellets of macrophages from several dishes (containing ~0.3 mg of cell protein) in 400 μl of buffer containing 50 mM potassium phosphate (pH 7.4) and 2 mM dithiothreitol, after which the suspension was homogenized at 4°C with 20 strokes of a Dounce homogenizer (Kontes Co., Vineland, N. J.). Aliquots of the cell extracts (25-70 μg of protein) were incubated in 200 μl of solution containing 50 mM potassium phosphate (pH 7.4), 2 mM dithiothreitol, 1.2 mg of bovine serum albumin, 2 mM ATP, 2 mM MgCl2, 4 mM coenzyme A, 100 μM [1-14C]oleate-albumin (10,000 cpm/nmol), and 1 μl of acetone containing either no cholesterol or 4 μg of cholesterol as indicated. After incubation at 37°C for 1 h, the reactions were terminated by addition of 4 ml of chloroform/methanol (2:1), and the cholesteryl [14C]oleate was isolated by thin-layer chromatography and quantified as described previously (24). Enzyme activity is expressed as the nanomoles of cholesteryl [14C]oleate formed per hour per milligram of total extract protein.

Protein Determination

The protein content of lipoproteins and cells was measured by the method of Lowry et al. (32) with bovine serum albumin as a standard.

RESULTS

Time Course of Cholesteryl Ester Accumulation in Macrophages

When mouse peritoneal macrophages were incubated for 4 d in the presence of fetal calf serum, the content of free cholesterol remained nearly constant (Fig. 1A). The content of esterified cho-
cholesterol was initially quite low (<1 µg sterol/mg protein) and remained low throughout the incubation (Fig. 1B). The addition of native LDL at 25 µg protein/ml did not affect the content of free or esterified cholesterol. The addition of the same concentration of acetyl-LDL caused a twofold increase in the concentration of cellular free cholesterol that was maximal on day 1 and remained constant thereafter (Fig. 1A). In the presence of acetyl-LDL, the content of esterified cholesterol increased at a nearly linear rate over the 4-d period, reaching an extremely high level of 300 µg sterol/mg of cell protein on day 4 (Fig. 1B). The increases in both free and esterified cholesterol in the presence of acetyl-LDL were blocked when 50 µg/ml of fucoidin was included in the incubation medium. We have previously shown that fucoidin blocks the uptake and degradation of acetyl-LDL that is mediated by the high affinity binding site on macrophages (26).

Influence of Acetyl-LDL on Synthesis of Cholesteryl Esters

Fig. 2 shows an experiment in which macrophages were incubated with increasing concentrations of either acetyl-LDL or native LDL in the presence of [14C]oleate. Acetyl-LDL caused a marked enhancement in the rate of [14C]oleate incorporation into cholesteryl [14C]oleate. The effective concentration range is similar to the concentration range over which acetyl-LDL enters macrophages via the high affinity binding site (26). Native LDL, which has been shown not to enter cells via the acetyl-LDL binding site (26), did not stimulate cholesteryl ester synthesis.

In cell-free homogenates prepared from macrophages that had been incubated for 5 h with 50 µg/ml of acetyl-LDL, the sp act of ACAT (7, 11, 28) was 16- to 20-fold higher than in cells not incubated with acetyl-LDL (Table I). The addition of exogenous cholesterol to the in vitro enzyme assay system did not increase enzyme activity in the homogenates from the cells that had not been incubated with acetyl-LDL. When the crude homogenate was subjected to ultracentrifugation at 100,000 g, 88% of the recovered ACAT activity was found in the membrane pellet. Omission of ATP-Mg⁺⁺ or coenzyme A from the incubation medium abolished cholesteryl [14C]oleate synthesis.

Hydrolysis of Cholesteryl Esters of Acetyl-LDL

To study the hydrolysis of the cholesteryl esters in acetyl-LDL, we prepared r-[3H-cholesteryl linoleate]acetyl-LDL in which the free and esterified
TABLE I

| Addition to medium | ACAT activity |
|--------------------|--------------|
| - Cholesterol in assay | + Cholesterol in assay |
| None | 127 | 91 |
| Acetyl-LDL | 2,072 | 1,892 |

10 x 10^6 peritoneal cells were dispensed into replicate 60-mm dishes. On day 0, each dish received 2 ml of medium A. On day 1, the medium was replaced with 2 ml of fresh medium A in the absence or presence of 50 µg/ml of acetyl-LDL as indicated. After incubation for 5 h at 37°C, each monolayer was washed twice with 3 ml of phosphate-buffered saline and the cells were harvested by scraping with a rubber policeman. The combined cell suspensions from four dishes were pooled together and cell-free extracts were prepared and assayed for ACAT activity in the absence or presence of exogenous cholesterol as described in Materials and Methods. Each value is the average of duplicate assays.

Uptake of Acetyl-LDL Labeled with I25I and [3H]Cholesteryl Linoleate

To determine whether the protein and cholesteryl ester components of acetyl-LDL were being taken up and hydrolyzed as a unit particle, we compared the rates of cellular uptake and hydrolysis of I25I-acetyl-LDL with those of r-[3H]-cholesteryl linoleate-acetyl-LDL in the experiment shown in Fig. 3. The ratio of cholesteryl linoleate to LDL-protein in the r-[3H]-cholesteryl linoleate-acetyl-LDL used in this experiment was 2.1 nmol/pg. At 7.5 h, the total uptake of [3H]cholesteryl ester was 138 nmol ([3H]cholesteryl ester in cells [55 nmol] + [3H]cholesterol in cells [30 nmol] + [3H]cholesterol in medium [53 nmol]). If the r-[3H]-cholesteryl linoleate-acetyl-LDL had been taken up and degraded as a unit particle, at 7.5 h a total of 65.6 µg of LDL-protein should have been taken up and degraded per mg cell protein. A total of 65.7 µg of I25I-acetyl-LDL-protein was actually observed to be taken up and degraded (data not shown).

Evidence for a Hydrolysis-Re-esterification Mechanism

Macrophages were incubated with r-[3H]-cholesteryl linoleate-acetyl-LDL in the presence of unlabeled oleate (Fig. 4). At various intervals, the cells were harvested, the cholesteryl linoleate was separated from cholesteryl oleate by thin-layer argentation chromatography, and the content of [3H]cholesterol in each of these ester fractions was determined. The data in Fig. 4A show that at early
FIGURE 4 Hydrolysis of r-[3H]-cholesteryl linoleate-acetyl LDL and re-esterification of the liberated [3H]cholesterol to form [3H]cholesteryl oleate (A) and inhibition of these processes by chloroquine (B) in macrophages. 2 x 10^6 peritoneal cells were dispensed into replicate 35-mm dishes. On day 0, each dish received 1 ml of medium A. On day 1, each monolayer was washed once with 2 ml of DMEM, after which was added 1 ml of medium C containing 10 μg/ml of r-[3H]-cholesteryl linoleate-acetyl-LDL (7,900 cpm/nmol of [3H]cholesteryl linoleate), 0.2 mM unlabeled oleate-albumin, and either no chloroquine or 20 μM chloroquine as indicated. After incubation at 37°C for the indicated interval, the cellular content of [3H]cholesterol (○), [3H]cholesteryl linoleate (▲), and [3H]cholesteryl oleate (△) was determined as described in Materials and Methods. Each value is the average of duplicate incubations.

Time points (<2 h) most of the accumulated [3H]-cholesteryl esters were composed of [3H]-cholesteryl linoleate. However, the level of intact [3H]-cholesteryl linoleate reached a steady-state plateau within 2 h. The subsequent accumulation of [3H]-cholesteryl esters was caused by an accumulation of [3H]-cholesteryl oleate that was generated via the hydrolysis and re-esterification of the exogenous [3H]-cholesteryl linoleate (Fig. 4A). Fig. 4B shows that in the presence of chloroquine this hydrolysis was inhibited and large amounts of intact [3H]-cholesteryl linoleate accumulated progressively during the 6-h experiment. Only small amounts of free [3H]cholesterol were generated in the presence of chloroquine, and there was no formation of [3H]-cholesteryl oleate.

To document the hydrolysis–re-esterification process in another way, we incubated macrophages with the r-[3H]-cholesteryl linoleate-acetyl-LDL in the presence of exogenous [14C]oleate. Fig. 4A shows that the cells initially accumulated free [3H]cholesterol, some of which was excreted into the medium after a lag. Fig. 5B shows that the content of [3H]cholesteryl esters increased progressively over 6 h. All of the rise in [3H]cholesteryl esters after the initial 2-h period could be accounted for by the measured accumulation of cholesteryl [14C]oleate (open circles, Fig. 5B). The amount of intact [3H]cholesteryl linoleate was estimated by subtracting the amount of cholesteryl [14C]oleate from the total amount of [3H]cholesteryl esters within the cells (X—X—in Fig. 5B). These data suggested that the cellular content of intact [3H]cholesteryl linoleate was constant after 2 h, thus supporting the measurements shown in Fig. 4. Taken together, the data in Figs. 4 and 5 indicate that the r-[3H]-cholesteryl linoleate-acetyl-LDL was rapidly taken up and hydrolyzed by the macrophages and that nearly all of the cholesteryl esters that accumulated within the cells after 2 h represented re-esterified cholesterol.

Light Microscope Studies of Cholesteryl Ester Accumulation in Macrophages

The accumulation of cholesteryl ester in macrophages incubated in the presence of acetyl-LDL.
produced striking alterations in the morphology of these cells. Fig. 6A shows that cells exposed to acetyl-LDL for 3 d had numerous droplets within the cytoplasm that stained with the lipid stain Oil Red O. On the other hand, cells exposed to the same concentration of native LDL were virtually devoid of Oil Red O positive droplets (Fig. 6B).

To establish that the fat-staining droplets represented sites of cholesteryl ester accumulation, we observed similarly treated sets of cells by polarized light microscopy (Fig. 7). At low power, the cells were seen to contain numerous birefringent droplets arranged primarily around the nucleus (N in Fig. 7A). At high power, these birefringent droplets showed a typical formée cross pattern characteristic of cholesteryl ester droplets (Fig. 7A, inset) (17, 34). In cells exposed to native LDL, no such birefringent droplets were present (Fig. 7B). The number of birefringent droplets was reduced when cells were incubated with acetyl-LDL in the presence of fucoidin (Fig. 7C). Fig. 7D shows a differential interference contrast picture of a cell treated with acetyl-LDL. The birefringent droplets, which appear white in Fig. 7D, were located primarily within the body of the cell, particularly in the region around the nucleus (N), whereas the outer margins of the cell (arrows) were free of these inclusions.

To study the time course of accumulation of birefringent droplets, we exposed macrophages to acetyl-LDL for various times and then processed them for polarized light microscopy. At zero time, no birefringent droplets were seen (Fig. 8A inset).

As early as 2 h after exposure to acetyl-LDL, a few scattered birefringent droplets were seen within the cytoplasm (not shown). At 4 h (Fig. 8C) and at 48 h (Fig. 8E inset), there was a progressive increase in both the number of birefringent droplets within each cell and in the percentage of cells that contained droplets. The time course of accumulation of birefringent droplets paralleled the time course of accumulation of esterified cholesterol within the cells (Fig. 1B).

**Electron Microscope Studies of Cholesteryl Ester Accumulation**

Fig. 9A–C shows several electron micrographs of macrophages exposed to acetyl-LDL for 6 d. These cells characteristically exhibited numerous cytoplasmic lipid droplets (L in Fig. 9). Although an occasional membrane-bounded lipid droplet was seen, most of the lipid droplets were not surrounded by a typical tripartite membrane. Many of the droplets appeared empty, the lipid having been extracted during the embedding procedure (Fig. 9C). In addition to the lipid droplets, the cells incubated with acetyl-LDL also had numerous membrane-bounded vacuoles (MBV) that contained small vesicles, electron-dense material, and myelin figures (V in Fig. 9). The morphology of these vacuoles is similar to that of vacuoles observed in foam cells isolated from the atheromatous aorta of cholesterol-fed rabbits (33). Such MBV in foam cells have been shown by Shio et al. (33) to represent secondary lysosomes on the basis
FIGURE 7  Polarized and differential interference contrast light microscopy of macrophages incubated with acetyl-LDL. 2.5 × 10⁶ peritoneal cells were dispensed onto a glass coverslip contained within a 35-mm dish. On day 0, each dish received 1 ml of medium A containing one of the following additions: 25 μg/ml of acetyl-LDL (A and D), 25 μg/ml of native LDL (B), or 25 μg/ml acetyl-LDL plus 100 μg/ml of fucoidin (C). The medium was replaced with fresh medium of identical composition on days 1 and 3. On day 4, the coverslips were removed and examined by either polarized light microscopy (A, B, and C) or differential interference contrast light microscopy (D) as described in Materials and Methods. Fig. 7A inset shows at high magnification a birefringent droplet that illustrates a typical formée cross. N, nucleus. (A) × 1,000; inset × 4,350. (B) × 850. (C) × 600. (D) × 1,650.
of cytochemical studies using acid phosphatase. By analogy, it seems likely that the MBV in Fig. 9 correspond to secondary lysosomes that are involved in digesting acetyl-LDL. Only an occasional MBV was seen in cells incubated with native LDL under the same conditions (not shown).

To trace the sequential change in morphology of macrophages exposed to acetyl-LDL, we treated cells with acetyl-LDL for periods of 4-48 h, after which we examined them by electron microscopy. Fig. 8A shows a low-power micrograph of a macrophage at zero time. These cells have a flattened basal surface, which was in contact with the substrata of the tissue culture dish, and a highly convoluted apical surface. Only a few MBV (V in Fig. 8A), reminiscent of residual bodies or secondary lysosomes, are seen. However, in cells that were exposed to acetyl-LDL for as little as 4 h (Fig. 8B), two changes were notable. First, some of the cells contained distinct lipid droplets that were not surrounded by a membrane. Second, there was an increase in the number of MBV. Neither of these changes was seen in cells incubated with native LDL for the same time in the same experiment (not shown). Fig. 8D shows at a high magnification the relationship of MBV and lipid droplets to other elements of the cell. Many of these membrane-bounded inclusions had a foamy appearance (solid arrows in Fig. 8D) that may be related to the accumulation of acetyl-LDL in this compartment. The cytoplasmic lipid droplets frequently were partially enveloped by rough...
Electron microscope appearance of macrophages incubated for 6 d with acetyl-LDL. 3 x 10^6 peritoneal cells were dispensed into each 35-mm dish. On day 0, each dish received 1 ml of medium A containing 50 μg/ml of acetyl-LDL. The medium was replaced with fresh medium of identical composition on days 3 and 5. On day 6, the monolayers were fixed in situ and processed for electron microscopy as described in Materials and Methods. L, neutral lipid droplet; V, membrane-bounded vacuole (MBV). (A) X 7,200. (B) X 5,200. (C) X 15,700.
endoplasmic reticulum (open arrows in Fig. 8D).

Fig. 8E shows at low power a cell from a culture that was exposed to acetyl-LDL for 48 h. These cells are filled with MBV and lipid droplets.

In combination, the light and electron microscopy studies in Figs. 6-9 indicate that both lipid and MBV accumulate in the cytoplasm of cells exposed to acetyl-LDL. While it appears that the majority of the accumulated cholesteryl ester is present in the non-membrane-bounded lipid droplets, it is impossible with present electron microscope data to determine quantitatively how much cholesteryl ester is associated with the MBV.

**Cholesteryl Ester Removal from Macrophages**

When macrophages were allowed to accumulate esterified cholesterol for 48 h and the acetyl-LDL was then removed, the cellular content of esterified cholesterol decreased rapidly (Fig. 10). All of the cholesterol released by such cells was found in the culture medium as free cholesterol. The hydrolysis and removal of the accumulated cholesteryl esters was not blocked by inclusion of 75 μM chloroquine in the medium, suggesting that this reaction is catalyzed by a nonlysosomal cholesterol esterase (data not shown).

Concomitant with the loss of cholesteryl esters upon removal of acetyl-LDL from the culture medium, the macrophages lost their birefringent inclusions. In the electron microscope, the lipid droplets were also diminished (data not shown).

**Influence of Acetyl-LDL on Cholesteryl Ester Removal**

The rapid hydrolysis of the accumulated cholesteryl esters that occurred on removal of the acetyl-LDL from the culture medium raised the question as to whether this hydrolysis was occurring continuously during the incubation, or whether it was suppressed during the period that acetyl-LDL was present in the medium. To distinguish these possibilities, we incubated macrophages with acetyl-LDL plus [14C]oleate for 24 h (Fig. 11). The exogenous [14C]oleate was then removed from the culture medium, and the cells were incubated for an additional 24 h in the absence or presence of acetyl-LDL. The incubations were conducted in the presence of two levels of fetal calf serum, 5 and 20%. When the acetyl-LDL was removed, the accumulated cholesteryl [14C]oleate was rapidly hydrolyzed in the presence of either 5 or 20% serum. On the other hand, when the acetyl-LDL remained in the incubation medium the decline in the cellular cholesteryl [14C]oleate was blocked. These data suggest that the activity of the nonlysosomal cholesteryl esterase that hydrolyzes cytoplasmic cholesteryl ester droplets is suppressed during the period when large amounts of cholesterol are entering the cell in the form of acetyl-LDL.

**DISCUSSION**

Recent studies of the LDL receptor pathway in cultured human fibroblasts have led to the concept that two functionally and morphologically distinct compartments exist for cholesteryl ester metabolism within cells (reviewed in references 11 and 19). The first compartment involves the lysosome which contains an active cholesterol esterase with
Inhibition by acetyl-LDL of the hydrolysis of preformed cholesteryl \[^{14}C\]oleate in macrophages. 2 \times 10^6 peritoneal cells were dispensed into replicate 35-mm dishes. On day 0, each dish received 1 ml of medium A. On day 1, each dish received 1 ml of medium A containing 25 \mu g/ml of acetyl-LDL and 0.1 mM \[^{14}C\]oleate-albumin (8,200 cpm/nmol). After incubation for 24 h (zero time in figure), each monolayer was washed twice with 2 ml of medium B and once with 2 ml of DMEM, after which was added 1 ml of DMEM containing either 5\% (vol/vol) fetal calf serum (○, ○) or 20\% fetal calf serum (△, △) in the absence (closed symbols) and presence (open symbols) of 25 \mu g/ml of acetyl-LDL without \[^{14}C\]oleate-albumin. At the indicated time, the monolayers were harvested and the cellular content of cholesteryl \[^{14}C\]oleate was measured as described in Materials and Methods. Each value is the average of duplicate incubations.

This lysosomal enzyme hydrolyzes the cholesteryl esters that enter the cell through the receptor-mediated uptake of LDL (11, 25). If this lysosomal cholesterol esterase is blocked, as is the case in normal fibroblasts incubated with chloroquine (22) or in mutant fibroblasts from patients with a genetic deficiency of this enzyme (11, 25), LDL-derived cholesteryl esters accumulate in lysosomes (11, 23). These cholesteryl ester-filled lysosomes appear in electron micrographs as large MBV (23). The second compartment for cholesteryl ester metabolism is non-lysosomal and involves the cytosol where cholesteryl esters accumulate in droplets not surrounded by a membrane. These esters are synthesized by a microsomal ACAT enzyme that is activated when cells are presented with excess cholesterol derived from the lysosomal hydrolysis of the cholesteryl esters of LDL (19). When the exogenous source of cholesterol is removed, these cytoplasmic cholesteryl esters are hydrolyzed by an enzyme that is distinct from the lysosomal cholesterol esterase in that its activity is not inhibited by chloroquine (23, 25) and its activity is not reduced in the mutant cells with a marked deficiency of the lysosomal cholesterol esterase (23, 25).

In contrast to fibroblasts and other types of nonmacrophage cells that specifically bind and take up native LDL, macrophages possess high affinity cell surface binding sites that recognize negatively charged LDL preparations, such as acetyl-LDL, rather than native LDL (26). Despite this difference in cell surface recognition between fibroblasts and macrophages, the current studies demonstrate that cholesteryl ester metabolism in mouse peritoneal macrophages, like that of human fibroblasts, can be considered in terms of a two-compartment hydrolysis-re-esterification model.

The hydrolysis-re-esterification mechanism for macrophages was demonstrated in the current study in several ways. First, the addition of acetyl-LDL produced a marked increase in the rate at which \[^{14}C\]oleate was incorporated into cellular cholesteryl \[^{14}C\]oleate. Indicating rapid re-esterification of acetyl-LDL-derived cholesterol. At a concentration of 25 \mu g/ml of acetyl-LDL, the rate of \[^{14}C\]oleate incorporation was 10 nmol-h\(^{-1}\)-mg protein\(^{-1}\) (Fig. 2). If sustained for 24 h, this rate would result in the formation of 240 nmol of cholesteryl \[^{14}C\]oleate/mg protein, which is equal to 92 \mu g of esterified cholesterol/mg protein. This latter value was within the range observed for the total increase in the mass of esterified cholesterol that occurred when macrophages were incubated for 24 h with 25 \mu g/ml of acetyl-LDL (see Fig. 1 B).

The above isotope data were also supported by mass measurement of the fatty acid composition of the cholesteryl esters that accumulated in cells incubated with acetyl-LDL. In an experiment not shown, we incubated macrophages for 30 h with 25 \mu g/ml of acetyl-LDL and 50 \mu M oleate bound to albumin. 65\% of the cholesteryl esters in the acetyl-LDL were cholesteryl linoleate and 19\% were cholesteryl oleate. Within the cells, however, cholesteryl oleate represented 85\% of the accumulated cholesteryl esters and cholesteryl linoleate represented <2\%. This switch in fatty acid composition of the cholesteryl esters strongly supports the concept that nearly all of the cholesteryl esters that accumulated in macrophages represented products of the endogenous esterification mechanism.
The second line of evidence supporting the hydrolysis-re-esterification process comes from experiments in which the metabolism of r-[\(^{3}H\)-cholestereryl linoleate]acyetyl-LDL was observed. These data showed that the [\(^{3}H\)-cholestereryl linoleate was rapidly hydrolyzed in a process that was competitively inhibited by excess unlabeled acetyl-LDL (which inhibits high affinity binding and hence cellular uptake) and was prevented by chloroquine (which inhibits the activity of lysosomal enzymes). Under the conditions of these experiments, about one-half of the free [\(^{3}H\)-cholesterol released from lysoosomal hydrolysis was excreted directly into the culture medium without undergoing re-esterification, and the other half was re-esterified and stored within the cell (Figs. 3 and 5). Thus, when the macrophages were taking up the r-[\(^{3}H\)-cholestereryl linoleate]acyetyl-LDL at a constant rate, the rate of excretion of free [\(^{3}H\)-cholesterol into the medium was equal to the rate of accumulation of [\(^{3}H\)]-cholesterol esters within the cell (Figs. 3 and 5).

Experiments in which acetyl-LDL was shown to stimulate ACAT activity provided the third line of evidence supporting the re-esterification process. From studies in other cell types (1, 4, 28), it is likely that the ACAT activity is present in the endoplasmic reticulum, but this remains to be formally demonstrated for macrophages. The ultrastructural demonstration of numerous cytoplasmic lipid droplets that were not surrounded by a limiting membrane provided a fourth line of evidence to support the hydrolysis-re-esterification mechanism.

Three different types of cells have now been induced to overaccumulate cholesteryl esters by incubation with derivatized human LDL in tissue culture: human fibroblasts (2) and human smooth muscle cells (17) incubated with polycationic LDL, and mouse peritoneal macrophages incubated with acetyl-LDL (current paper). In each case the biochemical and morphological characteristics of the lipid-laden cells are similar, although they vary somewhat in degree. In all three cell types, the striking feature is the accumulation of cytoplasmic lipid droplets that result from the lysosomal hydrolysis and cytosolic re-esterification of the LDL-cholesterol esters. Although the number and size of secondary lysosomes is increased and although some of the lipid is presumed to be present in these MBV, the biochemical and morphologic data suggest that most of the lipid is not present in the lysosomal compartment. The pathologic features of these lipid-laden cells in vitro generally resemble those of foam cells that accumulate in vivo in tissues of hyperlipidemic men and experimental animals (12, 21, 27, 33, 34, 39). The foam cells of tendon and cutaneous xanthomas are thought to be derived from macrophages (12, 16), whereas the foam cells of atheromas are thought to be derived from arterial smooth muscle cells (33, 38). Despite their differing origins, the similar appearance of these different types of foam cells in vivo is consistent with the findings in cell culture that smooth muscle cells and macrophages have similar pathways for intracellular cholesteryl ester metabolism, i.e., lysosomal hydrolysis and cytosolic re-esterification. That such a mechanism of cholesteryl ester accumulation also occurs in vivo is indicated by experiments showing that the rate of cholesteryl ester synthesis is markedly enhanced in arteries from cholesterol-fed animals (13, 29) and in atheromatous plaques from man (35, 36).

In a series of kinetic studies on cholesterol metabolism in mouse peritoneal macrophages, Werb and Cohn measured the response of these cells to the phagocytosis of particles consisting of free or esterified cholesterol complexes with albumin (37). When particles containing free [\(^{3}H\)]cholesterol were ingested by the cells, the cholesterol was subsequently excreted with a half-time of 20 h. It is not known whether any of the [\(^{3}H\)]cholesterol was stored in the form of [\(^{3}H\)]cholesterol esters before excretion. When the cells were allowed to ingest particles of [\(^{3}H\)]cholestereryl linoleate, the cellular radioactivity declined with a similar half-life (20 h). All of the [\(^{3}H\)]radioactivity that left the cell was in the form of free [\(^{3}H\)]cholesterol (37). In our current experiments, the decline in stored cholesteryl esters exhibited a half-time of \(~18\) h (Fig. 10), which is similar to both half-times observed in the Werb and Cohn study. Thus, it is possible that excess cholesterol entering macrophages by phagocytosis (i.e., as cholesteryl ester-albumin complexes) may be hydrolyzed in lysosomes and re-esterified in a manner similar to that of excess cholesterol entering macrophages by adsorptive endocytosis (i.e., through the acetyl-LDL-binding site).

The present results indicate that at least five cellular mechanisms play a role in determining the amount of cholesteryl esters that accumulate in macrophages incubated with acetyl-LDL. These include: (a) the high affinity binding site for acetyl-LDL (binding and uptake continue even when the cell has accumulated massive amounts of cholesteryl esters); (b) the factors mediating the direct
excretion of cholesterol derived from the lysosomal hydrolysis of acetyl-LDL (half of this cholesterol is excreted and the other half is re-esterified for storage); (c) the ACAT enzyme (its activity is enhanced by cholesterol released from acetyl-LDL); (d) the nonlysosomal enzyme that hydrolyzes cytosolic cholesteryl esters (this enzyme appears to be inhibited when the cells are actively taking up acetyl-LDL); and (e) the factors mediating the active excretion of cholesterol derived from the nonlysosomal hydrolysis of cytosolic cholesteryl esters (macrophages excrete enormous amounts of such cholesterol when the exogenous source of cholesterol is removed). Detailed studies of each of the above processes will be necessary before a complete understanding of the mechanism for macrophage cholesteryl ester accumulation is obtained.

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