# Cholesteryl ester synthesis in macrophages: stimulation by β-very low density lipoproteins from cholesterol-fed animals of several species

Robert W. Mahley,[1] Thomas L. Innerarity,[1] Michael S. Brown,[2] Y. K. Ho,[2] and Joseph L. Goldstein[2]

Gladstone Foundation Laboratories for Cardiovascular Disease, University of California, San Francisco, San Francisco, CA 94140, and Departments of Molecular Genetics and Internal Medicine, University of Texas Health Science Center at Dallas, Dallas TX 75235

**Abstract** Animals fed cholesterol accumulate several types of cholesterol-rich lipoproteins in their plasma and ultimately develop cholesteryl ester deposition in tissue macrophages. Previous studies in the cholesterol-fed dog have shown that one class of cholesterol-rich lipoproteins, β-migrating very low density lipoproteins (β-VLDL, density < 1.006 g/ml), possesses a unique ability to produce cellular cholesteryl ester accumulation when incubated with mouse peritoneal macrophages in vitro. This accumulation results from the receptor-mediated uptake of β-VLDL with subsequent lysosomal hydrolysis of the lipoprotein and re-esterification of the liberated cholesterol. In the current studies, we demonstrate that β-VLDL obtained from cholesterol-fed animals of several other species, including monkeys, rabbits, and rats, also causes cholesteryl ester accumulation in monolayers of mouse peritoneal macrophages, as monitored by an increase in the rate at which the cells incorporate exogenous [14C]oleate into cholesteryl [14C]oleate. Like canine β-VLDL, the β-VLDL from these three other species were effective at low concentrations and exhibited saturation kinetics, suggesting that they, too, entered macrophages by receptor-mediated endocytosis. Very low density lipoprotein (VLDL) from normal animals and low density lipoprotein (LDL) from normal and cholesterol-fed monkeys, rats, and rabbits did not stimulate cholesteryl ester synthesis in mouse peritoneal macrophages. In addition to their effects on mouse macrophages, the β-VLDL from cholesterol-fed dogs and rabbits stimulated cholesteryl ester synthesis in cultured human monocytes. The current findings suggest that β-VLDL from cholesterol-fed animals has the general property of stimulating cholesteryl ester synthesis and accumulation in macrophages.—**Mahley, R. W., T. L. Innerarity, M. S. Brown, Y. K. Ho, and J. L. Goldstein.** Cholesteryl ester synthesis in macrophages: stimulation by β-very low density lipoproteins from cholesterol-fed animals of several species. *J. Lipid Res.* 1980. **21:** 970–980.

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A characteristic accompaniment of profound hyperlipidemia in cholesterol-fed animals is the appearance of abnormal cholesterol-rich lipoproteins with the density of very low density lipoproteins (VLDL, density < 1.006 g/ml) (1, 2). In contrast to normal triglyceride-carrying VLDL, which shows pre-β-mobility on electrophoresis, these abnormal cholesterol-rich lipoproteins have β-mobility and are designated as β-VLDL (1). The accumulation of β-VLDL in the bloodstream of cholesterol-fed animals is associated with the deposition of large amounts of cholesteryl esters in macrophages in a variety of tissues in vivo (2–4).

In a previous study, we observed that β-VLDL from hyperlipidemic dogs has a specific ability to produce cholesteryl ester deposition in monolayers of mouse peritoneal macrophages (5). This effect was attributable to a high affinity binding site on the macrophage surface that recognized the β-VLDL and facilitated its uptake by the cell and degradation by cellular lysosomes (5). A portion of the cholesterol liberated from the degraded β-

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VLDL particles was re-esterified and stored by the cell as cholesteryl ester. The accumulation of these cholesteryl esters could be followed in two ways: *1)* by measurement of the mass of esterified cholesterol within the cell and *2)* by measurement of the rate at which the macrophages incorporated exogenous [$^{14}$C]oleate into cholesteryl [$^{14}$C]oleate. Stimulation of the latter re-esterification reaction was proportional to the amount of $\beta$-VLDL taken up and degraded by the cells (5). Other lipoproteins from hyper-lipidemic dogs, including LDL, HDL$_c$, apo E HDL$_c$, and typical HDL, were much less effective than $\beta$-VLDL in stimulating cholesteryl ester formation in macrophages. Normal VLDL and other lipoproteins from normal dogs and normal humans were similarly ineffective in stimulating cholesteryl ester accumulation in macrophages (5).

In the current studies, we have extended these data by investigating lipoproteins isolated from hyperlipidemic animals from three other species: monkeys, rats, and rabbits. We have investigated the effects of these lipoproteins on cholesterol metabolism in mouse and rabbit peritoneal macrophages and in human cells, including cultured fibroblasts and cultured monocytes. The results show that the ability to stimulate cholesteryl ester synthesis in macrophages is a general characteristic of cholesterol-rich $\beta$-VLDL from all of these animal species.

## MATERIALS AND METHODS

### Materials

Male and female NCS mice (25–30 g) were obtained from The Rockefeller University. [1-$^{14}$C]Oleic acid (56 mCi/mmol) was purchased from Amersham/Searle. Fetal calf serum (FCS), obtained from Flow Laboratories, was heat-inactivated (56°C, 30 min) prior to use. Dulbecco's modified Eagle's medium (DMEM, Cat. No. 320-1885), Eagle's minimum essential medium (MEM, Cat. No. 410-1100), RPMI 1640 medium (Cat. No. 320-1875), and Dulbecco's phosphate-buffered saline (Cat. No. 310-4190) were purchased from Grand Island Biological Co. Plastic petri dishes were obtained from Falcon, Division of Becton, Dickinson, and Co. All other supplies and reagents for assays were obtained from sources as previously reported (5, 6).

### Mouse macrophage monolayers

Peritoneal cells were harvested from unstimulated mice in phosphate-buffered saline as previously described (5–7). The fluid from 20 to 40 mice (6 to $10 \times 10^6$ cells/mouse) was pooled, and the cells were collected by centrifugation (400 $g$, 10 min, room temperature) and washed once with 30 ml of DMEM. The cells were resuspended in DMEM containing 20% (v/v) FCS, penicillin (100 U/ml), and streptomycin (100 $\mu$g/ml) at a final concentration of 2 to $3 \times 10^6$ cells/ml. One-ml aliquots of this cell suspension were dispersed into $35 \times 10$-mm plastic petri dishes and then incubated in a humidified $CO_2$ (5%) incubator at 37°C. After 1 to 2 hr, each dish was washed three times with 2 ml of DMEM without serum to remove nonadherent cells. The monolayers were incubated for 18 hr at 37°C in 1 ml of DMEM containing 20% FCS, after which the cells were washed with 2 ml of DMEM and then used for the experiment. Each dish of adherent macrophages contained about 30% of the total number of cells originally plated; each $10^6$ adherent cells contained approximately 60 to 75 $\mu$g of protein.

### Human monocyte monolayers

Human mononuclear cells were isolated from the peripheral blood of healthy subjects by the method of Böyum (8). The mononuclear cells were used to prepare monolayers of monocytes according to the method of Johnson, Mei, and Cohn (9). The isolated mononuclear cells were resuspended in RPMI 1640 medium containing 10% (v/v) autologous whole serum, penicillin (100 U/ml), and streptomycin (100 $\mu$g/ml) at a final concentration of $6 \times 10^6$ cells/ml. One-ml aliquots of the cell suspension were dispersed into each plastic petri dish ($35 \times 10$ mm) and then incubated in a humidified $CO_2$ (5%) incubator at 37°C. After 1 hr, each dish was washed three times with 2 ml of RPMI 1640 medium without serum to remove nonadherent lymphocytes. The adhering cells (90–95% monocytes) were incubated at 37°C in 1 ml of RPMI 1640 medium containing 10% autologous whole serum. On day 3, fresh RPMI 1640 medium containing 10% autologous whole serum was added and the incubations were continued for 2 more days. Experiments were carried out on day 5.

### Human fibroblast monolayers

Normal and mutant human fibroblasts were grown in monolayers as previously described (10). On day 0, confluent monolayers of cells from stock flasks were dissociated with 0.05% trypsin–0.02% EDTA solution, and $7.5 \times 10^4$ cells were seeded into each petri dish ($60 \times 15$ mm) containing 3 ml of medium A (Eagle's MEM supplemented with penicillin (100

U/ml), streptomycin (100 $\mu$g/ml), 20 mM Tricine (pH 7.4), 24 mM NaHCO$_3$, and 1% nonessential amino acids) and 10% (v/v) FCS. On day 3, the medium was replaced with fresh medium A containing 10% FCS. On day 5, each monolayer was washed with 3 ml of phosphate-buffered saline, after which 2 ml of fresh medium A containing 10% human lipoprotein-deficient serum (final protein concentration, 5 mg/ml) was added. Experiments were begun on day 7 after incubation for 48 hr in lipoprotein-deficient serum.

## Human lipoproteins

Human LDL (d 1.019–1.063 g/ml) and human lipoprotein-deficient serum (d > 1.215 g/ml) were isolated from the plasma of individual healthy subjects by ultracentrifugation as described (11). LDL was radiolabeled with $^{125}$I as previously described (12).

## Animal lipoproteins

Normal d < 1.006 lipoproteins (VLDL) were obtained from EDTA-anticoagulated plasma of rhesus monkeys, New Zealand white rabbits, and Sprague Dawley rats. The cholesterol-fed dogs were foxhounds. All animals except the rats were fasted overnight prior to obtaining blood, and in some cases the plasma from two or more animals was pooled to obtain larger quantities of the d < 1.006 g/ml lipoproteins. The normolipidemic d < 1.006 g/ml fraction was isolated at plasma density and washed by recentrifugation in saline at d 1.006 g/ml at 50,000 rpm for 15 hr in a 60 Ti rotor (Beckman Instruments). The hyperlipidemic lipoproteins were isolated from animals fed semisynthetic, semipurified, or commerical diets containing various fats (coconut oil, lard, egg yolk, or soybean oil) and cholesterol (see footnote, Table 1 for diet descriptions). The hyperlipidemic d < 1.006 g/ml fraction (principally, $\beta$-VLDL; Table 1) was isolated by centrifugation as described above for the normal d < 1.006 g/ml lipoprotein. Canine LDL and HDL$_c$ were isolated as described previously (5). Hyperlipidemic rabbit IDL (d 1.006–1.019 g/ml) and LDL (d 1.019–1.063 g/ml) were isolated by sequential ultracentrifugation at 59,000 rpm for 15 hr in a 60 Ti rotor. The hyperlipidemic rabbit HDL (d 1.063–1.21 g/ml) was isolated and washed by recentrifugation at 59,000 rpm for 48 and 24 hr, respectively.

Hyperlipidemic canine d < 1.006 g/ml lipoproteins were iodinated as described (5). More than 98% of the total $^{125}$I-radioactivity was precipitated by 10% trichloroacetic acid and less than 2% of the total $^{125}$I-radioactivity was extracted into chloroform–methanol. The chemical composition and apoprotein patterns on 11% polyacrylamide gels in 0.1% sodium dodecyl sulfate were obtained as described previously (5).

## Assays

The incorporation of [$^{14}$C]oleate–albumin (5,600–10,600 cpm/nmol) into cellular cholesteryl [$^{14}$C]oleate by cell monolayers (6) and the mass of cellular esterified cholesterol were measured as previously described (6) except that serum was omitted from the incubation medium. As previously reported, serum enhances the removal of cholesterol from macrophages and thereby diminishes the accumulation of cholesteryl esters when cells are incubated with lipoproteins (13). The proteolytic degradation of $^{125}$I-labeled lipoproteins by monolayers of human fibroblasts and mouse macrophages was measured by assaying the amount of $^{125}$I-labeled trichloroacetic acid-soluble (non-iodide) material formed by the cells and excreted into the culture medium (5). Corrections were made for small amounts of acid-soluble $^{125}$I-labeled material (<0.01% of total radioactivity added) that was present in parallel incubations without cells. The protein content of cell extracts and lipoproteins was determined by the method of Lowry, et al. (14), with bovine serum albumin as a standard. All data points represent the average of duplicate incubations.

## RESULTS

**Table 1** summarizes the characteristics of the various animal lipoproteins used in the current studies. The d < 1.006 g/ml fraction from the normolipidemic animals contained the triglyceride-rich, pre-$\beta$-migrating VLDL. With the consumption of high cholesterol diets, the striking change in the d < 1.006 g/ml lipoproteins was the occurrence of $\beta$-migrating, cholesterol-enriched particles, referred to as $\beta$-VLDL. The apoprotein content of the $\beta$-VLDL was also altered by a relative increase in the E apoprotein and a decrease in the C (low molecular weight) apoproteins. Apoprotein content was determined qualitatively by visual inspection of polyacrylamide gels. The similarities in the changes in the d < 1.006 g/ml fraction induced by cholesterol-feeding in dogs, rats, rabbits, and monkeys have been described previously (for review, see refs. 1, 2).

The d < 1.006 g/ml lipoproteins from a hyperlipidemic rhesus monkey produced a marked stimulation of cholesteryl ester synthesis in mouse peritoneal macrophages as evidenced by an increase in the rate of incorporation of [$^{14}$C]oleate into cholesteryl [$^{14}$C]oleate (**Fig. 1A**). The dose-response curve

TABLE 1. Characteristics of animal lipoproteins

| Animal Species | Diet | Plasma Cholesterol Level | Lipoprotein Fraction | Mass Ratio Cholesterol/Protein | Major Apoproteins |
|---|---|---|---|---|---|
| | | *mg/dl* | | | |
| Dog (No. 1) | Cholesterol[b] | 936 | d < 1.006 (>90%)[a] | 5.9 | B,E |
| Dog (No. 1) | Cholesterol[b] | 936 | HDL$_c$ | 2.9 | A-I,E |
| Dog (No. 1) | Cholesterol[b] | 936 | LDL | 2.5 | B |
| Dog (No. 2) | Cholesterol[b] | 1112 | d < 1.006 (100%) | 7.2 | B,E |
| Dog (No. 3) | Cholesterol[b] | 450 | d < 1.006 (~60%) | 2.8 | B,E |
| Monkey, rhesus | Normal[c] | 164 | d < 1.006 | 0.93 | B,C |
| Monkey, rhesus | Cholesterol[d] | 585 | d < 1.006 (>90%) | 5.0 | B,E |
| Rabbit | Normal (chow)[e] | 55 | d < 1.006 | 0.88 | B,C |
| Rabbit | Normal (semipurified)[f] | 150 | d < 1.006 | 1.8 | B,C |
| Rabbit (No. 1) | Cholesterol[g] | 1580 | d < 1.006 (>90%) | 10.7 | B,E |
| Rabbit (No. 1) | Cholesterol[g] | 1580 | IDL | 4.3 | B,E |
| Rabbit (No. 1) | Cholesterol[g] | 1580 | LDL | 2.0 | B |
| Rabbit (No. 1) | Cholesterol[g] | 1580 | HDL | 0.44 | A-I |
| Rabbit (No. 2) | Cholesterol[g] | 442 | d < 1.006 (~70%) | 10.0 | B,E |
| Rat | Normal[h] | 108 | d < 1.006 | 0.6 | B,E,C |
| Rat | Cholesterol[i] | 349 | d < 1.006 (~70%) | 5.3 | B,E |

[a] Values in parentheses represent the proportion of d < 1.006 g/ml lipoproteins that is β-VLDL as judged by visual inspection of overloaded paper strip electrophoretograms (21).

[b] Semisynthetic diet containing 16% hydrogenated coconut oil and 5% cholesterol (24).

[c] Semipurified diet (Bowman Gray Control diet 75-5) containing 42% of the calories as fat (egg yolk replacement without cholesterol and lard). The cholesterol content was 0.0013% by weight (0.03 mg/kcal) (Personal communication, L. L. Rudel).

[d] Semipurified diet (Bowman Gray Test diet 75-5) containing 42% of the calories as fat (egg yolk and lard). The cholesterol content was 0.31% by weight (0.74 mg/kcal) (Personal communication, L. L. Rudel).

[e] Purina laboratory rabbit chow.

[f] Semipurified diet (NIH Zeigler 701-79) containing 3% soybean oil by weight and no added cholesterol.

[g] Semipurified diet (NIH Zeigler 700-79) containing 3% soybean oil and 0.5% cholesterol by weight.

[h] Purina laboratory rat chow.

[i] Ground Purina rat chow containing 5% lard, 1.1% cholesterol, 0.3% taurocholate, and 0.1% propylthiouracil by weight (25).

showed saturation kinetics with half-maximal stimulation occurring at 5 to 10 μg protein/ml. On the other hand, the d < 1.006 g/ml lipoproteins from a normal rhesus monkey did not stimulate cholesteryl [14C]oleate formation at concentrations up to 50 μg protein/ml (Fig. 1A). The cholesterol to protein ratio of the hyperlipidemic d < 1.006 g/ml lipoproteins was about 5-fold higher than that of the normal d < 1.006 g/ml lipoproteins (Table 1). For this reason, the data of Fig. 1A are replotted in Fig. 1B to compare the two lipoproteins as a function of the cholesterol concentration added to the culture medium. The marked difference between the two lipoproteins persisted. At a cholesterol concentration of 50 μg/ml, the hyperlipidemic d < 1.006 g/ml lipoproteins produced a rate of cholesteryl ester synthesis that was 13-fold higher than that of the normal d < 1.006 g/ml lipoproteins (Fig. 1B).

A similar difference in stimulation of cholesteryl [14C]oleate synthesis in mouse macrophages was observed when hyperlipidemic rat d < 1.006 g/ml lipoproteins were compared with those of a normal rat (Fig. 2). Hyperlipidemia was induced by feeding diets containing lard, cholesterol, and taurocholate to

hypothyroid rats produced by propylthiouracil treatment.

The d < 1.006 g/ml lipoproteins from two hyperlipidemic rabbits also produced a marked stimulation in cholesteryl [14C]oleate formation in mouse macrophages (Fig. 3). The lipoproteins from hyperlipidemic rabbit No. 1 (total plasma cholesterol level, 1580 mg/dl) were more effective than those from hyperlipidemic rabbit No. 2 (total plasma cholesterol level, 442 mg/dl). Like the lipoproteins of the normal rhesus monkey and the rat, the d < 1.006 g/ml fraction from rabbits fed commercial rabbit chow had no significant effect on cholesteryl ester synthesis (shown by X in Figs. 3A and 3B). However, d < 1.006 g/ml lipoproteins from the rabbits fed a semipurified diet without added cholesterol showed a small but definite ability to stimulate cholesteryl ester synthesis (squares in Figs. 3A and B). Quantitatively, the d < 1.006 g/ml lipoproteins from the rabbits fed the semipurified diet were much less potent than those from either of the hyperlipidemic rabbits. It is noteworthy that rabbits fed the semipurified rabbit chow had a plasma cholesterol level more than twice the level observed in rabbits fed the commercial chow (Table 1). Although
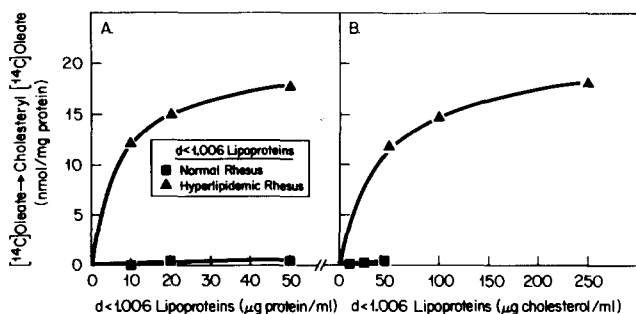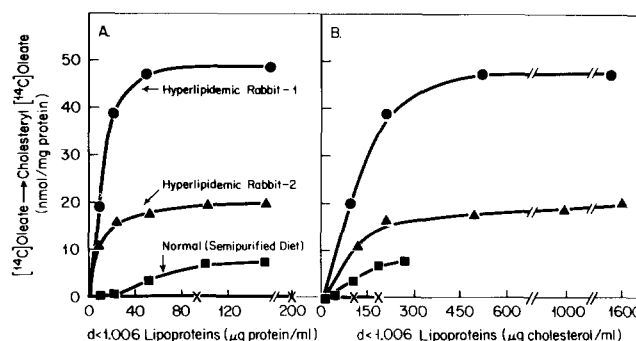
**Fig. 1.** Cholesteryl ester formation in mouse macrophages incubated with different concentrations of d < 1.006 g/ml lipoproteins from normal and hyperlipidemic monkeys. Each monolayer received 0.6 ml of DMEM containing 0.2 mM [14C]oleate–2.4 mg/ml albumin and the indicated concentration of d < 1.006 g/ml lipoproteins obtained either from a normal rhesus monkey (■) or a hyperlipidemic rhesus monkey (▲). After incubation for 6 hr at 37°C, the cellular content of cholesterol [14C]oleate was determined. The content of cholesteryl [14C]oleate in cells incubated without d < 1.006 g/ml lipoproteins was 0.5 nmol/mg protein. The values in Panels A and B represent the same data plotted in terms of the protein concentration (Panel A) and total cholesterol concentration (Panel B) of the d < 1.006 g/ml lipoproteins, respectively.

$\beta$-VLDL could not be detected by paper electrophoresis, the cholesterol/protein ratio of the d < 1.006 g/ml fraction from rabbits on the semipurified diet was greater than one, a consistent finding in cholesterol-fed animals with $\beta$-VLDL (Table 1). The slight stimulation of cholesteryl ester synthesis by the d < 1.006 g/ml fraction from rabbits on the semipurified diet may reflect the mild elevation in plasma cholesterol and the altered cholesterol/protein ratio of the d < 1.006 g/ml lipoproteins.
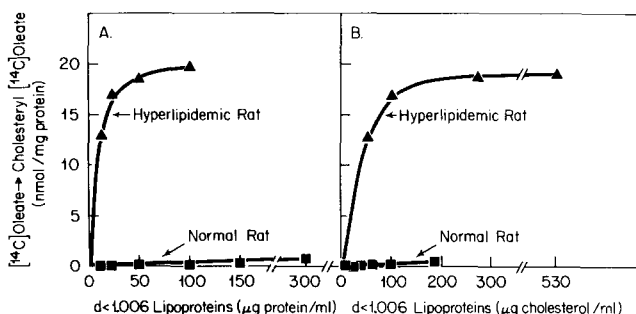


**Fig. 2.** Cholesteryl ester formation in mouse macrophages incubated with varying concentrations of d < 1.006 g/ml lipoproteins from a normal and a hyperlipidemic rat. Each monolayer received 0.6 ml of DMEM containing 0.2 mM [14C]oleate–2.4 mg/ml albumin and the indicated concentration of d < 1.006 g/ml lipoproteins obtained either from a normal rat (■) or from a hyperlipidemic rat (▲). After incubation for 6 hr at 37°C, the cellular content of cholesteryl [14C]oleate was determined. The content of cholesteryl [14C]oleate in cells incubated without d < 1.006 g/ml lipoproteins was 0.5 nmol/mg protein. The values in Panels A and B represent the same data plotted in terms of the protein concentration (Panel A) and total cholesterol concentration (Panel B) of the d < 1.006 g/ml lipoproteins, respectively.

**Fig. 3.** Cholesteryl ester formation in mouse macrophages incubated with d < 1.006 g/ml lipoproteins from two hyperlipidemic rabbits and two normal rabbits. Each monolayer received 0.6 ml of DMEM containing 0.2 mM [14C]oleate–2.4 mg/ml albumin and the indicated concentration of d < 1.006 g/ml lipoproteins obtained from one of the following animals: ●, hyperlipidemic rabbit No. 1; ▲, hyperlipidemic rabbit No. 2; ■, rabbit fed a semipurified diet without added cholesterol; or X, rabbit fed commercial rabbit chow. After incubation for 6 hr at 37°C, the cellular content of cholesterol [14C]oleate was determined. The content of cholesteryl [14C]oleate in cells incubated without d < 1.006 g/ml lipoproteins was 0.2 nmol/mg protein. The values in Panels A and B represent the same data plotted in terms of the protein concentration (Panel A) and the total cholesterol concentration (Panel B) of the d < 1.006 g/ml lipoproteins, respectively. Data for d < 1.006 g/ml lipoproteins of rabbits fed commercial chow (X) were obtained in a separate experiment under identical conditions.

Among the other lipoprotein fractions from hyperlipidemic rabbit No. 1, only IDL, which resembles $\beta$-VLDL in its apoprotein content, produced a significant stimulation of cholesteryl [14C]oleate formation in mouse peritoneal macrophages (**Table 2**). Hyperlipidemic LDL and HDL had no significant effect, even when added at very high cholesterol concentrations.

As expected from the above results, the addition to the culture medium of d < 1.006 g/ml lipoproteins from hyperlipidemic rabbit No. 1 produced a marked increase in the content of esterified cholesterol in the macrophages as measured by gas–liquid chromatography. The esterified cholesterol content rose from <1 $\mu$g sterol/mg cell protein to 19 $\mu$g per mg cell protein. In contrast, LDL from the same animal, when added to the medium at the same final cholesterol concentration of 150 $\mu$g/ml, produced no increase in cellular esterified cholesterol. Macrophages incubated with hyperlipidemic rabbit d < 1.006 g/ml lipoproteins showed numerous birefringent lipid droplets when examined under polarized light. LDL produced no such droplets.

The results obtained with rabbit lipoproteins in mouse peritoneal macrophages were reproduced with macrophages obtained from the peritoneal cavity of normal New Zealand white rabbits. The rabbit macrophages incubated with hyperlipidemic rabbit d

< 1.006 g/ml lipoproteins accumulated lipid droplets and incorporated [$^{14}$C]oleate into cellular cholesteryl esters. The d < 1.006 g/ml lipoproteins from the hyperlipidemic rabbits produced a 5-fold increase in cholesteryl [$^{14}$C]oleate formation as compared to values obtained with no added lipoproteins. The d < 1.006 g/ml lipoproteins were added at a concentration of 300 $\mu$g cholesterol/ml and were incubated for 16 hr. Normal rabbit d < 1.006 g/ml lipoproteins at the same cholesterol concentration did not significantly stimulate cholesteryl [$^{14}$C]oleate formation in the rabbit peritoneal macrophages.

**Fig. 4** compares the effects of hyperlipidemic rabbit d < 1.006 g/ml lipoproteins and LDL on cholesteryl ester synthesis in mouse peritoneal macrophages and in cells (cultured human fibroblasts) that express LDL receptors. The human fibroblasts were incubated with lipoprotein-deficient serum for 48 hr prior to the experiment in order to induce large numbers of LDL receptors, whereas the mouse macrophages were kept in 20% whole serum so as to prevent an induction of LDL receptors. As expected from the above data, the rabbit d < 1.006 g/ml lipoproteins stimulated cholesteryl [$^{14}$C]oleate synthesis in the mouse macrophages whereas the rabbit LDL did not (Fig. 4A). On the other hand, in human fibroblasts, both types of lipoprotein stimulated cholesteryl ester synthesis. In these cells, rabbit LDL achieved a maximal rate of cholesteryl ester formation that was about two-thirds that achieved by rabbit d < 1.006 g/ml lipoproteins (Fig. 4B). For comparison, the
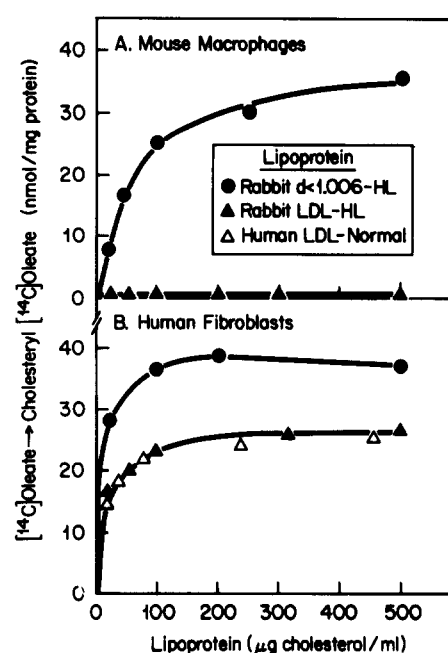


**Fig. 4.** Stimulation of cholesteryl ester formation in mouse macrophages (A) and human fibroblasts (B) by hyperlipidemic rabbit lipoproteins. Exp. A: each monolayer of mouse macrophages received 0.6 ml of DMEM containing 0.2 mM [$^{14}$C]oleate–2.4 mg/ml albumin and the indicated concentration of either d < 1.006 g/ml lipoproteins from hyperlipidemic rabbit No. 1 (●) or LDL from hyperlipidemic rabbit No. 1 (▲). After incubation for 6 hr at 37°C, the cellular content of cholesterol [$^{14}$C]oleate was determined. Exp. B: on day 7 of cell growth, after the cells had been incubated for 48 hr in lipoprotein-deficient serum, each monolayer of human fibroblasts received 1.5 ml of medium A containing 0.2 mM [$^{14}$C]oleate–albumin and the indicated concentration of one of the following lipoproteins: ●, d < 1.006 g/ml from hyperlipidemic rabbit No. 1; ▲, LDL from hyperlipidemic rabbit No. 1; or △, normal human LDL. After incubation for 6 hr at 37°C, the cellular content of cholesteryl [$^{14}$C]oleate was determined. The content of cholesteryl [$^{14}$C]oleate in cells incubated without lipoproteins was 0.8 and 0.4 nmol/mg protein for Exps. A and B, respectively. (Abbreviation in Fig. 4: HL, hyperlipidemic).
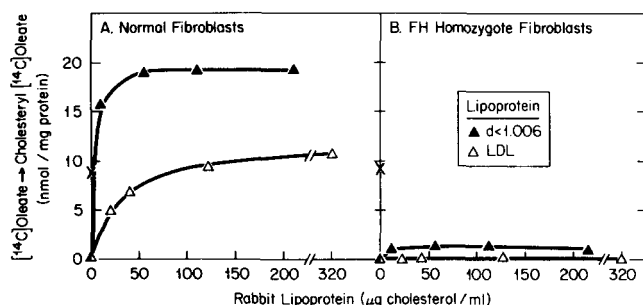
TABLE 2. Stimulation of cholesteryl ester formation in mouse macrophages by hyperlipidemic rabbit lipoproteins

| Lipoprotein Fraction Added to Medium | Concentration in Medium | | [$^{14}$C]Oleate → Cholesteryl [$^{14}$C]Oleate |
| --- | --- | --- | --- |
| | Protein | Cholesterol | |
| | $\mu$g/ml | | nmol/mg protein |
| None | 0 | 0 | 0.78 |
| d < 1.006 | 10 | 110 | 20.0 |
| d < 1.006 | 20 | 220 | 40.0 |
| IDL, d 1.006–1.02 | 10 | 43 | 3.4 |
| IDL, d 1.006–1.02 | 50 | 215 | 19.2 |
| LDL, d 1.02–1.063 | 100 | 200 | 1.1 |
| LDL, d 1.02–1.063 | 300 | 600 | 0.87 |
| HDL, d 1.063–1.21 | 100 | 44 | 0.49 |
| HDL, d 1.063–1.21 | 300 | 132 | 0.64 |

Each monolayer received 0.6 ml of DMEM containing 0.2 mM [$^{14}$C]oleate–2.4 mg/ml albumin and the indicated concentration of the indicated lipoprotein fraction. After incubation for 6 hr at 37°C, the cellular content of cholesteryl [$^{14}$C]oleate was determined. All lipoproteins were prepared from hyperlipidemic rabbit No. 1.

human fibroblasts were incubated with normal human LDL in the same experiment (open triangles, Fig. 4B). The human LDL was as effective as the hyperlipidemic rabbit LDL in stimulating cholesteryl [$^{14}$C]oleate synthesis.

To determine whether the stimulation of cholesteryl ester synthesis by hyperlipidemic lipoproteins in human fibroblasts required the action of the LDL receptor, we compared the results in normal cells and in cells from a patient with homozygous familial hypercholesterolemia (FH) that lack functional LDL receptors (**Fig. 5**). In the FH homozygote cells, the response to hyperlipidemic rabbit d < 1.006 g/ml lipoproteins and LDL was markedly attenuated (Fig. 5B). In the same experiment, 25-hydroxycholesterol stimulated cholesteryl [$^{14}$C]oleate formation equally in the normal and FH homozygote cells (shown by X
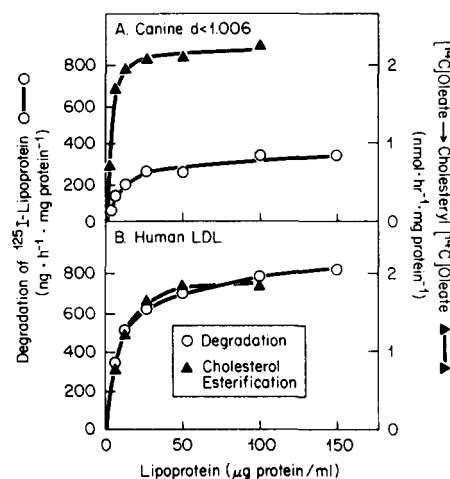
**Fig. 5.** Stimulation of cholesteryl ester formation by hyperlipidemic rabbit lipoproteins in normal human fibroblasts (A), but not in FH homozygote fibroblasts (B). On day 7 of cell growth, after the cells had been incubated for 48 hr in lipoprotein-deficient serum, each monolayer of human fibroblasts received 1.5 ml of medium A containing 0.2 mM [$^{14}$C]oleate–2.4 mg/ml albumin and the indicated concentration of one of the following: ▲, d < 1.006 g/ml lipoproteins from hyperlipidemic rabbit No. 1; △, LDL from hyperlipidemic rabbit No. 1; or X, 5 μg/ml of 25-hydroxycholesterol added in 4 μl of ethanol. After incubation for 6 hr at 37°C, the cellular content of cholesterol [$^{14}$C]oleate was determined. The content of cholesteryl [$^{14}$C]oleate in cells incubated without rabbit lipoproteins or 25-hydroxycholesterol was 0.2 nmol/mg for both Exps. A and B.

in Figs. 5A and 5B). This latter response does not require the action of the LDL receptor (15).

The hyperlipidemic d < 1.006 g/ml lipoproteins from all animal species have a several-fold higher cholesterol to protein ratio than does LDL (Table 1 and ref. 5). Thus, each particle of hyperlipidemic d < 1.006 g/ml lipoprotein that enters a cell brings in more cholesterol than does an entering particle of LDL. In human fibroblasts in which both LDL and the d < 1.006 g/ml lipoproteins enter through the LDL receptor, the higher cholesterol content of the hyperlipidemic d < 1.006 g/ml lipoproteins relative to LDL leads to a higher rate of cholesterol esterification for each milligram of protein that is degraded in lysosomes. For example, in the experiments shown in **Fig. 6A,** a maximum of 300 ng protein of canine hyperlipidemic $^{125}$I-labeled d < 1.006 g/ml lipoproteins was degraded per hr for each milligram of cell protein. Inasmuch as the mass ratio of cholesterol to protein for this particle was 2.8, this rate of degradation liberated 2.2 nmol/hr of cholesterol. The amount of cholesterol observed to be reesterified was 2.3 nmol/hr, as measured by the incorporation of [$^{14}$C]oleate into cholesteryl [$^{14}$C]oleate (Fig. 6A). Thus, essentially all of the liberated cholesterol was reesterified. Human LDL behaved in the same fashion (Fig. 6B). The maximal rate of $^{125}$I-LDL degradation was 800 ng protein per hr for each milligram of cell protein, which liberated 2.6 nmol/hr of cholesterol (mass ratio of cholesterol to protein for human LDL = 1.5). The observed reesterification rate was 2 nmol/hr (Fig. 6B).

In the mouse peritoneal macrophages, the canine hyperlipidemic d < 1.006 g/ml lipoproteins, which entered the cells through a high affinity mechanism (5), elicited a rate of cholesterol reesterification that was proportional to the amount of cholesterol liberated (**Fig. 7A**). For each milligram of cell protein, a maximum of 540 ng protein of hyperlipidemic canine $^{125}$I-labeled d < 1.006 g/ml lipoprotein was degraded per hour. Inasmuch as the mass ratio of cholesterol to protein for this particle was 7.2, the maximal amount of cholesterol liberated was 10 nmol/hr. The amount of cholesterol observed to be reesterified was 5.3 nmol/hr. Thus, a large fraction of the liberated cholesterol was available for reesterification.
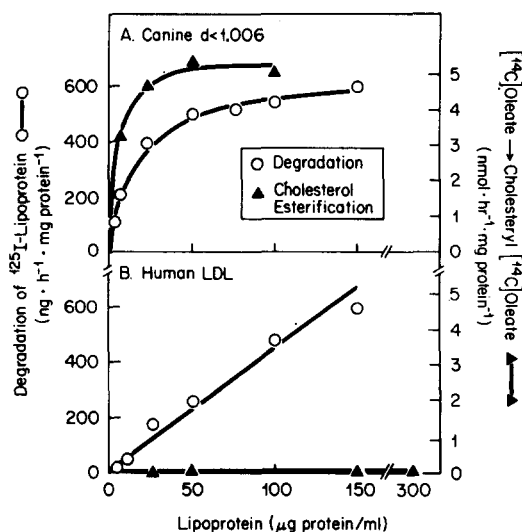
A strikingly different situation was obtained with regard to the metabolism of normal human LDL in mouse peritoneal macrophages (Fig. 7B). As previously reported (7, 16, 17), there was no evidence for a saturable high affinity degradation mechanism for $^{125}$I-LDL at concentrations up to 150 μg protein/ml

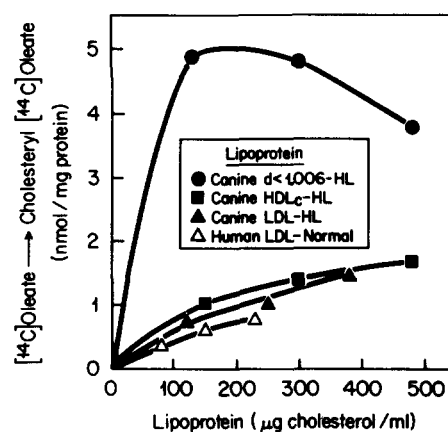

**Fig. 6.** Relation between the rate of degradation of $^{125}$I-labeled lipoproteins (○) and the rate of cholesteryl ester formation (▲) in normal human fibroblasts. All experiments were carried out on day 7 of cell growth after the cells had been incubated for 48 hr in lipoprotein-deficient serum. For degradation assays (○), each monolayer of fibroblasts received 1.5 ml of medium A containing 10% human lipoprotein-deficient serum and the indicated concentration of either hyperlipidemic canine $^{125}$I-d < 1.006 g/ml lipoproteins (106 cpm/ng protein) (Exp. A), or normal human $^{125}$I-LDL (89 cpm/ng protein) (Exp. B). After incubation for 5 hr at 37°C, the amount of $^{125}$I-labeled acid-soluble material in the medium was determined. For assays of cholesteryl ester formation (▲), each monolayer received 1.5 ml of medium A containing 0.1 mM [$^{14}$C]oleate–1.2 mg/ml albumin and the indicated concentration of either hyperlipidemic canine d < 1.006 g/ml lipoproteins (Exp. A) or normal human LDL (Exp. B). After incubation for 6 hr at 37°C, the cellular content of cholesteryl [$^{14}$C]oleate was determined. The rate of cholesteryl [$^{14}$C]oleate formation in cells incubated without lipoproteins was 0.05 nmol/hr per mg protein for both Exps. A and B. The hyperlipidemic canine lipoproteins were obtained from dog No. 3.

(Fig. 7B). However, the macrophages did degrade ^{125}I-LDL at a rate that rose linearly with increasing LDL concentrations. At an ^{125}I-LDL concentration of 150 μg protein/ml, the degradation rate was 600 ng/hr for each milligram of cell protein, which is equivalent to 2.3 nmol/hr of cholesterol liberated. However, at this concentration the observed amount of [^{14}C]oleate incorporated into cholesteryl [^{14}C]oleate was less than 0.05 nmol/hr, a value that was not different from the rate in cells incubated in the absence of lipoproteins. Furthermore, there was no increase when the LDL concentration was doubled to 300 μg protein/ml.

To determine if hyperlipidemic d < 1.006 g/ml lipoproteins were able to stimulate cholesteryl ester synthesis in cultured human monocytes, we prepared monolayers of human blood monocytes and incubated them for 5 days in 10% autologous whole serum. The subsequent addition of the d <1.006 g/ml lipoproteins from a hyperlipidemic dog markedly stimulated cholesteryl [^{14}C]oleate formation (**Fig. 8**). On the other hand, two other lipoprotein fractions from the



**Fig. 8.** Cholesteryl ester formation in human monocytes incubated with hyperlipidemic canine lipoproteins. On day 5, each monolayer of human monocytes received 0.6 ml of RPMI 1640 medium containing 0.2 mM [^{14}C]oleate–2.4 mg/ml albumin and the indicated concentration of one of the following: ●, hyperlipidemic canine d < 1.006 g/ml lipoproteins; ■, hyperlipidemic canine HDL$_c$; ▲, hyperlipidemic canine LDL; or △, normal human LDL. After incubation for 8 hr at 37°C, the cellular content of cholesteryl [^{14}C]oleate was determined. The content of cholesteryl [^{14}C]oleate in cells incubated without lipoproteins was 0.8 nmol/mg protein. The hyperlipidemic canine lipoproteins were obtained from dog No. 1. (Abbreviation in Fig. 8: HL, hyperlipidemic).

same hyperlipidemic dog, LDL and HDL$_c$, were much less potent, as was normal human LDL. In other experiments, we found that the d < 1.006 g/ml lipoproteins from hyperlipidemic rabbit No. 1 were about 6-fold more potent than LDL in stimulating cholesteryl ester synthesis in the cultured human monocytes (data not shown).

Throughout the current studies, we routinely measured the incorporation of [^{14}C]oleate into cellular [^{14}C]triglycerides as well as into cholesteryl [^{14}C]oleate. None of the lipoproteins from either normal or hyperlipidemic dogs affected the rate of [^{14}C]triglyceride formation significantly. This finding would tend to exclude the possibility of any significant dilution of the exogenous [^{14}C]oleate by endogenous fatty acids derived from the degradation of the hyperlipidemic lipoproteins.



**Fig. 7.** Relation between the rate of degradation of ^{125}I-labeled lipoproteins (○) and the rate of cholesteryl ester formation (▲) in mouse macrophages. For degradation assays (○), each monolayer received 0.6 ml DMEM containing 10% human lipoprotein-deficient serum and the indicated concentration of either hyperlipidemic canine ^{125}I-labeled d < 1.006 g/ml lipoproteins (47 cpm/ng protein) (Exp. A) or normal human ^{125}I-labeled LDL (129 cpm/ng protein) (Exp. B). After incubation for 5 hr at 37°C, the amount of ^{125}I-labeled acid-soluble material in the medium was determined. For assays of cholesteryl ester formation (▲), each monolayer received 1 ml of DMEM containing 0.2 mM [^{14}C]oleate–2.4 mg/ml albumin and the indicated concentration of either hyperlipidemic canine d < 1.006 g/ml lipoproteins (Exp. A) or normal human LDL (Exp. B). After incubation for 8 hr at 37°C, the cellular content of cholesteryl [^{14}C]oleate was determined. The rate of cholesteryl [^{14}C]oleate formation in cells incubated without lipoproteins was 0.04 and 0.08 nmol/hr per mg protein for Exps. A and B, respectively. The hyperlipidemic canine lipoproteins were obtained from dog No. 2.

## DISCUSSION

The current paper extends to three additional animal species the finding originally reported for cholesterol-fed dogs, namely, that the abnormal d < 1.006 g/ml lipoproteins that accumulate in plasma after cholesterol feeding have a specific ability to deposit cholesterol in macrophages (5). Inasmuch as these hyperlipidemic d < 1.006 g/ml lipoproteins consist predominantly of β-VLDL, they are hereafter

referred to as $\beta$-VLDL. It is noteworthy that the type of fat in the cholesterol-rich diets fed to the various animals differed (coconut oil, lard, egg yolk, or soybean oil); however, the characteristics of the d < 1.006 g/ml lipoproteins (Table 1) and the responses of the macrophages to the different cholesterol-induced d < 1.006 g/ml lipoproteins did not differ significantly. The presence of cholesterol in the diet appears to be the important determinant responsible for the change in the lipoproteins.

Previous studies have shown that the ability of canine $\beta$-VLDL to produce cholesteryl ester accumulation in macrophages is dependent on its ability to bind to cell surface receptors and thereby to be taken up by the cell. The internalized $\beta$-VLDL is digested in cellular lysosomes, and the liberated cholesterol is re-esterified and stored by the cell (5). That such a mechanism operates for the $\beta$-VLDL of the cholesterol-fed rhesus monkey, rat, and rabbit is supported by three observations: *1*) $\beta$-VLDL from each of these species stimulates cholesteryl ester formation as evidenced by an increase in incorporation of [$^{14}$C]oleate into cholesteryl [$^{14}$C]oleate; *2*) this stimulation by $\beta$-VLDL shows saturation kinetics with respect to the concentration of $\beta$-VLDL in the culture medium, a finding that is consistent with a limited number of high affinity surface binding sites; and *3*) the types of lipoprotein fractions that stimulate cholesteryl ester synthesis are similar in the rhesus monkey, rat, rabbit, and dog—that is, in each case hyperlipidemic $\beta$-VLDL is much more effective than are VLDL from normal animals and LDL from normal or cholesterol-fed animals.

Considered together with our previous report (5), the current data indicate that macrophages possess a surface binding site that recognizes some component that is present on $\beta$-VLDL but is either absent or masked on other lipoproteins. The comparative studies of macrophages and cultured fibroblasts (Figs. 4, 6, and 7) suggest that the macrophage binding site recognizes a lipoprotein component that differs from the one recognized by the previously-described LDL receptor. The LDL receptor of fibroblasts binds both LDL and $\beta$-VLDL as evidenced by the fact that both of these lipoproteins stimulated cholesteryl ester formation in normal fibroblasts but not in mutant FH homozygote cells (Fig. 5). On the other hand, the macrophage binding site recognizes $\beta$-VLDL, but not LDL, as evidenced by high affinity degradation of the former but not the latter lipoprotein in macrophages (Fig. 7 and refs. 7, 16, 17).

An important unresolved question relates to the nature of the nonsaturable degradation of $^{125}$I-LDL in macrophages (Fig. 7B). A similar nonsaturable degradation of $^{125}$I-LDL has been observed in FH homozygote fibroblasts that lack LDL receptors (18, 19). At high LDL concentrations, the rate of this degradation can be appreciable. In both macrophages and FH homozygote fibroblasts, this nonsaturable degradation process does not stimulate cholesteryl ester formation nor does it lead to the mass accumulation of cholesteryl esters within the cell (15). The nature of this process in both cell types is not known.

All the studies using mouse peritoneal macrophages were performed with freshly isolated cells that had been incubated for 24 hr with 20% whole fetal calf serum prior to the addition of lipoproteins. There was no attempt to induce LDL receptors by prior incubation in lipoprotein-deficient serum. This experimental design permits the biggest contrast between LDL receptor-bearing cells (i.e., induced cultured fibroblasts) and cells deficient in LDL receptors (i.e., noninduced mouse macrophages). In preliminary studies, we have observed that incubation of mouse peritoneal macrophages for 24 to 48 hr with lipoprotein-deficient serum leads to the expression of a small number of LDL receptors. The number of these induced receptors in macrophages (expressed on the basis of cell protein) is less than one-tenth the number seen in similarly induced fibroblasts.

The present study and our previous report (5) clearly demonstrate that cholesterol-induced $\beta$-VLDL from dogs, rats, rabbits, and monkeys is the major, if not the only, naturally occurring plasma lipoprotein from either normal or cholesterol-fed animals that is capable of inducing cholesteryl ester accumulation in macrophages under the current in vitro conditions. Likewise, normal human lipoproteins do not cause enhanced cholesteryl ester synthesis or accumulation. The potential biologic significance of these results relates to the possibility that the macrophage is an important cell type involved in the accumulation of cholesterol in atherosclerotic lesions (2, 20) and that $\beta$-VLDL may be the atherogenic lipoprotein responsible for cholesterol deposition and foam cell production in cholesterol-fed animals. An association between $\beta$-VLDL and macrophages has previously been implicated in the development of canine atherosclerosis. Cholesterol-fed dogs develop atherosclerosis only when the plasma cholesterol level exceeds 750 mg/dl (21). At non-atherogenic plasma levels of <750 mg/dl, the cholesterol is carried by LDL and HDL$_c$, and the d < 1.006 g/ml fraction contains primarily triglyceride-rich pre-$\beta$ VLDL. A major alteration in the plasma lipoprotein pattern that occurs with the atherogenic hypercholesterolemia is the appearance of cholesterol-rich $\beta$-VLDL as a

major lipoprotein fraction (21). The canine atherosclerosis that occurs when $\beta$-VLDL appears is characterized by the accumulation of foam cells which may be of macrophage origin (for review, see ref. 2).

In other species, such as the rabbit, atherosclerosis develops at all levels of hypercholesterolemia, and $\beta$-VLDL represents a significant cholesterol-carrying plasma lipoprotein at all levels. It has been shown previously that rabbit $\beta$-VLDL particles (d < 1.006 g/ml) of cholesterol-fed animals are remnants of chylomicron catabolism, and it has been suggested that these are the major atherogenic lipoproteins in rabbits (22). Several years ago Zilversmit (23) speculated that remnants may be important atherogenic lipoproteins in man as well. Whether human remnants, which resemble $\beta$-VLDL particles from hypercholesterolemic animals, produce cholesteryl ester deposition by a high affinity uptake mechanism in macrophages remains to be determined. Admittedly, atherogenesis is a complex process which may involve various plasma lipoproteins, e.g., high levels of LDL and/or low levels of HDL, and may involve several mechanisms responsible for cholesterol accumulation.■

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