Cholesterol efflux from cells enriched with cholesteryl esters by incubation with hypercholesterolemic monkey low density lipoprotein

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Abstract The mass efflux of free and esterified cholesterol was studied in skin fibroblasts loaded with cholesterol by incubation with low density lipoproteins (LDL) isolated from normal or hypercholesterolemic cynomolgus monkeys. Cells incubated with hypercholesterolemic LDL accumulated 2–3 times more cholesteryl ester than did cells incubated with the same amount of normal LDL. Cholesteryl oleate was the principal cholesteryl ester species to accumulate in cells incubated with both normal and hypercholesterolemic LDL. Efflux of this accumulated cholesterol was absolutely dependent on the presence of a cholesterol acceptor in the culture medium. Lipoprotein-deficient serum (LPDS) was the most potent promoter of cholesterol efflux tested, with maximum efflux occurring at LPDS concentrations greater than 1.5 mg protein/ml. Upon addition of efflux medium containing LPDS, there was a reduction in both the free and esterified cholesterol concentration of the cells. Greater than 90% of the cholesteryl esters that were lost from the cells appeared in the culture medium as free cholesterol, indicating that hydrolysis of cholesteryl esters preceded efflux. Efflux was not inhibited by chloroquine, however, suggesting a mechanism independent of lysosomes. Loss of cellular free cholesterol was maximum by 6 hr and changed very little thereafter up to 72 hr. Cholesteryl ester loss from cells decreased in a log linear fashion for efflux periods of 6–72 hr, with an average half-life for cholesteryl ester efflux of 30 hr, but with a range of 20–50 hr, depending upon the specific cell line. The rate of efflux of cellular cholesteryl esters was similar for cells loaded with normal or hypercholesterolemic LDL. In cells loaded with cholesteryl esters, cholesterol synthesis was suppressed and cholesterol esterification and fatty acid synthesis were enhanced. During efflux, cholesterol synthesis remained maximally suppressed while cholesterol esterification decreased for the first 24 hr of efflux, then plateaued at a level approximately 5-fold higher than control levels, while fatty acid synthesis was slightly stimulated. There was little difference in the rate of efflux of individual cholesteryl ester species. There was, however, the suggestion that reesterification of cholesterol principally to palmitic acid occurred during efflux. Since the rate of cellular cholesteryl ester efflux was similar regardless of whether the cells had been loaded with cholesterol by incubation with normal LDL or hypercholesterolemic LDL, the greater accumulation of cholesterol in cells incubated with hypercholesterolemic LDL cannot be explained by differences in rates of efflux.—**St. Clair, R. W., and M. A. Leight.** Cholesterol efflux from cells enriched with cholesteryl esters by incubation with hypercholesterolemic monkey low density lipoprotein. *J. Lipid Res.* 1983. **24:** 183–191.

Supplementary key words tissue culture • cholesterol synthesis • cholesterol esterification • fatty acid synthesis

Previous studies from our laboratory (1–4) and others (5–7) have shown that whole hypercholesterolemic serum or low density lipoproteins (LDL) isolated from hypercholesterolemic serum promotes enhanced cholesterol accumulation in cells in culture relative to the same concentration of normal serum or LDL. The mechanism of this enhanced cholesterol accumulation is unclear but is probably the result of several factors related to the abnormal composition of the hypercholesterolemic LDL. These LDL are larger than normal LDL due principally to an increased number of cholesteryl ester molecules per LDL particle (8–10). In addition, these abnormal LDL have an increase in the number of molecules of free cholesterol, phospholipid, and protein in the surface of the particle in order to cover the larger sphere. These abnormal LDL bind to LDL receptors on the surface of cells and are internalized and degraded by mechanisms apparently identical to normal LDL (3, 6). The cholesterol delivered to the cell acts normally to downregulate the synthesis of cholesterol and LDL receptors and to enhance cholesterol esterification (2, 4, 11, 12). In addition to the increased content of cholesteryl esters per particle of hypercholesterolemic LDL, there is a profound change in the fatty acid composition of these cholesteryl esters, being enriched in cholesteryl oleate relative to normal LDL, in which cholesteryl linoleate is the predominant cholesteryl ester species (10, 13).

The purpose of the present study was to determine if this difference in cholesteryl ester fatty acid composition of hypercholesterolemic LDL was reflected in differences in the cholesteryl ester composition of cells

Abbreviations: LDL, low density lipoproteins; LPDS, lipoprotein-deficient serum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); LCAT, lecithin:cholesterol acyltransferase; GLC, gas–liquid chromatography; ACAT, acyl CoA:cholesteryl acyltransferase; NLDL, normal low density lipoprotein; HLDL, hypercholesterolemic low density lipoprotein.

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incubated with this abnormal LDL, and if the resulting differences in the concentration or composition of cellular cholesteryl esters resulted in differences in cellular cholesteryl ester efflux. If such differences in cholesteryl ester efflux were present, then it might provide a partial explanation for the greater ability of hypercholesterolemic LDL to promote cellular cholesteryl ester accumulation relative to normal LDL.

MATERIALS AND METHODS

Cells and experimental protocol

Skin fibroblasts and aortic smooth muscle cells were grown from tissue explants from adult rhesus (*Macaca mulatta*) and cynomolgus (*M. fascicularis*) monkeys using procedures identical to those described previously (1). Normal human skin fibroblasts (Cat. No. GM-41) were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ. The basal tissue culture medium used for these studies consisted of Eagles' minimum essential medium (Auto-Pow) supplemented with twice the normal concentration of vitamins (Eagles' vitamins), 200 mM L-glutamine, 1.5 mg α-D (+)-glucose/ml, 23 mM sodium bicarbonate, 100 IU penicillin/ml, and 100 mg streptomycin/ml. Cells were routinely grown in 75-cm^2 flasks using this basal medium to which was added 10% fetal bovine serum for human cells and 10% calf serum for the monkey cells. For experiments, cells were dissociated from the flasks with 0.05% trypsin and 0.02% ethylenediaminetetraacetate (EDTA), transferred to 60-mm dishes and grown to confluency. The cells were then washed with phosphate-buffered saline (3) and incubated with the basal culture medium containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and 2.5 mg/ml of lipoprotein-deficient calf serum (LPDS). The LPDS was prepared as described previously (1). After 48–72 hr the cells were loaded with cholesterol by incubation with fresh tissue culture medium containing 20 mM Hepes, 2.5 mg/ml LPDS, and 50 μg LDL protein/ml. Hereafter, this will be referred to as the loading medium. After 24 hr the culture medium was poured off and the cells were washed three times with phosphate-buffered saline. A subgroup of dishes was harvested in order to determine the amount of cholesterol that had accumulated during the loading phase of the experiment. To the remaining cells were added the basal medium containing 20 mM Hepes and the various concentrations of LPDS used to promote efflux. This will be referred to hereafter as the efflux medium. Cells were incubated for up to 72 hr with the efflux medium, the medium was removed, the cells were washed three times with phosphate buffered saline, removed from the dishes with trypsin-EDTA, and washed again with phosphate buffered saline. The cells were stored frozen (−20°C) prior to being analyzed for cholesterol content or radioactivity. Results are expressed per mg cell protein. Similar results, however, were obtained if expressed per dish since there was no further growth of these confluent cultures when incubated for up to 72 hr with the efflux medium containing LPDS.

Lipoprotein isolation

LDL used in the loading medium was isolated from the pooled plasma of the same normal and hypercholesterolemic cynomolgus monkeys described previously (3). The LDL was isolated by the combined ultracentrifugal and agarose column procedure (3). The only modification of this previous method was that 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (0.4 mg/ml) was added to the tube in which blood was collected in order to inhibit lecithin:cholesterol acyltransferase (LCAT) activity. The DTNB was separated from the LDL during agarose chromatography.

The molecular weight of the LDL used in these studies averaged 2.82 ± 0.07 (S.D.) × 10^6 from ten separately isolated batches of normal LDL, and 3.83 ± 0.18 × 10^6 from eight separately isolated batches of hypercholesterolemic LDL.

LDL was labeled in vitro with [1,2-^{3}H]cholesterol using a modification of the procedure described previously (1). For this, 100 μCi of [1,2-^{3}H]cholesterol (54 Ci/mmol, Amersham), dissolved in 100 μl of acetone, was added to 4 mg of LDL protein dissolved in 40 ml of basal culture medium. This mixture was incubated overnight at 37°C and sterilized by filtration through a 0.45-μm Millipore filter.

Analyses

Cellular free and esterified cholesterol contents were determined by gas–liquid chromatography (GLC) as described previously (3). The protein content of cells and lipoproteins was determined by the method of Lowry et al. as modified by Kashyap, Hynd, and Robinson (14) using bovine serum albumin as the standard. Lipoprotein molecular weight and composition were determined as described previously (2). Sterol synthesis was determined by measuring the incorporation of radiolabeled acetate into sterols (12). Cholesterol esterification was determined by measuring the esterification of [1-^{14}C]oleate to cholesterol as described previously (1). All radioactivity determinations were counted to a 2 sigma error of <2% using a Beckman LS-230 liquid scintillation counter. An external standard channels ratio method was used to correct for quenching.

The fatty acid composition of cholesteryl esters from cells and LDL was determined as follows. Total lipids

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were extracted using the method of Bligh and Dyer (15) and the cholesteryl esters were separated by thin-layer chromatography as described previously (2). The cholesteryl esters were saponified for 1 hr at 60°C and the neutral lipids were extracted with redistilled Skellysolve B (Getty Oil Co., Tulsa, OK). The remaining aqueous phase was acidified to pH 2 with HCl and the fatty acids were extracted with Skellysolve B. This fraction was evaporated to dryness under N_2 at 45°C and the methyl esters were formed using BF_3-methanol (Applied Science Labs). Fatty acids were separated by GLC in 6′, 2 mm ID glass columns packed with 10% EGSS-X on 100/120 Gas Chrom P (Applied Science) essentially as described previously (16) except that separations were carried out using a Bendix 2500 Chromatograph at a column temperature of 215°C, inlet temperature of 190°C, and detector temperature of 215°C. Nitrogen was the carrier gas at 50 ml/min. Individual fatty acids were quantified using a Columbia Scientific, Model 38, digital integrator. The micrograms of individual cholesteryl esters were calculated by multiplying the total cellular cholesteryl ester content (esterified cholesterol × 1.7) by the percentage of individual fatty acids as measured by GLC.

RESULTS

The effect of LPDS concentration on cholesterol and cholesteryl ester efflux is shown in **Fig. 1.** LPDS isolated from calf serum as well as rhesus monkey plasma gave similar results. There was very little depletion of cellular cholesterol when no LPDS was added to the efflux medium. Instead, there appeared to be some hydrolysis of cellular cholesteryl esters as seen by the approximate 20% increase in the free cholesterol content with an equivalent decrease in cholesteryl ester content. There was little change in total cholesterol content, however. LPDS markedly stimulated the depletion of cellular free and esterified cholesterol. In this experiment about 30% of the free cholesterol of the loaded cells was lost in the first 24 hr with very little additional depletion of free cholesterol occurring after 48 and 72 hr. Cholesteryl esters, however, continued to be lost from the cells for the entire 72 hr of the experiment. With calf serum LPDS there was little evidence of an LPDS concentration effect on free or esterified cholesterol efflux during the first 24 hr. After 24 hr, however, cells incubated with the low concentration of LPDS (0.5 mg/ml) showed consistently less efflux than did cells incubated with the higher concentrations of LPDS. This effect was even more pronounced with the monkey LPDS. Since efflux proceeeded at a similar rate with 1.5 and 2.5 mg LPDS/ml it appeared that 2.5 mg LPDS/ml was a concentration sufficient to promote a maximum rate of cholesterol efflux. As a result, we used 2.5 mg LPDS/ml in subsequent experiments where we did not want efflux to be limited by the amount of acceptor in the efflux medium.

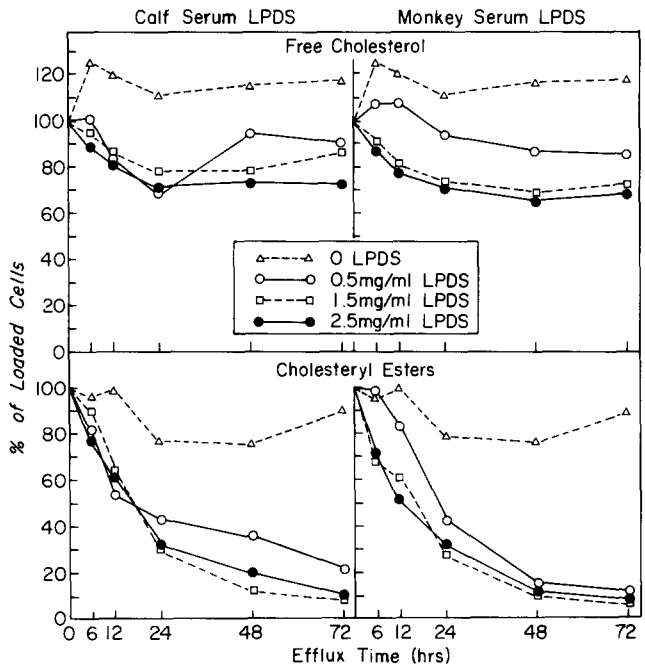

Fig. 1. Effect of lipoprotein-deficient serum (LPDS) concentration on cellular cholesterol efflux. Human skin fibroblasts were grown to confluence and incubated for 48 hr with basal medium containing LPDS. The cells were then loaded with cholesterol by incubation for 24 hr with 50 μg protein/ml of [^{3}H]cholesterol-labeled hypercholesterolemic LDL. After 24 hr, four dishes were harvested and analyzed for free and esterified cholesterol radioactivity. These loaded values averaged 949,100 ± 15,600 (SEM) and 107,650 ± 3,700 dpm/mg protein for free and esterified cholesterol, respectively, and were set at 100% for the 0-time values on the ordinate. The remaining cells were incubated for up to 72 hr with efflux medium containing 0, 0.5, 1.5, or 2.5 mg protein/ml of LPDS isolated from calf serum or normocholesterolemic rhesus monkey plasma. After the indicated efflux period, cells were harvested and analyzed for free and esterified cholesterol radioactivity. Results are the mean of duplicate cultures.

In preliminary experiments (data not shown), using cells loaded with [^{3}H]cholesterol-labeled LDL, we confirmed the results of others (17, 18) that virtually all (>90%) of the cholesterol that was lost from the cell during the efflux phase of the experiment could be recovered in the efflux medium as free cholesterol. Similar results were obtained with normal and hypercholesterolemic LDL and suggest that cellular cholesteryl esters must first be hydrolyzed to free cholesterol before efflux from the cell. Also consistent with the results of others (17), efflux of cholesteryl esters was found to be unaffected by chloroquine, suggesting that lysosomal pathways are not involved in the efflux process (data not shown).

Fig. 2 summarizes the results of five separate exper-

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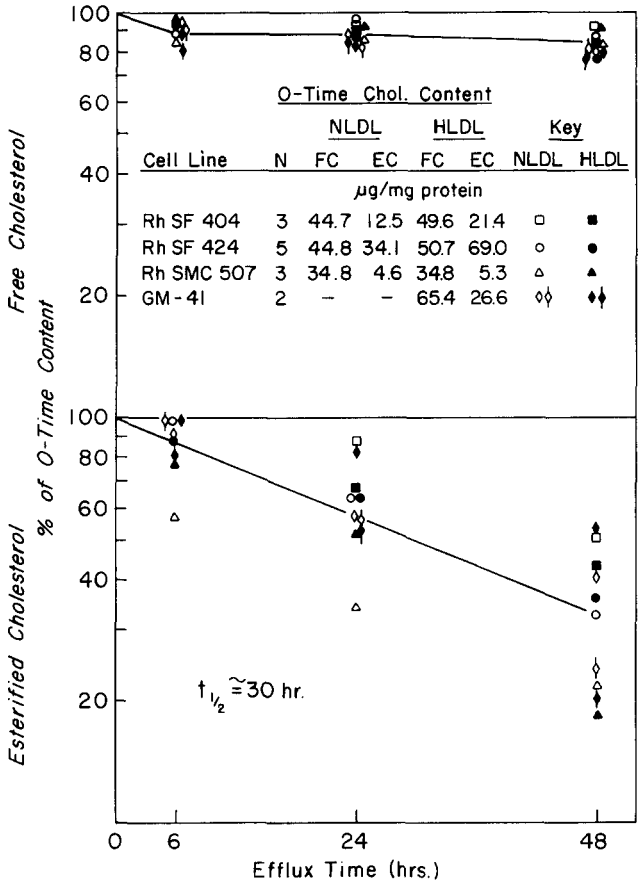

Fig. 2. Efflux of cholesterol from cells loaded with cholesterol by incubation with normal (NLDL) or hypercholesterolemic (HLDL) low density lipoprotein. Cells were grown to confluence and incubated for 48 hr with basal medium containing LPDS. For this study, four different lines of cells were used: two rhesus monkey skin fibroblast lines (Rh SF 404, Rh SF 424); one rhesus monkey smooth muscle cell line (Rh SMC 507); and a human skin fibroblast line (GM-41) for which the results of two experiments are included. The cells were then loaded with cholesterol by incubation with 50 μg protein/ml of normal (NLDL) or hypercholesterolemic (HLDL) low density lipoproteins. After 24 hr a group of cells was harvested and analyzed for free and esterified cholesterol mass by GLC. These values for the loaded cells are indicated above and expressed as 100% for the 0-time value on the ordinate. The remaining cells were incubated for up to 48 hr with efflux medium containing 2.5 mg protein/ml of LPDS. After the indicated efflux period, cells were harvested and analyzed for free and esterified cholesterol content. Results are the mean of two to five replicate cultures as indicated above. The line represents the best fit using all of the points.

iments using four different cell lines in which the relative rates of cholesterol and cholesteryl ester efflux were compared in the same cells loaded with cholesterol by incubation with either normal or hypercholesterolemic LDL. In all cell lines the free cholesterol mass decreased by 5–15% from the loaded value by 6 hr of efflux, and changed very little thereafter up to 48 hr. Cellular cholesteryl ester depletion showed considerable variability among the cell lines, but conformed best to a log linear rate of efflux with an average half-life of approximately 30 hr when considering all cell lines, but ranging from a low of approximately 20 hr to a high of 50 hr for individual cell lines. The differences in cholesteryl ester depletion rates were a function of the cells themselves since there were no consistent differences in the rates of cholesteryl ester removal from the same cells loaded with either normal or hypercholesterolemic LDL.

It is well documented that sterol synthesis is inhibited following enrichment of cellular cholesterol content after incubation with LDL. The purpose of the experiment summarized in **Fig. 3** was to determine whether sterol synthesis remained suppressed during efflux as

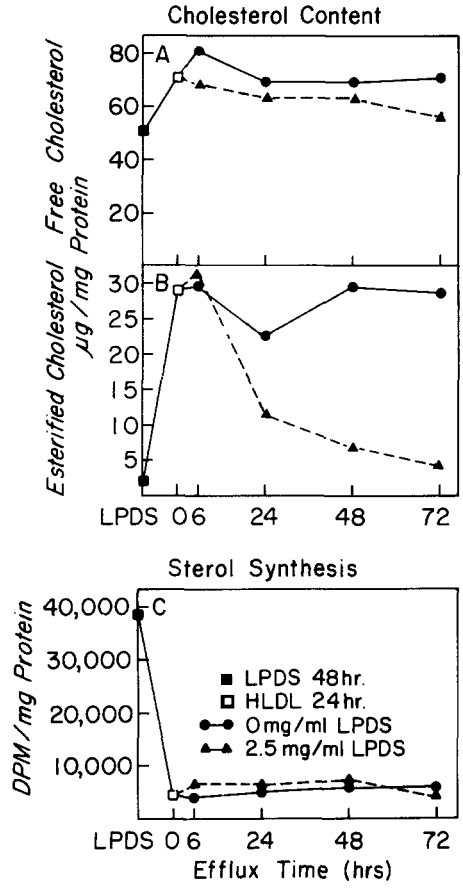

Fig. 3. Sterol synthesis during cholesterol efflux from cells previously loaded with cholesterol. Human skin fibroblasts were grown to confluence and incubated for 48 hr with basal medium containing LPDS. The cells from three dishes were harvested and analyzed for sterol synthesis and cholesterol mass and are represented above as LPDS. The remaining cells were incubated for 24 hr with 50 μg protein/ml of hypercholesterolemic low density lipoprotein (HLDL) and three dishes were harvested and analyzed for sterol synthesis and cholesterol mass. Sterol synthesis was measured after pulse-labeling the cells for 2 hr with [1-^{14}C]acetate. Cholesterol mass was determined by GLC. Results are indicated above as 0-time. The remaining cells were incubated for up to 72 hr with efflux medium containing either no LPDS or 2.5 mg/ml LPDS. After the indicated efflux period, cells were harvested and analyzed for free and esterified cholesterol content (panels A and B) and for sterol synthesis (panel C). For the efflux periods, results are the mean of duplicate cultures.

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well. As was expected, sterol synthesis was inhibited approximately 90% after 24 hr of incubation with hypercholesterolemic LDL during the loading phase. Sterol synthesis remined suppressed throughout the entire 72-hr period of efflux even after cellular cholesteryl ester content had returned to near normal levels, suggesting that the free cholesterol generated from the hydrolysis of the cholesteryl esters was effective in suppressing endogenous sterol synthesis.

In a separate experiment we also measured the changes in fatty acid synthesis and cholesterol esterification during efflux (**Fig. 4**). Panels A and B show the changes in free and esterified cholesterol content during efflux in the presence or absence of LPDS in the efflux medium. Panels C and D show the changes in cholesterol esterification and total fatty acid synthesis of the same cells. As expected, cholesterol esterification was low in cells depleted of cholesterol by incubation of 72 hr with LPDS, but increased more than 15-fold after 24 hr incubation with hypercholesterolemic LDL (Fig. 4C). This was associated with a greater than 25-fold increase in cellular cholesteryl ester content. Efflux in the presence of LPDS was accompanied by a marked decrease in both cellular cholesteryl ester content and in the rate of cholesterol esterification. By 24 hr the rate of esterification had plateaued at a rate still some 5-fold higher than the initial preloaded LPDS rate. In the absence of LPDS there was no loss of cellular cholesterol, but there was a decrease in the rate of cholesterol esterification to a plateau at about twice the rate seen in the LPDS efflux groups. This decrease probably reflects the absence of LDL cholesterol entry. The level of esterification remained higher, however, than when efflux was stimulated with LPDS. This may be due to the continuous hydrolysis and reesterification of free cholesterol, whereas in the presence of LPDS much of the hydrolyzed cholesterol is lost from the cell.

In the same cells there was a 3-fold increase in fatty acid synthesis after cholesterol loading (Fig. 4D). This rate remained relatively constant upon efflux for 72 hr in the presence of LPDS. Without LPDS, there was a drop in the rate of fatty acid synthesis for the first 6 hr of efflux followed by a stimulation to rates approximately twice that of the loaded cells. Greater than 80% of these newly synthesized fatty acids were esterified, with phospholipids accounting for 50%, triglycerides 17%, and cholesteryl esters 11% in the loaded cells. Where increases in fatty acid synthesis occurred upon efflux, the bulk of these fatty acids were esterified to phospholipids (data not shown).

The concentration of individual cholesteryl esters accumulating in cells incubated with normal or hyper-

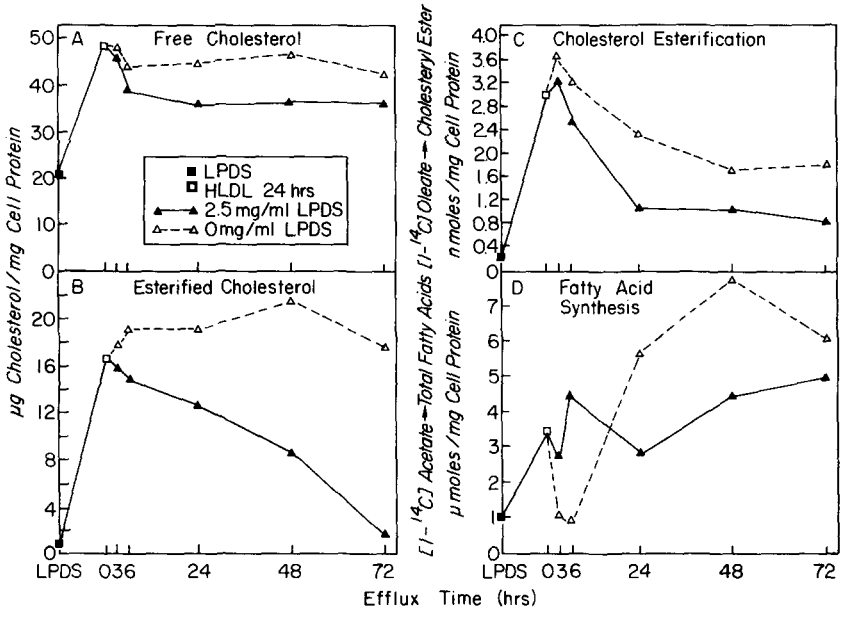

Fig. 4. Changes in cellular cholesterol content, fatty acid synthesis and cholesterol esterification during efflux. Skin fibroblasts from cynomolgus monkeys were incubated for 72 hr with the basal medium containing LPDS. Duplicate cultures were analyzed for cholesterol content, cholesterol esterification, and fatty acid synthesis and these results are represented above as LPDS. The remaining cells were incubated for 24 hr with 50 μg protein/ml of hypercholesterolemic LDL. A subgroup was removed for analysis after this loading phase and is represented as the 0-time point. The remainder of the cells were incubated with culture medium only, or with efflux medium containing 2.5 mg/ml of calf serum LPDS for the indicated times. At these times, one group of cells was pulse-labeled for 2 hr with [1-^{14}C]acetate and another group with [1-^{14}C]oleate. These cells were harvested for determination of fatty acid synthesis and cholesterol esterification. Results are the mean of duplicate cultures for all of the efflux times and the mean of four replicate cultures for the LPDS and 0-time samples.

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TABLE 1. Concentration of individual cholesteryl esters in monkey skin fibroblasts incubated with normal or hypercholesterolemic LDL

	Fatty Acids (Carbon No:Double Bonds)					
	16:0	18:0	18:1	18:2	20:4	Unidentified
	μg/mg cell protein					
Cellular cholesteryl esters						
Control	2.6	1.1	0.76	0.11	ND	
NLDL	4.1	7.1	17.7	6.6	1.6	
HLDL	9.8	7.9	22.7	3.7	1.8	
	% of total fatty acids					
LDL fatty acids						
NLDL	12.0	4.7	23.8	50.8	2.4	6.3
HLDL	9.4	3.6	38.7	39.4	3.5	5.4

Confluent T-75 flasks of skin fibroblasts from cynomolgus monkeys were incubated for 48 hr with LPDS-containing medium, then for an additional 24 hr with culture medium containing 50 μg protein/ml of normal (NLDL) or hypercholesterolemic (HLDL) low density lipoprotein. Control cells were maintained on medium containing calf serum prior to analysis. Results are the mean from duplicate flasks (duplicates differed by less than 5%) and duplicate analyses of the lipoproteins. The free and esterified cholesterol content of the cells were 52.8 and 23.7 μg/mg cell protein, respectively, for cells incubated with NLDL; 57.1 and 32.6 μg/mg cell protein, respectively, for cells incubated with HLDL; and 33.3 and 4.2 μg/mg cell protein, respectively, for control cells maintained on growth medium containing 10% calf serum. The micrograms of individual cellular cholesteryl esters were calculated by multiplying the total cellular cholesteryl ester content (esterified cholesterol × 1.7) by the percentage of individual esters as measured by GLC.

ND = none detected.

cholesterolemic LDL is shown in **Table 1.** As has been described previously (10, 13), the cholesteryl ester fatty acid composition of normal and hypercholesterolemic LDL differ substantially, with normal LDL being enriched in cholesteryl linoleate while hypercholesterolemic LDL is enriched in cholesteryl oleate. As shown in Table 1, the LDL cholesteryl esters used in this experiment exhibited a similar pattern of altered fatty acid composition. In cells incubated with either normal or hypercholesterolemic LDL, cholesteryl oleate represented the major cholesteryl ester species that accumulated. Relative to cells incubated with normal LDL, however, the cells incubated with hypercholesterolemic LDL contained more cholesteryl oleate and less cholesteryl linoleate.

The relative efflux rates of individual cholesteryl esters are shown in **Fig. 5.** The patterns of efflux were similar for cells loaded with cholesteryl esters by incubation with either normal or hypercholesterolemic LDL. It should be pointed out, however, that the total amount of cholesteryl ester accumulation was not as great as for the experiment shown in Table 1 and whether a similar pattern of depletion of individual cholesteryl esters would occur in cells loaded with larger amounts of cholesteryl esters is unknown. After 6 hr of efflux there was a rapid decline in the content of the 18:0 and 18:1 fatty acid cholesteryl esters with an associated increase in the 14:0 and 16:0 fatty acid cholesteryl esters. Thereafter, there was a decline in the concentration of the 16:0 fatty acid species. The concentration of 18:2 fatty acid cholesteryl esters that accumulated in the cells during the loading phase was too low to measure accurately, thus we did not attempt to quantify the efflux rate of the 18:2 fatty acid species.

DISCUSSION

Numerous studies have shown that cellular free cholesterol can exchange with cholesterol from a variety of sources including lipoproteins, biological membranes, and artificial vesicles. In addition, net amounts of cholesterol can be lost from cells or membranes to an appropriate acceptor in the surrounding milieu, resulting in depletion of membrane cholesterol (see Ref. 19 for recent review). Only a relatively few studies, however, have evaluated the characteristics of efflux of cholesterol from cells that have been enriched with cholesteryl esters.

Using a variety of cell types in which cellular cholesterol accumulation was induced by incubation with LDL or acetyl LDL (20–22) or LDL plus chloroquine (23–25), it has been shown that the accumulated cholesterol can be lost from cells to an appropriate acceptor in the culture medium. There is little information from these previous studies, however, on the effect of the efflux of the accumulated cholesterol on cellular cholesterol synthesis or esterification. In a similar manner, there is no

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published information on whether differences exist in the characteristics of cholesterol efflux from cells loaded with cholesterol by prior incubation with the abnormal hypercholesterolemic LDL.

Consistent with results shown previously by others (21, 26), cholesterol efflux demonstrated an absolute requirement for the presence of a cholesterol acceptor in the efflux medium. Within the first 6–24 hr after addition of the efflux medium, 10–30% of the accumulated cellular free cholesterol was lost, with little additional depletion of free cholesterol occurring with time for up to 72 hr as long as cellular cholesteryl ester stores were not fully depleted. Cellular cholesteryl ester content decreased for up to 72 hr after addition of the efflux medium. This decrease was log linear, suggesting that the rate of efflux is concentration-dependent, with the greatest rate occurring when cellular cholesteryl ester concentrations are highest. Similar results have been obtained in macrophages loaded with cholesteryl esters by phagocytosis (27). We cannot exclude the possibility that the initial rate of efflux measured over the first few hours may be linear as suggested by others (22, 28). Greater than 90% of the cholesterol lost from the cells could be accounted for in the efflux medium with virtually all of this occurring as free cholesterol. This is consistent with results by others using a variety of cell types (17, 22). Consequently, it appears that soon after addition of the efflux medium, cellular free cholesterol is lost to the LPDS acceptor. The extent of free cholesterol depletion is limited, however, perhaps due to the requirements of cell membranes for adequate amounts of cholesterol for normal membrane function, as has been described for red blood cell membranes (29). Since free cholesterol is the only form by which cholesterol effluxes from the cell, and since cholesterol synthesis remains suppressed, it is apparent that cholesteryl ester hydrolysis must occur prior to the efflux of the accumulated cholesteryl esters. In macrophages, cholesteryl ester hydrolysis and reesterification appear to occur in a continuous cycle (17). In the absence of a cholesterol acceptor in the medium, cholesteryl esters are continuously being hydrolyzed and reesterified without loss of cholesterol from the cell. In the presence of an acceptor, however, the free cholesterol that is generated is lost from the cell. Although in the present study we have no direct evidence of such a cycle occurring in the absence of the LPDS acceptor, the continual enhancement of cholesterol esterification during the efflux phase (relative to cholesterol depleted cells) is consistent with such a mechanism. Furthermore, in the presence of LPDS the temporal sequence of depletion of free and esterified cholesterol suggests that the free cholesterol generated from the hydrolysis of cholesteryl esters is first transferred to cell membranes prior

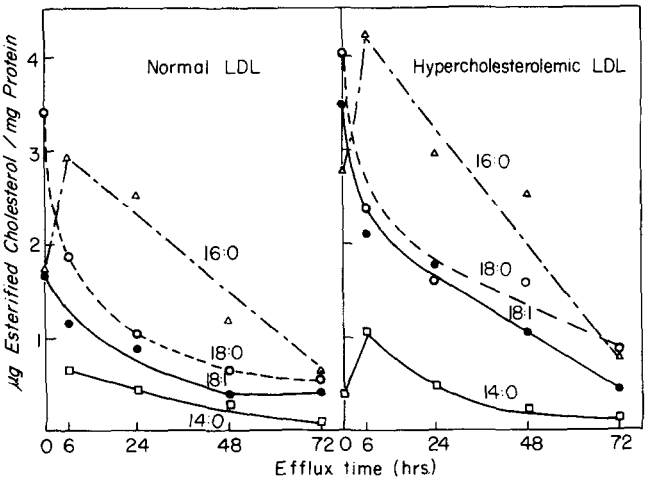

Fig. 5. Accumulation and removal of individual cholesteryl esters from skin fibroblasts loaded with cholesterol by incubation with normal or hypercholesterolemic LDL. Cells were incubated for 48 hr with LPDS, then for 24 hr with 50 μg/ml of normal or hypercholesterolemic LDL. A subgroup was analyzed for cholesteryl ester content after the loading phase and represents the 0-time point. The remainder of the cells were incubated with 2.5 mg/ml of calf serum LPDS for the indicated times and the efflux of the individual cholesteryl esters was measured. Individual cholesteryl ester concentrations were calculated from the total cholesteryl ester content as measured by GLC and the cholesteryl ester fatty acid composition was measured at each time point. Results are the mean of duplicate determinations. The 14:0 concentration was not included for the normal LDL 0-time sample due to the low concentrations of 14:0 that made quantification unreliable. The lines represent the best fit for all of the points for each of the cholesteryl ester species. Cholesteryl ester fatty acids are designated as number of carbon atoms:number of double bonds.

to being lost from the cell, which would account for the lack of further free cholesterol depletion after the initial 6–24 hr of efflux. As was true for macrophages (17) and aortic smooth muscle cells (30), the hydrolysis of the accumulated cellular cholesteryl esters does not appear to be carried out in the lysosomes as evidenced by the failure of chloroquine to alter the rate of efflux. In addition, the free cholesterol generated from cholesteryl ester hydrolysis during efflux apparently equilibrates with the regulatory pool of cellular cholesterol, since endogenous cholesterol synthesis remains maximally suppressed throughout the entire efflux period. Thus, it appears that when cells are stimulated to lose cholesterol, they respond differently depending on whether they contain stores of cholesteryl esters. If the cells are not enriched with cholesteryl esters, cholesterol synthesis is stimulated to compensate for the lost cholesterol, and measurement of cellular cholesterol depletion will underestimate total cellular cholesterol loss by the amount contributed to by synthesis (28). As shown in the present study, if the cells have been loaded with cholesteryl esters, cholesterol synthesis is not stimulated and the free cholesterol lost to efflux is instead replaced by cholesterol from hydrolyzed cholesteryl esters.

As mentioned previously, the LPDS was added to the

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efflux medium in excess so as not to limit efflux. Thus, the wide range of differences in the rate of cellular cholesterol depletion among cell lines cannot be readily explained by rate-limiting amounts of cholesterol acceptor, but instead is probably due to differences in the cells themselves. Phillips et al. (28) have suggested that in the presence of excess acceptor, processes such as the rate of dissociation of cholesterol from the plasma membrane into the surrounding aqueous milieu may control the rate of loss of cholesterol from the cell. Other cellular processes, such as the rate of cholesteryl ester hydrolysis, the rate of transfer of the generated free cholesterol from the site of hydrolysis to the plasma membrane, etc., have also been suggested to explain differences in rate of efflux among cells (22). It is not possible from the present study to distinguish among these possibilities.

After uptake of LDL by cells in culture, the LDL and its component cholesteryl esters are directed to the lysosomes where the cholesteryl esters are hydrolyzed by a lysosomal cholesteryl ester hydrolase (31, 32). The free cholesterol that is generated is rapidly reesterified in the endoplasmic reticulum by the enzyme acyl CoA:cholesterol acyltransferase (ACAT) (33). The resterification of cholesterol by ACAT occurs preferentially with oleic acid. As a result, if the cholesteryl esters that accumulate in the cells are the result of reesterification of cholesterol that has already been through the lysosomes, one would expect them to be enriched with cholesteryl oleate relative to the original LDL. Based on the marked enrichment of cellular cholesteryl esters with oleic acid and the paucity of cholesteryl linoleate, we would conclude that the bulk of the cellular cholesteryl esters that accumulate with both normal and hypercholesterolemic LDL are the result of resynthesis by ACAT.

Because the cholesteryl esters that accumulated in cells incubated with either normal or hypercholesterolemic LDL were similar in composition, it is perhaps not surprising that for the same cell line the rate of cholesteryl ester depletion was similar as well. This similarity in efflux rate was also seen in the depletion of several of the individual cholesteryl ester species (Fig. 5). Cholesteryl stearate and oleate were lost from the cells at similar rates, which is consistent with previous studies by others (34, 35). An unexpected finding, however, was the absolute increase in concentrations of cholesteryl palmitate, and to some extent cholesteryl myristate, after 6 hr of efflux, and the linear rather than curvilinear shape of the efflux curve for these cholesteryl ester species. As shown in Fig. 4, de novo fatty acid synthesis is stimulated approximately 3-fold in cells that are accumulating cholesteryl esters during the loading phase of the experiment. Since palmitic acid is the principal product of de novo fatty acid synthesis, these data are consistent with the conclusion that the increase in cholesteryl palmitate at 6 hr may be the result of synthesis of palmitic acid and its esterification to cholesterol by ACAT. Such a mechanism might also be expected to change the shape of the cholesteryl palmitate efflux curve, as appears to be the case (Fig. 5).

The results of this study indicate that the rate of cellular cholesteryl ester depletion is similar for the same cell line regardless of whether the cells have been loaded with cholesterol by prior incubation with normal or hypercholesterolemic LDL. Consequently, the greater accumulation of cholesterol in cells incubated with hypercholesterolemic LDL cannot be explained by differences in rates of efflux, but instead is probably best explained by enhanced delivery of cholesterol to the cells by the hypercholesterolemic LDL.

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REFERENCES

1. St. Clair, R. W., B. P. Smith, and L. L. Wood. 1977. Stimulation of cholesterol esterification in rhesus monkey arterial smooth muscle cells. *Circ. Res.* **40:** 166–173.
2. St. Clair, R. W., and M. A. Leight. 1978. Differential effects of isolated lipoproteins from normal and hypercholesterolemic rhesus monkeys on cholesterol esterification and accumulation in arterial smooth muscle cells in culture. *Biochim. Biophys. Acta.* **530:** 279–291.
3. St. Clair, R. W., J. J. Mitschelen, and M. A. Leight. 1980. Metabolism by cells in culture of LDL of abnormal composition from nonhuman primates with diet-induced hypercholesterolemia. *Biochim. Biophys. Acta.* **618:** 63–79.
4. St. Clair, R. W., and J. J. Mitschelen. 1980. Studies on the cellular mechanisms of the increased atherogenicity of low density lipoproteins from nonhuman primates with diet-induced hypercholesterolemia. *In* The Use of Nonhuman Primates in Cardiovascular Diseases. S. S. Kalter, editor. University of Texas Press, Austin and London. 147–177.
5. Bates, S. R. 1979. Accumulation and loss of cholesterol esters in monkey arterial smooth muscle cells exposed to normal and hyperlipemic serum lipoproteins. *Atherosclerosis.* **32:** 165–176.
6. Eisele, B., S. R. Bates, and R. W. Wissler. 1980. Interaction of low density lipoproteins from normal and hyperlipemic rhesus monkeys with arterial smooth muscle cells in culture. *Atherosclerosis.* **36:** 9–24.
7. Rothblat, G. H. 1974. Cholesteryl ester metabolism in tissue culture cells. I. Accumulation in Fu5AH rat hepatoma cells. *Lipids.* **9:** 529–535.
8. Rudel, L. L., L. L. Pitts II, and C. A. Nelson. 1977. Characterization of plasma low density lipoproteins of nonhuman primates fed dietary cholesterol. *J. Lipid Res.* **18:** 211–222.
9. Rudel, L. L., R. Shah, and D. G. Greene. 1979. Study of the atherogenic dyslipoproteinemia induced by dietary

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cholesterol in rhesus monkeys (*Macaca mulatta*). *J. Lipid Res.* **20:** 55–65.

10. Rudel, L. L., C. W. Leathers, M. G. Bond, and B. C. Bullock. 1981. Dietary ethanol-induced modifications in hyperlipoproteinemia and atherosclerosis in nonhuman primates (*Macaca nemestrina*). *Arteriosclerosis.* **1:** 144–155.
11. St. Clair, R. W. 1980. Differences in the metabolism of abnormal atherogenic low density lipoproteins from cholesterol-fed nonhuman primates by cells in culture. *In* Atherosclerosis V. Proceedings of the Fifth International Symposium on Atherosclerosis. A. M. Gotto, Jr., L. C. Smith, and B. Allen, editors. Springer-Verlag, New York. 541–545.
12. Guertler, L. S., and R. W. St. Clair. 1977. In vitro regulation of cholesterol metabolism by low density lipoproteins in skin fibroblasts from hypo- and hyper-responding squirrel monkeys. *Biochim. Biophys. Acta.* **487:** 458–471.
13. Tall, A. R., D. M. Small, D. Atkinson, and L. L. Rudel. 1978. Abnormal structure of atherogenic low density lipoproteins from *Macaca fascicularis*. *J. Clin. Invest.* **62:** 1354–1363.
14. Kashyap, M. L., B. A. Hynd, and K. Robinson. 1980. A rapid and simple method for measurement of total protein in very low density lipoproteins by the Lowry assay. *J. Lipid Res.* **21:** 491–495.
15. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
16. St. Clair, R. W., H. B. Lofland, Jr., and T. B. Clarkson. 1968. Composition and synthesis of fatty acids in atherosclerotic aortas of the pigeon. *J. Lipid Res.* **9:** 739–747.
17. Brown, M. S., Y. K. Ho, and J. L. Goldstein. 1980. The cholesteryl ester cycle in macrophage foam cells. Continual hydrolysis and re-esterification of cytoplasmic cholesteryl esters. *J. Biol. Chem.* **255:** 9344–9352.
18. Rothblat, G. H., and D. Kritchevsky. 1967. The excretion of free and ester cholesterol by tissue culture cells: studies with L5178Y and L-cells. *Biochim. Biophys. Acta.* **144:** 423–429.
19. Bell, F.P. 1978. Lipid exchange and transfer between biological lipid-protein structures. *Prog. Lipid Res.* **17:** 207–243.
20. Stein, O., J. Vanderhoek, and Y. Stein. 1976. Cholesterol content and sterol synthesis in human skin fibroblasts and rat aortic smooth muscle cells exposed to lipoprotein-depleted serum and high density apoprotein/phospholipid mixtures. *Biochim. Biophys. Acta.* **431:** 347–358.
21. 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.
22. Daniels, R. J., L. S. Guertler, T. S. Parker, and D. Steinberg. 1981. Studies on the rate of efflux of cholesterol from cultured human skin fibroblasts. *J. Biol. Chem.* **256:** 4978–4983.
23. Stein, O., J. Vanderhoek, G. Friedman, and Y. Stein. 1976. Deposition and mobilization of cholesterol ester in cultured human skin fibroblasts. *Biochim. Biophys. Acta.* **450:** 367–378.
24. Stein, O., J. Vanderhoek, and Y. Stein. 1977. Cholesterol ester accumulation in cultured aortic smooth muscle cells. *Atherosclerosis.* **26:** 465–482.
25. Stein, O., G. Halperin, and Y. Stein. 1979. Comparison of cholesterol egress from cultured cells enriched with cholesterol ester after exposure to cationized LDL or to LDL and chloroquine. *Biochim. Biophys. Acta.* **573:** 1–11.
26. Stein, Y., M. C. Glangeaud, M. Fainaru, and O. Stein. 1975. The removal of cholesterol from aortic smooth muscle cells in culture and Landschütz ascites cells by fractions of human high-density apolipoprotein. *Biochim. Biophys. Acta.* **380:** 106–118.
27. Werb, Z., and Z. A. Cohn. 1972. Cholesterol metabolism in the macrophage. III. Ingestion and intracellular fate of cholesterol and cholesterol esters. *J. Exp. Med.* **135:** 21–44.
28. Phillips, M. C., L. R. McLean, G. W. Stoudt, and G. H. Rothblat. 1980. Mechanism of cholesterol efflux from cells. *Atherosclerosis.* **36:** 409–422.
29. Cooper, R. A., E. C. Arner, J. S. Wiley, and S. J. Shattil. 1975. Modification of red cell membrane structure by cholesterol-rich lipid dispersions. A model for the primary spur cell defect. *J. Clin. Invest.* **55:** 115–126.
30. Stein, O., G. A. Coetzee, and Y. Stein. 1980. Modulation of cytoplasmic cholesteryl ester of smooth muscle cells in culture derived from rat, rabbit and bovine aorta. *Biochim. Biophys. Acta.* **620:** 538–549.
31. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1975. Receptor-dependent hydrolysis of cholesteryl esters contained in plasma low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **72:** 2925–2929.
32. Goldstein, J. L., S. E. Dana, J. R. Foust, A. L. Beaudet, and M. S. Brown. 1975. Role of lysosomal acid lipase in the metabolism of plasma low density lipoprotein. Observations in cultured fibroblasts from a patient with cholesteryl ester storage disease. *J. Biol. Chem.* **250:** 8487–8495.
33. Goldstein, J. L., S. E. Dana, and M. S. Brown. 1974. Esterification of low density lipoprotein cholesterol in human fibroblasts and its absence in homozygous familial hypercholesterolemia. *Proc. Natl. Acad. Sci. USA* **71:** 4288–4292.
34. Stein, Y., G. Halperin, and O. Stein. 1978. Intralysosomal hydrolysis of cholesterol esters of varying fatty acid composition in cultured human skin fibroblasts. *Biochim. Biophys. Acta.* **530:** 420–427.
35. Day, A. J., and M. Sheers. 1977. Synthesis and removal of different cholesterol esters by aortic smooth muscle cells in culture. *Atherosclerosis.* **26:** 483–492.

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