Lysosomal cholesterol derived from mildly oxidized low density lipoprotein is resistant to efflux

Patricia G. Yancey and W. Gray Jerome[1]

Department of Pathology, Wake Forest University School of Medicine, Winston-Salem, NC 27157

Abstract **In atherosclerotic lesions, macrophages store lipid in cytoplasmic inclusions and lysosomes. Regression studies show that lysosomal lipid is not as easily cleared as cytoplasmic inclusion lipid. Macrophages enriched with mildly oxidized low density lipoprotein (oxLDL) accumulate cholesteryl ester (CE) and free cholesterol (FC) in lysosomes. We examined whether lysosomal stores of cholesterol from oxLDL are cleared from THP-1 and mouse macrophages. As in previous studies, oxLDL-enriched THP-1 macrophages accumulated substantial lysosomal cholesterol. Surprisingly, less than 12% of oxLDL-derived lysosomal CE was cleared to efficient FC acceptors (e.g., cyclodextrins, apolipoprotein/phosphatidylcholine vesicles, and fetal bovine serum). Filipin staining showed that lysosomes of oxLDL-treated THP-1 cells contained FC, and despite removal of most of the cell FC (70–80%) by incubation with cyclodextrins, filipin staining of FC in lysosomes did not diminish. Also, when THP-1 macrophages were incubated with [^{3}H]CE oxLDL, 73–76% of the [^{3}H]CE was retained in a lysosomal hydrolysis resistant pool. In contrast, greater than 90% of acetylated low density lipoprotein (acLDL) [^{3}H]CE was hydrolyzed. Furthermore, [^{3}H]FC liberated from oxLDL [^{3}H]CE was released at a slower rate to cyclodextrins than was [^{3}H]FC from acLDL [^{3}H]CE. In contrast, only 27% of oxLDL [^{3}H]CE was resistant to hydrolysis in mouse macrophages, and the [^{3}H]FC generated from oxLDL and acLDL [^{3}H]CE was released to cyclodextrins at similar rates. We conclude that lack of hydrolysis and efflux of oxLDL cholesterol is not exclusively inherent in oxLDL, but also requires specific cell factors present in one cell type but not the other.**—Yancey, P. G., and W. G. Jerome. **Lysosomal cholesterol derived from mildly oxidized low density lipoprotein is resistant to efflux.** ***J. Lipid Res.*** **2001.** 42: **317–327.**

Supplementary key words cholesterol-engorged lysosomes • cholesterol efflux • atherosclerosis

An important early event in the development of atherosclerotic lesions is the appearance of macrophage foam cells. Initially, cholesteryl ester (CE) accumulation in foam cells is primarily localized to cytoplasmic inclusions. However, in the development of atherosclerosis at the transition point from a fatty streak to a fibrous plaque, CE and free cholesterol (FC) accumulate in lysosomes of macrophages (1–3). Subsequently, smooth muscle cells that migrate into the intima and become foam cells also store lipid lysosomally. This lysosomal lipid accumulation occurs in foam cells of lesions in many animal models besides humans, including pigeons, rabbits, and primates (1–3). The key question of why CE and FC are not cleared from lysosomes of foam cells remains to be answered.

Low density lipoprotein (LDL) is the main source of cholesterol found in atherosclerotic foam cells (4). Because uptake of native LDL is regulated, LDL requires modification in order to be processed by unregulated receptors, which load cells with levels of cholesterol typical of atherosclerotic foam cells (4). Acetylation and oxidation of LDL cause uptake by unregulated mechanisms, principally scavenger receptors (4–6). Oxidation is both physiologic and clearly linked to atherosclerosis (7–9). It is well known that acetylated LDL (acLDL) can induce macrophage CE accumulation (4, 10–12). The ability of oxidized LDL (oxLDL) to promote significant CE accumulation in macrophages, however, is more controversial (13–18). oxLDL studies have been confounded by using heavily oxLDL and short loading periods (13–18). Heavily oxLDL contains little or no unoxidized cholesterol (13, 19), and the use of short loading periods compromises the degree of CE accumulation. Studies by Greenspan et al. (20) have shown that when heavily oxLDL is enriched with FC, significant CE accumulation is obtained in mouse peritoneal macrophages. This is further evidence that it is lack of unoxidized cholesterol in heavily oxLDL that limits cholesterol accumulation. Our recent studies add further confirmation. We showed that LDL

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; acLDL, acetylated low density lipoprotein; BSA, bovine serum albumin; CE, cholesteryl ester; EC, esterified cholesterol; EM, electron microscopy; FBS, fetal bovine serum; FC, free cholesterol; LDL, low density lipoprotein; MEM, Eagle's minimum essential medium; oxLDL, oxidized low density lipoprotein; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid-reactive substances; TC, total cholesterol; TPA, phorbol ester.

[1] To whom correspondence should be addressed at current address: Department of Pathology, Vanderbilt University Medical Center, 1161 21st Ave South, Nashville, TN 37232-2561.

e-mail: jay.jerome@mcmail.vanderbilt.edu

that is mildly oxidized and retains much of its cholesterol in unmodified form can stimulate significant foam cell formation. In our studies, CE levels ranged from 83 to 336 μg/mg cell protein in mouse, pigeon, or THP-1 macrophages (21).

Although mildly oxLDL produced CE accumulation in mouse, pigeon, and THP-1 macrophages, there were differences in loading between different cell types (21). Mildly oxLDL cholesterol enrichment of THP-1 and pigeon macrophages produced lysosomal lipid engorgement similar to that found in atherosclerotic foam cells, with most oxLDL CE localized to lysosomes (21). In contrast, mouse peritoneal macrophages metabolized, for the most part, oxLDL cholesterol normally leading to CE accumulation in cytoplasmic inclusions (21). Thus, the ability of mildly oxLDL to induce lysosomal lipid engorgement is different between murine and nonmurine macrophages. In addition to CE accumulation, our studies indicated FC accumulation in lysosomes (21). This is consistent with reports that heavily oxLDL produces enrichment of FC in lysosomes of macrophages (16, 18). Our studies, however, demonstrated considerably more cholesterol accumulation using mildly oxidized LDL (21). These findings, combined with those studies localizing oxLDL to atherosclerotic lesions, suggest that oxLDL could be responsible for the lysosomal lipid engorgement found in foam cells of lesions.

Atherosclerotic foam cell cholesterol accumulation is dependent on the balance between influx of LDL cholesterol and efflux of cell cholesterol. The cholesterol efflux process is driven by the presence of extracellular FC acceptors (22). Sequestration of FC and CE within lysosomes may prevent it from being mobilized to plasma membrane for efflux. Indeed, studies on regression of atherosclerotic lesions have shown that lysosomal lipid is not as easily cleared as cholesterol in cytoplasmic inclusions (23, 24). This is true even when excess cholesterol acceptors are infused (25). To determine whether oxLDL mimics the FC and CE accumulation seen in atherosclerotic foam cells, we examined the availability of mildly oxLDL cholesterol for clearance from macrophages in the presence of acceptors. We combined quantitative microscopic and biochemical approaches to compare the efflux potential of oxLDL- and acLDL-derived cholesterol from THP-1 and mouse peritoneal macrophages. Our studies show that cholesterol from oxLDL in THP-1 macrophages is more resistant to hydrolysis and that free and esterified cholesterol sequestered in lysosomes is essentially unavailable for clearance even in the presence of cyclodextrins, a highly efficient cholesterol acceptor (26). In contrast, acLDL cholesterol was readily available for clearance from lysosomes of THP-1 macrophages. In mouse macrophages, where lysosomal accumulation of oxLDL cholesterol is only modest, oxLDL cholesterol was more available for efflux when compared with clearance from THP-1 macrophages. This suggests that conditions leading to lysosomal lipid accumulation are potentially more deleterious than those promoting cytoplasmic CE accumulation.

MATERIALS AND METHODS

Materials

Male $B_6C_3F_1$ mice were purchased from Charles River Laboratories, Raleigh, NC. Bovine serum albumin (BSA; fatty acid-free from fraction V), ethylenediaminetetraacetic acid (EDTA), triolein, and CE were purchased from Sigma Chemical Company, St. Louis, MO. Cholesterol and stigmasterol were obtained from Steraloids, Wilton, NH. Heat-inactivated FBS was purchased from Atlanta Biologicals, Norcross, GA. Eagle's minimum essential medium (MEM), RPMI, L-glutamine, Eagle's vitamins, streptomycin, and penicillin were obtained from Mediatech, Washington, DC. All tissue-culture plasticware was purchased from Falcon, Lincoln Park, NJ. The radioisotopes, cholesteryl [^{14}C]oleate (59.5 mCi/mmol), [^{3}H]cholesteryl oleate, and [^{3}H]cholesteryl linoleate were obtained from New England Nuclear, Boston, MA. All other chemical reagents and chemical solvents were obtained from Fisher Scientific, Pittsburgh, PA. acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, compound CP113,818, was a generous gift from Pfizer Central Research, Groton, CT.

Lipoprotein isolation, radiolabeling, and modification

Human LDL was isolated from plasma given by normocholesterolemic human volunteers following procedures approved by the Human Subjects Institutional Review Board. Pigeon LDL was isolated from plasma of White Carneau pigeons fed a diet of commercial pigeon pellets containing 0.5% cholesterol and 5% corn oil for at least 1 month. All animal procedures followed protocols approved by the University Animal Care and Use Committee. Apolipoprotein high density lipoprotein/phosphatidylcholine (apoHDL/PC) vesicles were prepared as previously described (27). Both human and pigeon LDLs ($1.006 \leqslant d \leqslant 1.063$) were isolated by sequential ultracentrifugation as previously described (10). LDL was dialyzed extensively against 0.9% NaCl containing EDTA (0.3 mmol/l) and sterilized by filtration through a millipore filter (0.45 μM). Before radiolabeling of human LDL with [^{3}H]cholesteryl oleate or [^{3}H]cholesteryl linoleate (40 μCi/mg LDL protein), LDL was dialyzed against buffer containing Tris-HCL (20 mmol/l), NaCl (150 mmol/l) and EDTA (1 mmol/l, pH 7.4). LDL was radiolabeled with [^{3}H]CE essentially as described by Brown, Dana, and Goldstein (28). Briefly, 640 μCi [^{3}H]CE was solubilized in 10 μl of toluene, and 1 ml of prewarmed dimethyl sulfoxide was added to the [^{3}H]CE solution. This mixture was incubated for 15 min at 40°C. The [^{3}H]CE solution was then diluted by addition of 1 ml of Tris-HCL buffer prewarmed to 40°C, and 16 mg of LDL in 6 ml of Tris-HCL buffer was added to the [^{3}H]CE solution. The mixture was incubated overnight at 40°C. Before modification, the labeled LDL was dialyzed extensively against 0.9% NaCl containing EDTA (0.3 mmol/l) and used within 2 weeks of preparation.

Pigeon, human, or [^{3}H]CE labeled LDL was acetylated following the procedure of Basu et al. (29). Before oxidation, LDLs (1–2 mg LDL protein/ml) were dialyzed three times against 0.9% NaCl to remove EDTA. To oxidize both native and [^{3}H]CE labeled human LDL, the lipoproteins were dialyzed at 37°C for 2–5 h against 0.9% NaCl containing 20 μmol/l of $CuSO_4$. Immediately before addition to cells, thin-layer chromatography (TLC) analysis was done on the [^{3}H]cholesteryl oleate oxLDL using hexane–ethyl ether–acetic acid 80:20:1 (v/v/v) as the solvent system. This confirmed that 85 ± 3% of the total tritium label was unoxidized CE. After oxidation of the [^{3}H]cholesteryl linoleate LDL, 55 ± 3% of the [^{3}H]CE was unoxidized. Pigeon LDL was oxidized by dialysis at 4°C for 18 h against 0.9% NaCl containing 20 μmol/l of $CuSO_4$. Oxidation of LDLs was ended by extensively dialyzing against 0.9% NaCl containing EDTA (0.3

mmol/l). All modified LDLs were sterilized by filtration through a Millipore filter (0.45 μM).

Oxidation or acetylation of LDL was confirmed by agarose gel electrophoresis as described (30). Oxidation of LDLs was also assayed by measuring thiobarbituric acid-reactive substances (TBARS) as described previously (21), using malonaldehyde bis(dimethyl acetal) (MDA) as a standard. Oxidation conditions were set to obtain thiobarbituric acid reactivity that ranged from 5 to 10 nmol MDA/mg LDL protein. Our previous studies have shown that mildly oxidized LDL is not toxic to cells and contains a sufficient amount of unoxidized cholesterol to induce a level of CE loading in macrophages that is typical of foam cells in atherosclerotic lesions (21).

Cell culture and isolation of mouse macrophages

Mouse peritoneal macrophages were isolated from $B_6C_3F_1$ mice by peritoneal lavage with phosphate buffered-saline (PBS) 4 days after intraperitoneal injection with 0.5 ml of 10% thioglycollate as described previously (10). Cells were plated onto 35-mm wells at a density of $1-2 \times 10^6$ cells and allowed to adhere by incubation for 2 h at 37°C in MEM containing 1% FBS. After removal of nonadherent cells by washing with PBS, the experiments were initiated. For experiments with THP-1 human monocyte/macrophage cell line, cells were plated onto 35-mm wells at a density of 1.5×10^6 cells and incubated for 3–4 days at 37°C in RPMI containing 10% FBS and phorbol ester (TPA), 50 ng/ml of medium, to allow for differentiation into macrophages (31). For the entire duration of the experiments, TPA was included in the incubation medium. Culture media for all cell incubations were supplemented with HEPES (20 mmol/l), bicarbonate (23 mmol/l), Eagle's vitamins, L-glutamine (200 mmol/l), glucose (1.5 mg/ml), streptomycin (100 μg/ml), and penicillin (100 IU/ml). All cells were incubated in a humid atmosphere at 37°C with 95% air and 5% CO_2.

Cholesterol loading of cells, cell cholesterol efflux, and lipid analyses

Cholesterol clearance was compared in THP-1 and mouse peritoneal macrophages loaded with either oxLDL or acLDL. Cholesterol clearance was examined by two methods. In some experiments, cells were first loaded with oxLDL or acLDL, then cellular cholesterol mass was measured during efflux. For cholesterol loading, THP-1 or mouse macrophages were incubated for 7 days at 37°C in culture medium containing 0.25% FBS and the ACAT inhibitor, compound CP113,818 (1.5 μg/ml of medium, 0.1% dimethyl sulfoxide final concentration) alone or with 150–200 μg of oxLDL or acLDL protein/ml (as is indicated in the figure legends). The ACAT inhibitor was included in the medium to provide a means to measure lysosomal CE clearance, and the low concentration of 0.25% FBS was used during loading to minimize the net efflux of cholesterol from acLDL loaded cells. The cholesterol loading medium was changed every 3–4 days to fresh medium containing the modified lipoproteins. At the end of loading, the cell monolayers were washed three times with MEM containing HEPES (20 mmol/l), and for efflux, cells were incubated for 1–24 h at 37°C in culture medium containing 1.5 μg/ml of compound CP113,818 alone or with the cholesterol acceptor (concentration as is indicated in the figure legends). FBS, apoHDL/PC vesicles, and 2-hydroxypropyl-β-cyclodextrins (2-OHρβ-CD) were used as cholesterol acceptors. At the end of the incubations, cells were washed three times with 2 ml of PBS. To quantitate cellular cholesterol, cell lipids were extracted by incubation at room temperature overnight with 2 ml of isopropanol containing 5–20 μg of stigmasterol as an internal standard. The lipid extract cholesterol content was then quantified by gas-liquid chromatography (GLC) following the procedure of Ishikawa et al. (32) as modified by Klansek and colleagues (33). Cell proteins were solubilized by addition of 1N NaOH to the wells, and the protein content was measured using the method of Lowry et al. (34).

Another approach to examining cholesterol clearance was to quantitate release of [^{3}H]cholesterol from THP-1 and mouse macrophages loaded with [^{3}H]CE labeled oxLDL or acLDL. Cell monolayers were incubated for 6 h to 3 days at 37°C in serum-free medium containing 1.5 μg/ml of compound CP113,818 and 20–50 μg of [^{3}H]CE labeled oxLDL or acLDL protein/ml (as is indicated in the figure legends). After cholesterol loading, cells were washed three times with MEM containing HEPES, then incubated for 1–7 h with culture medium containing 1.5 μg/ml of compound CP113,818 alone or with 2-OHρβ-CD (15 mmol/l). At times during both cholesterol loading and efflux, the medium was sampled for quantitation of [^{3}H]cholesterol. To remove any floating cells, the medium was centrifuged for 15 min at 2,500 rpm. Aliquots were then taken to measure both total [^{3}H]cholesterol content and distribution of [^{3}H]FC and [^{3}H]CE. Media lipids were extracted by the method of Bligh and Dyer (35). Cell lipids were extracted with isopropanol containing cholesteryl [^{14}C]oleate as an internal standard. After drying under N_2, cell and media lipids were dissolved in 25 μl of chloroform–methanol 2:1 (v/v) and plated onto LK6D silica gel 60 TLC plates. Free cholesterol and CE were separated from other lipids using hexane–ethyl ether–acetic acid 80:20:1 (v/v/v) as the solvent system and visualized with I_2. The FC and CE bands were scraped, and radioactivity was measured by liquid scintillation counting.

Microscopy

After cholesterol loading and efflux, microscopy was used to visualize cellular lipids. Fluorescence microscopy was used to visualize cellular filipin-stained FC, and transmission electron microscopy (TEM) was used to ultrastructurally quantitate cell lysosomal lipid volume. For filipin staining to visualize cell FC, macrophages were plated on sterile glass coverslips placed in the bottom of 35 mm tissue culture wells. After treatment with oxLDL or acLDL, cells were washed in PBS and fixed for 30 min in 10% neutral buffered formalin. Then, the coverslips were washed in PBS and stained by incubation at 37°C for 3 h in a filipin stain solution. The solution contained 1.25 mg of filipin dissolved in 0.5 ml of dimethyl sulfoxide (DMSO) diluted with 25 ml of PBS. After staining, the cells were washed with distilled water and mounted on glass slides with a phenylenediamine-glycerol solution to inhibit photobleaching. The glass slides were kept in the dark until viewing. The presence of filipin-positive FC was detected by epi-illumination with ultraviolet (UV) (UG-1 filter) excitation and viewed through a 510-nm barrier filter. Owing to the mild oxidation procedures used in these studies, the FC to protein ratios of the oxLDL and acLDL were similar (oxLDL = 0.41 ± 0.01 and acLDL = 0.42 ± 0.05). Thus, fluorescence observed with oxLDL-treated cells would be expected to be staining of mainly FC as opposed to oxidized FC. In addition, separation of cell lipids by TLC after treatment with oxLDL confirmed that fluorescence was associated only with FC.

For thin-section ultrastructural examination, cell monolayers were washed in cacodylate buffer and fixed in 2.5% glutaraldehyde. Lysosomes and related organelles were localized by the presence of acid phosphatase. To demonstrate acid phosphatase, samples were processed using a modification (2) of the Gomori lead precipitation method (36). Beta-glycerolphosphate was used as a substrate, and the reaction control was incubated in identical medium without the enzymatic substrate. After incubation, cells were postfixed in 1% osmium tetroxide, enbloc stained with uranyl acetate, dehydrated, and embedded in epoxy resin.

A Phillips EM400 operated at 300 keV was used to view thin sections (80 nm) of the embedded cells. Before further staining, electron microscopy (EM) sections were viewed to verify the enzymatic reaction, and then stained with uranyl acetate and lead citrate. The lipid volumes of lysosomes and cytoplasmic inclusions were estimated using point-counting sterologic techniques (37), as routinely done in our laboratory (2). Five thin sections from multiple parts of the embedded block were cut for each sample. From each thin section, 10–15 areas were randomly chosen by shifting the region of view a predetermined distance in both the x and y directions. The distances used were based on a random number generator algorithm. From these random fields, the average volume density (v/v cell cytoplasm) was computed for both lysosomal (acid phosphatase positive) and cytoplasmic inclusion (acid phosphatase negative) lipid stores.

RESULTS

Many studies have shown that serum can stimulate net removal of cholesterol from macrophage foam cells (38, 39). Initial studies were conducted using FBS as a cholesterol efflux promoter. The time courses of FC and CE clearance to FBS from THP-1 macrophages initially enriched with either oxLDL or acLDL cholesterol are shown in **Fig. 1**. The ACAT inhibitor, CP113,818, was included in the cholesterol enrichment medium to enable measurement of lysosomal CE clearance. Similar to our previous report, when THP-1 macrophages were loaded with oxLDL cholesterol in the presence of an ACAT inhibitor, cells accumulated significant CE (136 ± 10 μg/mg cell protein). During the 24-h incubation with FBS, only 10% of this oxLDL CE was cleared. In addition, cellular FC content that was initially 92 ± 3 μg FC/mg cell protein decreased only by 25% after 24 h of incubation with FBS. In contrast, acLDL cholesterol-enriched THP-1 cells initially contained higher FC than CE (65 ± 1 μg FC/mg cell protein vs. 37 ± 3 μg CE/mg cell protein). During incubation with FBS, 60% of the CE was cleared and the cell FC content was reduced by 45%. Most of the CE clearance occurred during the first few hours, and thereafter little clearance was observed. Thus, both FC and CE were readily cleared to FBS from acLDL-treated cells, but oxLDL cholesterol was resistant to clearance. Similar results were observed in CE clearance from oxLDL-treated THP-1 macrophages incubated with apoHDL/PC vesicles (data not shown).

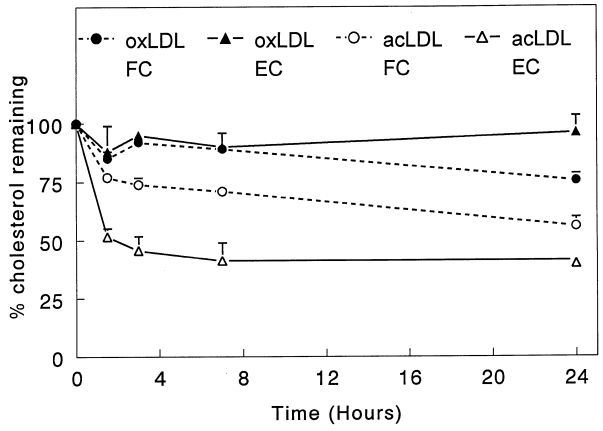

Fig. 1. Time courses of efflux of cholesterol from THP-1 macrophages loaded with oxLDL or acLDL in the presence of FBS. THP-1 macrophages were incubated for 7 days at 37°C in RPMI containing 0.25% FBS, 50 ng TPA/ml, and 1.5 μg CP113,818/ml alone or with 150 μg protein/ml of either pigeon oxLDL or acLDL. After cholesterol enrichment, cells were incubated for up to 24 h in RPMI containing 50 ng TPA/ml, 1.5 μg CP113,818/ml, and 10% FBS. At the end of each incubation, the cells were harvested and the cell cholesterol contents were measured by gas-liquid chromatography as described in Materials and Methods. The results are expressed as a percentage of the initial cholesterol content and are the mean ± SD for triplicate dishes. The initial FC and EC contents of the oxLDL-treated cells were 92 ± 3 and 136 ± 10 μg/mg cell protein, respectively. The initial FC and EC contents of the acLDL-treated cells were 65 ± 1 and 37 ± 3 μg/mg cell protein, respectively.

In recent years, much progress has been made in defining the mechanisms of cholesterol efflux. It is evident that specific cell factors including the SRB-1 receptor (40–42) and the ATP binding cassette (ABC-1) transporter (43) play a role. Studies have shown that the contribution of these two receptors to total cellular cholesterol efflux depends not only on the kind of acceptor, but varies extensively between types of cells (42). Cyclodextrins (CDs) are cyclic oligomers that are highly efficient at stimulating efflux, and previous studies have shown that any inherent differences in efflux between cell types are eliminated with efflux to CDs (26, 44). Therefore, CDs are a powerful tool for comparing the availability of exogenous sources of cholesterol for efflux from different cell types. **Figure 2** shows the time course of FC and CE clearance from THP-1 (Fig. 2A) or mouse macrophages (Fig. 2B) incubated with 2-OHρβ-CD. As when FBS was used as an acceptor, oxLDL cholesterol-enriched THP-1 macrophages did not clear CE to 2-OHρβ-CD, whereas acLDL-loaded cells cleared 51%. Similar results (range = 0–10%) were observed in THP-1 macrophages loaded with a wide range (16–216 μg CE/mg cell protein) of oxLDL CE, suggesting that the mechanism preventing lysosomal CE clearance is independent of the degree of CE loading. In contrast, CDs stimulated CE clearance from both oxLDL (59%) and acLDL (65%) cholesterol-enriched mouse macrophages. The 2-OHρβ-CD promoted a substantial decrease in FC content from both oxLDL and acLDL cholesterol-enriched THP-1 or mouse macrophages (Figs. 2A and 2B).

To examine the availability of oxLDL CE for clearance more accurately, [^{3}H]CE-labeled oxLDL was used in the next series of experiments. Besides being a more sensitive means of measuring lipoprotein-derived cholesterol than quantitating cell cholesterol mass by GLC, [^{3}H]CE-labeled oxLDL provides a way to examine clearance of unoxidized CE without including oxidized esters (13). The distribution of cell [^{3}H]CE and cell [^{3}H]FC over time in THP-1 macrophages incubated with either [^{3}H]CE-labeled oxLDL or [^{3}H]CE-labeled acLDL is shown in **Fig. 3A** and **B**. Also shown is the [^{3}H]FC content of the medium. During the first 6 h of incubation, the majority (55%) of the [^{3}H]CE derived from oxLDL was hydro-

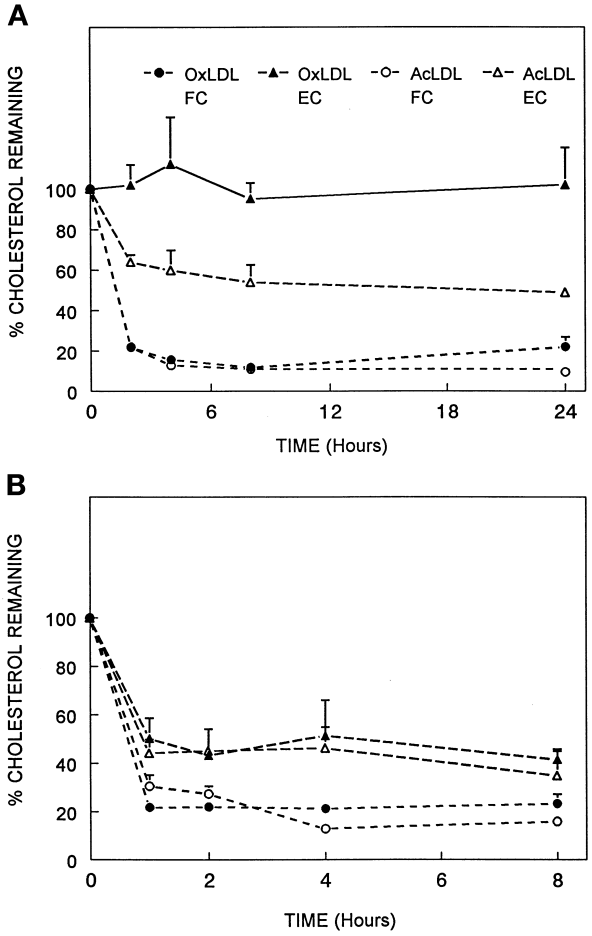

Fig. 2. Time courses of efflux of cholesterol from THP-1 and mouse macrophages loaded with oxLDL or acLDL in the presence of 2-OHρβ-CD. THP-1 (A) or mouse (B) macrophages were incubated for 7 days at 37°C in medium containing 0.25% FBS and 1.5 μg CP113,818/ml alone or with 200 μg protein/ml of either human oxLDL or acLDL. After cholesterol enrichment, cells were incubated for the indicated times in medium containing 1.5 μg CP113,818/ml and 15 mM 2-OHρβ-CD. During cholesterol loading and efflux with THP-1 cells, 50 ng TPA/ml of medium was present. At the end of each incubation, the cells were harvested and the cell cholesterol contents were measured by gas-liquid chromatography as described in Materials and Methods. The results are expressed as a percentage of the initial cholesterol content and are the mean ± SD for triplicate dishes. The initial FC and EC contents of the THP-1 cells were as follows: 1) oxLDL FC = 93 ± 8, 2) oxLDL EC = 95 ± 6, 3) acLDL FC = 166 ± 16, and 4) acLDL EC = 63 ± 7 μg/mg cell protein. The initial FC and EC contents of the mouse macrophages were as follows: 1) oxLDL FC = 135 ± 15, 2) oxLDL EC = 42 ± 6, 3) acLDL FC = 112 ± 0, and 4) acLDL EC = 39 ± 7 μg/mg cell protein.

lyzed, and most of the [^{3}H]FC generated was available for release to oxLDL in the medium. However, after 6 h, oxLDL [^{3}H]CE accumulated in the cells, and only 23–27% of the total oxLDL [^{3}H]CE internalized was hydrolyzed. Furthermore, the additional [^{3}H]FC generated was unavailable for release into the medium. In contrast, at all times the majority (90–95%) of the acLDL [^{3}H]CE taken up by cells was hydrolyzed. The release of acLDL-derived [^{3}H]FC into the medium increased linearly over time, and after 24 h, no additional [^{3}H]FC accumulated in the cells. Similar results were observed when THP-1 macrophages were incubated with [^{3}H]cholesteryl linoleate labeled oxLDL and acLDL (data not shown), suggesting that the type of fatty acid does not affect lysosomal sequestration.

Next, we compared efflux of [^{3}H]FC with 2-OHρβ-CD from THP-1 and mouse macrophages loaded for 3 days with either [^{3}H]CE oxLDL or [^{3}H]CE acLDL. **Figure 4A** shows the distribution of [^{3}H]FC and [^{3}H]CE in cells and medium at the end of the 3 days of loading. As observed in the previous experiment, the majority (71%) of oxLDL [^{3}H]CE was not hydrolyzed, and only 6% of the total [^{3}H]cholesterol appeared in the medium during the 3 days of incubation with THP-1 cells. The bulk of acLDL [^{3}H]CE (89%) internalized by THP-1 cells was hydrolyzed, with most of the generated [^{3}H]FC appearing in the medium (63%). Similarly, mouse macrophages hydrolyzed 95% of the internalized acLDL [^{3}H]CE. Metabolism of oxLDL [^{3}H]CE by mouse macrophages was more sluggish when compared with hydrolysis of acLDL [^{3}H]CE, but mouse macrophages still hydrolyzed 65% of the internalized oxLDL [^{3}H]CE. Despite the difference in oxLDL CE hydrolysis between mouse and THP-1 macrophages, the [^{3}H]FC generated from oxLDL in mouse macrophages, like that in THP-1 cells, was less available for release into the medium during loading than the [^{3}H]FC derived from acLDL [^{3}H]CE.

The time course of [^{3}H]CE and [^{3}H]FC clearance from THP-1 and mouse macrophages to 2-OHρβ-CD is shown in Fig. 4B and C. The appearance of [^{3}H]FC in the medium is shown in Fig. 4D. Although none of the oxLDL [^{3}H]CE in THP-1 macrophages was hydrolyzed during the 7-h incubation with 2-OHρβ-CD, 40% of the acLDL [^{3}H]CE was hydrolyzed. Thus, of the total acLDL[^{3}H]CE internalized by THP-1 cells during loading, only 7% was resistant to hydrolysis. In contrast, 73% of internalized oxLDL [^{3}H]CE existed in a hydrolysis-resistant pool. In addition, during the 7-h incubation with CDs, only 44% of the initial oxLDL [^{3}H]FC present in THP-1 macrophages was released into the medium, whereas 86% of the acLDL [^{3}H]FC appeared in the medium (Fig. 4B). This suggests that [^{3}H]FC derived from oxLDL was not readily transported to the plasma membrane and available for release to the CDs, whereas most of the acLDL-generated [^{3}H]FC was transported to the plasma membrane. During the 7-h incubation, 63% and 18% of the total cellular [^{3}H]cholesterol appeared in the medium from acLDL- and oxLDL-treated THP-1 cells, respectively (Fig. 4D).

In mouse macrophages, this difference in clearance to 2-OHρβ-CD between [^{3}H]FC derived from oxLDL and that derived from acLDL [^{3}H]CE was not seen (Fig. 4C). Of the initial [^{3}H]CE, 77% and 54% remained in oxLDL- and acLDL-treated mouse macrophages, respectively. Thus, of the total [^{3}H]CE internalized during cholesterol loading, 27% of the oxLDL and 7% of the acLDL [^{3}H]CE was resistant to hydrolysis. Consistent with this difference in [^{3}H]CE hydrolysis clearance, 61% of the total cellular [^{3}H]cholesterol from acLDL, but only 42% of the oxLDL [^{3}H]cholesterol, was released into the medium (Fig. 4D).

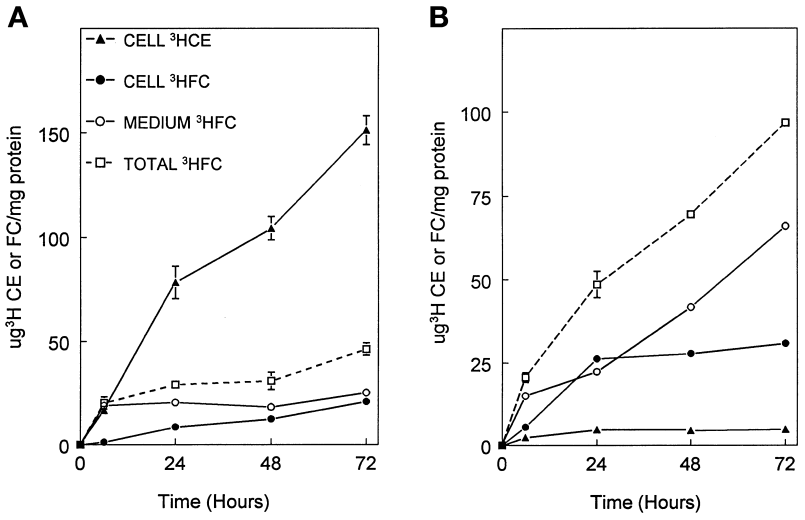

Fig. 3. Time courses of hydrolysis of oxLDL [^{3}H]CE and acLDL [^{3}H]CE in THP-1 macrophages. THP-1 macrophages were incubated for up to 3 days at 37°C in RPMI containing 50 ng TPA/ml, 1.5 μg CP113,818/ml, and 50 μg protein/ml of either human [^{3}H]CE-labeled oxLDL (A) or acLDL (B). At the indicated times, the cells were harvested and the [^{3}H]CE and [^{3}H]FC in both cells and medium were measured as described in Materials and Methods. The results are expressed as either μg [^{3}H]CE/mg cell protein or μg [^{3}H]FC/mg cell protein, and are the mean ± SD for triplicate dishes. The average total cpms/mg cell protein (cells [^{3}H]CE + [^{3}H]FC and medium [^{3}H]FC) after 3 days of incubation with [^{3}H]CE oxLDL or [^{3}H]CE acLDL were 1.6×10^6 and 1.2×10^6, respectively. After 3 days of treatment, the FC and EC contents of the [^{3}H]CE oxLDL-enriched cells were 81 ± 2 and 101 ± 11 μg/mg cell protein, respectively. The FC and EC contents of the [^{3}H]CE acLDL-treated cells were 56 ± 1 and 5 ± 1 μg/mg cell protein, respectively.

The studies presented in the previous figures suggest that the FC from oxLDL is not as readily available for release from cells as is acLDL FC. Next, we used fluorescence microscopy with filipin, a specific marker for FC, to demonstrate the presence of FC pools in oxLDL- and acLDL-treated cells (**Fig. 5**). The FC contents of cells after cholesterol enrichment and efflux are shown in **Table 1**. When THP-1 macrophages were loaded with oxLDL (FC content = 98 μg/mg cell protein), there was staining of both plasma membrane and large perinuclear pools (Fig. 5A). These perinuclear pools were confirmed by EM to be large, swollen, lipid lysosomes similar to those we have reported previously (21). In contrast, acLDL-treated cells (FC content = 74 μg/mg cell protein) exhibited substantial staining of plasma membrane and only minor staining of intracellular structures (Fig. 5B). The filipin staining of both plasma membranes and intracellular structures greatly diminished when acLDL cholesterol-enriched THP-1 macrophages were incubated with either FBS or CDs (Fig. 5D and F). In contrast, incubation of oxLDL cholesterol-enriched THP-1 cells with either FBS (Fig. 5C) or 2-OHρβ-CD (Fig. 5E) did not induce much diminution in fluorescence staining of perinuclear lysosomes. However, the FC content of both oxLDL- and acLDL-treated cells decreased similarly after incubation with either FBS or CDs (Table 1). In addition, only 27% of cell FC remained in oxLDL-enriched THP-1 cells after incubation with 2-OHρβ-CD. The fact that there was substantial staining of FC in large perinuclear pools after oxLDL-treated cells were incubated with CDs suggests that most of the small amount of FC remaining in the cells was localized in lysosomes.

oxLDL-treated mouse macrophages had some lysosomal fluorescence staining, confirmed by EM (Fig. 5G), although not as prominent as with oxLDL-treated THP-1 cells. The oxLDL-treated mouse macrophages initially contained 198 μg FC/mg cell protein; after incubation with 2-OHρβ-CD, 28% remained. However, unlike oxLDL-treated THP-1 macrophages, mouse macrophages showed minimal staining of FC in lysosomes after incubation with 2-OHρβ-CD (Fig. 5H). Therefore, like oxLDL CE, oxLDL FC is not as easily cleared from THP-1 cells as from mouse macrophages. Incubation of mouse or THP-1 cells in medium alone did not affect plasma membrane or intracellular staining.

DISCUSSION

During the early fatty streak phase of atherosclerosis, CE is stored in cytoplasmic inclusions of macrophages (1–3, 45, 46). However, as the disease advances from a fatty streak to a fibrous plaque, lipid is also stored in lysosomes (1–3, 45, 46). Our previous studies showed that lysosomal accumulation can be mimicked by loading of some macrophages with mildly oxLDL (21). THP-1 and pigeon macrophages accumulated substantial lysosomal stores, but mouse peritoneal macrophages stored only minor amounts of oxLDL cholesterol in lysosomes. The current studies extend these observations by examining lysosomal CE hydrolysis and the potential for oxLDL-derived lysosomal cholesterol to be cleared from THP-1 macrophages under conditions that are favorable for net efflux of cholesterol.

Clearance of acLDL and oxLDL cholesteryl ester

Normally, receptor-mediated uptake of LDL results in complete lysosomal hydrolysis of CE with the generated FC transported to the plasma membrane (28, 47, 48). Without FC acceptors, excess plasma membrane FC is transported to the endoplasmic reticulum, where it is re-esterified and stored in cytoplasmic CE droplets (47, 49). Our earlier studies showed that acLDL loading of THP-1 and mouse macrophages results in substantial cytoplasmic CE droplets (21). The current studies show that only a very minor pool of internalized acLDL CE (5–11%) is re-

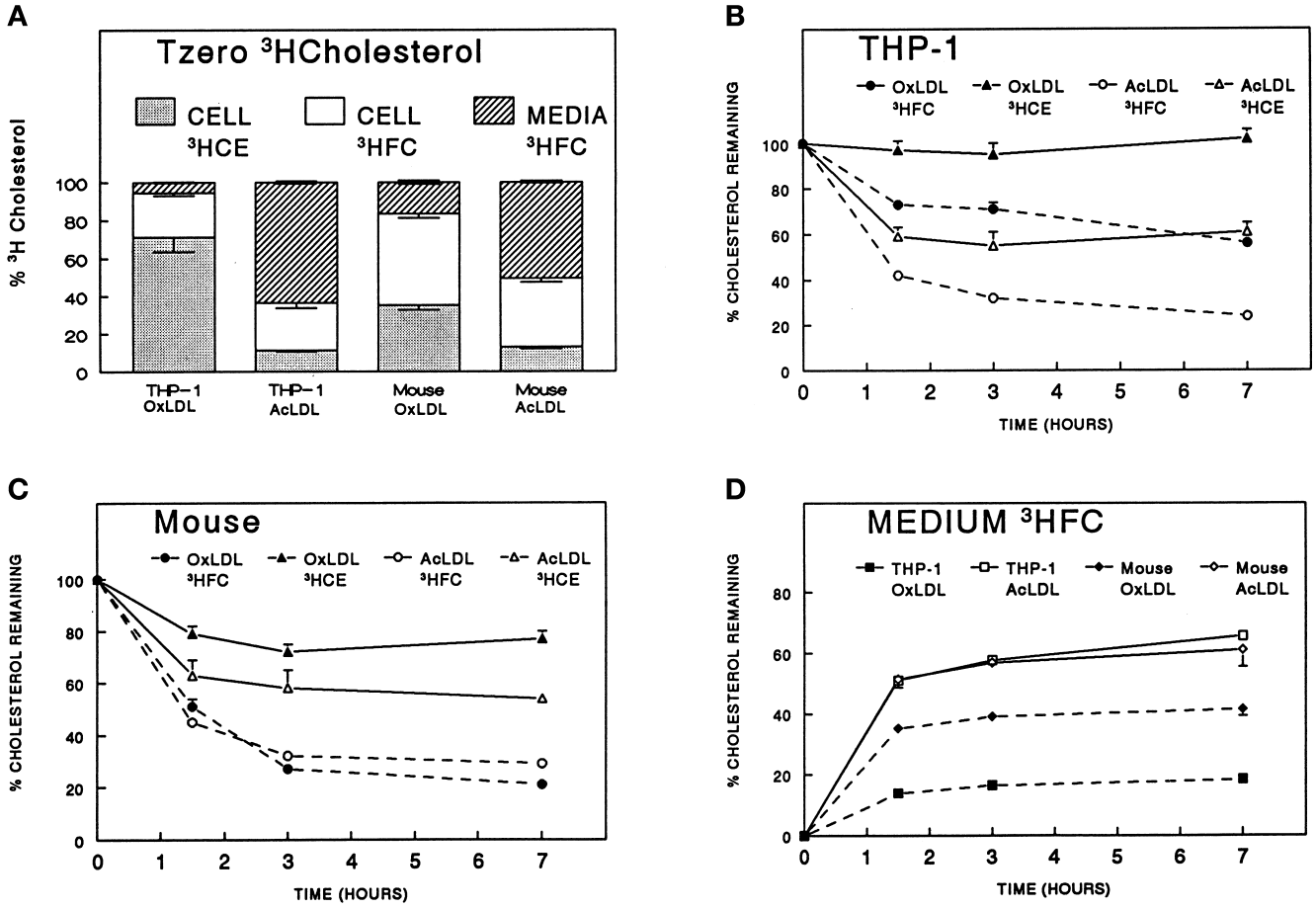

Fig. 4. Loading of THP-1 or mouse macrophages with [^{3}H]CE-labeled oxLDL or acLDL and efflux of the liberated [^{3}H]FC to 2-OHρβ-CD. THP-1 or mouse macrophages were incubated for 3 days at 37°C in medium containing 1.5 μg CP113,818/ml and 20 μg protein/ml of human [^{3}H]CE-labeled oxLDL or acLDL. After cholesterol loading, one set of cells was harvested and the [^{3}H]CE and [^{3}H]FC in both cells and medium were measured as described in Materials and Methods. After cholesterol loading, sets of THP-1 or mouse macrophages were harvested, and parallel sets were then incubated for up to 7 h in medium containing 1.5 μg CP113,818/ml and 15 mM 2-OHρβ-CD. After each incubation, the cells were harvested and [^{3}H]CE and [^{3}H]FC in both cells and medium were measured as described in Materials and Methods. A: The distribution of [^{3}H]CE and [^{3}H]FC in THP-1 or mouse macrophages and medium after 3 days of loading with [^{3}H]CE oxLDL or acLDL. B: The percent [^{3}H]CE or [^{3}H]FC remaining in THP-1 or (C) mouse macrophages during incubation with 2-OHρβ-CD. D: The percent [^{3}H]FC efflux ([^{3}H]FC cpms in medium/[^{3}H]FC and [^{3}H]CE in cells + [^{3}H]FC in medium) during incubation of THP-1 or mouse macrophages with 2-OHρβ-CD. In THP-1 cells, the average total cpms/mg cell protein (cells [^{3}H]CE + [^{3}H]FC and medium [^{3}H]FC) after 3 days of incubation with [^{3}H]CE oxLDL or [^{3}H]CE acLDL were 9.1×10^5 and 4.8×10^5, respectively. In mouse macrophages, the average total cpms/mg cell protein (cells [^{3}H]CE + [^{3}H]FC and medium [^{3}H]FC) after 3 days of incubation with [^{3}H]CE oxLDL or [^{3}H]CE acLDL were 19.6×10^5 and 3.7×10^5, respectively.

sistant to hydrolysis. In addition, FC generated from acLDL CE was readily released to acLDL (acting as a FC acceptor), suggesting that the FC is freely transported to the plasma membrane and available for efflux.

Unlike acLDL CE, a large pool of oxLDL CE was resistant to lysosomal hydrolysis in THP-1 cells and to a lesser extent in mouse macrophages. Seventy-three percent of the pool of internalized oxLDL CE in THP-1 macrophages remained undegraded in lysosomes, compared with only 27% in mouse macrophages. The mechanism of impaired oxLDL CE hydrolysis in THP-1 cells was independent of CE loading, because similar CE clearance was observed over a wide range of loading levels (16–215 μg CE/mg cell protein). In addition, the size of the hydrolysis-resistant pool increased over time, and the FC generated from oxLDL CE in THP-cells, unlike that generated from acLDL, was not readily transported to the plasma membrane and released. This increase in hydrolysis resistance is consistent with our earlier observation that FC initially builds up in lysosomes, but continued lipid accumulation produces lysosomal CE accumulation (21, 50).

There are many possible mechanisms for a hydrolysis-resistant pool of oxLDL CE. One is the retention of oxLDL in a prelysosomal compartment. The increasing size, over time, of the CE hydrolysis-resistant pool is consistent with this possibility. However, inconsistent with this possibility, our previous studies showed that most of the oxLDL lipid (70%) is localized to acid hydrolase-containing compartments (21), and recent studies of Lougheed et al. (51) demonstrated, both structurally and by cell fractionation,

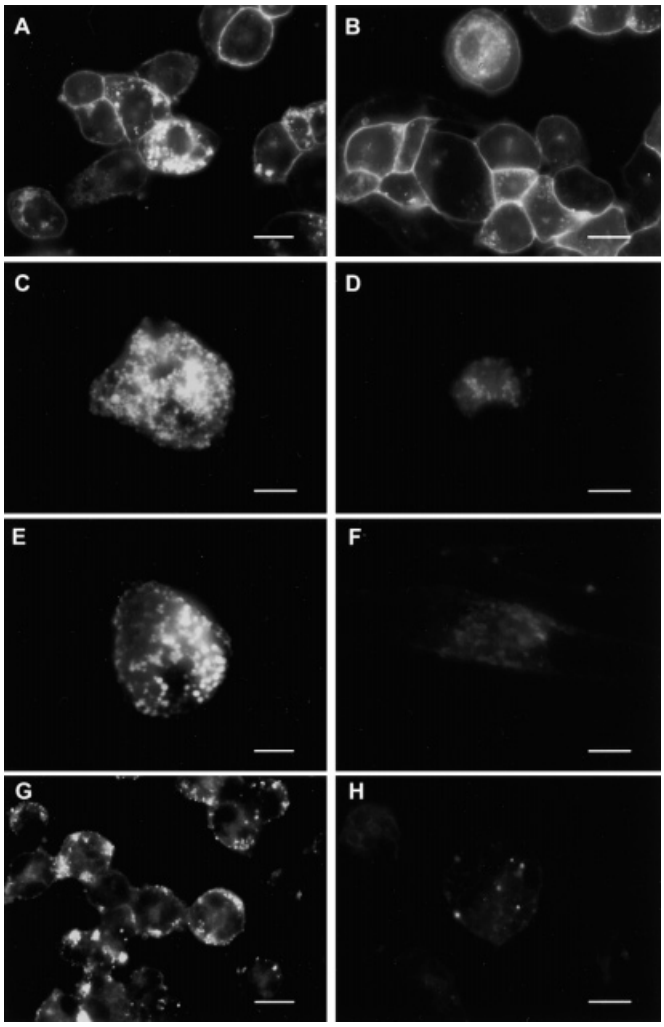

Fig. 5. Fluorescence photomicrographs of filipin-stained oxLDL or acLDL cholesterol-enriched THP-1 and mouse macrophages before and after incubation with FBS or 2-OHρβ-CD. THP-1 or mouse macrophages were cholesterol-enriched and treated with cholesterol acceptors as described in the legend of Table 1. After treatment, the cells were fixed and stained with filipin as described in Materials and Methods. All images were collected under the same conditions. There was no further manipulation of the images, except that contrast and brightness in panels F and H have been enhanced to display the very low levels of fluorescence. A: oxLDL cholesterol-enriched THP-1 macrophages. In addition to plasma membrane staining, bright fluorescence is seen in large perinuclear pools indicative of FC in lysosomes. Magnification = 400×, bar = 13 μm. B: acLDL cholesterol-enriched THP-1 macrophages. There is substantial staining of plasma membrane and only minor staining of intracellular structures. Magnification = 400×, bar = 13 μm. C: Substantial staining of FC in perinuclear pools remains in oxLDL cholesterol-enriched THP-1 cells after efflux to either FBS or (E) 2-OHρβ-CD. Magnification = 525×, bar = 10 μm. D: The fluorescence staining of both plasma membrane and intracellular structures is greatly diminished in acLDL-treated THP-1 cells after incubation with either FBS or (F) 2-OHρβ-CD. Magnification = 525×, bar = 10 μm. G: Filipin staining of oxLDL-enriched mouse macrophages. As with THP-1 cells, there is staining of FC in lysosomal perinuclear pools. Magnification = 400×, bar = 13 μm. H: In contrast to that in THP-1 macrophages, the staining of FC in lysosomes is greatly diminished after oxLDL-loaded mouse macrophages are incubated with 2-OHρβ-CD. However, in some cells, minor amounts of FC could still be seen in lysosomes. Magnification = 525×, bar = 10 μm.

TABLE 1. Cell FC contents before and after incubation with FBS or 2-OHρβ-CD

Foam Cell Type Initial FC Content	Cholesterol Acceptor	Cell FC Content After 8-h Efflux
μg/mg cell protein		*μg/mg cell protein*
THP-1 macrophages		
oxLDL: 98 ± 6	FBS	78 ± 3 (79)
	2-OHρβ-CD	26 ± 3 (27)
acLDL: 74 ± 1	FBS	49 ± 2 (67)
	2-OHρβ-CD	19 ± 0 (25)
Mouse macrophages		
oxLDL: 198 ± 3	2-OHρβ-CD	33 ± 2 (28)

THP-1 or mouse macrophages were cholesterol-enriched by incubation for 7 days at 37°C in medium containing 0.25% FBS and 150 μg of protein/ml of oxLDL or acLDL. After cholesterol loading, the cells were incubated for 7 h at 37°C in medium alone or with 10% FBS or 10 mM 2-OHρβ-CD. Shown are the cell free cholesterol (FC) contents after cholesterol enrichment and efflux. Values are the means ± SD for triplicate dishes. Shown in parentheses are the percent remaining values.

that oxLDL is trafficked to acidic compartments that have lysosomal enzyme activity. In addition, our recent studies show that colloidal gold-labeled oxLDL and acLDL are routed to lysosomes at similar rates and are extensively (60%) colocalized in the same lysosomes (P. G. Yancey and W. G. Jerome, unpublished observations).

Another possibility, consistent with a delay in inhibition of hydrolysis, is that fusion of vesicles carrying enzymes from the trans-golgi network (TGN) with lipid-containing lysosomes becomes compromised. Consistent with this, our previous studies demonstrated accumulation of acid phosphatase activity in the TGN of THP-1 and pigeon macrophages loaded with oxLDL (50). Also consistent with this hypothesis, Lougheed et al. (51) showed that oxLDL was localized to lysosomes with decreased density. This could be the result of both increased lipid accumulation and decreased fusion of hydrolase-containing vesicles with secondary lysosomes. Other studies have shown that fusion events are less likely to occur with lysosomes containing significant quantities of undigested material (52).

A final possibility, consistent with a delay in suppression of hydrolysis, is that oxidized lipids continue to accumulate until they reach a point that inhibits the acid lipase. Recent studies (53) showed that hydrolysis of unoxidized CE is compromised in mouse peritoneal macrophages incubated with mildly oxidized acLDL. It was proposed that this impairment resulted from accumulation of cholesteryl linoleate hydroperoxides that inhibit acid lipase (53, 54). In our current studies, mildly oxidized LDL, which should contain lipid hydroperoxides (13), was used. However, it is unclear why any of these proposed mechanisms would be more pronounced in THP-1 cells than in mouse macrophages, because in our studies, both were incubated with the same oxLDL. There must be other confounding factors to explain our results, such as differences in basal lysosomal function, retention of oxidized lipids, or additional oxidation of the oxLDL by THP-1 and mouse macrophages.

Removal of acLDL and oxLDL free cholesterol

There was substantial lysosomal sequestration of oxLDL FC in THP-1 macrophages, and some minor accumulation in mouse macrophages; this was not true of acLDL FC. Recent studies also showed that clearance of oxLDL FC is only minimally compromised, compared with that of acLDL FC, in mouse macrophages (55). Our current study extends these observations and shows that there is substantial lysosomal accumulation of oxLDL FC in THP-1 macrophages, but not in mouse macrophages, that remains sequestered in lysosomes even under conditions extremely favorable for net efflux of FC.

There are several possible mechanisms to explain the lysosomal sequestration of oxLDL FC. The CE that builds up in lysosomes could act as a sink for FC. Consistent with this, the differences in oxLDL CE hydrolysis between mouse and THP-1 cells paralleled the differences in lysosomal sequestration of oxLDL FC. A second possible mediator of lysosomal sequestration of FC is sphingomyelin. Indeed, studies by Maor, Mandel, and Aviram (17) demonstrated both FC and sphingomyelin buildup in lysosomes of oxLDL-treated J774 and human macrophages. These studies also showed that addition of 7-ketocholesterol to lysosomal extracts inhibits acid sphingomyelinase activity, resulting in accumulation of both FC and sphingomyelin. However, other factors would have to be considered to explain why such a mechanism would be more effective in THP-1 cells than in mouse macrophages. One possibility is that the lysosomal sequestration of 7-ketocholesterol is more pronounced in THP-1 macrophages than in mouse macrophages. Alternatively, the acid sphingomyelinase activity before oxLDL treatment could be different. Consistent with this, we have preliminary evidence that in mouse macrophages, not only is the basal activity of acid sphingomyelinase 7 times higher than in THP-1 cells, but the enzyme is not significantly down-regulated with oxLDL treatment (P. G. Yancey and W. G. Jerome, unpublished observations). In fact, the basal activity in THP-1 cells is similar to that in Nieman-Pick Type C cells, which also accumulate FC and sphingomyelin lysosomally (56).

Sphingomyelin accumulation in Nieman-Pick cells is due to inhibition of acid sphingomyelinase by lysosomal FC (56–59). In this regard, sequestration of oxLDL FC could be exacerbated and not readily reversed, as is suggested with our THP-1 cells incubated with 2-OHρβ-CD, in which most of cell FC was removed. It is also conceivable that lysosomal FC and sphingomyelin could affect fusion of lysosomes with vesicles and promote hypertrophy of TGN. It is worth noting that an abnormal, cholesterol-enriched TGN, as is seen in THP-1 cells, is also observed in Niemann-Pick cells (60). Recently, the lysosomal sequestration of FC in Nieman-Pick Type C cells was shown to result from a defect in the cholesterol trafficking protein, NPC-1 (61). Similarities between Nieman-Pick cells and oxLDL-treated cells raise the possibility that NPC-1 protein may also contribute to the lysosomal accumulation of oxLDL FC. Studies are ongoing in our laboratory to examine this possibility.

In summary, enrichment of macrophages with oxLDL promotes lysosomal cholesterol accumulation by both sequestration of FC and inhibition of oxLDL CE hydrolysis. In mouse macrophages, lipid sequestration is reversible, whereas in THP-1 cells, lysosomal FC and CE are trapped even in the presence of efficient FC acceptors. Thus, oxLDL enrichment of THP-1 macrophages mimics two important features of atherosclerotic foam cells: lysosomal lipid accumulation (1, 2) and irreversible sequestration (23).

These studies were supported by National Institutes of Health Grant RO1-HL49148 and Grant 97-FW05 from the North Carolina Affiliate of the American Heart Association. We thank the EM technologists, Mr. Ken Grant, Ms. Paula Moore, and Ms. Bilinda Dawson of Micromed, for their assistance. We also thank Ms. Tonya Walser for excellent technical assistance.

Manuscript received 6 April 2000 and in revised form 20 September 2000.

REFERENCES

1. Miller, B., and H. Kothari. 1969. Increased activity of lysosomal enzymes in human atherosclerotic aortas. *Exp. Molec. Pathol.* **10:** 288–294.
2. Jerome, W. G., and J. C. Lewis. 1985. Early atherogenesis in white carneau pigeons. II. Ultrastructural and cytochemical observations. *Am. J. Pathol.* **119:** 210–222.
3. Fowler, S., P. Berberian, H. Shio, S. Goldfischer, and H. Wolinsky. 1980. Characterization of cell populations isolated from aortas of rhesus monkeys with experimental atherosclerosis. *Circ. Res.* **46:** 520–530.
4. Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophage. *Ann. Rev. Biochem.* **52:** 223–261.
5. Steinberg, D., S. Parthasarathy, T. Carew, J. Khoo, and J. Witztum. 1989. Beyond cholesterol, modification of low-density lipoproteins that increase its atherogenicity. *New Eng. J. Med.* **320:** 915–924.
6. Steinbrecher, U., M. Lougheed, W. Kwan, and M. Dirks. 1989. Recognition of oxidized low density lipoprotein by the scavenger receptor of macrophages results from derivatization of apolipoprotein B by products of fatty acid peroxidation. *J. Biol. Chem.* **264:** 15216–15223.
7. Brown, A. J., and W. Jessup. 1999. Oxysterols and atherosclerosis. *Atherosclerosis.* **142:** 1–28.
8. Brown, A. J., S-L. Leong, R. T. Dean, and W. Jessup. 1997. 7-hydroperoxycholesterol and its products in oxidized low density lipoprotein and human atherosclerotic plaque. *J. Lipid Res.* **38:** 1730–1745.
9. Yla-Herttuala, S., W. Palinski, M. Rosenfield, S. Parthasarathy, T. Carew, S. Butler, J. Witztum, and D. Steinberg. 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* **84:** 1086–1095.
10. Yancey, P. G., and R. W. St. Clair. 1992. Cholesterol efflux is defective in macrophages from atherosclerosis-susceptible white carneau pigeons relative to resistant show racer pigeons. *Arterioscler. Thromb.* **12:** 1291–1304.
11. Bernard, D., A. Rodriques, G. Rothblast, and J. Glick. 1990. Influence of high density lipoprotein on esterified cholesterol stores in macrophages and hepatoma cells. *Arteriosclerosis.* **10:** 135–144.
12. Adelman, S. J., and R. W. St. Clair. 1988. Lipoprotein metabolism by macrophages from atherosclerosis-susceptible white carneau pigeons and resistant show racer pigeons. *J. Lipid Res.* **29:** 643–656.
13. Brown, A., R. Dean, and W. Jessup. 1996. Free and esterified oxysterol: formation during copper-oxidation of low density lipoprotein and uptake by macrophages. *J. Lipid Res.* **37:** 320–335.
14. Henriksen, T., E. Mahoney, and D. Steinberg. 1983. Enhanced

macrophage degradation of biologically modified low density lipoprotein. *Arteriosclerosis.* **3:** 149–159.
15. Klinkner, A., C. Waites, W. Kerns, and P. Bugelski. 1995. Evidence of foam cell and cholesterol crystal formation in macrophages incubated with oxidized LDL by fluorescence and electron microscopy. *J. Histochem. Cytochem.* **43:** 1071–1078.
16. Maor, I., and M. Aviram. 1994. Oxidized low density lipoprotein leads to macrophage accumulation of unesterified cholesterol as a result of lysosomal trapping of the lipoprotein hydrolyzed cholesteryl ester. *J. Lipid Res.* **35:** 803–819.
17. Maor, I., H. Mandel, and M. Aviram. 1995. Macrophage uptake of oxidized LDL inhibits lysosomal sphingomyelinase, thus causing the accumulation of unesterified cholesterol–sphingomyelin-rich particles in the lysosomes: a possible role for 7-ketocholesterol. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1378–1387.
18. Roma, P., A. Catapano, S. M. Bertulli, L. Varesi, R. Fumagalli, and F. Bernini. 1990. Oxidized LDL increase free cholesterol and fail to stimulate cholesterol esterification in murine macrophages. *Biochem. Biophys. Res. Comm.* **171:** 123–131.
19. Kritharides, L., W. Jessup, J. Gifford, and R. T. Dean. 1993. A method for defining the stages of low-density lipoprotein oxidation by the separation of cholesterol- and cholesteryl ester-oxidation products using HPLC. *Anal. Biochem.* **213:** 79–89.
20. Greenspan, P., H. Yu, F. Mao, and R. Gutman. 1997. Cholesterol deposition in macrophages: foam cell formation mediated by cholesterol-enriched oxidized low density lipoprotein. *J. Lipid Res.* **38:** 101–109.
21. Yancey, P., and W. Jerome. 1998. Lysosomal sequestration of free and esterified cholesterol from oxidized low density lipoprotein in macrophages of different species. *J. Lipid Res.* **39:** 1349–1361.
22. Phillips, M., W. Johnson, and G. Rothblast. 1987. Mechanisms and consequences of cellular cholesterol exchange and transfer. *Biochim. Biophys. Acta.* **906:** 223–276.
23. Jerome, W. G., and J. C. Lewis. 1990. Early atherogenesis in white carneau pigeons. Effect of a short-term regression diet. *Exp. Molec. Pathol.* **53:** 223–238.
24. Jerome, W. G., and C. Cash. 1995. Lysosomal lipid accumulation in atherosclerosis foam cells. *Molec. Biol. Cell.* **6:** 217a.
25. Badimon, J., L. Badimon, and V. Fuster. 1990. Regression of atherosclerotic lesions by high density lipoprotein plasma fraction in the cholesterol-fed rabbit. *J. Clin. Invest.* **85:** 1234–1241.
26. Yancey, P. G., W. V. Rodrigueza, E. P. C. Kilsdonk, G. W. Stoudt, W. J. Johnson, M. C. Phillips, and G. H. Rothblat. 1996. Cellular cholesterol efflux mediated by cyclodextrins. Demonstration of kinetic pools and mechanism of efflux. *J. Biol. Chem.* **271:** 16026–16034.
27. Adelman, S. J., J. M. Glick, M. C. Phillips, and G. H. Rothblat. 1984. Lipid composition and physical state effects on cellular cholesteryl ester clearance. *J. Biol. Chem.* **259:** 13844–13850.
28. Brown, M., S. Dana, and J. Goldstein. 1975. Receptor-dependent hydrolysis of cholesteryl esters contained in plasma low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **72:** 2925–2929.
29. Basu, S. K., J. L. Goldstein, R. Anderson, and M. S. Brown. 1976. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous hypercholesterolemia fibroblasts. *Proc. Natl. Acad. Sci. USA.* **73:** 3178–3182.
30. Barakat, H. A., and R. W. St. Clair. 1985. Characterization of plasma lipoproteins of grain- and cholesterol-fed white carneau and show racer pigeons. *J. Lipid Res.* **26:** 1252–1268.
31. Auwerx, J., and K. Schoonjans. 1991. New insights into apolipoprotein B and low density lipoprotein physiology; implications for atherosclerosis. *Acta. Clin. Belg.* **46:** 355–358.
32. Ishikawa, T. T., J. MacGee, J. A. Morrison, and C. J. Glueck. 1974. Quantitative analysis of cholesterol in 5 to 20 microliter of plasma. *J. Lipid Res.* **15:** 286–291.
33. Klansek, J. J., P. G. Yancey, R. W. St. Clair, R. T. Fischer, W. J. Johnson, and J. M. Glick. 1995. Cholesterol quantitation by glc: artifactual formation of short-chain steryl esters. *J. Lipid Res.* **36:** 2261–2266.
34. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
35. Bligh, E. G., and W. J. Dyer. 1959. A rapid method for total lipid extraction and purification. Protein measurement with the folin phenol reagent. *Can. J. Biochem. Physiol.* **37:** 911–917.
36. Gomori, G. 1952. Microscopic Histochemistry: Principles and Practice. University of Chicago Press, Chicago.
37. Weibel, E. R., G. S. Kistler, and W. F. Scherle. 1966. Practical stereological methods for morphometric cytology. *J. Cell Biol.* **30:** 23–38.
38. Mathur, S. N., F. J. Field, M. B. Megan, and M. L. Armstrong. 1985. A defect in mobilization of cholesteryl esters in rabbit macrophages. *Biochim. Biophys. Acta.* **834:** 48–57.
39. Ho, Y. K., M. S. Brown, and J. L. Goldstein. 1980. Hydrolysis and excretion of cytoplasmic cholesteryl esters by macrophages: stimulation by high density lipoprotein and other agents. *J. Lipid Res.* **21:** 391–398.
40. Ji, Y., B. Jian, N. Wang, Y. Sun, M. de la Llera-Moya, M. C. Phillips, G. H. Rothblat, J. B. Swaney, and A. R. Tall. 1997. Scavenger receptor B1 promotes high density lipoprotein-mediated cellular cholesterol efflux. *J. Biol. Chem.* **272:** 20982–20985.
41. Jian, B., M. de la Llera-Moya, Y. Ji, N. Wang, M. C. Phillips, J. B. Swaney, A. R. Tall, and G. H. Rothblat. 1998. Scavenger receptor class B type I as a mediator of cellular cholesterol efflux to lipoproteins and phospholipid acceptors. *J. Biol. Chem.* **273:** 5599–5606.
42. Rothblat, G. H., M. de la Llera-Moya, V. Atger, G. Kellner-Weibel, D. L. Williams, and M. C. Phillips. 1999. Cell cholesterol efflux: integration of old and new observations provides new insights. *J. Lipid Res.* **40:** 781–796.
43. Lawn, R. M., D. P. Wade, M. R. Garvin, X. Wang, K. Schwartz, and J. G. Porter. 1999. The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. *J. Clin. Invest.* **104:** R25–R31.
44. Kilsdonk, E. P. C., P. G. Yancey, G. W. Stoudt, F. W. Bangerter, W. J. Johnson, M. C. Phillips, and G. H. Rothblat. 1995. Cellular cholesterol efflux mediated by cyclodextrins. *J. Biol. Chem.* **270:** 17250–17256.
45. Goldfischer, S., B. Schiller, and H. Wolinsky. 1975. Lipid accumulation in smooth muscle cell lysosomes in primate atherosclerosis. *Am. J. Pathol.* **78:** 497–504.
46. Jerome, W. G., and J. C. Lewis. 1997. Cellular dynamics in early atherosclerotic lesion progression in white carneau pigeons. Spatial and temporal analysis of monocyte and smooth muscle invasion of the intima. *Arterioscler. Thromb. Vasc. Biol.* **17:** 654–664.
47. Brown, M., J. Goldstein, M. Krieger, Y. Ho, and R. Anderson. 1979. Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. *J. Cell Biol.* **82:** 597–613.
48. Neufeld, E. B., A. M. Cooney, J. Pitha, E. A. Dawidowicz, N. K. Dwyer, P. G. Pentchev, and E. J. Blanchette-Mackie. 1996. Intracellular trafficking of cholesterol monitored with a cyclodextrin. *J. Biol. Chem.* **271:** 21604–21613.
49. Brown, M. S., Y. K. Ho, and J. L. Goldstein. 1980. The cholesteryl ester cycle in macrophage foam cells. Continual hydrolysis and reesterification of cytoplasmic cholesteryl ester. *J. Biol. Chem.* **255:** 9344–9352.
50. Jerome, W., C. Cash, R. Webber, R. Horton, and P. Yancey. 1998. Lysosomal lipid accumulation from oxidized low density lipoprotein is correlated with hypertrophy of the golgi apparatus and trans-golgi network. *J. Lipid Res.* **39:** 1362–1371.
51. Lougheed, M., E. D. W. Moore, D. R. L. Scriven, and U. P. Steinbrecher. 1999. Uptake of oxidized LDL by macrophages differs from that of acetyl LDL and leads to expansion of an acidic endolysosomal compartment. *Arterioscler. Thromb. Vasc. Biol.* **19:** 1881–1890.
52. Montgomery, R. R., P. Webster, and I. Mellman. 1991. Accumulation of indigestible substances reduces fusion competence of macrophage lysosomes. *J. Immunol.* **147:** 3087–3095.
53. Kritharides, L., J. Upston, W. Jessup, and R. T. Dean. 1998. Accumulation and metabolism of low density lipoprotein-derived cholesteryl linoleate hydroperoxide and hydroxide by macrophages. *J. Lipid Res.* **39:** 2394–2405.
54. Maehira, F. 1994. Inhibitory effect of lipid hydroperoxide on cholesteryl esterases. *Biochem. Mol. Biol. Int.* **32:** 221–231.
55. Gelissen, I. C., K-A. Rye, A. J. Brown, R. T. Dean, and W. Jessup. 1999. Oxysterol efflux from macrophage foam cells: the essential role of acceptor phospholipid. *J. Lipid Res.* **40:** 1636–1646.
56. Pentchev, P., H. Kruth, M. Comly, J. Butler, M. Vanier, D. Wenger, and S. Patel. 1986. Type C Niemann-Pick disease: a parallel loss of regulatory responses in both the uptake and esterification of low density lipoprotein-derived cholesterol in cultured fibroblasts. *J. Biol. Chem.* **261:** 16775–16780.

57. Yoshikawa, H., and N. Sakuragawa. 1992. Removal of lipoprotein fraction from culture media corrects the reduction of acid sphingoyelinase activity induced by AY9944. *J. Inherit. Metab. Dis.* **15:** 155–156.
58. Maziere, J., C. Wolf, C. Maziere, L. Mora, G. Bereziat, and J. Polonovski. 1981. Inhibition of human fibroblasts sphingomyelinase by cholesterol and 7-dehydrocholesterol. *Biochem. Biophys. Res. Comm.* **100:** 1299–1304.
59. Liscum, L., and J. Faust. 1987. Low density lipoprotein (LDL)-mediated suppression of cholesterol synthesis and LDL uptake is defective in Niemann-Pick type C fibroblasts. *J. Biol. Chem.* **262:** 17002–17008.
60. Coxey, R. A., P. G. Pentchev, G. Campbell, and E. J. Blanchette-Mackie. 1993. Differential accumulation of cholesterol in Golgi compartments of normal and Niemann-Pick type C fibroblasts incubated with LDL: a cytochemical freeze-fracture study. *J. Lipid Res.* **34:** 1165–1176.
61. Carstea, E., J. Morris, K. Coleman, S. Loftus, D. Zhang, C. Cummings, J. Gu, M. Rosenfeld, W. Pavan, D. Krizman, J. Nagle, M. Polymeropoulos, S. Sturley, Y. Loannou, M. Higgins, E. Blanchette-Mackie, and P. Pentchev. 1997. Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. *Science.* **277:** 228–231.