Effects of Intracellular Free Cholesterol Accumulation on Macrophage Viability
A Model for Foam Cell Death


Abstract—This study was designed to identify cellular responses associated with free cholesterol (FC) accumulation in model macrophage foam cells. Mouse peritoneal macrophages (MPMs) or J774 macrophages were loaded with cholesteryl esters using acetylated LDL and FC/phospholipid dispersions and were subsequently exposed to an acyl coenzyme A:cholesterol acyltransferase (ACAT) inhibitor. This treatment produced a rapid accumulation of cellular FC. The FC that accumulated due to ACAT inhibition was more readily available for efflux to 2-hydroxypropyl-β-cyclodextrin (which removes cholesterol from the plasma membrane) than FC in untreated control cells. After a 3-hour exposure to an ACAT inhibitor, a significant increase in phospholipid synthesis was seen, followed by the leakage of LDH after 12 hours of treatment. We also observed, by electron and fluorescence microscopy, morphological indications of both apoptosis and necrosis in cells treated with an ACAT inhibitor. In addition, inhibition of ACAT for 48 hours resulted in the formation of FC crystals in MPMs but not in J774 cells. If compound 3β-[2-(diethylamino)ethoxy]androst-5-en-17-one (U18666A), which modulates intracellular trafficking of cholesterol, was added together with the ACAT inhibitor, each of the metabolic changes elicited by the accumulation of excess FC was either diminished or eliminated. The protective affect of U18666A was not due to a decrease in cellular FC concentrations, because cells treated with an ACAT inhibitor accumulated similar amounts of FC in the presence or absence of U18666A. Thus, treatment with U18666A results in the sequestering of FC in a pool that prevents it from causing various responses to FC deposition in macrophages. The metabolic changes that were produced when these model foam cells were treated with the ACAT inhibitor parallel the pathological events that have been shown to occur in the developing atherosclerotic plaque. (Arterioscler Thromb Vasc Biol. 1998;18:423-431.)

Key Words: cholesterol • macrophage • foam cell

It has been established that accumulation of excess FC is toxic to cells.1-2 Many cell types, including macrophages, have a mechanism to “detoxify” excess FC and store it as CE in cytoplasmic inclusions through the actions of ACAT.3 This CE may be hydrolyzed back to FC by a CE hydrolase, and if the cholesterol is not effectively removed by an acceptor, it is reesterified by the actions of ACAT, establishing a CE cycle.3,4 In vitro perturbation of the CE cycle in CE-loaded macrophages results in the accumulation of high levels of FC.1 This may be physiologically relevant because, in advanced atherosclerotic lesions, macrophages accumulate an abundance of FC as well as CE.5,6 Furthermore, endogenous ACAT inhibitors that could provoke such FC accumulation have been identified.7,8

Deposition of excess FC in macrophages has been shown to cause a number of cellular responses. Increased phospholipid synthesis, through the dephosphorylation of CTP:phosphocholine cytidylyltransferase, occurs as a result of loading macrophages with cholesterol using acLDL in the presence of an ACAT inhibitor.9 Tabas et al2 have suggested that this transient increase in phospholipid synthesis may be an adaptive response to accommodate excess FC; failure of this response results in cellular toxicity. An increase in toxicity also occurs in CE-loaded macrophages treated with an ACAT inhibitor.9 This toxicity is eliminated when cells are cotreated with the cholesterol transport inhibitor U18666A, even though similar intracellular concentrations of FC are reached in both treatments.

We have extended these studies to identify additional responses to increased levels of cellular FC and to gain a better understanding of the events that occur leading to macrophage death. The effect of U18666A cotreatment on these responses was also evaluated.

Methods

Materials
FBS, BSA (essentially fatty acid free), gentamicin, unesterified cholesterol (FC), cholesteryl methyl ether, sodium pyruvate, and NADH
were purchased from Sigma Chemical Co. Organic solvents were products of Fisher Scientific. Tissue culture flasks and plates were obtained through Corning or Falcon. [1,2-3H]Cholesterol and [meth-yl-H]cholesterol chloride were purchased from New England Nuclear. Tissue culture medium was obtained from GIBCO. PC was purchased from Avanti Polar Lipids. FC and PC dispersions were made by the method of Arhagast et al.10 Human LDL (1.019 g/mL < d < 1.063 g/mL) was fractionated by sequential ultracentrifugation11 and acetylated according to Basu et al.12 ACAT inhibitors Sandoz 58035 and 113–818 were gifts from Sandoz and Pfizer Pharmaceuticals, respectively. 2OH–βCD was a gift from Crestar (Hammond, Ind). Compound U18666A was a gift from the Upjohn Corp (Kalamazoo, Mich).

**Cell Culture**

MPMs were prepared as previously described.1 J774 macrophages were routinely grown in RPMI 1640 medium containing 50 mmol/L HEPES buffer and 50 μg/mL gentamicin (RPMMI) and supplemented with 10% FBS. To cholesterol-load the macrophages, RPMI containing 1% FBS, acLDL (100 μg/mL of protein per milliliter), and FC/PC dispersions10,13 (250 μg/mL of FC per milliliter) were added to the incubation media for 48 hours. Monolayers were then washed three times with MEM containing 2 g/L sodium bicarbonate and 50 μg/mL gentamicin. Monolayers were equilibrated in RPMI containing 0.2% BSA for 18 hours. After this period, cells were ready to be incubated with an ACAT inhibitor (58035 or 113–818, 2 μg/mL), or a combination of an ACAT inhibitor and U18666A (2 μg/mL each). The ACAT inhibitors 113–818 and 58035 were used interchangeably, since preliminary studies demonstrated that there was no difference in efficacy between the two compounds at 2 μg/mL (data not shown). Control incubations contained RPMI with 0.2% BSA. Cells were incubated in a humidified atmosphere containing 95% air/5% CO2 at 37°C.

**Protein Determination**

After lipid extraction, cell monolayers were solubilized in SDS, and protein was determined by a Lowry assay, as modified by Markwell et al.14

**Measurement of [3H]Choline Incorporation**

Monolayers of J774 macrophages treated with the compounds indicated above were pulsed for 1 hour with 2 μCi/mL [3H]choline chloride in the appropriate treatment medium. Cell lipids were extracted with isopropanol and reextracted two times by the method of Bligh and Dyer15 to ensure removal of free choline. Total radioactivity was determined in the lipid extract using a liquid scintillation counter (model LS 3801, Beckman Instruments Inc). [3H]Choline incorporation into phosphatidylcholine was calculated by measuring H label incorporated into a total lipid extract during a 1-hour pulse. In most experiments control incubations showed no increase in phospholipid synthesis. However, in some experiments the control incubations showed a modest transient increase in phospholipid synthesis. Thus, the data presented in Fig 3 were expressed as a percentage of the control for each experiment.

**Measurement of Cell Toxicity and Apoptosis**

Measurement of cellular toxicity was evaluated by the release of LDH into the extracellular medium. Aliquots (150 μL) of experimental media were removed from the wells at the times indicated, after which the samples of the media were filtered (Multiscreen filtration system, Millipore Corp) and incubated at room temperature for 1 hour in the presence of 0.97 mmol/L sodium pyruvate, 0.21 mmol/L NADH, and 0.1% Triton X-100.16 Decreases in absorbance relative to a negative control (without NADH) were measured at 340 nm. The LDH data were expressed as a decrease in absorbance at 340 nm as an indication of the presence of LDH activity in the extracellular medium. Acridine orange staining of cell nuclei was used to assess apoptosis.17 In this assay, cells were grown on culture slides, treated, and then fixed and dried after a 20-hour incubation with the test media. Cells were rehydrated in PBS for 10 minutes and incubated in acridine orange at 10 μg/mL for 5 minutes. Slides were washed briefly in water and coverslipped with Slow Fade Light (Molecular Probes). Apoptosis was visualized by fluorescence microscopy with a narrow-band blue filter. Cells positive for apoptotic nuclei were counted in 10 random fields at ×400 magnification. Cells cultured in 0.2% BSA served as the control group. The percentage of cells exhibiting apoptosis and values for treated monolayers compared with control monolayers were determined. Counts were performed by two independent investigators, and the results reported are the average of the two.

**Cellular Cholesterol Quantification**

J774 macrophages were treated as described under cell culture. To stop the incubation, the treatment medium was removed, and the monolayer was washed three times with cold PBS. The lipids were extracted from the monolayers with isopropanol with the addition of cholesterol methyl ether as an internal standard. Unesterified and total cholesterol was quantified using gas-liquid chromatography as previously described.18 An increase in FC mass in treated monolayers is expressed as a percent change relative to the FC mass in untreated normal control cells at each designated time point.

**Crystal Isolation and Identification**

FC crystals were isolated from monolayers of MPMs by ultracentrifugation. Cells were scraped into 0.25 mol/L sucrose containing 10 mmol/L Tris-HCl and 1 mmol/L EDTA. The cells were then layered over the 0.25 mol/L sucrose buffer to which 10% Percoll had been added, and PBS was layered on top of the cell fraction. The gradient was centrifuged for 45 minutes at 13,000 rpm in an SW40Ti rotor (Beckman). The crystals banded at a density of 1.02 g/mL.

The crystals isolated from the gradient described above were first examined by polarizing light microscopy and were observed to have the morphology of cholesterol monohydrate.19-21 A slurry of crystals was placed in two 0.6-mL Qualitron microcentrifuge tubes and spun at 6000 rpm for 3 hours. The sediment was aspirated and placed in standard 1-mm quartz x-ray diffraction tubes and gently centrifuged in a special adapter for x-ray capillaries to sediment the crystals in the tube. After centrifugation, the tubes were observed under a dissecting microscope using polarizing optics, and when the crystals were adequately concentrated in the tube, they were adjusted to be in the beam of the x-ray diffractometer and irradiated for 24 hours, using an Ellicott GX6 rotating anode x-ray generator producing a nickel-filtered Cu alpha radiation (wavelength, 0.15418 nm). Diffraction patterns were recorded on photographic film.

**Electron Microscopy**

MPMs for electron microscopy were grown on Formvar (Montanto Co)—coated coverslips. At the times indicated in the results, cells were washed with 0.1 mol/L sodium cacodylate (pH 7.4) and then fixed with glutaraldehyde diluted to 4% with cacodylate buffer. After fixation, the cells were washed in cacodylate, postfixed in 1% osmium tetroxide in 0.1 mol/L cacodylate buffer, and washed again in cacodylate buffer. After the cell were washed, the Formvar was gently removed from the coverslip. The cells were progressively dehydrated in a graded series of ethanol/water washes and embedded in Spurr’s resin (Polysciences) by transferring the Formvar sheet containing the
cells between the various solutions. The use of Formvar provided a substrate that was easy to section and allowed us to gently dehydrate and embed the cells without the need to either scrape the cells from a plastic dish or pellet the cells during embedding. Embedded preparations were thin-sectioned (60 to 80 nm) and viewed at 80 keV using a Philips 400 transmission electron microscope.

Microscopic quantification of cell death is problematic since the resulting cell debris could be formed from a few or many cells. Consequently, a rapid semiquantitative scheme was used to determine differences between treatment conditions. Sections were made from three different levels within the embedded block for each condition. Fifteen fields were analyzed from each level (45 total fields). The grid bars of the 200 mesh grid were used as the boundaries of the field. Thus, each field represented an area of 0.03 mm². The fields were chosen using a computer-generated randomization scheme. Dying cells or cell debris was taken as evidence of cell death in the field. Cell debris could be either extracellular or within phagocytic vacuoles of adjacent macrophages. The percentage of fields containing evidence of cell death was used as an estimate of the extent of death occurring in the sample. As a test of how well the sampling scheme described the total sample, the ACAT-inhibited sample taken at 12 hours was resectioned and quantified using the same sampling scheme. The second result was within 2% of the initial result.

**Efflux of FC to Cyclodextrins**

J774 macrophages were plated in 12-well dishes and loaded for 48 hours with acLDL (100 μg of protein per milliliter), FC/PC dispersions (250 μg of FC per milliliter), and 2 μCi/mL [3H]cholesterol. After this loading and labeling period, monolayers were incubated for 18 hours in RPMI containing 0.2% BSA to allow for equilibration of the FC and EC pools. Cells were then treated for 24 hours in RPMI containing 0.2% BSA and supplemented with one of the following: an ACAT inhibitor, U18666A, a combination of an ACAT inhibitor and U18666A, or nothing (control). Initial cholesterol measurements were made at the time of the addition of these compounds. One set of monolayers (time zero) was washed three times with cold PBS and extracted with isopropanol. Total radioactivity was determined in the lipid extract. FC was separated from EC by thin-layer chromatography and expressed as a percentage of the total radioactivity. Other sets of monolayers were washed three times with MEM HEPES and placed in efflux media. The efflux media consisted of MEM HEPES containing 100 mmol/L of 2OH-CD, which was 50% saturated with FC. To measure efflux of cellular cholesterol, the media were removed at desired times, and an aliquot was saved to determine radioactivity. Cholesterol efflux was determined by dividing the FC [3H]counts in the medium after efflux by the amount that was originally in the cell at time zero. The GraphPad Prism 2.28 software (version 2.0, GraphPad Software Inc) was used to analyze the efflux kinetics. The data were fitted to a biexponential equation by nonlinear regression as described previously. The equation used describes efflux from two pools, assuming that all of the cholesterol is available for efflux. A and B are the fractional sizes of the independent pools 1 and 2, respectively. The apparent rate constants for fractional efflux from pools 1 and 2 are $k_1$ and $k_2$, respectively. The apparent half-times were calculated as $t_{1/2} = \ln2/k$.

**Statistical Analysis**

Values are expressed as mean±SD unless otherwise stated. Student’s t test was used to determine statistical differences between treatments and controls. The criterion for significance was set at $P<.05$. Calculations were performed using the SigmaStat statistical program (Jandel Corp).

**Results**

**Accumulation of FC**

Previous studies reported that CE-loaded MPMs that were exposed to an ACAT inhibitor for 24 hours showed signs of toxicity and that this toxicity was attenuated when cells were cotreated with compound U18666A.1 The first part of the present study was designed to address the possibility that compound U18666A causes a lag in the accumulation of FC in CE-loaded cells that have been ACAT-inhibited. If compound U18666A causes slow hydrolysis of stored CE within a cell, the toxicity associated with excess FC accumulation may not be apparent within the time frame used in the previous study.1

Loading J774 macrophages for 48 hours with acLDL and FC/PC dispersions resulted in an 18-fold increase in the amount of total cholesterol in the cells (29±1 μg total cholesterol per milligram protein) compared with unloaded macrophages (17±1 μg total cholesterol per milligram protein). Fig 1 illustrates that subsequent inhibition of ACAT caused a twofold increase in the amount of FC in the cells after 48 hours of treatment; cells cotreated with an ACAT inhibitor plus U18666A accumulated similar amounts of FC. The level of FC in untreated CE-loaded J774 macrophages remained constant over 48 hours (data not shown).

**Cellular Toxicity**

Release of LDH from cells was used to measure toxicity. There was no toxicity associated with any treatment up to 6 hours in CE-loaded J774 macrophages (Fig 2). The cells incubated with an ACAT inhibitor had a significant release of LDH into the extracellular medium after 12 and 24 hours of treatment. There...
was no significant LDH release in monolayers cotreated with an ACAT inhibitor plus U18666A or with U18666A alone or in control incubations.

**Rate of Phospholipid Synthesis**

Tabas et al demonstrated that increased phospholipid synthesis is a mechanism cells use to detoxify excess FC. It is believed that as FC accumulates in cells, phospholipids are synthesized to accommodate excess sterol. One possible explanation for the lack of toxicity in cells cotreated with an ACAT inhibitor plus U18666A is that U18666A further stimulates phospholipid synthesis so that excess FC is more efficiently solubilized. Fig 3 demonstrates that when CE-loaded J774 macrophages were exposed to an ACAT inhibitor, the rate of phospholipid synthesis increased within 3 hours of the addition of the ACAT inhibitor. The rate of phospholipid synthesis continued to rise over time, resulting in a fivefold increase by 12 hours compared with CE-loaded untreated control cells. Rather than further stimulating phospholipid synthesis, the addition of U18666A together with the ACAT inhibitor blunted the FC-stimulated increase in phospholipid synthesis. As can be seen in Fig 3, there was a moderate increase in phospholipid synthesis in monolayers cotreated with an ACAT inhibitor plus U18666A starting at 6 hours, producing a twofold increase at 12 hours. Exposure to U18666A alone did not have any effect on the rate of phospholipid synthesis (data not shown).

**Apoptosis**

In our experimental system, there is clear evidence (release of LDH and changes in cell morphology) indicating cellular necrosis after 24 hours of treatment with the ACAT inhibitor 113–818. However, unique morphological changes suggesting apoptosis were also evident in the ACAT–inhibited cells during the first 20 hours of treatment. In MPMs after 12 hours of treatment with an ACAT inhibitor, membrane blebs and condensed cytosol were seen in the cells by light microscopy (data not shown). In addition, loaded MPMs exposed to the ACAT inhibitor for 20 hours showed a significant increase over control MPMs in nuclear condensation (Fig 4). Acridine orange staining revealed highly condensed chromatin that was uniformly stained. In contrast, viable cells showed variations in the chromatin staining pattern, reflecting the distribution of euchromatin and heterochromatin. The majority of cells positive for apoptosis had condensed nuclei, with very few cells containing apoptotic bodies. There was a smaller increase in nuclear condensation in the cells cotreated with U18666A and an ACAT inhibitor. Cells treated with U18666A alone had less nuclear condensation than the untreated control cells (Fig 4). In a more detailed study, using electron microscopy, the morphol...
Figure 5. Electron micrographs depicting the effect of ACAT inhibition and cotreatment with U18666A on MPMs. a, Necrotic cells from culture treated for 12 hours with both 58035 and U18666A. The cell at the top shows a late stage of necrosis. The cell is vacuolated, the plasma membrane is no longer intact, and the nucleus shows clear evidence of chromatin clumping along the periphery of the nucleus. The cell at the bottom shows a much earlier stage of necrosis. There is minor swelling of the cytoplasm, giving the cell an open, clear appearance. Mitochondrial organization has been lost (arrowheads), but the nucleus appears fairly normal. Magnification ×5000. Bar=1 μm. b, Necrotic cell from culture treated for 24 hours with 58035. Membrane integrity has been lost, and cellular organelles are swollen. The chromatin in the nucleus is aggregated (arrowheads), and the mitochondria are no longer distinguishable. Pieces of cellular debris have been phagocytized by one of the adjacent cells (arrow). Magnification ×8000. Bar=0.75 μm. c, Cells from culture treated with 58035 plus U18666A. Most cells appear normal. However, an apoptotic cell is present on the right. The plasma membrane is intact, and the cell is distinguished by the characteristic nuclear condensation and fragmentation (arrow) as well as the vacuolization of the cytoplasm. Adjacent cells have phagocytized apoptotic bodies (arrowhead) and “apoptotic nuclear ghosts” (asterisk). Magnification ×4000. Bar=1 μm. d, Cells from culture treated with 58035 for 12 hours. Except for the two cells at the bottom, all the cells in the field show evidence of cell death. The central cell (asterisk) shows condensation of the cytoplasm and condensation and fragmentation of the nucleus characteristic of apoptosis. The cells to the right and left of this apoptotic cell show vacuolization, loss of mitochondrial architecture, and disruption of the plasma membrane. These characteristics indicate a more necrotic type of cell death. The bottom two relatively normal-appearing cells have phagocytized cellular debris and “nuclear ghosts.” Magnification ×3500. Bar=2 μm. e, Cell from culture incubated for 6 hours with 58035. This cell shows minor chromatin clumping and cytoplasmic vacuolization, but it still phagocytized an apoptotic body (arrowhead). Magnification ×10 500. Bar=1 μm.

particularly at 12 and 24 hours, had many fields with only a few viable cells left (Fig 5d). The remainder of the field was occupied by dying cells or cell debris. In contrast, in U18666A-cotreated cultures most fields with dying cells had only one or two such cells with minimal debris (Fig 5c).

**Cholesterol Efflux**

To further investigate the possibility that U18666A sequesters FC in a subcellular location that prevents it from eliciting the effects associated with FC accumulation, we investigated the efflux of cholesterol to 2OH-βCD. Normally, cholesterol efflux from cells exposed to cyclodextrin indicates the presence of fast and slow pools of plasma membrane cholesterol. 20 The effects of an ACAT inhibitor and U18666A on cholesterol efflux from J774 macrophages were examined (Fig 6). The half-time of the fast pool in J774 macrophages was 2 to 3 minutes and was not changed under any of the incubation conditions (Fig 6). In addition, the sizes of the fast and slow pools both remained at 50±3%. However, efflux was enhanced when cells were treated with an ACAT inhibitor. The monolayers cotreated with U18666A plus an ACAT inhibitor and the cells treated with U18666A alone had efflux kinetics similar to the control cells.

**FC Crystal Formation**

During a prolonged incubation of CE-loaded MPMs with an ACAT inhibitor, the formation of intracellular crystals was observed (Fig 7). Crystals appeared in plate and elongated ("rod") forms, and they started to appear after 48 hours in the ACAT inhibitor–treated cells. The crystals were isolated from the macrophages by ultracentrifugation as described in “Methods.” The diffraction pattern of the concentrated crystals after 24 hours clearly indicated the presence of 3.42-nm and 1.67-nm spacings, indicative of the 001 and 002 spacings of cholesterol monohydrate. A short 0.57-nm spacing characteristic of cholesterol monohydrate was also present. These data demonstrate that the crystals are cholesterol monohydrate. There was no crystal formation in the other treatment groups at 48 hours. After 72 hours of treatment, there were many crystals in the cells treated with the ACAT inhibitor, whereas the cells treated with an ACAT inhibitor plus with U18666A had only a few crystals. No crystals were evident in control conditions (Fig 6).

![Figure 6. FC efflux from J774 macrophages to cyclodextrins. J774 macrophages were loaded with [3H]cholesterol and treated as described in Fig 1, but 113–818 was used instead of 58035 for 24 hours. Efflux medium containing 100 mM of 2OH-βCD was applied for up to 1.5 hours. Cholesterol efflux was determined by dividing the FC [H] counts in the medium after efflux by the amount that was originally in the cell at time zero. The half-time of the fast pool in J774 macrophages was 2 to 3 minutes and was not changed under any of the incubation conditions (Fig 6). In addition, the sizes of the fast and slow pools both remained at 50±3%. However, efflux was enhanced when cells were treated with an ACAT inhibitor. The monolayers cotreated with U18666A plus an ACAT inhibitor and the cells treated with U18666A alone had efflux kinetics similar to the control cells.](image-url)
monolayers or cells treated with U18666A alone. There was no evidence of crystal formation in J774 macrophages incubated under similar conditions.

**Discussion**

The presence of CE-loaded macrophages, known as foam cells, is the hallmark of the atherosclerotic plaque.23,24 The development of a macrophage into a foam cell is promoted, at least in part, by the unregulated receptor-mediated uptake of lipoproteins.25,26 The excess FC delivered to a cell by this process is subsequently esterified by ACAT, with the excess FC stored in cytoplasmic inclusions as CE.25,26 Under these conditions, if appropriate cholesterol acceptors are present, the macrophages remain viable and functional, with the stored CE being hydrolyzed to FC by the action of neutral CE hydrolases and the newly generated FC being removed from the cells.27–29 If FC is not removed by an acceptor, it becomes reesterified by ACAT, thus establishing a CE cycle.3,4 However, within the atherosclerotic lesion this protective homeostatic mechanism does not always operate efficiently, and there is often death of macrophage-derived foam cells, accompanied by production of cellular debris, including FC and CE.4,28,30 This process of cell death with the production of cell debris leads to the development of the necrotic core of the atherosclerotic plaque.31 Such lipid-rich plaques are unstable and responsible for the majority of cardiovascular events.32–34 Because of the key role played by both cholesterol deposition and cell death in the development of the plaque, we1,35 and others3,9,36 have used macrophages in culture to establish in vitro experimental conditions that reproduce the sequence of metabolic events that could occur within the developing atherosclerotic lesion. These previous studies have indicated that the cellular accumulation of excess FC might serve as the trigger for cell death.1,2 In the present investigation, we have extended our studies of the cellular events occurring when macrophages accumulate excess FC.

Two general experimental approaches have been used to supply excess FC to macrophages in culture. In the first approach, best represented by the studies of Tabas et al.,2 macrophages are incubated in the presence of acLDL, and the excess FC is directly generated by the uptake of LDL cholesterol and subsequent lysosomal processing of the lipoprotein. The experimental approach we have used has been to preload the macrophages with cholesterol, primarily in the form of CE, and then to generate excess FC by inhibiting ACAT while the cells are maintained in a cholesterol acceptor–free medium. In this approach, the FC is produced from the CE stored in lipid inclusions in these model foam cells. Both experimental protocols have clearly demonstrated that the accumulation of excess cell FC leads to cell toxicity.1,2 In addition, we have demonstrated that the toxic effect of FC can be eliminated, or moderated, by the treatment of the macrophages with compounds such as U18666A, which have been shown to affect intracellular cholesterol transport.37 In the present study, we have extended these observations and established an experimental time line illustrating some of the metabolic responses produced on FC accumulation (Fig 8). The events that we have observed are consistent with the changes that occur in the developing atherosclerotic plaque.24,31,32,38,39

**Cholesterol Accumulation and Phospholipid Synthesis**

To establish the relationship between FC accumulation and subsequent metabolic changes in the macrophages, we determined the time course of FC accumulation in the presence of an ACAT inhibitor, with and without cotreatment with U18666A. In contrast to other studies,2,40 we are initiating the accumulation of excess FC in cells that have already been enriched in cholesterol. Thus, the inhibition of ACAT produces a rapid increase in FC during the first 12 hours of exposure, resulting in a sevenfold enrichment in FC content.
compared with unloaded J774 macrophages. Importantly, the accumulation of FC in the cultures treated with both the ACAT inhibitor and U18666A is similar. Therefore, the protective effect produced by U18666A cannot be attributed to an inhibition of FC accumulation.

Previously, Shiratori et al. demonstrated that phospholipid synthesis increases when FC accumulates within a cell. It has been proposed that this newly synthesized phospholipid serves as a cellular reservoir that solubilizes excess FC. Consistent with this model are the early observations of McGookey and Anderson demonstrating the appearance of myelin bodies in model foam cells. In the present study, there was a rapid response in the rate of phospholipid synthesis on the addition of the ACAT inhibitor, with a significant increase demonstrated after only 3 hours of ACAT inhibitor treatment. Since it is believed that increased phospholipid synthesis serves to protect the cells from excess FC, it is possible that the protection produced by treatment with U18666A is a result of further stimulation of phospholipid synthesis. However, the data in Fig 3 illustrate that this is not the case: rather than stimulating phospholipid synthesis, the cotreatment with U18666A actually attenuates the enhanced phospholipid synthesis provoked by excess FC.

**Cholesterol Efflux**

To investigate the distribution of the excess FC among cellular pools, we examined the kinetics of FC efflux to cyclodextrins. Previous studies indicated that cells exhibit two pool kinetics of efflux when cyclodextrin is used as the extracellular acceptor. At the present time we do not know the precise location of the cholesterol in these kinetic pools. We believe that the fast pool represents plasma membrane cholesterol, whereas the location of the slow pool remains to be determined. Although the location of these pools needs to be established, any change in the efflux kinetics of cholesterol from cells treated with an ACAT inhibitor or U18666A would be consistent with a redistribution of intracellular FC. Indeed, this is the case, since the kinetic data presented in Fig 6 indicate that by 24 hours ACAT inhibition changes the cellular distribution of FC and that the presence of U18666A prevents this change. Further experiments will be required to establish whether the pool(s) of excess cholesterol that induces cell death is exclusively in the plasma membrane.

**Cell Death and Apoptosis**

The cellular changes that we observed after the addition of an ACAT inhibitor, ie, nuclear condensation, membrane blebs, and condensed cytosol, are indications of apoptosis. These cellular changes were not evident in the monolayers treated with U18666A plus an ACAT inhibitor or with U18666A alone or in the control cells. Cell death is not easy to quantify except in synchronized cultures; thus, elaborate schemes have been devised to precisely quantify the extent of cell death. In the present study, however, such precision was not warranted, since our semiquantitative scheme was sufficient to highlight clearly that cell death was more prevalent in ACAT-inhibited cultures than in U18666A-treated cultures. Knowledge of cell death has greatly expanded in the last 5 years, and the previous strict distinction between apoptosis and necrosis is becoming blurred. At the biochemical level, it now appears that some forms of both apoptosis and necrosis can use similar pathways. However, the basic distinction that apoptosis is an energy-requiring process whereas necrosis is due to loss of the capacity of the cell to generate energy remains important for analyzing the initial injury that induces cell death. In this regard, morphological evidence can be important for confirming the presence of key traits distinguishing necrosis from apoptosis. In the present study, the cellular appearance indicated that both necrosis and apoptosis were occurring in the same culture. It is not clear why both types of cell death were observed, although necrotic cell death secondary to apoptosis is known to occur. It may be that apoptosis was inducing necrosis in adjacent cells. Alternatively, necrosis could have been initiated by stimuli unrelated to those inducing apoptosis. Previous studies indicated that cells exhibit two pool kinetics of efflux when cyclodextrin is used as the extracellular acceptor. At the present time we do not know the precise location of the cholesterol in these kinetic pools. We believe that the fast pool represents plasma membrane cholesterol, whereas the location of the slow pool remains to be determined. Although the location of these pools needs to be established, any change in the efflux kinetics of cholesterol from cells treated with an ACAT inhibitor or U18666A would be consistent with a redistribution of intracellular FC. Indeed, this is the case, since the kinetic data presented in Fig 6 indicate that by 24 hours ACAT inhibition changes the cellular distribution of FC and that the presence of U18666A prevents this change. Further experiments will be required to establish whether the pool(s) of excess cholesterol that induces cell death is exclusively in the plasma membrane.

**Cholesterol Crystal Formation**

Another effect of inhibiting ACAT in CE-loaded macrophages was FC crystal formation. In our present system, cells are preloaded with cytoplasmic CE and subsequently allowed to hydrolyze the stored CE by neutral CE hydrolase. Reesterification was prevented by the addition of an ACAT inhibitor. Thus, FC crystals may be formed in a subcellular location other than the lysosome. Cotreatment with U18666A delayed and diminished the formation of crystals in MPMs, and this was not due to a difference in cellular FC concentration. Under the same conditions, cholesterol crystals did not form in J774 macrophages. This may be due to several factors. J774 macrophages hydrolyze stored CE slower than primary mouse macrophages, and J774 macrophages are a dividing cell population in which FC accumulation may be moderated by dilution due to cell growth. Both of these characteristics may serve to limit the FC accumulation and thereby prevent a nucleating event.

In summary, we have now demonstrated that many of the toxic events that are thought to occur within the atherosclerotic lesion, such as FC accumulation, increased phospholipid synthesis, FC crystallization, and apoptosis and necrosis, can be reproduced in a model foam cell culture system. It is evident that in FC content in a foam cell can disturb the homeostatic balance and provoke toxic responses, such as necrosis and apoptosis. Also, although the rate of phospholipid synthesis increases, apparently as a protective response, it is not sufficient to prevent toxicity. A treatment that does diminish or eliminate toxic response is exposure to U18666A, a compound that has been shown to inhibit some
intracellular cholesterol transport steps. It appears that U18666A exerts its protective effect by sequestering the FC in an isolated intracellular pool, the location of which remains to be determined. Although in the present study we have used a pharmacological approach to block ACAT and generate intracellular FC, physiological inhibitors of ACAT have been identified, and such inhibitors could affect foam cells in the lesion, producing the sequence of toxic responses that we have observed in cultured macrophages. In addition, other compounds (such as progesterone) that have characteristics similar to that of U18666A may play a role in maintaining cell integrity in the face of excess FC.

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