Hydrolysis and excretion of cytoplasmic cholesteryl esters by macrophages: stimulation by high density lipoprotein and other agents

Y. K. Ho, Michael S. Brown, and Joseph L. Goldstein[1]

Departments of Molecular Genetics and Internal Medicine, University of Texas Health Science Center at Dallas, 5323 Harry Hines Blvd., Dallas, Texas 75235

Abstract The ability of mouse peritoneal macrophages to hydrolyze and excrete cytoplasmic cholesteryl ester droplets was studied. The macrophages were loaded with cholesteryl esters by incubation with acetylated low density lipoprotein (acetyl-LDL), which is internalized by adsorptive endocytosis. The cholesteryl esters of acetyl-LDL are hydrolyzed within lysosomes and the liberated cholesterol is re-esterified in the cytoplasm where it accumulates as cytoplasmic cholesteryl ester droplets. Hydrolysis and excretion of these stored cholesteryl esters were quantified by gas-liquid chromatographic measurement of the content of free and esterified cholesterol in cells and in medium. After removal of acetyl-LDL from the culture medium, the cytoplasmic cholesteryl esters were rapidly hydrolyzed and large amounts of free cholesterol were excreted from the cells. Hydrolysis and excretion required a cholesterol acceptor in the culture medium. The following agents were shown to be effective as cholesterol acceptors: high density lipoprotein (HDL), whole serum, the density > 1.215 g/ml fraction of whole serum, intact erythrocytes, casein, and thyroglobulin. The following agents did not promote the hydrolysis and excretion of cholesteryl esters under these experimental conditions: LDL, serum albumin, serum γ-globulins, and phosphatidylcholine/sphingomyelin liposomes. The results indicate that net hydrolysis of cytoplasmic cholesteryl esters in macrophages is coupled to the process of cholesterol excretion and that net hydrolysis does not occur unless an effective cholesterol acceptor is present in the culture medium.—**Ho, Y. K., M. S. Brown, and J. L. Goldstein.** Hydrolysis and excretion of cytoplasmic cholesteryl esters by macrophages: stimulation by high density lipoprotein and other agents. *J. Lipid Res.* 1980. **21:** 391–398.

Supplementary key words low density lipoprotein · erythrocytes · modified lipoproteins · membrane cholesterol · liposomes

Macrophages play an important role in body cholesterol metabolism. They normally ingest and degrade effete erythrocytes and other cells, releasing cholesterol from the ingested membranes. Moreover, under certain normal and pathologic conditions macrophages may take up and degrade significant amounts of plasma lipoproteins (1–3). The cholesterol that macrophages liberate from cell membranes and plasma lipoproteins must ultimately be excreted from the cell.

The mechanism by which macrophages excrete large amounts of cholesterol has recently begun to be explored. In an early series of experiments, Werb and Cohn (4) loaded monolayers of mouse peritoneal macrophages with cholesterol by incubating the cells with free or esterified [^{3}H]cholesterol bound to albumin. The cells ingested these particulate suspensions of cholesterol-albumin by phagocytosis. The [^{3}H]cholesteryl esters were hydrolyzed within lysosomes by an active acid lipase. When the exogenous source of cholesterol was eliminated, the macrophages promptly excreted the excess [^{3}H]cholesterol. Excretion required the presence of serum in the culture medium. Only free [^{3}H]cholesterol was excreted by the macrophages irrespective of whether they took up free or esterified [^{3}H]cholesterol (4).

In more recent experiments, mouse peritoneal macrophages have been loaded with cholesteryl esters by incubation with plasma lipoproteins, including human low density lipoprotein (LDL) that is chemically modified by acetylation (acetyl-LDL) (5, 6), human LDL that is bound to dextran sulfate (7), and β-migrating very low density lipoproteins (β-VLDL) obtained from cholesterol-fed dogs.[2] Macrophages take up these three lipoproteins by adsorptive endocytosis, each lipoprotein being recognized by a different cell surface binding site (5–7).[2] In each case, the lipoprotein is delivered to lysosomes where its cholesteryl esters are hydrolyzed. In the case of acetyl-LDL, about 50% of the liberated cholesterol is ex-

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HDL, high density lipoprotein; LDL, low density lipoprotein.

[1] To whom correspondence should be addressed.

[2] Goldstein, J. L., Y. K. Ho, M. S. Brown, T. Innerarity, and R. W. Mahley. 1980. *J. Biol. Chem.* **255:** 1839–1848.

Downloaded from www.jlr.org by guest, on June 19, 2012

creted by the cell, the remaining 50% being re-esterified by a membrane-bound acyl-CoA: cholesterol acyltransferase enzyme (6). This re-esterified cholesterol accumulates within homogeneous cytoplasmic lipid droplets that lack a classical limiting membrane. When the source of incoming lipoprotein-cholesterol is removed, these stored cholesteryl esters are hydrolyzed and the cytoplasmic lipid inclusions disappear (6).

The above lysosomal hydrolysis–microsomal re-esterification mechanism for cholesteryl ester storage has been termed the two-compartment model (6, 8). Its relevance to normal macrophage physiology is emphasized by the observation that cholesteryl ester-laden macrophages in vivo exhibit large numbers of lipid droplets in the cytoplasm, suggesting that these esters have been synthesized endogenously by cholesterol re-esterification (2, 3, 9).

In the current experiments, we have investigated the requirements for excretion of endogenously synthesized, cytoplasmically stored cholesteryl esters. As a model system, we have used mouse peritoneal macrophages that are loaded with cholesteryl esters by incubation with acetyl-LDL. The cholesteryl esters that accumulate under these conditions are derived almost entirely from cytoplasmic re-esterification of lipoprotein-derived cholesterol that enters the cell by adsorptive endocytosis (5, 6). The data demonstrate that macrophages can hydrolyze and excrete large amounts of stored cholesteryl esters, but to achieve this they require an acceptor in the medium. Of the two major lipoproteins in human plasma, high density lipoprotein (HDL) is a much more effective acceptor of this excreted cholesterol than is LDL.

MATERIALS AND METHODS

Materials

Male and female NCS mice (25–30 g) were obtained from The Rockefeller University. [1,2-^{3}H]Cholesterol (43 Ci/mmol) was purchased from Amersham/Searle. [^{3}H]Cholesteryl oleate was synthesized from [1,2-^{3}H]-cholesterol and oleyl chloride (10). Human albumin (crystallized) was purchased from Miles Biochemicals (Cat. No. 82-301-1). Thyroglobulin (Type 1, bovine) and γ-globulins (Cohn Fraction II, human) were purchased from Sigma Chemical Co. Casein (purified, Cat. No. 0336-15) was purchased from DIFCO Laboratory. Egg phosphatidylcholine and egg sphingomyelin were obtained from Avanti Biochemicals, Inc. Fetal calf serum (FCS) was obtained from Flow Laboratories. Dulbecco's modified Eagle medium (Cat. No. 320-1885) was purchased from Grand Island Biological Co. Plastic petri dishes were obtained from Falcon, Division of Becton, Dickinson, and Co. All other supplies and reagents for assays were obtained from sources as previously reported (6).

Mouse macrophage monolayers

Peritoneal cells were harvested from unstimulated mice in phosphate-buffered saline as previously described (5, 6). The fluid from 20 to 40 mice (6 to 10 $\times 10^6$ cells/mouse) was pooled, and the cells were collected by centrifugation (400 *g*, 10 min, room temperature) and washed once with 30 ml of Dulbecco's modified Eagle medium (DMEM). The cells were resuspended in DMEM containing 20% (v/v) FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at a final concentration of 4 to 5 $\times 10^6$ cells/ml. One-ml aliquots of this cell suspension were dispensed into 35 mm $\times$ 10 mm plastic petri dishes and then incubated in a humidified CO_2 (5%) incubator at 37°C. After 1–2 hr, each dish was washed three times with 2 ml of DMEM without serum to remove nonadherent cells, after which the experiment was initiated. Each dish of adherent macrophages contained about 30% of the total number of cells originally plated, and each 10^6 adherent cells contained approximately 60–75 μg of protein (5, 6).

Lipoproteins

Human LDL (d 1.019–1.063 g/ml) and HDL_3 (d 1.125–1.215 g/ml) were isolated from the plasma of individual healthy human subjects by ultracentrifugation (11). Fetal calf lipoprotein-deficient serum (d $>$ 1.215 g/ml) was prepared by ultracentrifugation (11). LDL was acetylated with repeated additions of acetic anhydride as previously described (5, 6). Acetyl-LDL radiolabeled with [^{3}H]cholesteryl oleate was prepared by a previously described reconstitution method in which the endogenous neutral lipids of acetyl-LDL were removed by heptane extraction and replaced with [^{3}H]cholesteryl oleate (6, 10). The resulting reconstituted lipoprotein, designated r-[^{3}H-cholesteryl oleate]acetyl-LDL, was shown to comigrate with acetyl-LDL in agarose gel electrophoresis at pH 8.6 (6). For LDL, acetyl-LDL, and HDL_3, the mass ratio of total cholesterol to protein was 1.5:1, 1.5:1, and 1:4, respectively. Fetal calf lipoprotein-deficient serum contained 0.39–0.44 μg cholesterol per mg protein.

Assays

The cellular content of free and esterified cholesterol in macrophages was measured by gas–liquid

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH

chromatography (12) as previously described (6). The hydrolysis of r-[^{3}H-cholesteryl oleate]acetyl-LDL and excretion of the liberated [^{3}H]cholesterol by macrophage monolayers was quantified by thin-layer chromatography as previously described (6). The protein content of lipoproteins and cells was determined by the procedure of Lowry et al. (13) with bovine serum albumin as a standard. Unless otherwise stated, each data point in the Tables and Figures represents the average of duplicate incubations.

RESULTS

To study the excretion of cholesterol from cholesteryl ester-loaded macrophages, we incubated mouse peritoneal macrophages for 24 hr with 50 μg protein/ml of human acetyl-LDL. At the end of this interval, the cells had accumulated 100–250 μg of esterified cholesterol per mg of cell protein. The cellular content of free cholesterol remained relatively constant at 30–50 μg of cholesterol per mg protein. Previous studies have shown that the esterified cholesterol that accumulates under these conditions is derived from lipoprotein-bound cholesteryl esters that have been taken up by adsorptive endocytosis, hydrolyzed within lysosomes, and re-esterified within the cytoplasmic compartment (5, 6). Most of these esters are stored in the cytoplasm as homogeneous lipid droplets that lack a classical limiting membrane (6).

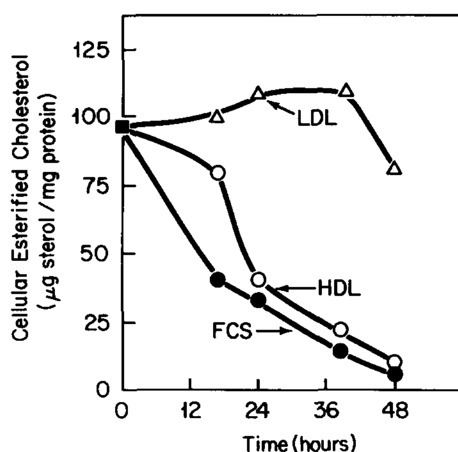

Fig. 1. Decline in cholesteryl ester content of macrophages: effect of time of incubation with whole serum and plasma lipoproteins. After an initial incubation for 24 hr at 37°C with 1 ml of DMEM containing 10% FCS and 50 μg/ml of acetyl-LDL, each monolayer was washed twice with 2 ml of DMEM containing 10% FCS followed by one wash with 2 ml of DMEM. The cells were then incubated at 37°C with 1 ml of DMEM containing one of the following: ●, 10 mg protein/ml of FCS; ○, 0.1 mg protein/ml of HDL; or △, 1 mg protein/ml of LDL. After incubation for the indicated time, the monolayers were washed and harvested, and their content of esterified cholesterol was measured by gas–liquid chromatography.

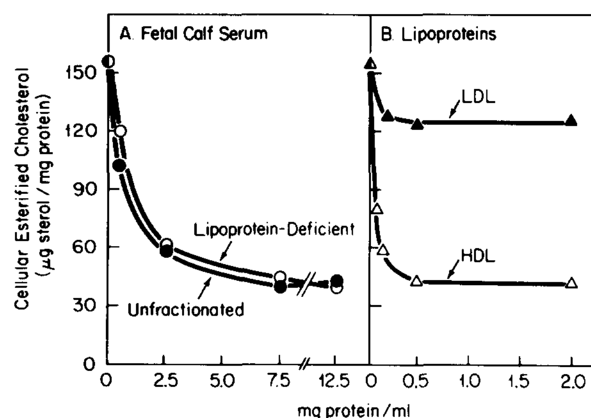

Fig. 2. Decline in cholesteryl ester content of macrophages after incubation with varying concentrations of fetal calf serum (A) and isolated lipoprotein fractions (B). After an initial incubation for 24 hr at 37°C with 1 ml of DMEM containing 10% FCS and 50 μg/ml of acetyl-LDL, each monolayer was washed as described in the legend to Fig. 1. The cells were then incubated at 37°C for 24 hr with 1 ml of DMEM containing the indicated concentration of one of the following: ●, unfractionated FCS; ○, lipoprotein-deficient FCS; ▲, LDL; or △, HDL_3. Following the second incubation, the monolayers were washed and harvested, and their content of esterified cholesterol was measured by gas–liquid chromatography.

When macrophages were loaded with cholesteryl esters in the above manner and subsequently incubated in medium containing whole serum (FCS) but devoid of acetyl-LDL, the cellular content of esterified cholesterol declined rapidly (**Fig. 1**). By 48 hr, nearly all of the cholesteryl esters had disappeared from the cell. More than 90% of the released cholesterol appeared as unesterified cholesterol in the culture medium (see below). The FCS (10 mg protein/ml) could be replaced with small amounts of HDL_3 (0.1 mg protein/ml) without affecting the excretion. On the other hand, LDL (1 mg protein/ml) could not replace the FCS. After 48 hr in the presence of LDL, there was only a minimal decline in the cellular cholesteryl ester content.

Fig. 2A shows the effect of varying concentrations of whole serum on cholesterol excretion by the cholesteryl ester-loaded macrophages. In the absence of protein in the medium, the cells continued to show a high cholesteryl ester content (150 μg sterol/mg protein) even after 24 hr incubation in the absence of acetyl-LDL. The addition of increasing amounts of unfractionated FCS caused a progressive decrease in the cholesteryl ester content. Fifty percent of the maximal decline was achieved at an FCS concentration of 1 mg protein/ml. Removal of the lipoproteins from the FCS by ultracentrifugation did not affect

Downloaded from www.jlr.org by guest, on June 19, 2012

JOURNAL OF LIPID RESEARCH ASBMB

ASBMB
JOURNAL OF LIPID RESEARCH

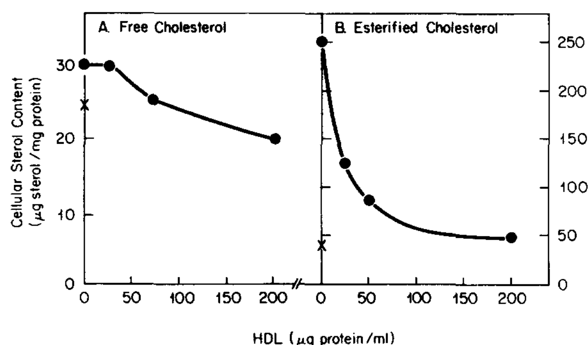

Fig. 3. Decline in content of free (A) and esterified (B) cholesterol in macrophages after incubation with different concentrations of HDL_3. After an initial incubation for 24 hr at 37°C with 1 ml of DMEM containing 10% FCS and 50 μg/ml of acetyl-LDL, each monolayer was washed as described in the legend to Fig. 1. The cells were then incubated for 24 hr at 37°C with 1 ml of DMEM containing the indicated concentration of HDL_3. Following the second incubation, the monolayers were washed and harvested, and their content of free (A) and esterified (B) cholesterol was measured. The content of free and esterified cholesterol in macrophages incubated in the absence of HDL_3 but with 10% FCS (5 mg protein/ml) was 25 and 40 μg sterol/mg protein, respectively (indicated by X).

the ability of the serum to stimulate cholesteryl ester excretion (lipoprotein-deficient serum, Fig. 2A).

Human HDL_3 was much more potent on a protein basis than whole serum in its ability to enhance cholesterol excretion from macrophages, 50% of maximal excretion occurring at an HDL_3 protein concentration of 0.05 mg/ml (Fig. 2B). In contrast, LDL permitted only minimal excretion of cholesteryl esters, even at protein concentrations as high as 2 mg/ml (Fig. 2B).

After incubation of macrophages with acetyl-LDL, the cellular content of esterified cholesterol was generally much higher than the content of free cholesterol (250 versus 30 μg sterol/mg protein in **Fig. 3**). Subsequent incubation of the cells with increasing amounts of HDL_3 led to a net loss of 200 μg of esterified cholesterol but only 10 μg of free cholesterol per mg of cell protein (Fig. 3). Similar reductions were obtained when macrophages were incubated with 10% FCS in the absence of HDL_3 (X in Fig. 3).

Although most of the decline in cellular sterol content represented the loss of esterified cholesterol, nearly all of the cholesterol that appeared in the medium was free cholesterol. **Table 1** shows an experiment in which macrophages were incubated for 24 hr with acetyl-LDL that had been reconstituted with ^{3}H-labeled cholesteryl oleate (6, 10). The cells were then washed and switched to medium containing no protein or 200 μg protein/ml of HDL_3. When incubated with HDL_3 alone, the cellular content of [^{3}H]cholesterol declined by 450 nmol/mg protein, of which 420 nmol/mg represented a decline in esterified [^{3}H]cholesterol and 30 nmol/mg represented a decline of free [^{3}H]cholesterol. Nearly all of the [^{3}H]cholesterol found in the medium, however, was free [^{3}H]cholesterol (390 nmol/mg of free versus 15 nmol/mg of esterified).

Treatment of FCS with *N*-ethylmaleimide, which inactivates the lecithin:cholesterol acyltransferase enzyme (14), did not affect the ability of the serum to remove cholesterol from cholesteryl ester-loaded macrophages (**Fig. 4A**). Formed elements of the blood were also able to remove cholesterol from macrophages. Intact washed human erythrocytes added to the culture medium were extremely potent in this regard. Half-maximal cholesterol excretion was observed when less than 1 μl of packed erythrocytes was added to 1 ml of medium (Fig. 4B).

Although serum, HDL_3, and erythrocytes all contain phospholipids, their effectiveness in removing macrophage cholesterol could not be attributed solely to their phospholipid content. Pure phospho-

TABLE 1. Removal of [^{3}H]cholesterol from macrophages previously incubated with r-[^{3}H-cholesteryl oleate]acetyl-LDL: stimulation by HDL

Addition to Medium	Cellular Content of [^{3}H]Cholesterol		Medium Content of [^{3}H]Cholesterol	
	Free	Esterified	Free	Esterified
	nmol/mg protein		*nmol/mg protein*	
None	180	1050	21	6.5 (1260)[a]
HDL_3, 200 μg protein/ml	150	630	390	15 (1190)

[a] Values in parentheses represent the total amount of free and esterified [^{3}H]cholesterol contained within the cells and medium.

After an initial incubation for 24 hr with 0.8 ml of DMEM containing 50 μg protein/ml of r-[^{3}H-cholesteryl oleate]acetyl-LDL (5,600 cpm/nmol of cholesteryl oleate; mass ratio of cholesterol to protein, 1.4), each monolayer was washed as described in the legend to Fig. 1. The cells were incubated at 37°C for 24 hr with 1 ml of DMEM containing the indicated addition. Following this second incubation, the content of free and esterified [^{3}H]cholesterol for the cells and the medium was determined by thin-layer chromatography.

Downloaded from www.jlr.org by guest, on June 19, 2012

lipid liposomes did not remove large amounts of cholesterol from macrophages. **Fig. 5A** shows an experiment in which cholesteryl ester-loaded macrophages were incubated with increasing concentrations of sonicated phosphatidylcholine/sphingomyelin (10:1) liposomes in the absence or presence of 1 mg/ml of albumin. The liposomes were ineffective at concentrations up to 200 μg phospholipid/ml. Higher concentrations were toxic to the macrophages. In another experiment, addition of unsonicated phosphatidylcholine liposomes to limiting amounts of FCS did not increase the amount of cholesterol removed by the serum itself (Fig. 5B).

A variety of purified proteins were also tested for their ability to remove cholesterol from macrophages. Albumin and γ-globulins had no effect at concentrations as high as 10 mg/ml. On the other hand, casein and thyroglobulin, at concentrations above 1 mg/ml, did facilitate the hydrolysis and excretion of cholesterol (**Fig. 6A**). The casein and thyroglobulin did not lose any ability to remove cholesterol when they were subjected to lipid extraction (Fig. 6B), indicating that the effect was due to the proteins themselves and not to attached lipids.

To trace the fate of the cholesterol released from

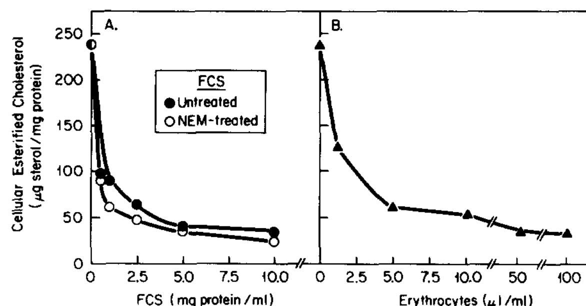

Fig. 4. Decline in cholesteryl ester content of macrophages after incubation with different concentrations of *N*-ethylmaleimide-treated fetal calf serum (A) and human erythrocytes (B). After an initial incubation for 24 hr at 37°C with 1 ml of DMEM containing 10% FCS and 50 μg/ml of acetyl-LDL, each monolayer was washed as described in the legend to Fig. 1. The cells were then incubated for 24 hr at 37°C with the indicated concentration of one of the following: ●, untreated FCS; ○, FCS that had been subjected to prior treatment with *N*-ethylmaleimide (NEM); or ▲, human erythrocytes. Following the second incubation, the monolayers were washed and harvested, and their content of esterified cholesterol was measured. *N*-Ethylmaleimide-treated FCS (○) was prepared by incubating FCS with 1 mM *N*-ethylmaleimide for 1 hr at 37°C, after which the FCS was dialyzed extensively against 0.15 M NaCl. The untreated FCS (●) was incubated at 37°C for 1 hr in the absence of *N*-ethylmaleimide and was dialyzed extensively against 0.15 M NaCl. Human erythrocytes (▲) were obtained from the peripheral venous blood of a healthy individual. The blood was collected in EDTA, the plasma and erythrocytes were separated by centrifugation (800 rpm, 10 min, 4°C), erythrocytes were washed twice with DMEM, and the washed cells were resuspended in DMEM (2 volumes of DMEM per 1 volume of packed cells).

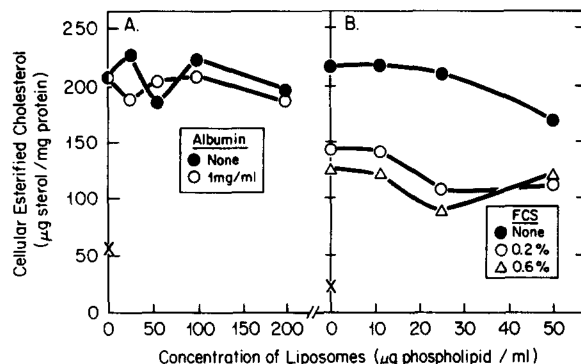

Fig. 5. Failure of phospholipid liposomes to promote the decline in cholesteryl ester content of macrophages. After an initial incubation for 24 hr at 37°C with 1 ml of DMEM containing 10% FCS and 50 μg/ml of acetyl-LDL, each monolayer was washed as described in the legend to Fig. 1. In Exp. A, the cells were incubated at 37°C for 24 hr with 1 ml of DMEM containing the indicated concentration of sonicated unilamellar phosphatidylcholine/sphingomyelin (10:1) liposomes in the absence (●) or presence (○) of 1 mg/ml of human albumin. In Exp. B, the cells were incubated at 37°C for 24 hr with 1 ml of DMEM containing the indicated concentration of multilamellar phosphatidylcholine liposomes in the absence (●) or presence of either 0.2% FCS (○) or 0.6% FCS (△). Following the second incubation, the monolayers were washed and harvested, and their content of esterified cholesterol was measured. The content of esterified cholesterol in macrophages incubated with 10% FCS (5 mg protein/ml) was 55 and 20 μg sterol/mg protein for Exps. A and B, respectively (indicated by the X). For Exp. A, liposomes were prepared by drying 10 mg of egg phosphatidylcholine and 1 mg of egg sphingomyelin in a glass container. Ten ml of 0.15 M NaCl solution was added and the mixture was sonicated for a total of 12 min on ice. The suspension was centrifuged for 15 min at 100,000 *g*, and aliquots of the clear supernatant were added to cells. For Exp. B, 30 mg of egg phosphatidylcholine was dried as a monolayer on a round-bottom flask, after which 1 ml of 0.15 M NaCl was added and the mixture was agitated on a Vortex mixer for 5 min without sonication. Aliquots of the cloudy suspension were added to cells as indicated.

macrophages incubated in the presence of serum, cells were preincubated with r-[^{3}H-cholesteryl oleate]-acetyl-LDL. The cholesteryl ester-loaded cells were then incubated with medium containing 5% lipoprotein-deficient serum, after which the medium was subjected to ultracentrifugation at density 1.215 g/ml (**Table 2**). The mass and the radioactivity of the excreted sterol were measured independently (see legend to Table 2). The mass of cholesterol in the unfractionated medium increased from 5.3 μg before incubation (the amount of cholesterol initially contained in the lipoprotein-deficient serum) to 17.4 μg after incubation. About half of the cholesterol mass in the medium after incubation was found in the fraction of medium that floated in the ultracentrifuge at d < 1.215 g/ml. On ultracentrifugation the distribution of the [^{3}H]cholesterol released from the cells into the medium was the same as the distribution of mass. On thin-layer chromatography, about 93% of

Downloaded from www.jlr.org by guest, on June 19, 2012

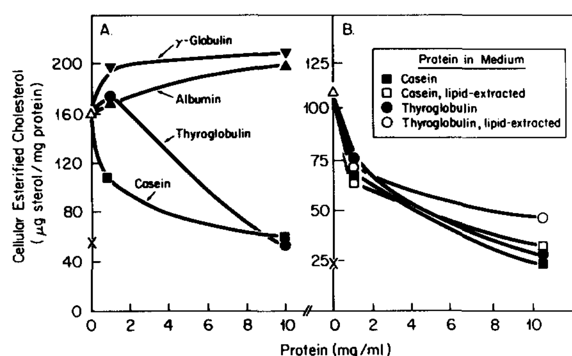

Fig. 6. Decline in cholesteryl ester content of macrophages after incubation with different types of proteins. After an initial incubation for 24 hr at 37°C with 1 ml of DMEM containing 10% FCS and 50 μg/ml of acetyl-LDL, each monolayer was washed as described in the legend to Fig. 1. The cells were then incubated for 24 hr at 37°C with the indicated concentration of one of the following: Exp. A: ▲, albumin; ▼, γ-globulins; ●, thyroglobulin; or ■, casein. Exp. B: ●, untreated thyroglobulin; ○, lipid-extracted thyroglobulin; ■, untreated casein; or, □, lipid-extracted casein. Following this second incubation, the monolayers were washed and harvested, and their content of esterified cholesterol was measured. Each value represents the mean of triplicate incubations. The content of esterified cholesterol in macrophages incubated with 10% FCS (5 mg protein/ml) was 55 and 23 μg sterol/mg protein for Exps. A and B, respectively (indicated by the X). Lipid-extracted thyroglobulin and casein were prepared as follows: 50 mg of protein was suspended in 6 ml of cold ethanol–ether 1:1, agitated on a Vortex mixer, and kept at 4°C for 1 hr. The mixture was then centrifuged (2000 rpm, 10 min, 4°C), and the organic phase was discarded. The residue was dried under a stream of air and then redissolved in 5 ml of DMEM, and the resulting solution was passed through a Millipore filter (0.45 μm).

the [3H]cholesterol in both ultracentrifugal fractions of medium was free cholesterol and about 7% was present as cholesteryl ester (Table 2).

DISCUSSION

The current results demonstrate that: *1*) mouse peritoneal macrophages loaded with cholesteryl esters hydrolyze and excrete the esters rapidly when the source of cholesterol is removed; *2*) the excretion does not occur unless the medium contains a substance that is capable of binding or accepting the liberated cholesterol; *3*) net hydrolysis and excretion are coupled so that the lack of an acceptor for free cholesterol prevents the net hydrolysis of stored cholesteryl esters; and *4*) a variety of substances can serve as acceptors for cholesterol, and these substances exhibit a wide range of effectiveness.

Among human plasma lipoproteins, HDL_3 was much more effective in removing macrophage cholesterol than was LDL. The lipoprotein-deficient fraction of serum was also potent in its ability to remove cholesterol from cholesteryl ester-laden macrophages, indicating that the activity of serum is due in large part to components other than lipoproteins of d < 1.215 g/ml. Erythrocytes also had a striking ability to stimulate the excretion of cholesterol from macro-

TABLE 2. Distribution of [3H]cholesterol excreted from macrophages previously incubated with r-[3H-cholesteryl oleate]acetyl-LDL

Fraction of Medium	Protein Content	Total Cholesterol Mass	[3H]Cholesterol Content
	mg/fraction	*μg/fraction*	*cpm × 10^{-3}/fraction*
Medium before incubation			
(d > 1.215 g/ml)	12	5.3	
Medium after incubation			
(d < 1.215 g/ml)	0.02	8.5	351
(d > 1.215 g/ml)	8.3	8.9	338
Total	8.3	17.4	689

Each of ten monolayers received 1 ml of DMEM containing 10% FCS and 25 μg protein/ml of r-[3H-cholesteryl oleate]acetyl LDL (28,000 cpm/nmol of cholesteryl oleate; mass ratio of cholesterol to protein, 0.67). After incubation for 24 hr at 37°C, the cells were washed as described in the legend to Fig. 1, after which each monolayer received 1 ml of DMEM containing 1.5 mg protein/ml of fetal calf lipoprotein-deficient serum (cholesterol content, 0.66 μg sterol/ml). Following incubation at 37°C for 24 hr, the medium from the ten dishes was removed and pooled together. After aliquots were removed for analyses, the remainder of the medium (8 ml) was adjusted to a final density of 1.215 g/ml with solid KBr (11) and subjected to ultracentrifugation in a 65 Ti Beckman rotor (64,000 rpm, 36 hr, 4°C) in a 12 ml-polyallomer tube. After completion of the centrifugation, the top 1.3 ml of the medium (d < 1.215 g/ml fraction) and the bottom 10.3 ml (d > 1.215 g/ml fraction) were separated by the tube slicing technique. Aliquots from both fractions were removed for determination of protein content, total cholesterol mass by gas–liquid chromatography, and content of [3H]cholesterol by scintillation counting. About 93% of the [3H]radioactivity in both fractions co-migrated with authentic free cholesterol on thin-layer chromatography, and the remaining 7% of the [3H]radioactivity co-migrated with authentic cholesteryl esters.

ASBMB JOURNAL OF LIPID RESEARCH
Downloaded from www.jlr.org by guest, on June 19, 2012

phages. The results indicate that in 1 ml of whole blood the red cells were about 6-fold more potent in accepting cholesterol than was the whole serum.

Previous studies have demonstrated that HDL can remove cholesterol from cultured cells, including arterial smooth muscle cells and fibroblasts (15–18). Moreover, complexes of the major HDL apoprotein, A-I, and phospholipid are also effective in this regard (16, 18). In these previous studies the excreted cholesterol was derived either from cell membrane cholesterol or from intralysosomal cholesteryl esters that accumulated in the presence of the lysosomal inhibitor chloroquine (15–18). The current studies differ from these earlier studies in that we have measured the excretion of cytoplasmic cholesteryl esters that accumulated in macrophages as a result of endogenous cholesterol esterification. One of our major conclusions is similar to that of the earlier experiments: excretion of cellular cholesterol requires a cholesterol acceptor. A new conclusion is that net hydrolysis of cytoplasmic cholesteryl esters will not take place unless such an acceptor is present. Moreover, when an acceptor is present the macrophages are shown to excrete massive amounts of cholesterol—up to 200 μg sterol per mg protein per day, an amount that is greater than excreted by other cell types (15–18).

The substances in the d > 1.215 g/ml fraction of serum that remove cholesterol from macrophages are yet to be defined. In experiments not shown, we have found that human lipoprotein-deficient serum was as effective as fetal calf lipoprotein-deficient serum. The two major proteins of this fraction, albumin and γ-globulins, do not accept large amounts of cholesterol (Fig. 6). Extraction of the d > 1.215 g/ml fraction with butanol–diisopropyl ether 40:60, which removes virtually all of the lipids of serum (19), did not affect the ability of this fraction to remove macrophage cholesterol (data not shown). Moreover, serum that was rendered lipoprotein-deficient by manganese phosphate precipitation, which removed all of the LDL and half of the HDL, was as effective as whole serum and ultracentrifugally prepared lipoprotein-deficient serum (data not shown). It is well known that the lipoprotein-deficient fraction of human serum contains apoproteins of plasma lipoproteins (especially apoprotein A-I), and it is possible that these apoproteins are responsible for the activity of this fraction.

Additional studies will be necessary to define the mechanism by which the stored cholesteryl ester exits from the macrophage. The most likely mechanism would involve hydrolysis of the cholesteryl esters with transient incorporation of the liberated cholesterol into the cell's plasma membrane. If the cholesterol were continually being removed from the membrane by cholesterol-binding agents in the medium, this might trigger the net hydrolysis of additional cholesteryl esters. In this model the agents in the medium would provide the primary force for the excretory process by pulling cholesterol from the cell membrane. In their studies of [^{3}H]cholesterol excretion from macrophages, Werb and Cohn concluded that such a mechanism was operative (4).

Alternatively, the cholesterol liberated from stored cholesteryl esters might be actively secreted by the cell, perhaps by exocytosis. In this case it is difficult at this point to envision the role played by the cholesterol acceptor in the medium. It will be important to determine whether cholesterol leaves the macrophage as individual molecules, or whether it leaves in some sort of cholesterol-phospholipid or cholesterol-protein complex.

It is of interest that phosphatidylcholine/sphingomyelin liposomes were not able to trigger the excretion of macrophage cholesterol, even though these liposomes are known to bind cholesterol. Similarly, LDL binds cholesterol, but it too did not remove large amounts of macrophage cholesterol. Thus, the simple ability to bind cholesterol is not sufficient for cholesterol removal from macrophages.

Future studies of macrophage cholesterol excretion will be of importance because they may have relevance to the cholesteryl ester deposition that occurs in macrophages and foam cells during the formation of atherosclerotic lesions. In the face of a source of macrophage cholesterol input, such as modified lipoproteins, a deficiency of a cholesterol acceptor in plasma and interstitial fluid might accelerate the development of atherosclerosis by preventing the hydrolysis and excretion of cytoplasmic cholesteryl esters.

Wendy Womack and Michael Gaisbauer provided excellent technical assistance. This research was supported by USPHS Grant PO 1 HL-20948 from the National Institutes of Health.

Manuscript received 19 October 1979 and in revised form 17 December 1979.

REFERENCES

1. Goodman, D. S. 1965. Cholesterol ester metabolism. *Physiol. Rev.* **45:** 747–839.
2. Small, D. M. 1977. Liquid crystals in living and dying systems. *J. Colloid Interface Sci.* **58:** 581–602.
3. Goldstein, J. L., and M. S. Brown. 1978. Familial hypercholesterolemia: pathogenesis of a receptor disease. *Johns Hopkins Med. J.* **143:** 8–16.
4. Werb, Z., and Z. A. Cohn. 1972. Cholesterol metabolism in the macrophage. III. Ingestion and intracellular

Downloaded from www.jlr.org by guest, on June 19, 2012

fate of cholesterol and cholesterol esters. *J. Exp. Med.* **135:** 21–44.
5. Goldstein, J. L., Y. K. Ho, S. K. Basu, and M. S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* **76:** 333–337.
6. Brown, M. S., J. L. Goldstein, M. Krieger, Y. K. Ho, and R. G. W. Anderson. 1979. Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. *J. Cell Biol.* **82:** 597–613.
7. Basu, S. K., M. S. Brown, Y. K. Ho, and J. L. Goldstein. 1979. Degradation of low density lipoprotein-dextran sulfate complexes associated with deposition of cholesteryl esters in mouse macrophages. *J. Biol. Chem.* **254:** 7141–7146.
8. Brown, M. S., M. K. Sobhani, G. Y. Brunschede, and J. L. Goldstein. 1976. Restoration of a regulatory response to low density lipoprotein in acid lipase-deficient human fibroblasts. *J. Biol. Chem.* **251:** 3277–3286.
9. Fredrickson, D. S., J. L. Goldstein, and M. S. Brown. 1978. The familial hyperlipoproteinemias. Chapter 30. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill Book Co., New York. 604–655.
10. Krieger, M., M. S. Brown, J. R. Faust, and J. L. Goldstein. 1978. Replacement of endogenous cholesteryl esters of low density lipoprotein with exogenous cholesteryl linoleate: reconstitution of a biologically active lipoprotein particle. *J. Biol. Chem.* **253:** 4093–4101.
11. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1974. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured human fibroblasts: comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* **249:** 789–796.
12. Brown, M. S., J. R. Faust, and J. L. Goldstein. 1975. Role of the low density lipoprotein receptor in regulating the content of free and esterified cholesterol in human fibroblasts. *J. Clin. Invest.* **55:** 783–793.
13. 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.
14. Glomset, J. A. 1968. The plasma lecithin: cholesterol acyltransferase reaction. *J. Lipid Res.* **9:** 155–167.
15. Bailey, J. M. 1973. Regulation of cell cholesterol content. Atherogenesis: Initiating Factors. *Ciba Found. Symp.* **12** (new series): 63–92.
16. Stein, Y., M. C. Glangeaud, M. Fainaru, and O. Stein. 1975. The removal of cholesterol from aortic smooth muscle cells in culture and Landschutz ascites cells by fractions of human high-density apolipoprotein. *Biochim. Biophys. Acta.* **380:** 106–118.
17. Bates, S. R., and G. H. Rothblat. 1974. Regulation of cellular sterol flux and synthesis by human serum lipoproteins. *Biochim. Biophys. Acta.* **360:** 38–55.
18. Stein, O., V. Vanderhoek, and Y. Stein. 1977. Cholesterol ester accumulation in cultured aortic smooth muscle cells. *Atherosclerosis.* **26:** 465–482.
19. Cham, B. E., and B. R. Knowles. 1976. A solvent system for delipidation of plasma or serum without protein precipitation. *J. Lipid Res.* **17:** 176–181.

Downloaded from www.jlr.org by guest, on June 19, 2012