Hypertriglyceridemic Very Low Density Lipoproteins Induce Triglyceride Synthesis and Accumulation in Mouse Peritoneal Macrophages

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ABSTRACT Triglyceride-rich lipoproteins may be responsible for the lipid accumulation in macrophages that can occur in hypertriglyceridemia. Chylomicrons and very low density lipoproteins (VLDL, total and with flotation constant [S_f] 100–400) from fasting hypertriglyceridemic subjects induced a massive accumulation of oil red O-positive inclusions in unstimulated peritoneal macrophages. Cell viability was not affected. The predominant lipid that accumulated in cells exposed to hypertriglyceridemic VLDL was triglyceride. Hypertriglyceridemic VLDL stimulated the incorporation of [^{14}C]oleate into cellular triglyceride up to ninefold in 16 h, but not into cholesteryl esters. Mass increase in cellular triglyceride was 38-fold. The stimulation of cellular triglyceride formation was dependent on time, temperature, and concentration of hypertriglyceridemic VLDL. By contrast, VLDL, low density, and high density lipoproteins from fasting normolipemic subjects had no significant effect on oleate incorporation into neutral lipids or on visible lipid accumulation.

^{125}I-Hypertriglyceridemic VLDL (S_f 100–400) were degraded by macrophages in a dose-dependent manner, with 50 and 100% saturation observed at 3 and 24 μg protein/ml (2.5 and 20 nM), respectively. Hypertriglyceridemic VLDL inhibited the internalization and degradation of ^{125}I-hypertriglyceridemic VLDL (4 nM) by 50% at 3 nM. Cholesteryl ester-rich VLDL from cholesterol-fed rabbits gave 50% inhibition at 5 nM. Low density lipoproteins (LDL) inhibited by 10% at 5 nM and 40% at 47 nM. Acetyl LDL at 130 nM had no effect. We conclude that the massive triglyceride accumulation produced in macrophages by hypertriglyceridemic VLDL is a direct consequence of uptake via specific receptors that also recognize cholesteryl ester-rich VLDL and LDL but are distinct from the acetyl LDL receptor. Uptake of these triglyceride-rich lipoproteins by monocyte-macrophages in vivo may play a significant role in the pathophysiology of atherosclerosis.

INTRODUCTION

Some forms of hypertriglyceridemia are associated with premature atherosclerosis and with the accumulation of lipid-filled macrophages throughout the body (1). Macrophages have been implicated in atherogenesis; some of the foam cells found in arterial plaques may be derived from blood-born monocyte-macrophages (2, 3). Elevated levels of functionally abnormal triglyceride-rich lipoproteins may contribute to these pathologic conditions. We have found that very low density lipoproteins (VLDL)[1] from hypertriglyceridemic but not normal subjects suppress the activity of 3-hydroxy-3-methylglutaryl-CoA reductase in cultured human fibroblasts and endothelial cells via the low density lipoprotein (LDL) receptor pathway (4–6). Moreover, low levels of the hypertriglyceridemic VLDL, but not normal VLDL, are toxic to cultured vascular endothelial cells; this, potentially, is an atherogenic consequence of abnormal receptor-me-

A preliminary report of this work has appeared in abstract form in 1980. *Circulation.* **62:** III–17a.

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Received for publication 28 August 1981 and in revised form 8 March 1982.

[1] *Abbreviations used in this paper*: CER, cholesteryl ester-rich; HDL, high density lipoproteins; HTG, hypertriglyceridemic; LDL, low density lipoproteins; LPDS, lipoprotein-deficient serum; S_f 100–400 VLDL, $VLDL_1$; S_f 60–100 VLDL, $VLDL_2$; S_f 20–60 VLDL, $VLDL_3$; TCM, tissue culture medium; VLDL, very low density lipoproteins.

diated uptake (6). Macrophages, however, including those from mouse peritoneal cavity, have few classical LDL cell surface receptors (7). Peritoneal macrophages take up only small amounts of normal LDL, but internalize large quantities of LDL that have been modified chemically so that their net charge is more negative, as by acetylation (7, 8), or LDL that is complexed to high molecular mass (500,000 daltons) dextran sulfate (9). In addition to recognizing the chemically modified or complexed LDL, mouse peritoneal macrophages have a cell-surface receptor that appears to be specific for cholesteryl ester-rich (CER) β-VLDL, a lipoprotein that accumulates in the blood of cholesterol-fed animals (10). After uptake by any of these receptor-mediated pathways, the CER lipoproteins are hydrolyzed in the lysosomes. The released cholesterol is reesterified in the cytoplasm where it accumulates as lipid droplets. These droplets can be visualized after staining with the neutral lipid stain oil red O or by their birefringence under polarized light. Human monocyte-derived macrophages possess both the scavenger pathway, which recognizes acetylated or malondialdehyde-treated LDL, as well as the classical LDL receptor pathway (11–13).

We now report that VLDL from the plasma of hypertriglyceridemic subjects, with no chemical modification and in the absence of dextran sulfate as a carrier, cause a massive accumulation of oil red O-positive, osmiophilic triglyceride inclusions in freshly isolated peritoneal macrophages from unstimulated mice. Moreover, hypertriglyceridemic VLDL, but not normal VLDL, stimulate up to a ninefold increase in the incorporation of [^{14}C]oleate into cellular triglyceride and a 38-fold increase in the mass of cellular triglyceride. These effects of hypertriglyceridemic VLDL on macrophage lipid metabolism are a consequence of uptake and degradation via saturable receptors that also recognize abnormal VLDL from cholesterol-fed rabbits.

METHODS

Animals. 6–8-wk-old Balb/c, C3H/HeJ, and C3HeB/FeJ mice of both sexes were purchased from Jackson Laboratories, Bar Harbor, ME. Mice were housed in cages equipped with barrier filters and fed standard laboratory chow and water ad lib.

Preparation and culture of macrophage monolayers. Detailed descriptions for isolation of mouse peritoneal leukocytes and preparation of adherent cell (macrophage) monolayers have been published previously (14, 15). Cells were collected from normal, unstimulated mice by repeatedly injecting 3 ml of tissue culture medium (TCM) intraperitoneally and aspirating the fluid contents with a syringe. The TCM consisted of Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with 5% (vol/vol) human lipoprotein-deficient serum, $d > 1.21$ (LPDS), 2 mM glutamine, 36 mM $NaHCO_3$, 100 U/ml potassium penicillin G, and 100 μg/ml streptomycin sulfate. Harvests from 10 to 24 mice were pooled, pelleted by centrifugation (400 *g* for 10 min) at 4°C, washed in Hanks' balanced salt solution or TCM and resuspended in TCM at 2×10^6 cells/ml. 1 ml was added to each 35-mm culture dish (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) and cultures were incubated for 1 h at 37°C in a humidified chamber containing an atmosphere of 83% N_2, 10% CO_2, and 7% O_2. After gentle agitation, nonadherent leukocytes were removed by aspirating the supernatant with a sterile Pasteur pipette and by washing monolayers two times with 1-ml portions of TCM. To initiate each experiment, 1 ml TCM and serum lipoproteins were added to macrophage monolayers and incubated under conditions described above for the designated time periods.

Characterization of lipoprotein-induced cytoplasmic inclusions. Macrophage monolayers were prepared as described (15) by overlaying 22×22-mm sterile glass cover slips with 0.3 ml of the peritoneal leukocyte suspension. Following 1-h incubation at 37°C, cover slips were rinsed in a beaker containing TCM and placed in a 35-mm culture dish containing 1 ml TCM. Lipoproteins were added and cultures were incubated under standard conditions for varying lengths of time. At termination, cover slips were removed and thoroughly washed by three successive transfers to culture dishes containing 1 ml phosphate-buffered saline with 2% bovine serum albumin. Cover slips were then fixed in 60% isopropyl alcohol, stained with oil red O, counterstained with Harris' hematoxylin, and mounted, face-down, on glass microscope slides using prewarmed (45°C) glycerin jelly. Slide preparations were examined by light microscopy at $\times$1,000 magnification; the number of oil red O-staining inclusions per cell in 100 randomly observed cells were tabulated with each slide.

Conditions for the massive accumulation of cholesteryl esters in acetyl LDL-treated macrophages were followed in order to analyze hypertriglyceridemic VLDL-induced cytoplasmic lipids by polarized light microscopy (8). Macrophage monolayers were prepared on cover slips and incubated with the indicated lipoproteins. After 24 h, the medium was removed and replaced with fresh TCM and lipoprotein. After an additional 24-h incubation, cover slips were washed and mounted as described above, heated to 45°C on a slide warmer and examined immediately at room temperature using an Olympus light microscope equipped with polarizing lenses (Olympus Corporation of America, New Hyde Park, NY).

Lipoproteins. Normal lipoprotein fractions and LPDS ($d > 1.21$) were isolated from the plasma of fasting (12–14 h) adult normolipemic males (ages 21–26, plasma triglyceride level $\leq$ 100 mg/dl). Hypertriglyceridemic VLDL were obtained from the plasma of fasting patients with type IV and type V lipoprotein profiles. The diagnoses were based on commonly used criteria (1). Lipoprotein fractions were isolated from fresh plasma containing 10 μM phenylmethylsulfonyl fluoride and 1 mM NaN_3 according to standard sequential flotation techniques in a 60 Ti rotor at 45,000 rpm and 14°C for indicated times in a Beckman preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) using KBr for density adjustment (16). Total VLDL were isolated after chylomicrons were removed (0.5 h at 35,000 rpm) in a second centrifugation (18 h) without adjusting the density of plasma ($d < 1.006$); LDL were isolated (18-h spin) at d 1.006–1.063 or d 1.02–1.063; high density lipoproteins (HDL) were isolated at d 1.063–1.21. Flotation constant (S_f) 100–400 VLDL ($VLDL_1$), S_f 60–100 VLDL ($VLDL_2$), and S_f 20–60 VLDL ($VLDL_3$) were prepared from

the $d < 1.006$ fraction by cumulative flotation (17) as described in detail elsewhere (5). CER-VLDL were isolated from the plasma of rabbits fed a diet containing 1% cholesterol for ≥8 d. The hypercholesterolemic plasma was diluted with an equal volume of Tris-buffered saline then ultracentrifuged for 18 h. These VLDL contained 58–71% cholesteryl ester, ≤5% triglyceride, apoB, elevated apoE, and little to no apoC peptides, and had beta mobility on agarose electrophoresis. CER-VLDL, relative to LDL, exhibited enhanced affinity for the human skin fibroblast LDL receptor (18).

Lipoproteins and LPDS were dialized against three changes of 50 vol of 0.15 M NaCl containing 50 mM Tris–HCl, pH 7.4, and 0.3 mM EDTA at 4°C for 36–48 h. Lipoproteins and thrombin-treated LPDS (19) were sterilized by filtration through a 0.45 μm Millex (lipoproteins) or a 0.22 μm filter unit (LPDS) (Millipore Corporation, Bedford, MA); LPDS was stored at −20°C and lipoproteins at 4°C.

Total protein content of the lipoproteins was determined by a modification of the Lowry method (20); sodium dodecyl sulfate, at a final concentration of 0.1%, was included to prevent interference by opalescence and light scattering (21). ApoB was determined as the difference between the total protein and the protein soluble in 4.2 M tetramethylurea (22). Soluble apoprotein composition was determined after delipidation with 4.2 M tetramethylurea. The apoproteins were separated by electrophoresis on 7.5% polyacrylamide gels containing 8 M urea, stained with amido-Schwartz, and quantitated by scanning densitometry (22). Cholesterol, free and esterified, was quantified enzymatically (23). Phospholipid phosphorus was assayed by the method of Bartlett (24). Triglycerides were determined in the clinical laboratory of the Baylor Lipid Research Clinic (25). Lipoprotein particle concentrations were calculated on the basis of the following values: hypertriglyceridemic VLDL, 5% protein, 23.6×10^6-dalton particle mass (17); CER-VLDL, 6.4% protein and 50.4×10^6 daltons, calculated from the Stokes radius as determined by laser-light scattering techniques (26); LDL and acetyl LDL, 25% protein and 2.2×10^6 daltons. LDL was acetylated with acetic anhydride.

The incorporation of [1-^{14}C]-oleic acid (Amersham Corp., Arlington Heights, IL) into cellular triglyceride and cholesteryl ester was measured as described (28) except that 30-μl aliquots were used for Lowry protein determinations; triolein, 10 μg/ml, was included in the chloroform/methanol extraction step; and silica gel coated plastic sheets without fluorescent indicator, 3.5 × 6.5 cm (Eastman Kodak Co., Rochester, NY), were used for thin-layer chromatography of the lipid extracts. Regions of the chromatograms containing cholesteryl esters, triglycerides, and the [^{3}H]-cholesterol, used as internal standard, were visualized with iodine vapor, cut out, and counted by liquid scintillation. Each point represents the average of duplicate to quadruplicate determinations. VLDL were iodinated by a modification of the iodine monochloride method of McFarland (29). Excess, unbound iodine was removed from iodinated lipoproteins by gel filtration and extensive dialysis. Samples were filtered (0.45 μm Millex) immediately before use; specific activities ranged from 42 to 200 cpm/ng protein. Less than 13% of the label was extractable into organic solvent. Uptake of ^{125}I-VLDL was measured as the cell-associated radioactivity after extensive washing with chilled albumin-containing buffer (30). The amount of noniodide, nonlipid, trichloroacetic acid-soluble radioactivity in the medium was used as a measure of iodinated lipoprotein degradation (30); these values were corrected for nonspecific degradation by subtracting the amount degraded in control dishes that contained no cells. For mass analysis of cellular triglyceride, cholesterol, and cholesteryl ester, the cells of 24 mice were pooled and seeded in nine 60-mm dishes. After nonadherent cells were removed, three dishes of macrophages were incubated in the absence of lipoproteins (group A); three dishes with acetyl-LDL, 45 μg protein/ml (group B); and three dishes (group C) with hypertriglyceridemic $VLDL_1$ (subject J.P., type V pattern, 29 μg protein, 218 μg triglyceride/ml). After incubation at 37°C for 22 h the dishes were chilled on ice. To ensure that the increase in cell-associated triglyceride in group C were not due to surface bound VLDL rather than intracellular triglyceride, the equivalent amount of hypertriglyceridemic $VLDL_1$ previously fed to group C cells was added to each of the chilled control dishes. The medium from each dish was then aspirated, the cells washed five times with ice-cold buffered saline containing 0.2% bovine serum albumin (fatty acid free) and two times with cold buffered saline. The cells were harvested and total lipids were extracted with chloroform/methanol (2:1). The total lipid extracts from dishes in each group were combined, evaporated, and dissolved in 10 μl of isopropanol before separation by thin-layer chromatography on rods covered with a thin layer of scintered silicic acid (Chromarods, Iatron Laboratories, Inc., Tokyo, Japan). The lipids were quantified by means of a flame ionization detector (Iatron TH-10 analyzer, Iatron Laboratories, Inc.) (31).

RESULTS

Foam cell formation. When unstimulated mouse peritoneal macrophages are incubated in lipoprotein-deficient medium for 24 h and then stained with the neutral lipid stain oil red O, ~30–40% of the cells contain no detectable oil red O inclusions, as in Fig. 1A. In sharp contrast, these macrophages accumulate massive amounts of oil red O-positive droplets when incubated in the presence of VLDL isolated from the plasma of subjects with hypertriglyceridemia. The cells in Fig. 1B were incubated for 24 h in the presence of the VLDL from a subject with endogenous hypertriglyceridemia, type IV lipoprotein pattern (45 μg protein and 321 μg triglyceride/ml). Exposure of macrophages to hypertriglyceridemic VLDL causes a striking increase in the average number of oil red O-positive droplets per cell. A differential cell count based on the number of lipid droplets per cell shows that hypertriglyceridemic VLDL produce an increase in the percentage of cells containing numerous lipid droplets (Table I). This relatively simple visual method represents a rapid, semiquantitative way of comparing the abilities of various lipoproteins to induce lipid accumulation in the macrophages. Chylomicrons isolated from the plasma of a patient with type V hyperlipoproteinemia also induced lipid accumulation in macrophages (Table I).

The experiment illustrated in Fig. 2 typifies the effects of lipoproteins isolated from fasting normolipemic subjects on visible lipid accumulation in the macrophages. After a 16-h incubation in the absence

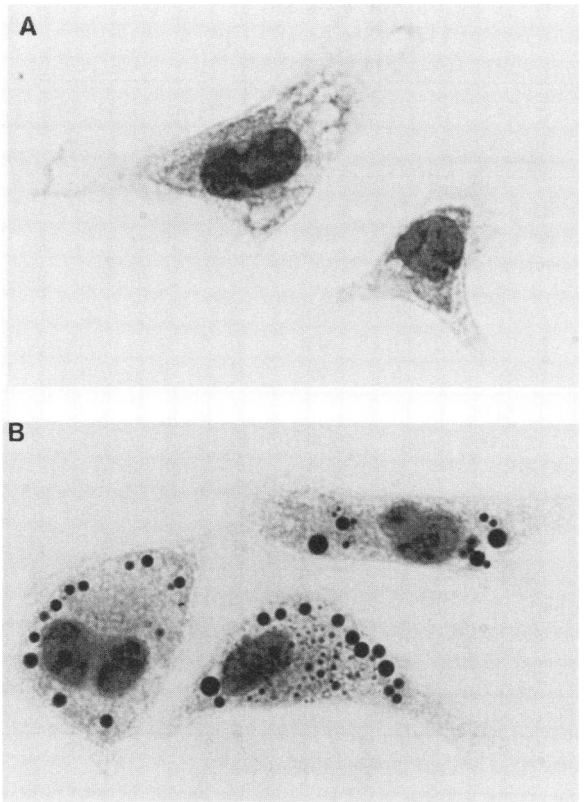

FIGURE 1 Light microscopic appearance of oil red O-stained macrophages incubated for 24 h in TCM containing LPDS in the absence (A) or in the presence (B) of hypertriglyceridemic VLDL, 45 μg protein/ml, isolated from the plasma of a subject with endogenous hypertriglyceridemia (type IV hyperlipoproteinemia, subject L.F.). Cells were isolated and cultured on glass cover slips and stained with oil red O (Methods). ×1,475.

of lipoproteins, <40% of the cells contained five or more lipid droplets per cell. LDL, HDL, and VLDL (even at triglyceride concentrations as high as 650 μg/ml) from a normolipemic subject had little effect on the percentage of cells containing five or more lipid droplets per cell. By contrast, there was a striking reversal of the distribution of stained cells after incubation with hypertriglyceridemic VLDL, so that 83% of the cells contained five or more lipid droplets. Acetyl LDL, the effects of which have been well documented (7, 8), is included as a control in each experiment. Like the hypertriglyceridemic VLDL, acetyl LDL produces intracellular lipid engorgement. Here 95% of the cells incubated with acetyl LDL contained numerous lipid droplets. In six separate experiments, VLDL from four normolipemic subjects failed to induce pronounced visible lipid accumulation in macrophages, even when treated for 24 h with VLDL concentrations as high as 650 μg triglyceride/ml. By contrast, VLDL from four subjects with a type IV lipoprotein pattern, four with a type V pattern, and two with a type IIb pattern stimulated lipid accumulation in macrophages (19 experiments).

VLDL are heterogeneous with respect to size, density, and composition. A potential explanation for the differences in effects of normal and hypertriglyceridemic VLDL on triglyceride accumulation by macrophages is that the abnormal total VLDL contain more large, triglyceride-rich particles than normal VLDL. We therefore isolated more homogeneous subclasses by a cumulative flotation procedure devised by Lindgren and associates (17) and tested these for their effects on visible lipid accumulation. As we have previously reported (5), the composition of VLDL subclasses from normal subjects are similar to the comparable subclasses from hypertriglyceridemic subjects with hyperlipoproteinemia types IV and V: the percent weight of the major components of S_f 100–400 normal VLDL (hypertriglyceridemic type IV VLDL) were 5.9±0.7 (5.0±1.0) protein; 18.5±1.9 (15.5±1.4) phospholipid; 5.9±1.4 (5.8±1.1)cholesterol; 6.1±1.6 (8.4±1.9) cholesteryl ester; and 63.6±2.6 (65.4±3.4) triglyceride (mean±SD; n = 5, normal; n = 4, hypertriglyceridemic subjects). There were no apparent differences in total apoB, apoE, or C peptide content as judged by polyacrylamide gel electrophoresis (5).

Thus, normal and hypertriglyceridemic VLDL subclasses at equal protein contents are equivalent both in triglyceride contents and in particle numbers. The largest VLDL subclass (S_f 100–400, $VLDL_1$) from hypertriglyceridemic subjects produced the greatest increase in the percentage of cells with increased visible lipid content and could account for most of the accumulation noted in cells exposed to the unfractionated VLDL (Table II). It is noteworthy that the pres-

TABLE I
Effects of Lipoproteins on Lipid Accumulation in Macrophages

Culture additions	Time of incubation	Oil red O-positive droplets/cell				
		0	1–4	5–8	9–12	>12
	h					
Saline	24	33‡	37	18	5	7
	48	12	42	36	7	3
HTG VLDL*	24	0	0	0	0	100
	48	0	0	0	0	100
Chylomicrons	24	0	7	35	25	33
	48	0	0	0	0	100

* Final concentration of type IV HTG VLDL (L.F.) and type V chylomicrons (D.G.) was 45 and 32 μg protein/ml, respectively.
‡ Percentage of cells in each differential.

ence of the smaller VLDL (S_f 60–100, $VLDL_2$; S_f 20–60, $VLDL_3$) in the unfractionated hypertriglyceridemic VLDL did not inhibit lipid accumulation. At similar concentrations, however, the largest normal VLDL subclass ($VLDL_1$), like the total normal VLDL, failed to induce lipid accumulation. Thus, macrophages, like normal human skin fibroblasts (4, 5) and endothelial cells (6), can distinguish between normal and hypertriglyceridemic VLDL.

The visible lipid accumulation induced by hypertriglyceridemic VLDL in the peritoneal macrophages increases with time, as does that produced by acetyl LDL, until all cells contain numerous lipid droplets. Control cells incubated in the absence of lipoproteins also show a moderate increase in lipid accumulation which plateaus between 16 and 24 h. By 24–48 h the hypertriglyceridemic VLDL-treated cells became so engorged that they presented a rounded form and foamy appearance. There was no decrease in cell viability in hypertriglyceridemic VLDL-treated macrophages relative to macrophages maintained in lipoprotein-deficient medium for up to 72 h, as judged by trypan blue exclusion.

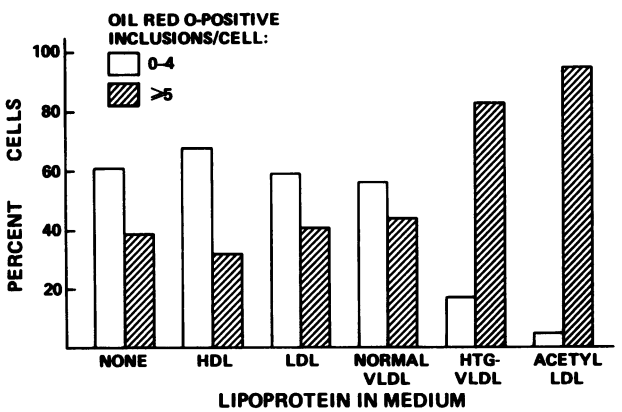

FIGURE 2 Effects of lipoproteins on lipid accumulation in macrophages. Cells were isolated and cultured on glass cover slips (Methods). Immediately after nonadherent cells were rinsed away, each coverslip was placed in a 35-mm tissue culture dish; 1 ml of medium containing 5% LPDS and the indicated lipoproteins, in 0.05 ml, were added. The VLDL preparations ($d < 1.006$) were from a normolipemic volunteer (O.P.) and a patient with endogenous hypertriglyceridemia (L.F.). The cells were incubated for 24 h before they were fixed and stained with oil red O (Methods). Slide preparations were examined by light microscopy at ×1,000 magnification; the number of oil red O-staining droplets in each of 100 ramdomly chosen cells were counted. Open bars represent the percent of cells with 0 to 4 oil red O-positive droplets per cell and hashed bars represent the percentage of cells containing ≥5 droplets per cell. The concentration of lipoproteins were in micrograms lipoprotein protein per millileter (micrograms triglyceride per milliliter) medium: HDL, 176; LDL, 80; normal VLDL, 43 (220); hypertriglyceridemic VLDL, 43 (307); acetyl LDL, 21.

TABLE II
Effects of VLDL Subclasses on Lipid Accumulation in Macrophages

Culture additions		Oil red O-positive droplets/cell 0–4	5–8	9–12	13–16	>16
Experiment 1						
Saline		100*	0	0	0	0
Normal VLDL						
$d < 1.006$	13‡	94	4	2	0	0
$VLDL_1$	5	100	0	0	0	0
$VLDL_2$	7	94	6	0	0	0
$VLDL_3$	8	98	2	0	0	0
HTG VLDL						
$d < 1.006$	37	20	16	6	22	36
$VLDL_1$	7	16	18	18	14	34
$VLDL_2$	8	78	12	4	2	4
$VLDL_3$	5	86	12	2	0	0
Experiment 2						
Saline		82*	6	2	6	4
Normal $VLDL_1$	21	74	16	8	0	2
Normal $VLDL_2$	28	78	8	6	4	4
HTG $VLDL_1$	29	2	0	8	0	90
HTG $VLDL_2$	33	6	14	10	16	54

* Percentage of cells in each differential.
‡ Final concentrations of VLDL subclasses in micrograms protein per milliliter.

A plot of the percentage of cells containing five or more lipid droplets per cell against the concentration of hypertriglyceridemic $VLDL_1$ to which the cells had been exposed was distinctly curvilinear after brief (3–4-h) incubations, with saturation at 320 μg triglyceride/ml. Saturability suggests that the visible lipid accumulation is a result of interaction of the hypertriglyceridemic VLDL with a limited number of cell sites rather than due to a nonspecific pinocytotic mechanism.

Thus, unstimulated macrophages assume the morphology of foam cells when incubated with hypertriglyceridemic VLDL but not when incubated with normal LDL, HDL, or VLDL, even at normal VLDL triglyceride levels of 650 μg/ml, far above saturating levels for hypertriglyceridemic VLDL. The ability of total hypertriglyceridemic VLDL to induce lipid accumulation resides primarily in the largest subclass, $VLDL_1$.

Macrophages accumulate triglyceride. The lipid that accumulates in macrophages exposed to acetyl LDL is cholesteryl ester and, as such, shows typical birefringence when observed by polarized light microscopy (8). By comparison, cells exposed to hypertriglyceridemic VLDL become filled with oil red O-

TABLE III
Lipid Composition of Murine Macrophages

Lipid	A. Control	B. Acetyl LDL* (B/A)	C. HTG $VLDL_1$* (C/A)
		μg/mg cell protein‡	
Cholesteryl ester	10.4	110 (10.6)	17.2 (1.7)
Triglyceride	10.4	31.4 (3.0)	397 (38.2)
Cholesterol	317	805 (2.5)	938 (3.0)

* Final concentration of acetyl LDL was 45 μg protein/ml; HTG $VLDL_1$ was 29 μg protein and 218 μg triglyceride/ml.
‡ Values express micrograms lipid per milligram cell protein. Macrophages from six animals were used for each group, incubated for 22 h, combined, and extracted for lipid separation and quantification (Methods).

positive, osmiophilic droplets, but they are not birefringent. This lack of birefringence suggests that the oil red O-positive material in these cells is not cholesteryl ester. Indeed, the predominant species from total lipid extracts of macrophages exposed to hypertriglyceridemic VLDL proved to be triglyceride as determined by thin-layer chromatography.

Corroborative chemical evidence for the accumulation of triglyceride in macrophages was obtained by preparative thin-layer chromatography of lipid extracts of macrophages treated with hypertriglyceridemic VLDL and [^{14}C]oleate in the standard solvent. Material that had the same mobility as authentic triolein (R_f 0.53) was eluted and rechromatographed in two different solvent systems. The visible lipid and 85% of the radioactivity comigrated with authentic triolein (R_f 0.72 in hexanes/diethyl ether/acetic acid, 80:20:1, solvent 2; R_f 0.26 for triolein and 0.23 for cell lipid in hexanes/diethyl ether, 95:5, solvent 3). After hydrolysis, this cellular lipid had the same mobility as authentic oleic acid (R_f 0.17 in solvent 2 and R_f O in solvent 3).

The mass increase in cellular triglyceride, cholesterol, and cholesteryl ester in cells incubated with acetyl LDL or with hypertriglyceridemic $VLDL_1$ are given in Table III. The cells were exposed to hypertriglyceridemic $VLDL_1$ at a level near saturation both for visible lipid accumulation and for specific uptake and degradation, as described below (29 μg protein and 218 μg triglyceride/ml). Relative to control cells incubated in the absence of lipoproteins, hypertriglyceridemic $VLDL_1$-treated cells contained 38-fold more triglyceride and threefold more cholesterol; cholesteryl ester was not significantly elevated. As expected, acetyl LDL-treated cells contained markedly increased cholesteryl ester (11-fold over control cells); cholesterol and triglyceride content were elevated 2.5- and threefold, respectively. Thus, the major lipid that macrophages accumulated was the predominant lipid carried by the lipoprotein to which they were exposed.

Experiments measuring the incorporation of [^{14}C]oleate into cellular lipids revealed that hypertriglyceridemic VLDL stimulate the incorporation of oleate not into cholesteryl esters but into triglyceride (Fig. 3). Oleate incorporation into cellular triglyceride induced by hypertriglyceridemic VLDL is linear for at least 4 h. This process increases in a dose-dependent, saturable manner (Fig. 4) and parallels visible lipid accumulation as measured by staining with oil red O. Saturation occurred at ~25μg protein/ml (180 μg triglyceride/ml).

Stimulation of oleate incorporation into cellular triglyceride by hypertriglyceridemic VLDL, like the incorporation of oleate into cellular cholesteryl esters induced by acetyl LDL, is temperature dependent. There is no detectable incorporation of oleate into either cellular triglycerides or cholesteryl esters at 4°C.

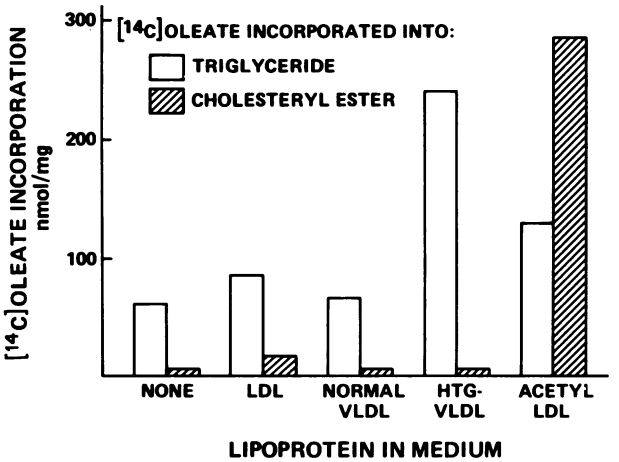

FIGURE 3 Effects of lipoproteins on the incorporation of [^{14}C]oleate into triglyceride and cholesteryl esters in macrophages. Cells were isolated and cultured in 35-mm tissue culture dishes (Methods). When the nonadherent cells were washed away, each dish received 1 ml of tissue culture medium, 0.02 ml of 10 mM [^{14}C]oleate/12% bovine serum albumin (sp act 10,000 cpm/nmol), and the indicated lipoproteins or saline (0.1 ml). The dishes were incubated at 37°C for 18 h before the cells were harvested. The data are expressed as nanomoles oleate incorporated per milligram cell protein into celluar triglyceride (open bars) and into cholesteryl esters (hashed bars). Each value represents the average of duplicate plates, except for the control values (cells that received no lipoprotein), which are the average of four dishes. The lipoproteins are the same as those used in the experiment described in the legend to Fig. 2 and were used at the same concentrations.

At 37°C, acetyl LDL stimulated cholesteryl ester synthesis by approximately threefold (from 2.6 to 7.0 nmol/mg cell protein) while hypertriglyceridemic VLDL stimulated triglyceride synthesis by twofold (from 54.3 to 101.2 nmol/mg) in the 3.5 h incubation.

Receptor-mediated uptake and degradation produce triglyceride accumulation. Dextran sulfate inhibits the binding of acetyl LDL to its specific receptor in macrophages (7). Fig. 5 shows that dextran sulfate at 5 μg/ml completely inhibited the enhanced incorporation of [^{14}C]oleate into cholesteryl ester induced by acetyl LDL but not the incorporation of [^{14}C]-oleate into triglycerides induced by hypertriglyceridemic VLDL. Rather than inhibiting uptake of hypertriglyceridemic VLDL, increasing concentrations of dextran sulfate stimulated uptake (Fig. 6).

We have also examined the uptake and degradation of iodinated hypertriglyceridemic VLDL$_1$ by macrophages to determine if uptake is facilitated by a specific receptor and is, therefore, saturable. Uptake and degradation occurred in a curvilinear, dose-dependent manner, with 50 and 100% saturation at 3 and 24 μg protein/ml (20 and 160 μg triglyceride/ml), respectively (Fig. 7). When based on particles of VLDL$_1$/ml medium, these values are equivalent to 2.5 and 20 pmol VLDL$_1$/ml (nM).

Competition studies showed that hypertriglyceridemic VLDL$_1$ were highly effective in inhibiting the internalization and degradation of ^{125}I-hypertriglyceridemic VLDL$_1$, with 50% inhibition at 3 nM (Fig. 8). CER-VLDL from cholesterol-fed rabbits were nearly as effective, giving 50% inhibition at a level of 5 pmol/ml (5 nM). LDL were less effective, with 10% inhibition at 5 nM and 40% inhibition at 47 nM. Acetyl LDL did not compete at 130 nM. These results indicate that hypertriglyceridemic VLDL uptake and degradation are mediated via specific receptors that recognize CER-VLDL but are distinct from the receptors for acetyl LDL.

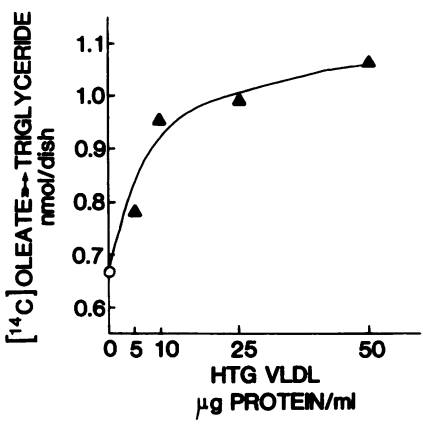

FIGURE 4 Concentration-dependent incorporation of oleate into triglyceride in macrophages exposed to hypertriglyceridemic VLDL. Cells were isolated and grown in 35-mm tissue culture dishes (Methods). After the nonadherent cells were removed, each dish of macrophages was incubated at 37° for 3.5 h in 1 ml of TCM plus 0.02 ml of 10 mM [^{14}C]oleate/albumin and 0.2 ml of saline (four dishes) or hypertriglyceridemic VLDL (S_f 100–400, subject H.W., type IV profile) at the indicated final concentrations. Each point represents the average of values of two dishes, except the control value which is the average of four dishes. The protein content averaged 22 μg protein/dish.

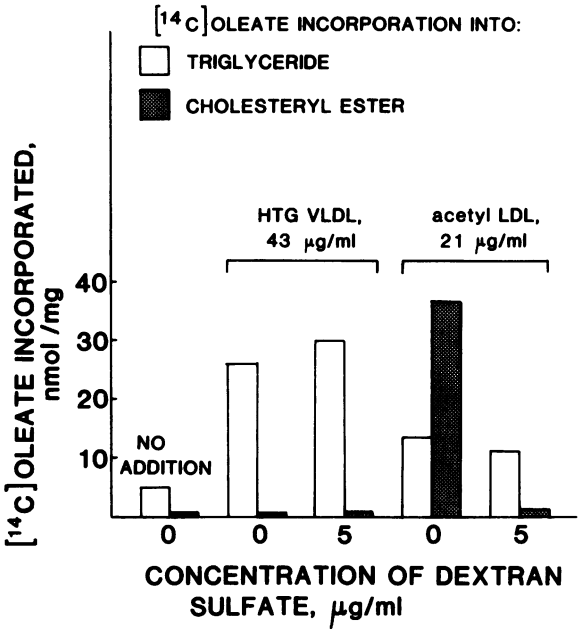

FIGURE 5 Dextran sulfate (500,000 daltons) inhibits the incorporation of oleate into cholesteryl ester induced by acetyl LDL but not the incorporation of oleate into triglyceride induced by hypertriglyceridemic VLDL. Leukocytes were isolated and placed in 35-mm tissue culture dishes as described. When the nonadherent cells were rinsed away, the macrophages received 1 ml of tissue culture medium containing 0.18 mM [^{14}C]oleate, 0.05 ml of saline or dextran sulfate, 110 μg/ml, and 0.05 ml of acetyl-LDL (final concentration 21 μg protein/ml), hypertriglyceridemic VLDL (type IV, subject L.F., $d < 1.006$, final concentration 43 μg protein/ml and 307 μg triglyceride/ml) or saline. The cells were incubated at 37°C for 16 h. Each value represents the average from duplicate dishes (four dishes for the control values).

To determine whether or not lysosomal hydrolysis of the hypertriglyceridemic VLDL is required for the stimulation of oleate incorporation, the cells were incubated at 37°C for 16 h in the presence and absence of 20 μM chloroquine with and without hypertriglyceridemic VLDL or acetyl LDL. In the absence of added lipoprotein, the basal incorporation of oleate into cellular triglyceride was 53 nmol/mg (57 in the presence of chloroquine). Hypertriglyceridemic VLDL

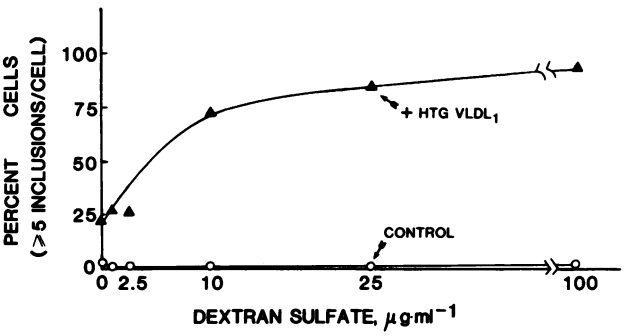

FIGURE 6 Dextran sulfate (500,000 daltons) stimulates cellular lipid accumulation induced by hypertriglyceridemic VLDL. Cells were isolated, cultured on glass cover slips, stained with oil red O, and counted (Methods). The freshly isolated macrophages were incubated with hypertriglyceridemic VLDL (type IV subject L.F., S_f 100–400, 71 μg protein/ml final concentration) and the indicated concentrations of dextran sulfate for 4 h at 37°C before the cells were washed, fixed, and stained. The percentage of cells containing 5 or more oil red O-positive droplets/cell is plotted as a function of the concentration of dextran sulfate in the medium.

(S_f 100–400, type IV, 40 μg protein/ml) stimulated triglyceride formation by 2.3-fold, to a level of 122 nmol/mg; 20 μM chloroquine inhibited the enhanced oleate incorporation by 41%, so that 92 nmol/mg of oleate were incorporated in the presence of hypertriglyceridemic VLDL plus chloroquine. In the same experiment, 20 μM chloroquine also inhibited the incorporation of oleate into cellular cholesteryl ester induced by acetyl LDL (21 μg/ml) by 56%, from 34.5 to 15.5 nmol/mg (basal level in the absence of lipoproteins was 0.7 nmol/mg without and 1.3 nmol/mg with chloroquine).

DISCUSSION

We have shown that receptor-mediated uptake and degradation of hypertriglyceridemic VLDL induced a reesterification and massive accumulation of triglycerides in mouse peritoneal macrophages that was not observed with VLDL from normolipemic subjects. Thus, in this report we detail for the first time a rapid, receptor-mediated foam cell formation induced in vitro by a native human lipoprotein. In previous studies, massive lipid accumulation was produced in macrophages only by chemically modified or complexed lipoproteins (7–9, 11, 12), by abnormal lipoproteins induced by cholesterol feeding (10), or by prolonged (≥4 d) incubation (13). Our results suggest a possible cellular mechanism for the relationship between hypertriglyceridemia and atherosclerosis (1, 34). Pronounced triglyceride reesterification and accumulation were apparent after only 4-h incubation of macrophages with hypertriglyceridemic VLDL. These phenomena were not observed with VLDL obtained from fasting normolipemic subjects, even when incubated for 24 h with 650 μg triglyceride/ml. Chylomicrons isolated from normal as well as hypertriglyceridemic subjects, however, produced oil red O-positive droplets in the macrophages. The presence of chylomicrons in the VLDL or serum from pooled normal plasma might account for the visible lipid accumulation induced by these preparations in human monocyte-derived macrophages observed after prolonged (≥4 d) incubation, as recently reported by Traber and Kayden (13). Alternatively, differences in cell type, culture conditions, or lipoprotein isolation techniques or the potential secretion of lipoprotein lipase by these cells (33) could explain our different results.

The evidence shows that triglyceride accumulates in macrophages treated with hypertriglyceridemic VLDL. First, lipid droplets were not birefringent when examined under polarized light. Second, thin-layer chromatography in three different solvent systems showed that the predominant cellular lipid was triglyceride rather than cholesteryl ester. Third, exogenous [^{14}C]oleate was incorporated into cellular triglyceride rather than into cholesteryl ester. Fourth, macrophages exposed to saturating levels of hypertriglyceridemic VLDL contained 38-fold more triglyceride mass than did control cells incubated in the absence of lipoproteins.

For several reasons, lipoprotein lipase does not appear to be responsible for the rapid, massive triglyceride accumulation induced by hypertriglyceri-

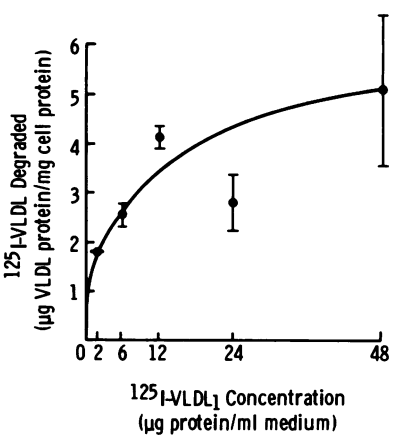

FIGURE 7 Degradation of hypertriglyceridemic ^{125}I-VLDL$_1$ by macrophages is saturably dependent on VLDL concentration. Adherent cells were incubated with the indicated concentrations of ^{125}I-VLDL$_1$ (subject J.P., type V) for 16 h at 37°C before the media were processed as described in Methods to determine acid-soluble, noniodide radioactivity. Each value represents the mean±SD of triplicate dishes.

demic VLDL in these unstimulated peritoneal macrophages. First, normal VLDL failed to produce visible lipid accumulation or to stimulate the incorporation of oleate into cellular triglyceride, although both normal and hypertriglyceridemic VLDL are good substrates for lipoprotein lipase. Second, heparin failed to enhance triglyceride accumulation. Heparin augments the lipoprotein lipase-mediated triglyceride accumulation by preadipocytes incubated with VLDL (32) and stimulates the release of lipoprotein lipase from ascites macrophage lines (33). We attempted to augment production and release (32, 33) of lipoprotein lipase by incubating peritoneal macrophages in the presence and absence of heparin with stimulatory and nonstimulatory $VLDL_1$. Heparin at 10 and 100 μg/ml failed to enhance triglyceride accumulation in all cases. Third, chloroquine, an inhibitor of lysosomal acid hydrolases, inhibited the oleate incorporation into cellular triglyceride stimulated by hypertriglyceridemic VLDL. Thus, lysosomal hydrolysis of hypertriglyceridemic VLDL appears to precede the incorporation of oleate into triglyceride stimulated by hypertriglyceridemic VLDL. Fourth, studies with iodinated hypertriglyceridemic $VLDL_1$ clearly indicate that these VLDL are taken up and degraded by macrophages via specific receptors. Fifth, the saturation analyses for ^{125}I-hypertriglyceridemic-VLDL degradation, for hypertriglyceridemic-VLDL-stimulated oleate incorporation into cellular triglyceride, and for visible lipid accumulation are remarkably similar, suggesting that these processes are mediated by the same pathway. Our observations indicate that the formation and accumulation of triglyceride in macrophages exposed to hypertriglyceridemic VLDL depend on a specific uptake mechanism that delivers VLDL to lysosomes for hydrolysis, followed by cytoplasmic reesterification to form triglyceride droplets. This is analogous to the formation and accumulation of cholesteryl esters in macrophages induced by acetyl LDL, by LDL complexed to dextran sulfate, or by canine β-VLDL (7–10).

Hypertriglyceridemic VLDL are not taken up by the scavenger pathway, since acetyl LDL failed to compete with iodinated hypertriglyceridemic VLDL and since dextran sulfate, an efficient inhibitor of this pathway, did not inhibit hypertriglyceridemic VLDL-induced triglyceride reesterification or accumulation. CER-VLDL (β-VLDL) from cholesterol-fed rabbits, however, effectively competed with VLDL uptake and degradation, indicating internalization via the same receptors. Partial inhibition by LDL (Fig. 8) suggests that some uptake is mediated by receptors equivalent to the fibroblast B/E (LDL) receptor, which recognizes both hypertriglyceridemic VLDL and CER-VLDL (4, 5, 10, 18).

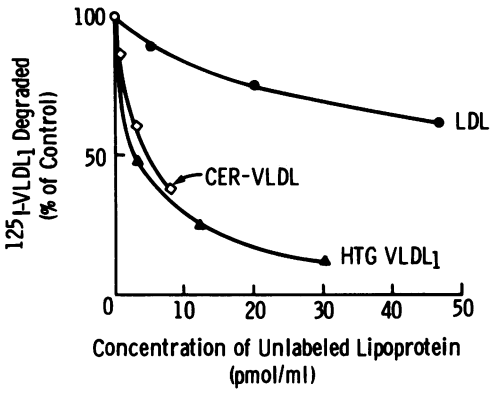

FIGURE 8 Degradation of hypertriglyceridemic $VLDL_1$ is competitively inhibited by hypertriglyceridemic $VLDL_1$, CER-VLDL and, to a lesser extent, by LDL. Freshly isolated adherent cells were incubated with ^{125}I-hypertriglyceridemic $VLDL_1$ (J.P., type V; 4.6 μg protein/ml, equivalent to 4 pmol $VLDL_1$/ml) and the indicated concentrations of unlabeled lipoproteins: (▲) homologous hypertriglyceridemic $VLDL_1$; (◇) CER-VLDL from cholesterol-fed rabbits; (●) normal LDL, *d* 1.02–1.063. Each point represents the mean from three dishes and is expressed as percentage of control, the amount degraded in the absence of competing unlabeled lipoproteins. For the LDL competition curve, this control value was 1,757 ng/mg cell protein and SD was <12%, for the CER-VLDL curve the control value was 647 ng/dish and SD was <5%. Hypertriglyceridemic (HTG) VLDL was run in each experiment and gave essentially identical competition curves (50% inhibition at 3 pmol HTG $VLDL_1$/ml).

The distinctive features of VLDL from patients with type IV and type V hyperlipoproteinemia, which result in enhanced interactions with cells, have not yet been identified. We have found no consistent changes in major lipid or apoprotein components between the normal and the type IV or type V VLDL that could account for their differences in reactivity with the fibroblast LDL receptor and now with macrophages (4, 5). The receptor recognition factors present in the hypertriglyceridemic VLDL probably are due to apoB or apoE or both, since rabbit CER-VLDL apoproteins are almost entirely apoB and apoE, with little or no apoC present. A configuration of apoB/E similar to that of β-VLDL on the surface of hypertriglyceridemic VLDL that is masked or lacking in normal VLDL may account for the cellular uptake of hypertriglyceridemic but not normal $VLDL_1$; this would not necessarily be reflected by major compositional abnormalities.

Macrophages store the major lipid carried by the lipoproteins that they actively internalize. Thus, acetyl LDL and CER-VLDL produce cholesteryl ester reesterification and accumulation. By contrast, but in an analogous fashion, the triglyceride-rich hypertriglyceridemic VLDL induce triglyceride reesterifica-

tion and storage. The moderate increase in cellular cholesterol mass after incubation with hypertriglyceridemic VLDL reflects the lower amounts of cholesterol relative to triglyceride present in these lipoproteins. Lysosomal hydrolysis of VLDL triglycerides would produce free fatty acids. Reesterification and storage of the fatty acids as nontoxic glycerides would protect the cells from toxic effects of free fatty acids. Indeed, even prolonged exposure to high levels of hypertriglyceridemic VLDL failed to reduce cell viability. The effect, however, was to produce cells with all the morphologic characteristics of foam cells.

Lipid-filled macrophages accumulate in hyperlipoproteinemia types I, III, and V, all of which are characterized in part by hypertriglyceridemia (1). Normally, catabolism of triglycerides in large VLDL is mediated by lipoprotein lipase. In hypertriglyceridemia, however, the lipoprotein lipase pathway is overloaded, either because of an absolute or a relative deficiency (such as saturation) of the enzyme. Triglyceride-rich lipoproteins accumulate in the plasma and catabolism of these particles may be diverted through the macrophage pathway, resulting in foam cell accumulation. In eruptive xanthomas associated with hypertriglyceridemia, the predominant lipid found in macrophage-derived foam cells is triglyceride (35). With lesion regression, the triglyceride is rapidly lost but cholesterol remains as the major lipid. Since some foam cells in atheromatous lesions may be derived from blood-born monocytes (2, 3), uptake of triglyceride-rich lipoproteins by monocyte-macrophages in vivo in hypertriglyceridemic subjects potentially could initiate a similar sequence of lipid accumulation and be a significant factor in the pathophysiology of atherosclerosis.

ACKNOWLEDGMENTS

We thank Ms. Flora Brown for excellent technical assistance and Sue Floyd for preparation of the manuscript. We are grateful to Ms. Emma Osherovich of Gulf Research and Development for quantitative analysis of macrophage lipids.

This research was supported by grants from the American Heart Association, Texas Affiliate, Inc.; US Public Health Service HL-17269, HL-27341, and RR-05425; and by grant IM-138A from the American Cancer Society.

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