Stimulation of esterified cholesterol accumulation in tissue culture cells exposed to high density lipoproteins enriched in free cholesterol

George H. Rothblat, Loretta Y. Arbogast, and Eva K. Ray

Department of Physiology/Biochemistry, The Medical College of Pennsylvania, 3300 Henry Ave., Philadelphia, PA 19129

Abstract Human high density lipoprotein enriched in free cholesterol was obtained by exposing the lipoprotein to lipid dispersions having a free cholesterol/lecithin molar ratio greater than two. The metabolism of cholesterol was studied in tissue culture cells exposed to normal and cholesterol-enriched lipoproteins. Incubation of Fu5-AH rat hepatoma cells in medium containing cholesterol-enriched lipoprotein resulted in the accumulation of cellular cholesterol whereas normal high density lipoprotein produced no change in cellular content. The accumulated sterol was recovered primarily as esterified cholesterol and was derived almost entirely from lipoprotein free cholesterol. The esterification of incorporated free cholesterol and the cellular cholesterol content were directly related to the molar ratio of free cholesterol to phospholipid in the lipoprotein and to the concentration of lipoprotein in the culture medium. Isotopic experiments utilizing lipoprotein labeled with ^{125}I or [4-^{14}C]cholesteryl oleate demonstrated that a large fraction of the cholesterol incorporated from lipoprotein enriched in free cholesterol occurred by mechanisms that did not result in lipoprotein internalization and degradation. The response of other tissue culture cells to cholesterol/phospholipid dispersions is presented. The data indicate that the lipid composition of a lipoprotein can regulate free cholesterol uptake and esterification as well as cellular cholesterol content.

Supplementary key words hepatoma · cholesterol esterification · phospholipids

The incorporation of serum cholesterol by cells can occur via two possible mechanisms, internalization of intact lipoprotein molecules and transfer of cholesterol from serum lipoproteins to the cellular plasma membrane without concurrent incorporation of the lipoprotein itself (surface transfer). The binding and internalization of the intact lipoprotein would carry the entire complement of lipoprotein lipid into the cell. This process, which has been studied in a variety of cell systems, is thought to involve endocytosis mediated through cellular receptors and has been shown to result in the incorporation of both exogenous free cholesterol (FC) and esterified cholesterol (EC) (1–4). The incorporated cholesterol is an effective regulator of both de novo sterol biosynthesis and cellular esterification of cholesterol (1, 2).

The extent to which cellular cholesterol content and metabolism are influenced by the second process involving surface transfer mechanisms is not clearly understood. Evidence has accumulated from studies conducted on both whole tissues and isolated cells demonstrating the incorporation of labeled unesterified cholesterol into cells, apparently without uptake of the entire lipoprotein (5–8). In most of these experimental systems, the movement of cholesterol into the cell may not be associated with significant increases in the cellular content of cholesterol (1, 9). Surface transfer that resulted in a one-for-one molecular exchange of sterol would not alter cellular cholesterol content; however, a shift in the equilibrium between serum lipoprotein and the plasma membrane could result in net movement of FC. This surface transfer of FC could result in either net cellular uptake (influx), net cellular loss (efflux), or no change in the cellular concentration (exchange). Efflux of sterol resulting in cellular cholesterol depletion has been shown with cells incubated with high density lipoprotein (HDL) or delipidated apoproteins (10, 11). Net cellular uptake is the subject of this report.

Previous studies in our laboratory have shown that the flux of cholesterol between cells and their

Abbreviations: MEM, minimal essential medium; FC, free cholesterol; EC, esterified cholesterol; PL, phospholipid; HDL, high density lipoprotein; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; LCAT, lecithin: cholesterol acyltransferase.

growth medium could be markedly influenced by the protein, phospholipid, and FC content of the medium (10, 12–14). More recent investigations have established the ability to produce an increase in the FC content of red blood cells and platelets by incubation with dispersions having cholesterol/phospholipid (FC/PL) molar ratios greater than one (15, 16). Similar dispersions have also been employed to produce an increase in the EC content of Fu5-AH rat hepatoma cells (17). Increased cellular EC content occurred only when dispersions were presented to cells together with serum or isolated serum lipoproteins, suggesting that the uptake of exogenous FC from the dispersions was mediated through serum lipoproteins (17).

Since Cooper et al. (15) had previously shown that incubation of HDL with dispersions having a FC/PL molar ratio greater than one produced an increase in the FC content of the HDL, the present study was initiated to determine if modification of the FC content of lipoproteins can cause net accumulation of either FC or EC by cells and to gain further insight into whether such accumulation proceeds through surface transfer of FC or internalization of lipoproteins.

MATERIALS AND METHODS

Cells and growth conditions

The Fu5-5 and Fu5-AH rat hepatoma cell lines were derived from the Reuber H-35 rat hepatoma (18). Cultures of rabbit aortic medial cells were obtained from aortic explants and used between the 5th and 8th passages. IMF diploid human lung fibroblasts were obtained from Dr. E. Levine, Wistar Institute, and used between the 20th and 30th passages. Hepatocytes were taken from adult rats following the method of Laishes and Williams (19) and used 18 hr after plating.

Monolayers of hepatomas, fibroblasts, and medial cells were grown in Eagle's minimal essential medium (MEM, Auto-Pow, Flow Labs, Rockville, MD) supplemented with BME vitamins and 10% fetal bovine serum. Hepatocytes were grown in Williams Medium E supplemented with 10% fetal bovine serum (19). Cells from a single 35 or 60-mm petri dish (Falcon Plastics, Oxnard, CA) provided sufficient material for both isotopic and gas–liquid chromatographic (GLC) analysis.

Free cholesterol–lecithin dispersions

Cholesterol–lecithin dispersions having a FC/PL ratio greater than one were prepared as previously described (15–17). Briefly, 40 mg of L-α-dipalmitoyllecithin (Grand Island Biological Co., Grand Island, NY) and 80 mg of free cholesterol (Sigma Chemical Co., St. Louis, MO) were added to 10 ml of physiological saline and sonicated for 20 min at 45°C with a Branson Sonifer (70 watts) to yield dispersions with molar ratios ranging from 2.5 to 3.5. Reduction in both the amount of cholesterol (35 mg) and the time of sonication (10 min) produced dispersions with molar ratios of 1.5–1.8. After sonication, dispersions were centrifuged at 26,000 *g* for 30 min and the supernatant material was sterilized by filtration through a 0.45 μm Millipore filter. In certain experiments free [4-^{14}C]cholesterol (10 mCi/mmol, New England Nuclear, Boston MA) was added to an ethanolic solution, to preformed lipid dispersions, or to the cholesterol–lecithin mixtures prior to sonication (17).

High density lipoproteins

Human plasma was obtained from blood supplied by the blood bank of the Medical College of Pennsylvania. Rabbit serum was purchased from Flow Laboratories. High density lipoprotein (HDL) was isolated by density centrifugation techniques following the procedure described by Havel, Eder, and Bragdon (20). The lipoproteins were washed by recentrifugation at d 1.21 g/ml.

HDL was delipidized and reassembled following the method of Scanu et al. (21). Reassembly was accomplished using HDL lipids to which were added 9.38 μCi of [7α-^{3}H]cholesterol (New England Nuclear) and 0.17 μCi of [4-^{14}C]cholesteryl oleate (New England Nuclear) per mg of HDL protein. Following reassembly by sonication of the apoproteins and the isotopically labeled lipids, doubly labeled lipoprotein of d 1.063–1.21 g/ml was obtained following the procedure of Scanu et al. (21).

Human HDL was iodinated with ^{125}I following the iodine monochloride method of MacFarlane as modified by Bilheimer, Eisenberg, and Levy (22) and employing 7 mol ^{125}I per mol HDL (^{125}I specific activity, 82 mCi/mol). All HDL preparations were iodinated prior to treatment with dispersions. More than 99% of the ^{125}I-HDL precipitated with 10% trichloroacetic acid (TCA) and from 1–3% of the ^{125}I was associated with lipid. All preparations of HDL (native, reassembled, and iodinated) were extensively dialyzed against 0.15 M NaCl, 0.001 M EDTA and sterilized by filtration through 0.45 μm Millipore filters prior to use. In certain experiments indicated in the text, [4-^{14}C]cholesterol (10 mCi/mmol) was added as an ethanolic solution to iodinated preparations of HDL.

The apoprotein compositions of the control and dispersion-treated ^{125}I-HDL were compared using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis employing the procedure of Weber, Pringle, and Osborn (23) as modified by Swaney, Reese, and Eder (24). Samples were electrophoresed on a single 10% polyacrylamide slab and compared using Coomassie Brilliant Blue staining and gel slicing techniques. The radioactivity distribution of the sliced, stained gels was determined by counting individual slices for ^{125}I radioactivity.

Dispersion–modification of HDL

HDL was modified by incubating the lipoprotein with a 20-fold excess of dispersion FC for 24 hr at 37°C. Dispersions were separated from HDL by flotation at d 1.064 g/ml (100,000 *g*, 20 hr). Dispersion-treated HDL was dialyzed against 0.15 NaCl, 0.001 M EDTA, analyzed for protein and lipid composition, then redialyzed against tissue culture medium. This material was sterilized by filtration and reanalyzed for protein, phospholipid, and FC content prior to addition to cell cultures.

Cellular incubation conditions and analysis

Monolayers of Fu5-AH cells were refed with medium containing either control or dispersion-treated HDL at concentrations indicated in the text. In the experiments comparing the response of various cell types, FC–lecithin dispersions sufficient to supply 150 μg FC/ml were added directly to the culture medium together with 5% whole human serum. Following incubation at 37°C, media were removed and cell monolayers were washed twice with phosphate buffered saline (PBS). Cells were detached with 0.25% trypsin, resuspended in PBS, and washed twice by centrifugation.

Analysis for protein, FC and EC content, and incorporation and metabolism of exogenous radiolabeled cholesterol were conducted as previously described (25, 26). In experiments utilizing ^{125}I-HDL, metabolism of the lipoproteins was quantitated using minor modifications of published procedures (1–4). TCA-soluble and insoluble materials were assayed following precipitation with 10% TCA. TCA precipitates were collected on glass fiber filters and ^{125}I-labeled lipids were removed by chloroform extraction. TCA-soluble ^{125}I counts in the culture media were corrected for ^{125}iodide using the H_2O_2 oxidation method of Bierman, Stein, and Stein (27).

Analytical procedures

Proteins were assayed by the method of Lowry et al. (28) and lipids were extracted as described by Bligh and Dyer (29). Cholesterol was quantitated by GLC (25, 26). Phospholipid phosphorus was measured by the method described by Sokoloff and Rothblat (10). Thin-layer chromatographic separation of lipids was performed using silica gel IB2 plates (Baker Chemical Co., Phillipsburg, NJ) developed in petroleum ether–ethyl ether–acetic acid 75:24:1. ^{14}C and ^{3}H were quantitated by liquid scintillation techniques in a Packard Tri-carb liquid spectrometer using LSC complete scintillator, (Yorktown Research, Hackensack, NJ). ^{125}I was quantitated using a Packard Gamma counter.

RESULTS

FC enrichment of HDL

Twenty-four-hour exposure of HDL to FC/lecithin dispersions having molar ratios greater than 2 and subsequent re-isolation of the HDL by centrifugation resulted in a marked increase in the FC content of the lipoprotein (**Table 1**). Accompanying the increase in FC, small decreases in phospholipid and esterified cholesterol content were noted. The apoprotein pattern of ^{125}I-HDL was not quantitatively or qualitatively altered by dispersion treatment using SDS-polyacrylamide gel electrophoretic analysis. The fourfold increase that occurred in the FC content of HDL after treatment with dispersions resulted in a FC/phospholipid molar ratio greater than one compared to a ratio of 0.2 in the control HDL. Dispersion treatment of HDL isolated from rabbit serum also resulted in an enrichment of FC content similar to that observed with human lipoprotein (data not shown).

Cellular response to cholesterol-enriched HDL

Fig. 1 illustrates the FC and EC content of Fu5-AH cells incubated in medium containing increasing concentrations of untreated, control human HDL and dispersion-treated HDL enriched in cholesterol. In contrast to the lack of response obtained with

TABLE 1. Alteration of HDL composition by cholesterol-rich dispersions

HDL	Free Cholesterol	Esterified Cholesterol	Phospholipids	Molar ratio FC/PL
	mg/mg HDL Protein (±SE)			
Untreated	0.05 ± 0.01	0.23 ± 0.01	0.40 ± 0.02	0.25
Treated[a]	0.20 ± 0.01[b]	0.18 ± 0.02[c]	0.33 ± 0.02[c]	1.23

[a] HDL incubated for 18 hr at 37°C with FC/PL dispersions (FC/PL > 2). Ratio of dispersion FC/HDL FC = 20. N = 8.
[b] $P < 0.001$.
[c] $P < 0.05$.

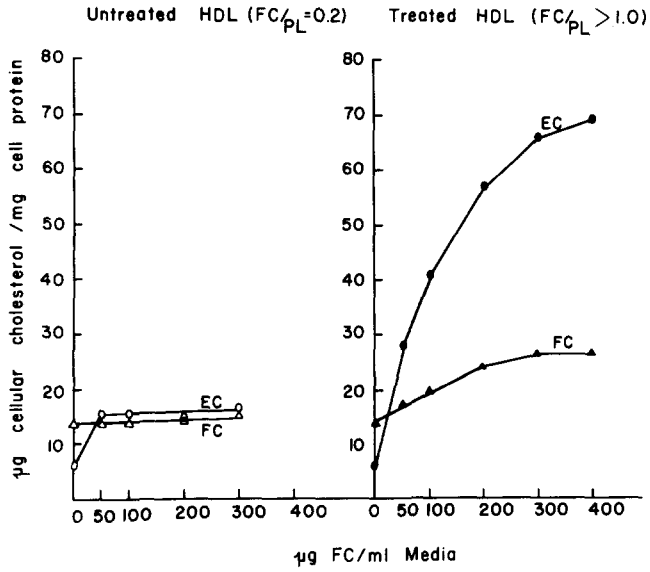

Fig. 1. Free cholesterol (FC) and esterified cholesterol (EC) content of Fu5-AH hepatoma cells grown for 24 hr in medium supplemented with increasing concentrations of either untreated human HDL or FC-enriched HDL. Average of duplicate determinations.

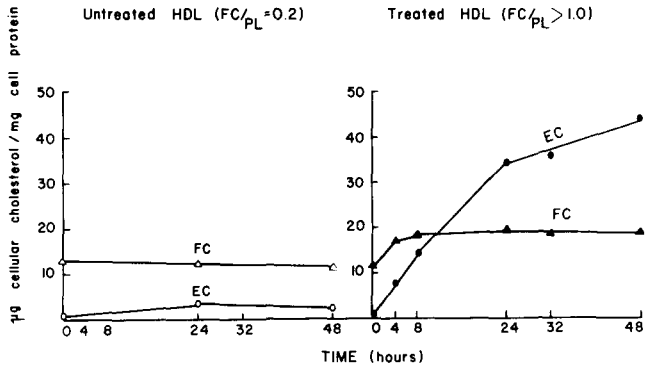

Fig. 2. Free cholesterol (FC) and esterified cholesterol (EC) content of Fu5-AH hepatoma cells grown in normal human HDL and FC-enriched HDL. Both lipoprotein preparations added to culture medium to supply 150 μg FC/ml. Average of duplicate determinations.

untreated HDL, there was a dose-dependent increase in the cholesterol content of cells incubated in the presence of dispersion-treated HDL. Although there was a small increase in the FC content of the cells, the most dramatic change was observed in the cellular EC content. Increasing the concentration of HDL above 400 μg FC/ml gave little further increase in the FC and EC content of the cells.

The time course of the cellular response to treated HDL is shown in **Fig. 2**. A small increase in FC content occurred in the first 4–8 hr of incubation after which no additional increase was noted. The cellular EC content increased in a linear manner for 24 hr and continued to increase, although at a slower rate, upon further incubation. Incubation of Fu5-AH cells in the presence of control HDL resulted in no increase in either FC or EC content during the course of the experiment. A cellular response similar to that shown in Figs. 1 and 2 was produced by dispersion-treated, FC-enriched rabbit HDL (data not shown) indicating that there was no species specificity associated with the cellular response.

To assess if the extent of FC-enrichment could alter cellular accumulation of EC, cells were incubated in media containing HDL having molar ratios of 0.2 (control), 0.5, and 1.1 (**Fig. 3**). As previously noted in Fig. 1, increasing concentration of FC-enriched HDL produced a dose-dependent increase in cellular EC content in contrast to the lack of response produced by control HDL. Moreover, the amount of EC that accumulated was correlated with the extent to which the FC/PL molar ratio of the lipoprotein had been increased (Fig. 3). Heating the HDL at 56°C for 30 min to remove LCAT activity did not alter the cellular response.

Origin of the EC and mechanism of accumulation

In an effort to establish the origin of the EC that accumulates in the Fu5-AH rat hepatoma cell upon exposure to HDL enriched in FC, HDL was treated with dispersion and subsequently labeled with [4-^{14}C]-cholesterol (see Methods), then added to culture medium at increasing concentrations. Following incubation, the cellular uptake of radiolabeled FC was

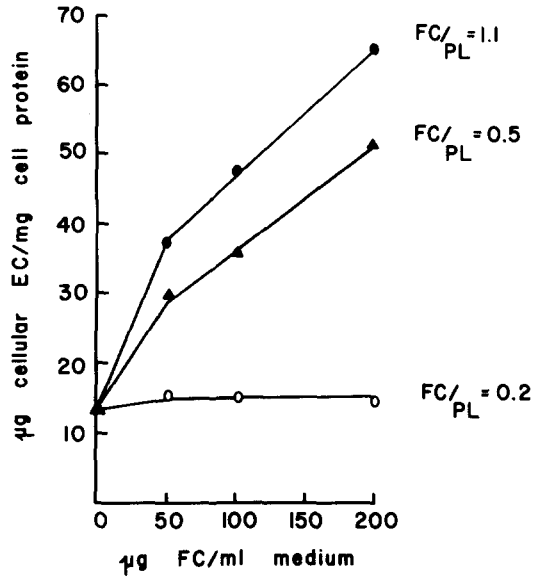

Fig. 3. Esterified cholesterol (EC) content of Fu5-AH hepatoma cells grown for 24 hr in medium supplemented with increasing concentrations of normal, human HDL (FC/PL = 0.2) and HDL preparations enriched with two different concentrations of free cholesterol. Average of duplicate determinations.

TABLE 2. Contribution of free cholesterol from free cholesterol-enriched HDL to cellular cholesterol in FU5-AH cells

μg HDL-FC / ml media	HDL-FC Incorporated: μg/mg Cell Protein	HDL-FC Incorporated: % Esterified	Cellular FC: μg/mg Cell Protein	Cellular FC: % from HDL-FC	Cellular EC: μg/mg Cell Protein	Cellular EC: % from HDL-FC
50	30.2	47	16.0	102	14.2	58
100	49.0	66	16.7	95	32.3	81
200	71.4	73	19.3	100	52.1	93
300	89.9	75	22.5	104	67.4	96
400	91.0	77	20.9	98	70.1	93

Cells were incubated for 24 hr in various concentrations of HDL enriched in FC (FC/PL = 1.1), labeled with [4-^{14}C]cholesterol. FC incorporation was determined by quantitating cellular radioactivity and the data calculated from the specific activity of the HDL-FC at the start of the experiment. Cellular cholesterol content was determined by GLC analysis. Results are averages of duplicate determinations. Zero time cells had 12.3 μg FC/mg protein and 2.2 μg EC/mg protein.

quantitated and compared to the net accumulation of cellular sterol as determined by GLC analysis. As shown in **Table 2**, essentially all of the FC recovered from the cells had been derived from exogenous radiolabeled FC. A large fraction (47–77%) of the incorporated FC was esterified by the cells during the 24-hr incubation and, at concentrations greater than 100 μg HDL-FC/ml, the esterification of exogenous FC contributed over 80% of the EC recovered in the cells. The cellular EC that was not derived from exogenous radiolabeled FC may reflect unlabeled cholesterol present in the cells at the start of the experiment and cholesterol that was synthesized during the incubation. The contribution of these unlabeled pools of cholesterol would be largest at low HDL concentrations where net EC accumulation was minimal. Previous studies have shown that endogenous or "zero time" free cholesterol is rapidly and extensively esterified upon exposure of Fu5-AH cells to hyperlipemic rabbit serum (26).

TABLE 3. Cellular uptake and metabolism of free and esterified cholesterol from reconstituted HDL having normal and elevated free cholesterol composition

Reconstituted HDL Added to Culture Medium[a]	HDL Cholesterol in Cells (24 hr incubation): μg/mg Cell Protein FC[b]	μg/mg Cell Protein EC[c]	FC Esterified[d]	Apparent EC Hydrolysis[e]
			%	%
Normal FC (FC/PL = 0.25)	5.2	2.0	17.1	65.6
Enriched FC (FC/PL = 1.31)	29.4	1.7	61.7	25.8

[a] Composition of medium (μg/ml): normal HDL: EC = 100, FC = 32, PL = 236, protein = 455; enriched HDL: EC = 100, FC = 180, PL = 280, protein = 625.
[b] Calculated from specific activity of [^{3}H]cholesterol in HDL.
[c] Calculated from specific activity of [^{14}C]cholesteryl ester in HDL.
[d] Percentage of the [^{3}H]cholesterol incorporated by cells and recovered as EC.
[e] Percentage of the [^{14}C]cholesteryl oleate incorporated by cells and recovered as FC.

To obtain further information on the metabolism of both FC and EC, the hepatoma cells were incubated in the presence of reassembled HDL that had been doubly labeled with [7-α-^{3}H]-cholesterol and [4-^{14}C]cholesteryl oleate (**Table 3**). In these experiments the uptake of exogenous EC was taken as a marker for lipoprotein internalization. When exposed to HDL that had a normal FC/PL ratio, 2.0 μg of [^{14}C]cholesteryl ester were incorporated. Based on the composition of the normal HDL, the incorporation of 2.0 μg EC would be accompanied by the uptake of 0.6 μg FC/mg cell protein if the whole lipoprotein was internalized. However, actual cellular uptake of free [^{3}H]cholesterol was 5.2 μg/mg protein of which 17% was recovered as EC. The cholesterol content of the cells did not change significantly during the incubation. Thus, in the presence of normal HDL, a small amount of FC apparently was taken up by surface transfer; however, this uptake represented a one-for-one exchange and produced no change in cellular cholesterol concentration.

In the presence of doubly labeled, reconstituted HDL enriched in FC, the cellular uptake of [^{14}C]-cholesteryl ester was 1.7 μg/mg protein (Table 3). Internalization of enriched HDL with a FC/EC ratio of 1.8 would have provided 3.1 μg FC/mg protein. Actual uptake of free [^{3}H]cholesterol was 29.4 μg/mg cell protein. Thus, under these experimental conditions, approximately 90% of the FC recovered in the cells after a 24-hr incubation in enriched HDL was incorporated by surface transfer. In addition, 61.7% of this FC was esterified by the cells providing 18.1 μg of cellular EC. This uptake was not simply an exchange process, because the cellular content of both FC and EC increased by 5.7 μg/mg protein and 34.2 μg/mg protein, respectively.

The apparent hydrolysis of the incorporated [4-^{14}C]-cholesteryl oleate as measured by the recovery of cellular free [^{14}C]cholesterol differed considerably between cells incubated in normal or in enriched HDL. With normal HDL 66% of the incorporated labeled EC was present as cellular FC, whereas with HDL enriched in FC the apparent hydrolysis was only 26% (Table 3). Although it is possible that the enriched HDL reduced cellular EC hydrolysis, it is more likely that similar extensive EC hydrolysis oc-

curred in the presence of both normal and enriched HDL. The observed elevated esterification of FC in cells treated with enriched HDL would result in a greater re-esterification of the FC produced by hydrolysis.

To further distinguish the role of lipoprotein internalization from surface transfer in delivering FC to the cell, experiments were conducted using ^{125}I-labeled HDL enriched in FC. In a series of experiments ^{125}I-HDL which also contained [4-^{14}C]-cholesterol (see Methods) was added to the culture medium at a concentration of 200 μg FC/ml. After 24 hr of incubation, the amount of free [^{14}C]-cholesterol incorporated from the FC-enriched HDL (80 μg/mg cell protein, range 71–88), the cellular EC derived from ^{14}C-labeled free cholesterol (55 μg/mg cell protein, range 62–75) were all similar to values obtained in previous experiments (Table 2, Figs. 1 and 2). Quantitation of cell-associated ^{125}I indicated that 1.4 μg (range 1.0–2.1) of HDL protein/mg cell protein was present in the cells, this value being equivalent to only 0.3 μg HDL-FC/mg cell protein. The culture medium was assayed for TCA-soluble ^{125}I after the 24-hr incubation period. Because of the possibility that monoiodotyrosine generated from HDL metabolism may have been deiodinated during the prolonged incubation, the calculation of HDL metabolism was not corrected for the presence of ^{125}iodide in the medium and reflects total TCA-soluble radioactivity. As such it may represent a considerable overestimate of HDL internalization. With this maximum estimate of HDL degradation, it was determined that 120 μg of HDL could have been degraded and that lipoprotein internalization would have supplied 28 μg FC/mg cell protein. This value is 35% of the observed 80 μg/mg cell protein of radiolabeled FC recovered in the cells.

Cell types

Our previous investigations on the accumulation of EC in tissue culture cells exposed to hyperlipemic rabbit serum revealed the existence of considerable variation among different cell types as to their ability to esterify FC and accumulate EC (26). **Table 4** illustrates a similar variation among cell types in their response to FC-rich dispersions. As was the case with hyperlipemic rabbit sera, the Fu5-AH rat hepatoma amassed the greatest amount of EC following growth in media supplemented with human serum and FC-rich dispersions. A closely related clone of hepatoma cell, the Fu5-5, accumulated only low levels of EC. Primary rat hepatocytes yielded intermediate levels of EC, while no significant EC accumulation was observed with either rabbit aortic medial cells or human lung fibroblasts. Thus the ability of FC-rich dispersions to provoke extensive cellular EC accumulation appears not to be a universal phenomenon common to all cells in culture but rather a selective and quantitatively variable response of specific cells.

DISCUSSION

In recent years much evidence has accumulated establishing the ability of cells to incorporate serum lipoproteins (1–4). A number of these studies have shown that the process of lipoprotein internalization results in the incorporation of lipoprotein free and esterified cholesterol which can result in a net accumulation of cellular cholesterol (1, 30, 31). This process is accompanied by a reduction in de novo sterol synthesis and, in some instances, a stimulation in cholesterol esterification (1, 30). While the details involved in lipoprotein internalization are currently receiving intensive investigation, alternative mech-

TABLE 4. Response of cells to cholesterol-rich dispersions

Medium Supplement[a]	Cellular Cholesterol Content[b]			
	Human Serum		Human Serum + Dispersions	
Cell	FC	EC	FC	EC
Rabbit aortic medial	34.0 ± 2.4	0.8 ± 0.3	36.1 ± 2.5	1.9 ± 0.7
IMF fibroblasts	33.5 ± 2.4	3.2 ± 1.6	39.5 ± 3.0	5.7 ± 2.3
Fu5-5 hepatoma	17.1 ± 1.5	0.5 ± 0.3	21.5 ± 1.4	4.3 ± 0.3
Rat hepatocytes	18.7 ± 1.9	3.4 ± 0.6	22.9 ± 3.1	11.7 ± 0.9
Fu5-AH hepatoma	15.9 ± 0.9	18.3 ± 0.9	18.9 ± 1.4	77.5 ± 2.4

[a] Human serum at 5%. Free cholesterol/lecithin dispersions (FC/PL = 2.1–2.5) at 150 μg FC/ml. Incubation time, 44 hr.

[b] μg cholesterol/mg cell protein ± SE. Average value for at least five independent determinations.

anisms of cellular cholesterol transport are receiving less emphasis. One such mechanism is surface transport without incorporation of the entire lipoprotein moiety. Although suggested a number of years ago, (32, 33), it has been difficult to establish cell surface transfer of cholesterol as a physiologically important phenomenon. Free cholesterol exchange has been demonstrated using tissues, red blood cells, cells in culture, and isolated lipoproteins (5–8, 15, 16, 34–36). In many instances this movement is thought to represent a one-for-one molecular exchange which results in no net change of cholesterol concentration in any of the interacting components. At the cellular level, a strict exchange process would produce neither cellular cholesterol accumulation nor depletion and thus, presumably, no regulation of the cellular cholesterol metabolism.

To demonstrate cellular surface transfer of cholesterol as a physiologically important process, it is necessary to produce cellular accumulation of lipoprotein cholesterol without the metabolism of the carrier lipoprotein. In addition, the incorporation of the exogenous cholesterol should result in the regulation of cellular sterol metabolism in such a way as to confirm that the incorporated cholesterol becomes functionally available to the cell. The experimental system described in this report, utilizing Fu5-AH hepatoma cells exposed to HDL enriched in FC, satisfied the above criteria. The hepatoma cells accumulate large amounts of exogenous HDL-bound free cholesterol in excess of that which could have been supplied by HDL internalization and degradation. In addition, the incorporated FC is recovered primarily from the cellular EC pool, showing that the exogenous FC is not merely associated with the cell surface but becomes available to cellular enzyme(s), presumably acyl-CoA: cholesterol acyltransferase (ACAT).

The ability of HDL enriched in FC to stimulate a net uptake of FC by the cells via surface transfer is supported by the studies using either ^{125}I-labeled protein or [^{14}C]cholesteryl ester as markers for lipoprotein internalization. These experiments indicated that from 65% (^{125}I as marker) to 90% ([^{14}C]cholesteryl ester as marker) of the incorporated FC was derived by processes other than lipoprotein uptake. Although these results are consistent with a cell surface membrane mechanism for FC uptake, they do not exclude the possibility that the HDL-FC is selectively removed from internalized lipoprotein followed by HDL release without extensive degradation.

Studies have been conducted to establish the origin of the esterified cholesterol that accumulates in cells and tissues (2, 5, 6, 30, 31, 37). These investigations have shown that both esterification of free cholesterol and uptake of serum EC can contribute to cellular EC. Although the relative contribution of the two processes to EC accumulation probably differs considerably depending on the experimental conditions, the results from the present study indicate that the EC present in Fu5-AH cells after exposure to the HDL enriched in FC is derived almost entirely from the esterification of FC (Table 2). Thus the physical-chemical transfer of free cholesterol can result in the accumulation of large amounts of cellular EC by the process of cellular esterification. It is probable that the differences in response observed among various cell types (Table 4) is linked, in part, to the level of ACAT in these cells. Previous studies have revealed that ACAT activity is higher in Fu5-AH cells than in Fu5-5 hepatomas (38) and aortic medial cells.[1] Cellular esterification of FC could regulate transfer of FC into the cell by allowing EC to accumulate as cellular inclusions (39) thus removing the cholesterol from the FC pool. A favorable equilibrium would be generated allowing for further incorporation of FC with a net transfer of FC from the external environment to the cell membranes.

The data shown in Fig. 3 illustrate that the accumulation of EC in Fu5-AH cells is directly related to the FC/PL ratio of the HDL. These results are consistent with our previous studies which indicated that the relative cholesterol/phospholipid composition of serum and cell membrane lipoproteins could regulate the net flux of cholesterol between medium and cells (33). Modifying the FC/PL ratio of the serum lipoprotein by enriching it with FC, as in the present experiments, results in a shift in the physical-chemical equilibrium resulting in the net transfer of cholesterol to the cells. The transfer to the Fu5-AH cells is accentuated by the ability of these cells to esterify the FC, thus preventing the FC pool, presumably in cellular membranes, from becoming saturated. A net transfer of cholesterol from untreated HDL could be anticipated if cellular FC could be depleted. We have previously observed a limited net accumulation of HDL cholesterol in L-cell mouse fibroblast that had been partially depleted in FC by continued growth in a sterol-free medium (40). These results support the conclusions of Lange and D'Allessandro (41) who have recently proposed that cholesterol movement between erythrocytes and plasma, whether exchange or net movement, is governed by the same process and is based on the

[1] Rothblat, G. H., unpublished observation.

cholesterol to phospholipid ratios in the interacting components.

In this study we have utilized dispersion-treated HDL because of the ease with which HDL can be separated from the dispersion. It is probable, however, that other serum lipoproteins can also become enriched in FC and mediate a transfer of excess cholesterol to the cells, as indicated from studies in which Fu5-AH cells accumulated EC when exposed to FC-enriched dispersions together with either low density lipoproteins or very low density lipoproteins (17). The extent to which FC transfer can contribute to EC accumulation in such pathological conditions as atherosclerosis remains to be established. Lipoproteins having elevated FC/PL ratios are present in cholesterol-fed rabbits[1] and in some hypercholesteremic humans, particularly in Type II hyperlipoproteinemia (42–45). Such FC-enriched lipoproteins could be potential contributors of cholesterol to cells.

Recently Shattil et al. (45) determined that platelets from Type II hyperlipoproteinemic patients have elevated FC/PL ratios that correlate with similar elevated ratios in the low density lipoproteins. Although platelets exhibited an elevated cholesterol content, red blood cells from these individuals had a normal FC/PL. Thus the specificity of the response of various cell types in culture to elevated exogenous FC/PL ratios, as shown in the present study (Table 4), and the similar differences between platelets and red blood cells (45) may reflect fundamental differences in membrane structure and composition. Our previous studies with Fu5-AH cells exposed to lipoproteins obtained from hypercholesteremic rabbits have indicated that up to 60% of the esterified cholesterol accumulated in the cells is derived from exogenous EC, presumably incorporated via lipoprotein uptake followed by EC hydrolysis and reesterification (26, 46). Thus cellular cholesterol accumulation probably represents a balance between uptake of cholesterol by lipoprotein internalization and by surface transfer from lipoproteins without uptake. In atherosclerosis the balance may be disrupted by abnormal lipoproteins or by disordered cellular mechanisms. The differences observed among cells in the uptake of cholesterol by surface transfer implies a more important role for this mechanism of cellular cholesterol accumulation than is commonly suspected.

This research was supported in part by USPHS research grant HL-20608. The authors wish to thank Genevieve Stoudt and Mary Porter for their excellent technical assistance.

Manuscript received 21 June 1977; accepted 9 November 1977.

REFERENCES

1. Weinstein, D. B., T. E. Carew, and D. Steinberg. 1976. Uptake and degradation of low density lipoprotein by swine arterial smooth muscle cells with inhibition of cholesterol biosynthesis. *Biochim. Biophys. Acta.* **424:** 404–421.
2. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1975. Receptor-dependent hydrolysis of cholesteryl esters contained in plasma low density lipoproteins. *Proc. Nat. Acad. Sci.* **72:** 2925–2929.
3. Ho, Y. K., M. S. Brown, H. J. Kayden, and J. L. Goldstein. 1976. Binding, internalization, and hydrolysis of low density lipoproteins in long-term lymphoid cell lines from a normal subject and a patient with homozygous familial hypercholesterolemia. *J. Exp. Med.* **144:** 444–455.
4. Stein, O., D. B. Weinstein, Y. Stein, and D. Steinberg. 1976. Binding, internalization and degradation of low density lipoprotein by normal human fibroblasts and by fibroblasts from a case of homozygous familial hypercholesterolemia. *Proc. Nat. Acad. Sci.* **73:** 14–18.
5. Newman, H. A. I., and D. B. Zilversmit. 1966. Uptake and release of cholesterol by rabbit atheromatous lesions. *Circulation Res.* **18:** 293–302.
6. Hashimoto, S., and S. Dayton. 1966. Transfer of cholesterol and cholesteryl esters into wall of rat aorta in vitro. *J. Atheroscler. Res.* **6:** 580–590.
7. Rothblat, G. H., R. W. Hartzell, H. Miahlle, and D. Kritchevsky. 1966. The uptake of cholesterol by L5178Y tissue-culture cells: Studies with free cholesterol. *Biochim. Biophys. Acta.* **116:** 133–145.
8. Bailey, J. M., and J. Butler. 1973. Cholesterol uptake from doubly-labeled α-lipoproteins by cells in tissue culture. *Arch. Biochem. Biophys.* **159:** 580–581.
9. 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.
10. Sokoloff, L., and G. H. Rothblat. 1972. Regulation of sterol synthesis in L cells: steady-state and transitional responses. *Biochim. Biophys. Acta.* **280:** 172–181.
11. 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 apolipoprotein/phospholipid mixtures. *Biochim. Biophys. Acta.* **431:** 347–358.
12. Rothblat, G. H., M. K. Buchko, and D. Kritchevsky. 1968. Cholesterol uptake by L5178Y tissue culture cells: Studies with delipidized serum. *Biochim. Biophys. Acta.* **164:** 327–338.
13. Burns, C. H., and G. H. Rothblat. 1969. Cholesterol excretion by tissue culture cells: Effect of serum lipids. *Biochim. Biophys. Acta.* **176:** 616–625.
14. Rothblat, G. H. 1969. The effect of serum components on sterol biosynthesis in L cells. *J. Cell. Physiol.* **74:** 163–170.
15. Cooper, R. A., C. Arner, J. S. Wiley, and S. J. Shattil. 1975. Modification of red cell membrane structure by cholesterol-rich lipid dispersions. *J. Clin. Invest.* **55:** 115–126.
16. Shattil, S. J., R. Anaya-Galindo, J. Bennett, R. W.

Coleman, and R. A. Cooper. 1975. Platelet hypersensitivity induced by cholesterol incorporation. *J. Clin. Invest.* **55:** 636–643.
17. Arbogast, L. Y., G. H. Rothblat, M. H. Leslie, and R. A. Cooper. 1976. Cellular cholesterol ester accumulation induced by free cholesterol-rich lipid dispersions. *Proc. Nat. Acad. Sci.* **73:** 3680–3684.
18. Pitot, H. C., C. Peraino, P. A. Morse, and V. R. Potter. 1964. Hepatomas in tissue culture compared with adapting liver in vivo. Nat. Cancer Inst. Monograph **13:** 229–245.
19. Laishes, B. A., and G. M. Williams. 1976. Conditions affecting primary cell cultures of functional adult rat hepatocytes. *In Vitro.* **12:** 521–532.
20. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
21. Scanu, A., E. Cump, J. Toth, S. Koga, E. Stiller, and L. Albers. 1970. Degradation and reassembly of a human serum high-density lipoprotein. Evidence for differences in lipid affinity among three classes of polypeptide chains. *Biochemistry.* **9:** 1327–1335.
22. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein protein. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.
23. Weber, K., J. R. Pringle, and M. Osborn. 1972. Measurement of molecular weights by electrophoresis on SDS–acrylamide gel. *In* Methods in Enzymology. C. H. W. Hirs and S. N. Timashesi, editors. Vol. XXVI. Academic Press, New York, N. Y.
24. Swaney, J. B., H. Reese, and H. A. Eder. 1974. Polypeptide composition of rat high density lipoprotein: Characterization by SDS–gel electrophoresis. *Biochem. Biophys. Res. Commun.* **59:** 513–519.
25. Rothblat, G. H. 1974. Cholesterol ester metabolism in tissue culture cells. I. Accumulation in Fu5AH rat hepatoma cells. *Lipids.* **9:** 526–535.
26. Rothblat, G. H., L. Arbogast, D. Kritchevsky, and M. Naftulin. 1976. Cholesteryl ester metabolism in tissue culture cells. II. Source of accumulated esterified cholesterol in Fu5AH rat hepatoma cells. *Lipids.* **11:** 97–108.
27. Bierman, E. L., O. Stein, and Y. Stein. 1974. Lipoprotein uptake and metabolism by rat aortic smooth muscle cells is tissue culture. *Circ. Res.* **35:** 136–150.
28. Lowry, O. H., D. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
29. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
30. 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.
31. Stein, O., J. Vanderhoek, and Y. Stein. 1977. Cholesterol ester accumulation in cultured aortic smooth muscle cells. *Atherosclerosis.* **26:** 465–482.
32. Bailey, J. M. 1967. Cellular lipid nutrition and lipid transport. *In* Lipid Metabolism in Tissue Culture Cells. G. H. Rothblat and D. Kritchevsky, editors, Monograph #6, Wistar Institute Press. Philadelphia, Pa. 85–109.
33. Rothblat, G. H., and D. Kritchevsky. 1968. The metabolism of free and esterified cholesterol in tissue culture cells: A review. *Exp. Mol. Pathol.* **8:** 314–329.
34. Murphy, J. R. 1962. Erythrocyte metabolism. IV. Equilibration of cholesterol-4-C^{14} between erythrocytes and variously treated sera. *J. Lab. Clin. Med.* **60:** 571–578.
35. Bell, F. P. 1974. Studies on physico-chemical sterol exchange: Esterification of exchangeable membrane-bound cholesterol by intact aortas and cell-free arterial homogenates from cholesterol-fed rabbits. *Artery.* **1:** 86–96.
36. Bell, F. P. 1976. Lipoprotein lipid exchange in biological systems. *In* Low Density Lipoproteins. C. E. Day and R. S. Levy, editors. Plenum Publishing Corp. N.Y., N.Y. 111–133.
37. St. Clair, R. W., and G. J. Harpold. 1975. Stimulation of cholesterol esterification in vitro in organ cultures of normal pigeon aortas. *Exp. Mol. Pathol.* **22:** 207–219.
38. Rothblat, G. H., M. Naftulin, and L. Y. Arbogast. 1977. Stimulation of acyl-CoA: cholesterol acyltransferase activity by hyperlipemic serum lipoproteins. *Proc. Soc. Exp. Biol. Med.* **155:** 501–506.
39. Rothblat, G. H., J. M. Rosen, W. Insull, A. O. Yau, and D. M. Small. 1977. Production of cholesterol-ester rich anisotropic inclusions by mammalian cell in culture. *Exp. Mol. Pathol.* **26:** 318–324.
40. Bates, S., and G. H. Rothblat. 1974. Regulation of cellular sterol flux and synthesis by human serum lipoproteins. *Biochim. Biophys. Acta.* **360:** 38–55.
41. Lange, Y., and J. S. D'Allessandro. 1977. Characterization of mechanisms for transfer of cholesterol between human erythrocytes and plasma. *Biochemistry.* **16:** 4339–4343.
42. Howard, A. N., V. Blaton, D. Vandamme, N. Van Landschoot, and H. Peeters. 1972. Lipid changes in the plasma lipoproteins of baboons given an atherosclerotic diet. *Atherosclerosis.* **16:** 257–272.
43. Slack, J., and G. L. Mills. 1970. Anomalous low density lipoproteins in familial hyperbetalipoproteinemia. *Clin. Chim. Acta.* **29:** 15–25.
44. Brown, H. B., L. A. Lewis, and I. H. Page. 1973. Mixed hyperlipemia, a sixth type of hyperlipoproteinemia. *Atherosclerosis.* **17:** 181–196.
45. Shattil, S. J., J. S. Bennett, R. W. Coleman, and R. A. Cooper. 1976. Abnormalities of cholesterol–phospholipid composition in platelets and low-density lipoproteins of human hyperbetalipoproteinemia. *J. Lab. Clin. Med.* **89:** 341–353.
46. Rosen, J. M., and G. H. Rothblat. 1977. Origin of fatty acids of cholesteryl esters accumulated by Fu5-AH cells in culture. *Lipids.* **12:** 222–227.