The Accumulation of Serum Lipoprotein Cholesterol by Tissue Culture Cells[1] (36018)

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When serum β-lipoproteins are incubated with mouse fibroblasts, sudanophilic droplets develop within the cytoplasm of these cells (1). The composition of these droplets has not been fully defined. Since β-lipoproteins contain a high content of cholesterol, it is possible that these lipid inclusion droplets might also be rich in cholesterol. Should this be the case, this incubation system would provide a suitable model for some studies of atherogenesis. β-lipoprotein infiltration of the intima has been related to the causation of atherosclerosis (2), a disease characterized by both free and ester cholesterol accumulation in the arterial intima (3–5). In the present study, we incubated mouse fibroblasts with labeled cholesterol bound either to β-lipoprotein or bovine albumin. These cells and their inclusion bodies were analyzed subsequently for free cholesterol and cholesterol ester content. Evidence was obtained concerning the origin of the cholesterol components contained in these cellular inclusions.

Methods and Materials. The tissue culture cells were derived from a continuous line of mouse fibroblasts (NCTC No. 2445) which has been adapted to and routinely cultured in 1% bovine albumin (Sigma) in Waymouth No. 752 synthetic medium. These cells were subsequently incubated with several cholesterol-4-^{14}C preparations composed of various mixtures of free and ester cholesterol contained in β-lipoprotein or else of only free cholesterol bound to bovine serum albumin. The period of incubation was 48 hr during which time the number of cells usually multiplied two- to fourfold.

β-Lipoproteins were isolated either from human or bovine serum by precipitation with dextran sulfate of molecular weight 60,000–90,000 (Sigma). After resuspension of the lipoproteins in 20% NaCl containing 1 *N* sodium oxalate the dextran sulfate was removed partially by protamine sulfate and completely by DEAE-Sephadex 50 as previously reported (6). This lipoprotein preparation was dialyzed first against distilled water and then against saline for 2 hr. Finally it was sterilized by Millipore filtration. By paper electrophoresis, this preparation was found to be free of α-lipoproteins and other contaminating serum proteins.

Human β-lipoproteins were labeled by two methods with cholesterol-4-^{14}C obtained from New England Nuclear, Boston and whose purity by thin-layer chromatography was about 98%. By one method, β-lipoprotein was first separated from the serum and reprecipitated once before labeling. Five-tenths microcurie of cholesterol-4-^{14}C was then incubated with 5 ml of the β-lipoprotein preparation at 4° for 2 hr. In the second method, 0.5 μCi of cholesterol-4-^{14}C was added directly to 5 ml of serum and incubated for 2 hr at 37°. By this method, the amount of labeled cholesterol ester was increased through subsequent esterification of the labeled free cholesterol by the plasma cholesterol esterase. In both procedures the β-lipoprotein was removed by dextran precipitation after labeling and then prepared for tissue culture as already described. An average of 11.7% of the cholesterol-4-^{14}C was esterified with the second method as compared to only 4.2% esterification by the first method.

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TABLE I. The Uptake of β-Lipoprotein Cholesterol by Cultured Mouse Fibroblasts (μg of cholesterol/10^6 cells).

Source of β-lipoprotein (βLP)	Cholesterol in whole cells after addition of β-lipoprotein				Cholesterol within intracellular inclusions				Depletion of β-lipoprotein cholesterol during inclusion formation		
	Total	Free[a]	Ester	% Ester	Total	Free[b]	Ester	% Ester	Total	Free	Ester
Human	5.77	1.52[c] (1.34–1.81)	4.25 (4.01–5.05)	74	4.58	1.35 (0.50–2.25)	3.32 (3.01–3.56)	71	16.1	5.2	10.9
Bovine	6.15	1.87 (1.68–2.05)	4.28 (3.97–4.58)	70	4.33	1.84 (0.41–2.93)	2.49 (2.33–2.83)	58	29.0	4.3	24.7

[a] Free cholesterol within 10^6 treated cells minus free cholesterol within 10^6 control cells (4.0 μg).
[b] Free cholesterol retained on filter from 10^6 treated cells minus free cholesterol retained on filter from 10^6 control cells (0.91 μg).
[c] Mean for 3 individual runs with the range in parentheses.

Cholesterol was bound to bovine serum albumin by incubating 1% cholesterol and 0.5 μCi of free cholesterol-4-^{14}C in ethanol with 1% bovine albumin in the synthetic medium. The incubation of this sterile suspension was carried out for 24 hr at 37° and then any excess microcrystals of cholesterol were removed by filtration through a Millipore filter with pore size of 0.45 μ. The cholesterol was further diluted by synthetic medium to provide a final concentration of 4 mg/100 ml.

For the determination of the cholesterol content of the tissue culture cells, the cells were washed with and resuspended in Hanks' balanced salt solution. A small aliquot was counted with a Coulter counter Model B while the majority of the cells were concentrated by centrifuging at 2000 rpm for 15 min. The cell pellet was extracted with ethanol–acetone (1:1). The cellular content of free and esterified cholesterol was determined by the method of Sperry and Webb (7) which employs digitonin precipitation. The extracts of digitonin-precipitated free and total cholesterol were mixed in a scintillation mixture (4 g of PPO and 0.1 g of POPOP in 1 liter of toluene) and counted with a Packard Tricarb liquid scintillation spectrometer with an efficiency of 58%. The recovery of a known added amount of labeled cholesterol-4-^{14}C by this technique was 98%.

The cellular inclusions (lipid droplets) were collected after digesting the cells with 1% pronase for 30–60 min at 37°. After vigorous agitation and centrifugation at 2000 rpm for 5 min, the supernatant was passed through a Millipore filter (0.45 μ pore size) which collected the inclusions and also a small amount of cellular debris. The filter was washed with physiological saline, air dried, and then extracted with chloroform. This extract was then analyzed as above for cholesterol content or for cholesterol radioactivity.

Results. The effects of human and bovine β-lipoproteins upon the cellular cholesterol uptake. Nine individual sets of control fibroblasts grown in 1% bovine albumin alone contained an average of 4.0 μg of free cholesterol/10^6 cells with a range of 3.6 to 5.2 μg. Ester cholesterol could not be detected. The

addition of either human or bovine β-lipoprotein to the incubation medium greatly increased the total cholesterol content of the fibroblasts (Table I). The cells accumulated 5.77 and 6.15 μg additional amounts of cholesterol, respectively, a several hundred percent increase. The cellular content of cholesterol was located chiefly in the sudanophilic inclusion bodies which contained an average of 74.5 and 70.5% of the total accumulated cellular cholesterol after human and bovine β-lipoprotein incubation, respectively. The presence of cholesterol esters in the inclusion bodies could explain their sudanophilic staining, a property that cholesterol has only when esterified. This, however, does not exclude the possibility that the sudanophilia is due to some other lipid such as triglycerides.

The range of values for free cholesterol content in the inclusion bodies was greater than that for esterified cholesterol. This resulted from the variation in the amount of free cholesterol contained in the cellular debris that was retained on Millipore filter during the collection of the inclusion. The contaminating cholesterol as determined by using control cells averaged 0.91 μg of free cholesterol/10^6 with a range of 0.74 to 1.06 μg.

The amounts of human and bovine β-lipoprotein-bound cholesterol depleted from the medium during incubation was 16.1 and 29.0 μg/10^6 cells, respectively. As shown from Table I, these amounts of cholesterol could only partly be recovered from the tissue culture cells after the incubation period. The failure to recover some of the cholesterol removed from the medium suggested that it might have been metabolized by the cells to a sterol or other products no longer reactive with digitonin or with Liebermann-Burchard colorimetric reagent.

The cellular uptake of cholesterol-4-^{14}C from β-lipoprotein. Table II summarizes the results obtained when human β-lipoprotein labeled with cholesterol-4-^{14}C was incubated with cells for 48 hr. The inclusion clearly accumulated the labeled cholesterol, both in the free and esterified forms. When cells were exposed to β-lipoprotein containing between 3.4 and 5.1% of the total ^{14}C-cholesterol in the ester form (Expt. A in Table II), the amount of labeled ester within the inclusions averaged 9098 cpm which was similar to the ester count of 8838 cpm removed from the medium during incubation. When the labeled ester of the β-lipoprotein was increased from 4.2 (Expt. A) to 11.7% (Expt. B), the cholesterol ester counts of the lipoprotein increased accordingly. The amount of ester counts now removed from the medium by the cells was 20,448 compared to 8383 cpm in Expt. A. This increase in ester uptake was associated with an average increase in ester counts, from 9098 to 12,071, as well as an increase in the percentage of cholesterol ester within the intracellular inclusions (from 18 to 33%). Thus an increase in the labeled cholesterol ester content of the β-lipoprotein in the medium was associated with an increase in cholesterol ester within the inclusions. These results also suggest that the cholesterol ester within the inclusions originated largely from the direct deposition of transported lipoprotein-bound cholesterol ester instead of intracellular esterification of transported free cholesterol.

The cellular uptake of albumin-bound cholesterol and cholesterol-4-^{14}C. Fibroblasts cultured in medium containing free cholesterol bound to bovine albumin developed sudanophilic inclusions identical to those produced by β-lipoprotein. In two experiments, the cells contained 1.10 and 1.01 μg of esterified cholesterol/10^6 cells. When this preparation contained tracer amounts of ^{14}C-free cholesterol the cells were found to contain 42 and 43%, respectively, of the total ^{14}C-cholesterol radioactivity added to the medium. They esterified 10.2 and 11.5%, respectively, of the ^{14}C-cholesterol taken up. If cholesterol was added to the medium in the microcrystalline form without albumin, the cellular uptake of the iostope was only 1% of the total cholesterol added; and inclusion bodies did not form. It then appears that small portions of transported free cholesterol (only when in the solubilized form), can be esterified by intact cells and deposited as sudanophilic inclusions identical to those produced by β-lipoproteins.

Discussion. This study clearly demonstrated that the addition of β-lipoprotein to

TABLE II. The Incorporation of Cholesterol Ester by Cultured Mouse Fibroblasts After Incubation with Cholesterol-4-^{14}C Ester Bound to β-Lipoprotein. Results are expressed as total counts per minute (cpm) of cholesterol-4-^{14}C contained in cholesterol esters and free cholesterol.

	Cholesterol counts in medium before culture				Cholesterol ester counts removed from medium during inclusion formation;	Cholesterol counts in intracellular inclusions			
	Total	Free	Ester	% Ester	Ester	Total	Free	Ester	% Ester
Expt. A[a]									
1	870,400	825,139	45,261	5.1	13,237	84,547	70,174	14,373	17
2	351,937	339,971	11,966	3.4	5702	31,675	25,340	6335	20
3	395,138	379,332	15,806	4.0	7575	36,589	30,003	6586	18
Mean				4.2	8838			9098	18
Expt. B[b]									
1	268,926	233,966	34,960	13.0	18,562	29,760	20,832	8928	30
2	396,576	352,954	43,622	11.0	17,563	31,624	20,872	10,752	34
3	583,372	519,001	64,371	11.0	25,219	51,700	35,156	16,544	32
Mean				11.7	20,448			12,071	33

[a] β-Lipoprotein was first removed from whole serum before it was labeled with ^{14}C-free cholesterol.

[b] β-Lipoprotein was labeled with ^{14}C-free cholesterol before it was isolated from the parent serum.

the culture medium of fibroblasts caused the formation of intracytoplasmic inclusion bodies which contained significant quantities of cholesterol ester. Although cholesterol esters have previously been found within cultured mouse cells (8–10), the formation of inclusion bodies containing cholesterol and the role played by β-lipoprotein in this process have not been previously described.

Other investigators have shown that cultured mouse fibroblasts can take up cholesterol esters from the medium (10 11) and can esterify small amounts of free cholesterol transported from the medium (12). Likewise, the fibroblasts used in this study also were able to accumulate esterified cholesterol either by esterification of ingested free cholesterol or by direct deposition of transported ester from the medium. For example, when inclusion body formation was induced by albumin-bound free cholesterol added to the medium, the cholesterol esters within the inclusions could only have resulted from intracellular esterification. When a small amount of cholesterol ester was present along with a much larger amount of free cholesterol in the β-lipoprotein of the medium, then the amount of labeled ester accumulated within these inclusions was not significantly different than the quantity of ester removal from the medium. This suggested that the cholesterol ester within the inclusions originated in large part from transported cholesterol ester bound to β-lipoprotein instead of by intracellular esterification of free cholesterol. When more of the total labeled ^{14}C-cholesterol was in the esterified form, then about ½ of the labeled ester depleted from the medium during culture could be recovered from the inclusion. In the experiments using unlabeled human β-lipoprotein which contained an even larger percentage of cholesterol ester, the amount of ester recovered from the cells or inclusions was now only ⅕ of the ester removed from the medium.

These observations then suggest that if cells are exposed to cholesterol only in the free form, then they can accumulate a small quantity of cholesterol ester by virtue of intracellular esterification. If, however, the medium contains esterified cholesterol, the cells preferentially deposit the already formed cholesterol ester into the inclusion bodies. Even under this circumstance the possibility that some of the cholesterol ester originated as the result of intracellular esterification of transported free cholesterol cannot be excluded. These fibroblasts apparently contain an enzyme that can either esterify free cholesterol or hydrolyze cholesterol ester (13). Its action is perhaps dependent upon the amount of esterified cholesterol within the system.

In our experiments, much of the transported β-lipoprotein-bound cholesterol could not be recovered from the cells or medium either as a digitonin precipitable fraction or as one that reacts with the Liebermann-Burchard reagent. This finding is in agreement with the findings of Bensch *et al.* (14), who also could not recover all of the cholesterol ingested by cells. Recently, it has been shown that the cells used in this study can metabolically degrade the terminal carbon of cholesterol to 26-$^{14}CO_2$ (15). It has not been determined whether further catabolic alterations occur such as hydrogenation of the 5 double bond which would render this steroid nonreactive with the Liebermann-Burchard reagent.

Why the lipid of the medium becomes deposited as an inclusion droplet is a question of considerable significance. One explanation might be a simple overloading phenomenon in which the cells have been exposed to excessive amounts of protein-bound cholesterol in the medium. The overloaded cells can not mobilize or metabolize this excess lipid, consequently it coalesces into droplet form. The importance of a cholesterol-binding protein in cholesterol metabolism and its mobilization from cells *in vitro* has been demonstrated by Bailey (16) and Day *et al.* (17). Furthermore, Dixon (18, 19) has stressed the significance of lipotropic proteins and phospholipids in preventing the conversion of invisible micellar fat to visible droplet fat. Thus, it is conceivable that a relative lack of such substances may well contribute to the coalescing of newly ingested lipid into droplet form seen as cytoplasmic inclusion bodies. This might be especially so if lipid such as cholesterol

ester from β-lipoprotein might depend upon an intracellular carrier for its transport within the cytoplasm after being separated from its protein carrier at the cell surface (20, 14).

The β-lipoprotein-induced cholesterol ester accumulation within fibroblasts has certain similarities to the cholesterol ester accumulation with the intimal cells of arteries developing atherosclerosis. β-Lipoprotein has been incriminated in atherogenesis (2), has been found in the intimal fatty streak (21–23) and more specifically within smooth muscle cells (20–24). The same accumulation of cholesterol ester which occurs in the inclusion bodies is also seen in diseased intimal cells and in the foam cells of the early fatty streak lesion (3–5). By electron microscopy, these inclusions are morphologically identical to the predominant form of lipid found within the intimal cell of early atherogenesis.[2] The smooth muscle cells of arteries that accumulate sudanophilic inclusions are believed also to have differentiated from fibroblasts.

This analogy can be carried further because the extracellular medium (*i.e.*, the plasma) in which atherosclerosis develops usually contains increased β-lipoprotein cholesterol concentration. It is hypothesized that this hypercholesterolemia leads to the formation of "foam cells" and their cholesterol ester accumulation in the arterial intima. Thus, in both tissue culture cells and in the intimal cells of arteries, cholesterol overloading occurs with lipid droplet formation whenever the medium contains a high concentration of cholesterol. The uptake of cholesterol from the medium becomes greater than the capacity of the cell to excrete cholesterol; and cholesterol then accumulates within the cell.

Summary. β-Lipoprotein isolated from human or bovine serum elicited sudanophilic inclusion bodies within cultured fibroblasts. Such sudanophilia was accompanied by an increase in cellular cholesterol, about ⅔ of which was in the esterified form. The origin of the cholesterol appears to be largely from the deposition of β-lipoprotein-bound cholesterol ester instead of newly esterified cholesterol. Much of the accumulated cholesterol, especially the cholesterol ester, was found within the inclusion bodies. They should be particularly considered as being formed as the result of cholesterol ester uptake. Free cholesterol bound to bovine albumin also elicited intracellular sudanophilic inclusion bodies containing cholesterol ester. Free cholesterol in microcrystalline form did not lead to similar sudanophilia or increase in cellular cholesterol content.

This tissue culture system provided analogies to atherosclerosis in that cholesterol accumulated in the cells in both instances when the extracellular medium or plasma contained high concentrations of cholesterol bound to protein. In both instances, also, lipid droplet formation occurred and cholesterol ester accumulation was the chief form of the cholesterol overload.

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