Evidence that cholesteryl ester and triglyceride accumulation in J774 macrophages induced by very low density lipoprotein subfractions occurs by different mechanisms

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Abstract The present investigations have examined the mechanism(s) whereby S_f 60–400 very low density lipoproteins (VLDL) from Type IV hypertriglyceridemic subjects cause cholesteryl ester and triglyceride accumulation in J774 macrophages. Both apolipoprotein (apo) E-poor and apoE-rich Type IV VLDL subfractions, isolated by heparin-Sepharose chromatography, were capable of enhancing cellular cholesterol and triglyceride content. The apoE-rich fraction was significantly more effective at inducing cholesterol esterification ($P < 0.05$) and accumulation of esterified cholesterol ($P < 0.05$), whereas both subfractions caused similar increases in cellular triglyceride content. Thus, the amount of VLDL-associated apoE determined the extent to which Type IV VLDL loaded J774 cells with cholesterol but not triglyceride. Two VLDL subfractions, S_f 60–400 and S_f 20–60, isolated from Type III subjects homozygous for $apoE_2$, caused little or no effect on cellular esterified cholesterol content, whereas both fractions induced the same degree of cellular triglyceride accumulation as Type IV VLDL. Type IV VLDL-induced cholesteryl ester accumulation was blocked by an anti-apoE monoclonal antibody, known to block the binding of apoE to the LDL receptor; however, the increase in cellular triglyceride was unaffected. Therefore, VLDL-induced triglyceride accumulation in this cell line can occur without apoE-mediated uptake of intact VLDL particles. The addition of heparin to J774 cells resulted in a 6-fold increase in lipoprotein lipase (LPL) activity in the media, and significantly enhanced the ability of Type IV VLDL to induce cellular triglyceride accumulation ($P < 0.01$), but significantly decreased cellular cholesteryl ester content ($P < 0.025$). Finally, S_f 60–400 VLDL from two subjects homozygous for apoC-II deficiency failed to increase cellular lipid content. However, the addition of exogenous apoC-II to C-II-deficient VLDL resulted in significant increases of both triglyceride and esterified cholesterol in J774 cells. In the presence of apoC-II, the anti-apoE monoclonal antibody blocked the cellular cholesteryl ester increase induced by C-II-deficient VLDL, but had no effect on the increase in cellular triglyceride. Collectively, these experiments demonstrate that extracellular lipolysis of S_f 60–400 VLDL by LPL is required for cholesteryl ester and triglyceride accumulation in J774 macrophages. After interaction with cellular LPL, VLDL triglycerides are hydrolyzed. The resulting free fatty acids are readily taken up by the macrophage, and re-esterified into triglyceride. Lipolysis proceeds until apoE epitopes are exposed, allowing the triglyceride-depleted remnant, containing all the cholesteryl ester, to be taken up via an apoE-mediated process. —**Evans, A. J., C. G. Sawyez, B. M. Wolfe, P. W. Connelly, G. F. Maguire, and M. W. Huff.** Evidence that cholesteryl ester and triglyceride accumulation in J774 macrophages induced by very low density lipoprotein subfractions occurs by different mechanisms. *J. Lipid Res.* 1993. **34:** 703–717.

Supplementary key words hypertriglyceridemia • foam cells • lipoprotein lipase

The presence of cholesterol-laden macrophages, or foam cells, in developing atherosclerotic plaques is well established (1) and several groups of investigators have shown that human very low density lipoproteins (VLDL) are capable of inducing foam cell formation in a variety of macrophage culture systems (2–6). Type IV hypertriglyceridemia is a disorder associated with elevated plasma levels of VLDL, due to overproduction and decreased catabolism (7, 8). The importance of hypertriglyceridemia as a risk factor for coronary artery disease remains a controversial issue (9); however, it is possible that the elevated cholesterol content of Type IV VLDL particles (10, 11) contributes to the atherosclerosis that has been repeatedly associated with this disorder (12–14). In addition, metabolic studies in Type IV subjects have demonstrated that large amounts of S_f 60–400 VLDL are directly removed from the circulation by a catabolic shunt

Abbreviations: LDL, low density lipoproteins; VLDL, very low density lipoproteins; LPL, lipoprotein lipase; LPDS, lipoprotein-deficient serum; BSA, bovine serum albumin; TLC, thin-layer chromatography.

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that bypasses the conversion of VLDL into low density lipoproteins (LDL) (15–17). The tissue site for this abnormal VLDL removal pathway and its role in atherogenesis are unknown. The fact that macrophages can catabolize VLDL in vitro suggests that these cells may be involved in direct VLDL catabolism in vivo and that this process is potentially atherogenic.

It is thought that VLDL particles are internalized by macrophages by receptor-mediated endocytosis (18), although the receptor involved has not been definitively identified. Previous work from our laboratory (5) and from other investigators (19–21) has strongly suggested that the ligand for VLDL uptake by these cells is apolipoprotein E (apoE). These studies have all involved some modification of VLDL-associated apoE using approaches such as proteolytic treatment (19), monoclonal antibodies (5), or the addition of exogenous apoE (20, 21). Conversely, none of these studies have demonstrated a relationship between the amount of apoE associated with Type IV, S_f 60–400 VLDL, as isolated from plasma, and the extent of uptake by cultured macrophages.

Studies that assess the importance of functional isoforms of apoE in VLDL-induced cholesterol deposition in cultured macrophages have yielded conflicting observations. The apoE2 isoform, for which most subjects with Type III hyperlipoproteinemia are homozygous (22), has been shown to be markedly defective in its ability to bind to the LDL receptor (23, 24). The cholesteryl ester-rich β-VLDL that accumulates in Type III subjects is thought to play a major role in the deposition of cholesterol in macrophages by a mechanism involving apoE. The primary evidence supporting this idea is derived from studies using β-VLDL from cholesterol-fed animals (19, 25, 26); however, unlike apoE2, the apoE from experimental animals is not defective. In addition, β-VLDL from typical Type III subjects has been shown to induce cholesteryl ester accumulation in mouse peritoneal macrophages (26, 27). However, these cells have been shown to secrete functional apoE (28, 29) which precludes a clear understanding of the role of apoE2 in the uptake process. Conversely, we have recently shown that VLDL from typical Type III subjects had no effect on the total cholesterol content of a murine macrophage-like cell line that does not secrete apoE (5). The effects of Type III VLDL on macrophage triglyceride content have not been reported.

Finally, the role of extracellular lipolysis by macrophage-secreted lipoprotein lipase (LPL) on VLDL uptake and lipid deposition has not been fully elucidated. The use of VLDL from subjects homozygous for apolipoprotein C-II (apoC-II) deficiency provide an ideal approach for addressing this issue; however, such studies have also yielded inconsistent results. Lindqvist et al. (3) reported that extracellular lipolysis only modestly enhanced the uptake of VLDL by mouse peritoneal macrophages, whereas, Ishibashi et al. (20) showed that the uptake of radiolabeled, C-II-deficient VLDL by human monocyte-derived macrophages was enhanced considerably by the addition of exogenous apoC-II.

The interpretation of studies using ^{125}I-labeled VLDL to investigate the uptake of intact VLDL particles by cells in culture may be complicated by the dissociable nature of labeled apoC and E associated with VLDL, especially during lipolysis. We reasoned that measuring increases in the mass of cellular cholesteryl ester and triglyceride as well as increases in oleate incorporation into cellular cholesteryl esters would better reflect the uptake of VLDL particles by macrophages.

The experiments described in the present report were designed to define further the role of VLDL-associated apoE in the ability of large Type IV VLDL particles to cause cholesteryl ester and triglyceride accumulation in J774A.1 cells (J774). In addition, experiments were carried out to clarify some of the inconsistencies concerning the role of extracellular lipolysis by macrophage-secreted LPL in the uptake process.

MATERIALS AND METHODS

Subjects

The lipid and lipoprotein profiles of the subjects used in these experiments are summarized in **Table 1.** The

TABLE 1. Plasma and lipoprotein lipid concentrations of VLDL donors[a]

	Plasma		VLDL		LDL	HDL
Lipoprotein Phenotype	C	TG	C	TG	C	C
Type IV (n = 13)[b]	6.4 ± 0.2	8.2 ± 0.9	2.7 ± 0.3	6.7 ± 0.9	2.8 ± 0.4	0.8 ± 0.1
Type III (n = 4)[c]	10.2 ± 0.8	5.6 ± 0.2	5.4 ± 0.5	3.4 ± 0.5	3.3 ± 0.7	1.1 ± 0.1
ApoC-II-deficient (n = 4)[d]	9.2 ± 3.0	29.1 ± 8.2	1.6 ± 0.5	4.8 ± 2.0	0.5 ± 0.2	0.6 ± 0.1

[a]Values are expressed as means ± SEM in mmol/l; C, cholesterol; TG, triglyceride.
[b]The distribution of apoE phenotypes in the Type IV subjects used in these studies was as follows; six were E3E3, three were E4E2, two were E3E2, one was E4E3, and one was E4E4.
[c]The Type III subjects used in these studies were all homozygous for apoE2.
[d]Two subjects, both homozygous for apoC-II deficiency and for apoE3, were studied. One subject was studied once and the other was studied three times on three different occasions within 18 months. Therefore, lipid values represent the mean ± SEM of four separate samples. Values for fasting plasma C and TG for these subjects include the lipids present in the chylomicron fraction: C = 5.6 ± 1.5 and TG = 20.6 ± 4.2 mmol/l.

Type IV hypertriglyceridemic subjects and the Type III hyperlipoproteinemic subjects were classified according to the criterion of Schaefer and Levy (30) after visits to the Outpatient Endocrinology Clinic at University Hospital in London, Ontario. The Type IV subjects all presented with primary hypertriglyceridemia and none displayed fasting chylomicronemia or had a metabolic disorder such as obesity, diabetes, renal dysfunction, or hypothyroidism known to cause hypertriglyceridemia. In addition, none of these subjects were being treated with drugs known to influence hypertriglyceridemia. The Type III subjects were all homozygous for apoE2. VLDL samples from two subjects homozygous for apoC-II deficiency were also used in specific experiments. These subjects were members of the C-II$_{Toronto}$ kindred that has been described previously (31, 32). These studies were approved by the University of Western Ontario Health Sciences Standing Committee on Human Research and all subjects gave informed consent. Prior to blood sampling all subjects fasted 12–14 h.

Lipoprotein isolation

Approximately 50 ml of blood was collected from the fasted donors and placed in tubes containing Na_2EDTA at a final concentration of 0.15%. Plasma was obtained by centrifugation at 1000 *g* for 25 min at 4°C. Approximately 25 ml of plasma was immediately layered under buffer A (1.006 g/ml density solution containing 0.195 M NaCl, 1 mM Tris, pH 7.4, 1 mM EDTA-Na_2, 10 μM phenylmethane sulfonyl fluoride, 3 mM NaN_3, 0.10 mM merthiolate) in a 37.5 ml Beckman Quickseal tube. VLDL (S_f 60–400) was isolated by ultracentrifugation in a Beckman 60 Ti rotor for 2 h at 40,000 rpm at 12°C using a Beckman L8 ultracentrifuge. For the Type III subjects only, the S_f 60–400 ($VLDL_1$) infranatant was transferred to a new 37.5 ml Quickseal tube and overlaid with buffer A. The S_f 20–60 ($VLDL_2$) fraction was then obtained by ultracentrifugation at 40,000 rpm in the 60 Ti rotor for 16 h at 12°C. The $VLDL_1$ fraction was used to provide a comparison of large VLDL containing only apo E2 to VLDL particles of similar density isolated from Type IV subjects. The $VLDL_2$ fraction was studied because this fraction contains most of the smaller, abnormally cholesterol-rich β-VLDL particles (33). Plasma from the C-II$_{Toronto}$ patients was centrifuged in a Beckman 50.3 rotor for 30 min, 12°C, at 20,000 rpm to isolate the chylomicron fraction. The VLDL fraction (S_f 60–400) was isolated from the infranatant in the 50.3 rotor, spun at 40,000 rpm, 12°C, for 2 h. All of the VLDL samples were washed through an equal volume of buffer A in a Beckman 70.1 Ti rotor spun at 40,000 rpm, 12°C, for 18 h. Lipoprotein-deficient serum (LPDS) was isolated from the plasma of fasting, healthy laboratory personnel at d > 1.21 g/ml using a 60 Ti rotor and dialyzed against 0.15 M NaCl containing 1 mM Tris, pH 7.4, 1 mM EDTA-Na_2, clotted with thrombin (200 units/ml), heat-inactivated at 56°C for 30 min, filtered using sterile 0.22 μm filters, and stored at −20°C. The LPDS was assayed for apoC-II and E (17) and found to be free of these apolipoproteins.

Type IV, S_f 60–400 VLDL was subfractionated into apoE-poor and apoE-rich subfractions using heparin-Sepharose chromatography exactly as described previously (34, 35). The VLDL subfractions from duplicate columns were pooled together and re-isolated by ultracentrifugation at 40,000 rpm, at 12°C in buffer A in a Beckman 50.3 Ti rotor. The ratios of apoE to apoC of the re-isolated VLDL subfractions were determined by analytical isoelectric focusing gel electrophoresis as described previously (35).

All lipoprotein samples were analyzed for protein content by a modification of the Lowry method (36) and for total cholesterol and triglyceride using diagnostic kits from Boehringer Mannheim GmbH Diagnostica, Montreal, Quebec (triglycerides without free glycerol for triglycerides and CHOD-PAP system kit for cholesterol). All lipoprotein samples were stored at 4°C and used for tissue culture experiments within 1 week. Apolipoprotein E phenotypes were determined on all VLDL donors used in these studies by analytical isoelectric focusing gel electrophoresis (35, 37).

Purification of apoC-II

Human apoC-II was purified from acetone-soluble VLDL by ion-exchange HPLC. VLDL (S_f 20–400) was isolated from lipemic plasma, obtained from the Red Cross (Toronto, Ontario), after ultracentrifugation and washing as described above. VLDL was diluted to 1 mg/ml of total protein using buffer A, whereupon acetone was added at room temperature in a ratio of 1.2:1 (acetone–buffer A, v/v) (38). The solution was vortexed, centrifuged at 1500 *g*, and the supernatant was filtered twice through glass wool, dialyzed against 5 mM NH_4HCO_3, and lyophilized. Acetone-soluble VLDL was extracted three times using ethanol–diethyl ether 3:1 (v/v) followed by two washes with diethyl ether alone. Acetone-soluble VLDL (20 mg) was dissolved in 2 ml of buffer B (0.03 M Tris, 40% isopropanol, pH 7.45) and 0.5 ml of 0.01 M Tris, 8 M urea, pH 8.2, and filtered through a Gelman LC3A filter (Mandel Scientific Company Ltd., Guelph, Ontario). The filtered solution (500 μl) was applied to a Brownlee Prep-10, AX-300 (10 mm I.D. × 10 cm) anion exchange HPLC column (Chromatographic Specialties, Brockville, Ontario) that had been equilibrated to buffer B containing 1% buffer C (0.3 M Tris, 50% acetonitrile, pH 7.45) at a flow rate of 5 ml/min. Two minutes after the injection the percent of buffer C was increased linearly from 1 to 100% over the next 30 min. At 50 min the percentage of buffer C was decreased to 1% over a 2-min period. Fractions containing apoC-II were dialyzed against 5 mM NH_4HCO_3 and lyophilized. Purity of the apoC-II was

verified by reverse phase HPLC as described previously (39) and by immunoblotting of analytical isoelectric focusing gels with antibodies against human apoC-II and apoC-III (39).

Tissue culture

J774A.1 cells, a murine macrophage-like cell line, were used in this study. These macrophages do not secrete apoE (40, 41) and have been reported to secrete a 2- to 3-fold greater amount of a neutral triglyceride hydrolase compared to mouse peritoneal macrophages (42). J774 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown on 100-mm culture dishes (Falcon, Canlab, Mississauga, Ontario) in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. The cells were maintained in 10 ml of Dulbecco's Modified Eagle's Medium (DMEM) (low glucose) (Gibco, Burlington, Ontario) containing 0.37% sodium bicarbonate, 0.1 mg/ml Garamycin (Schering, Pointe Claire, Quebec), 0.25 μg/ml Fungizone (Squibb, Flow Laboratories Inc., Mississauga, Ontario), and 10% fetal bovine serum (FBS) (Flow Laboratories Inc.). The cells were maintained on a 7-day cycle, being split 1:3 on days 1 and 5 and having fresh media added on day 3. For all of the experiments in the present report, J774 cells were plated in six-well (35 mm) culture plates (Linbro, Flow Laboratories Inc.) in 2.0 ml of DMEM containing 10% FBS and grown for 1–2 days. When the monolayers had become 70–80% confluent, the media were replaced with DMEM containing 5% LPDS. The final albumin concentration in the media was 0.13%. The appropriate concentrations of lipoproteins were added to duplicate dishes of cells and were incubated for 24 h unless otherwise indicated. The cells were not pre-incubated in DMEM containing LPDS prior to the experiments as we had previously shown that such treatment has no influence on the results (5). In specific experiments heparin (Hepalean, Organon Canada Ltd.) was added to the media (10 I.U./ml) in the absence or in the presence of lipoproteins and incubated for 24 h.

Monoclonal antibodies directed against apoE that *i)* specifically inhibit binding by apoE to the LDL (apoB/E) receptor (1D7), and *ii)* that react with an epitope near the N-terminal of apoE that is not involved with receptor recognition (6C5) were generously provided by Drs. Ross Milne and Yves Marcel, Clinical Research Institute of Montreal (43). Fab fragments, prepared as described previously (44), were used to avoid interaction with the Fc receptor of macrophages. The Fab fragments were incubated with VLDL preparations for 0.5 h at 37°C before addition to cells.

Determination of cellular lipid content

For experiments in which total cellular cholesterol was measured, cells were given three washes with buffer B (0.15 M NaCl, 50 mM Tris, 0.2% bovine serum albumin (BSA), pH 7.4) and three additional washes with buffer B without BSA. To each dish, 1.0 ml of 0.1 N NaOH was added and incubated overnight at 37°C to digest the cells. Cell protein was determined by the modified Lowry procedure (36), and 20-μg aliquots of 5α-cholestane in ethyl acetate (Supelco, Toronto, Ontario) were added to the remaining cell extract. The cell extract was saponified in 5 ml of 0.5 N KOH in 90% ethanol at 70°C for 2 h, and the cholesterol was extracted after the addition of 5 ml of water and 5 ml of petroleum ether. The petroleum ether phase was separated, dried under N_2, and the trimethylsialyl ether derivatives of cholesterol were prepared using Syl-Prep (Supelco). Cholesterol mass was measured by gas–liquid chromatography (GLC) on a Hewlett Packard 5830A gas chromatograph, with a flame ionization detector, on a 2 mm (internal diameter) × 1.2 m column packed with 3% SP 2250 on 100/120 mesh Supelcoport (Supelco). The injection, column, and detector temperatures were set at 300, 280, and 300°C, respectively. The cholesterol content was calculated by comparison of the peak areas of derivatized cholesterol to those of the 5α-cholestane internal standard. For the determination of cellular esterified cholesterol and triglyceride mass, the cells were washed three times in buffer B without BSA and the lipids were extracted in situ after two 30-min incubations with 1.0 ml of hexane–isopropanol 3:2 (v/v). The solvents from each extraction were pooled and the cells were digested in 0.1 N NaOH. The lipid extracts were dried under N_2, resuspended in chloroform–methanol 2:1 (v/v), and separated by thin-layer chromatography (TLC) on Fisherbrand Silica Gel G plates (Fisher Scientific, Toronto, Ontario) using a petroleum ether–diethyl ether–acetic acid 84:15:1 (v/v/v) solvent system. A standard lipid mixture (Supelco) was used to identify the cholesteryl ester and triglyceride on the TLC plates, while [1α,2α(n)^{3}H]cholesteryl oleate and tri[1-^{14}C]oleoyl glycerol (Amersham, Oakville, Ontario) were used to assess recovery. The plates were dried in air and the bands were visualized by exposure to I_2 vapour. After complete disappearance of the I_2 color, the bands co-migrating with the appropriate standards were scraped from the plates into 16 × 100 mm borosilicate glass tubes. To each cholesteryl ester sample, 2 μg 5α-cholestane in ethyl acetate was added as an internal standard. The cholesteryl ester was eluted from the silica gel with hexane, the solvent was evaporated under N_2, and the residue was saponified as described above. The esterified cholesterol mass in each sample was determined by GLC. The triglyceride was eluted from the silica gel by isopropanol (Ultrapure Analar, BDH, Toronto, Ontario), dried under N_2, and resuspended in 1.0 ml of isopropanol. The triglyceride mass in each sample was then determined using the method of Neri and Frings (45).

Cholesterol esterification assay

The incorporation of [1-^{14}C]oleic acid into cellular cho-

lesteryl esters was determined as described previously (5, 46). Lipoproteins were added to duplicate dishes of J774 cells and incubated for 5 h in DMEM containing 5% LPDS. Each dish received 0.04 μCi [1-^{14}C]oleic acid (Amersham, Oakville, Ontario) complexed with fatty acid-free BSA (Sigma). The molar ratio of oleic acid to BSA was 6.8:1. The cells were washed three times with buffer B without BSA, the lipids were extracted as described above, and the cell protein was determined. The lipids were separated by TLC as described above, using [1α,2α(n)^{3}H]cholesteryl oleate to assess recovery. The cholesteryl ester bands were scraped from the plates and counted in Aquasol-2 (Dupont Canada, Mississauga, Ontario) using a Beckman LS 3801 counter.

J774 cell lipoprotein lipase assay

J774 cells (80% confluent) were incubated with DMEM containing 5% LPDS in the presence or absence of 10 I.U./ml of heparin (Hepalean, Organon Canada Ltd.) for 24 h. The effect of heparin on the LPL activity secreted by J774 cells was also assessed in the presence of Type IV VLDL (50 μg cholesterol/ml of media). The conditioned media were collected in sterile tubes and stored at −80°C until assayed for LPL activity. The cells were washed three times with buffer B without BSA and the cell protein was measured. The LPL activity in the conditioned media was determined using a stable tri[1-^{14}C]oleoyl glycerol emulsion as described previously (47). Duplicate 200-μl aliquots of conditioned or unconditioned media and 100 μl of the substrate were added to the incubation buffer (0.15 M NaCl, 0.2 M Tris, pH 8.2, 0.05 g/ml fatty acid-free BSA, containing 12% normolipidemic human plasma (v/v) as a source of apoC-II). As a negative control, the conditioned medium was also assayed in the presence of 1 M NaCl, conditions known to inhibit LPL (47). Samples were incubated for 1 h at 37°C and the release of [1-^{14}C]oleic acid was determined (47). LPL activity was expressed in units/mg of cell protein (one unit = 1 μmol of free fatty acid released per ml of media per h.

Statistical analyses

The data were analyzed using an unpaired Student's *t*-test.

RESULTS

Experiments were conducted to assess the effect of the amount of apoE, relative to apoC, on large Type IV VLDL in terms of its ability to induce lipid accumulation in J774 macrophages. Large Type IV VLDL was subfractionated into apoE-poor and apoE-rich fractions by heparin-Sepharose chromatography. **Table 2** shows that the apoE-rich fraction had a 5-fold greater ratio of apoE to apoC than did the apoE-poor fraction. **Fig. 1** demonstrates that both of these subfractions are capable of inducing increments in the total cholesterol (Fig. 1A), esterified cholesterol (Fig. 1C), and triglyceride (Fig. 1D) content of J774 cells. The cells to which no lipoproteins were added (referred to as control cells) did not have detectable levels of esterified cholesterol mass. Fig. 1 also shows that the apoE-rich fraction caused significantly greater increases (2-fold) in cellular total and esterified cholesterol content when compared to the apoE-poor fraction ($P < 0.05$). Fig. 1B shows that this trend was maintained over a range of VLDL concentrations. The addition of more than 37.5 μg VLDL protein/ml of media for either fraction did not result in further increases in the total cholesterol content of the cells. In contrast, both the apoE-poor and apoE-rich VLDL induced marked triglyceride mass accumulation in these cells; however, there

TABLE 2. Characteristics of VLDL samples used in J774 cell studies[a]

Lipoprotein Type	C/TG	C/Protein	TG/Protein	ApoE/ApoC	ApoE/Protein
Type IV[b]	0.16 ± 0.02	1.28 ± 0.34	7.99 ± 1.43	ND	0.22 ± 0.07
Type IV subfractions[c]					
EP	0.13 ± 0.01	1.16 ± 0.22	9.19 ± 1.37[d]	0.11 ± 0.04[e]	0.07 ± 0.02
ER	0.20 ± 0.04	1.13 ± 0.17	5.83 ± 0.97	0.51 ± 0.10[e]	0.31 ± 0.03[e]
Type III VLDL$_1$[f]	0.31 ± 0.06	2.24 ± 0.43	7.61 ± 2.02	ND	0.20 ± 0.05
Type III VLDL$_2$[g]	0.52 ± 0.07	2.49 ± 0.33	5.11 ± 1.26	ND	0.25 ± 0.06
ApoC-II-deficient[h]	0.14 ± 0.03	1.41 ± 0.35	9.41 ± 1.31	ND	0.18 ± 0.04

[a]Values are expressed as means ± SEM; C, cholesterol; TG, triglyceride; ND, not determined. The units of measurement for C, TG, and Protein were μg/μl.
[b]Characteristics of S_f 60–400 VLDL isolated from all 13 Type IV subjects used in these studies.
[c]S_f 60–400 VLDL from 6 of the 13 Type IV subjects studied was subfractionated as described in Materials and Methods; EP, apoE-poor fraction; ER, apoE-rich fraction.
[d]$P < 0.025$ relative to the apoE-rich fraction.
[e]$P < 0.005$ relative to the apoE-poor fraction.
[f]$VLDL_1$ corresponds to S_f 60–400 VLDL.
[g]$VLDL_2$ corresponds to S_f 20–60 VLDL.
[h]Characteristics of S_f 60–400 VLDL isolated from two subjects homozygous for apoC-II deficiency. One of these subjects was studied once and the other three times on three different occasions within 18 months. Therefore, lipid values represent the mean ± SEM of four separate samples.

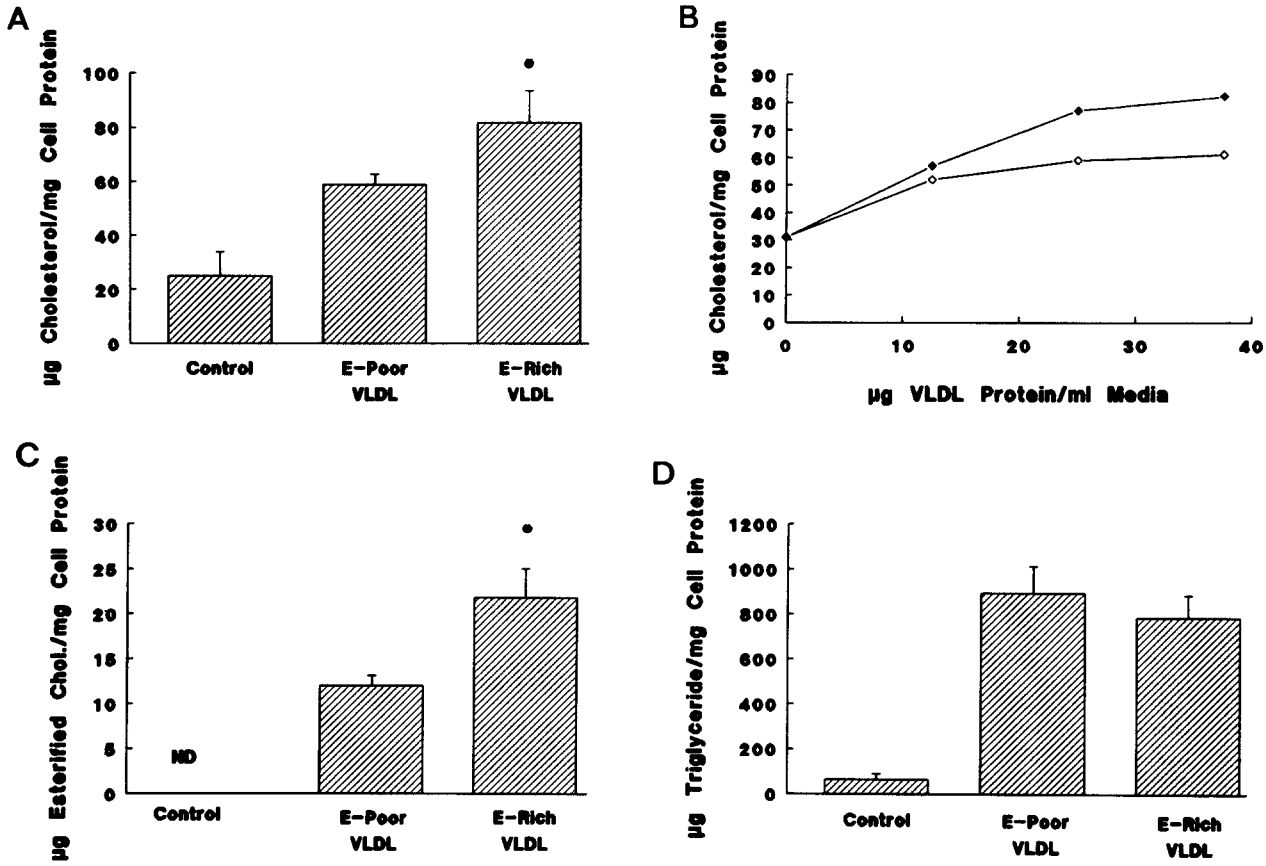

Fig. 1. Lipid content of J774 macrophages incubated with subfractions of Type IV, S_f 60–400 VLDL. (A) ApoE-poor and apoE-rich VLDL subfractions (from five Type IV subjects) (50 µg protein/ml of media), separated by heparin-Sepharose chromatography, were incubated with J774 cells for 24 h and the total cellular cholesterol content was determined as described in Materials and Methods. (B) Various concentrations of the apoE-poor (◊) and apoE-rich (♦) VLDL subfractions from one Type IV subject (apoE3E3 phenotype) were incubated with J774 cells as in (A). ApoE-poor and apoE-rich VLDL subfractions from three Type IV subjects were incubated with J774 cells as in (A) and the cellular contents of esterified cholesterol (C) and triglyceride (D) were determined as described in Materials and Methods. The values are the results of duplicate determinations for five experiments in (A) and three experiments in (C) and (D), expressed as means ± SEM. ND, not detectable; *, $P < 0.05$ relative to apoE-poor VLDL.

was no significant difference between the two fractions.

Both the apoE-poor and apoE-rich subfractions of S_f 60–400 Type IV VLDL stimulated acyl-CoA:cholesterol acyl transferase (ACAT) activity in J774 macrophages. As was the case with cellular esterified cholesterol mass, the apoE-rich fraction induced a significantly greater increase ($P < 0.05$) in ACAT activity (4-fold over control cells) than did the apoE-poor fraction (2.5-fold over control cells) (data not shown). This was a consistent finding over the range of VLDL concentrations used (12–36 mg VLDL protein/ml of media).

As shown in **Fig. 2,** apoE was responsible for the majority of the cholesteryl ester accumulation in J774 cells after incubation with Type IV VLDL. Incubation of Type IV VLDL at 50 µg/ml of media resulted in a 23-fold increase in cellular cholesteryl ester ($P < 0.002$). Pre-incubation of the Type IV VLDL with Fab fragments of monoclonal antibody 1D7, which specifically inhibits apoE-mediated binding to the LDL receptor, significantly inhibited cholesteryl ester accumulation by approximately 76% ($P < 0.024$). Pre-incubation of VLDL with anti-apoE monoclonal antibody 6C5, which binds to an epitope of apoE close to the N-terminal group, had no effect (data not shown). In contrast to its effect on cellular esterified cholesterol, the monoclonal antibody 1D7 failed to block the increase in cellular triglyceride induced by Type IV VLDL (Fig. 2). Also, antibody 6C5 had no effect on the increase in cellular triglyceride (data not shown).

Experiments were conducted to compare the abilities of large Type IV VLDL and two VLDL fractions from typical Type III subjects, possessing only apoE2, to induce esterified cholesterol and triglyceride mass accumulation in J774 cells. **Fig. 3** demonstrates that while S_f 60–400 Type IV VLDL caused significant accumulation of esterified cholesterol relative to control cells ($P < 0.01$), $VLDL_1$ and $VLDL_2$ from the Type III subjects did not (Fig. 3A). This result was in spite of the fact that the Type III VLDL particles had a 2- to 3-fold greater ratio of cholesterol to protein (Table 2). Fig. 3B shows that this difference between Type III and Type IV VLDL was maintained over the range of VLDL concentrations assessed. The accumulation of esterified cholesterol induced

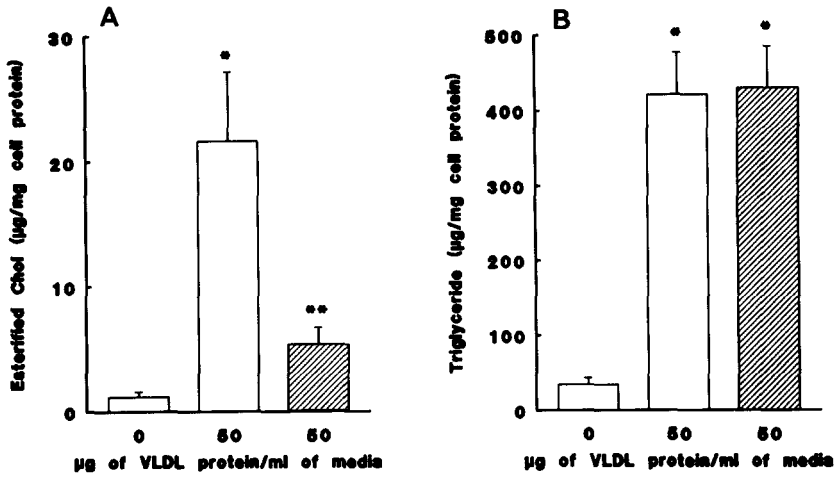

Fig. 2. Comparison of the effects monoclonal anti-apoE antibody (1D7) on the accumulation of (A) esterified cholesterol and (B) triglyceride content of J774 cells incubated with Type IV VLDL for 16 h. Fifty μg of VLDL protein/ml of media was pre-incubated in the presence (hatched bars) or absence (open bars) of 50 μg of 1D7/ml of media for 30 min at 37°C before addition to the cells. Cellular esterified cholesterol and triglyceride contents were determined as described in Materials and Methods. The values (mean ± SEM) are the results of duplicate determinations from five experiments using VLDL from five patients. *: significantly different from no lipoproteins, $P < 0.002$. **: significantly different from VLDL without 1D7, $P < 0.025$.

by Type IV VLDL reached a maximum at 18 h of incubation and did not increase further up to 36 h of incubation. Extending the incubation time up to 36 h with Type III $VLDL_1$ did not result in esterified cholesterol accumulation. In contrast to the results obtained for cellular esterified cholesterol content, Fig. 3C shows that the addition of equal amounts of Type IV and Type III VLDL triglyceride resulted in similar degrees of triglyceride ac-

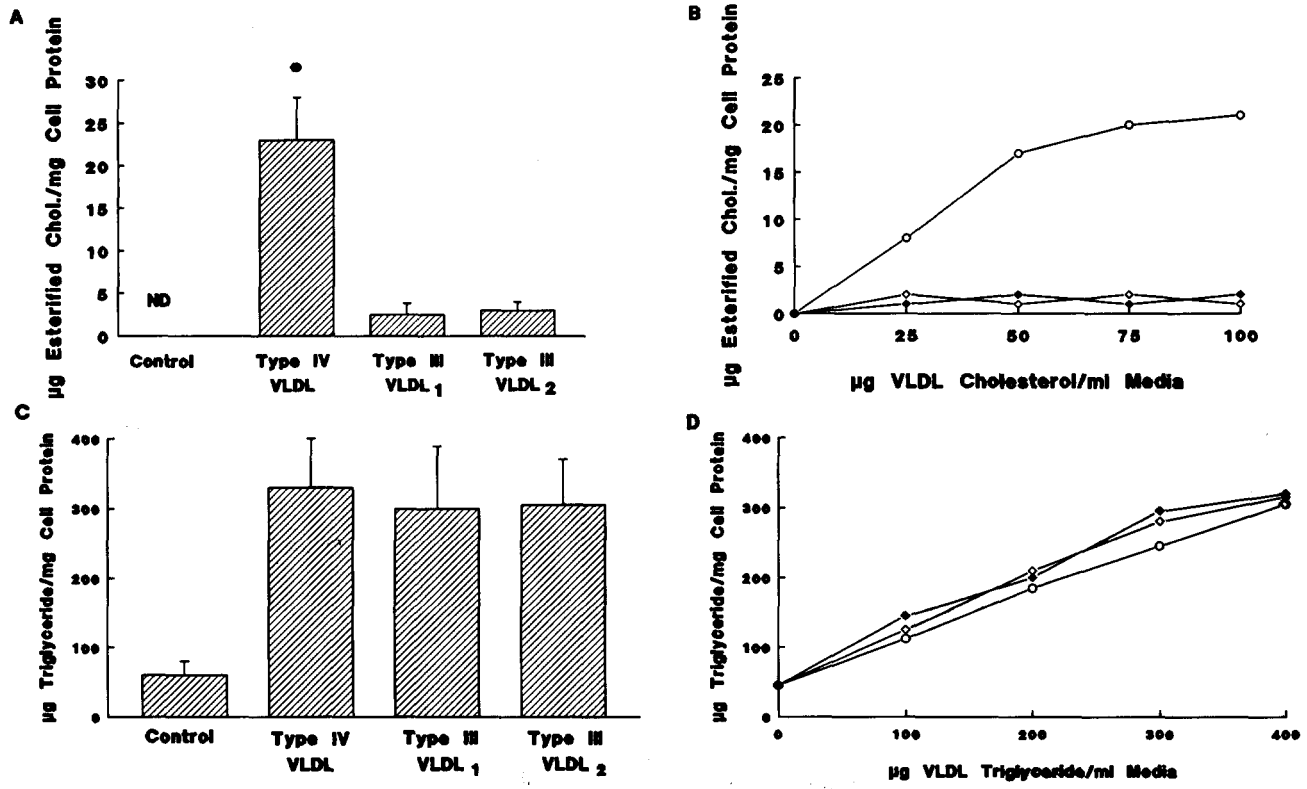

Fig. 3. Comparison of the effects of VLDL from Type IV subjects and Type III subjects on the esterified cholesterol (A and B) and triglyceride (C and D) content of J774 macrophages. (A and C) fifty μg cholesterol/ml of media of S_f 60-400 VLDL from four Type IV subjects and $VLDL_1$ (S_f 60-400) and $VLDL_2$ (S_f 20-60) from four Type III subjects was incubated with J774 cells for 24 h. The values are the results of duplicate determinations for four experiments expressed as means ± SEM. ND, not detectable; *, $P < 0.01$ relative to control cells. (B and D) Various concentrations of S_f 60-400 VLDL from two Type IV subjects (○) and $VLDL_1$ (◇) and $VLDL_2$ (◆) from two Type III subjects were incubated with J774 cells as in (A and C). The values are the results of duplicate determinations for two experiments. Cellular esterified cholesterol and triglyceride contents were determined as described in Materials and Methods.

TABLE 3. Measurement of lipoprotein lipase activity secreted by J774 macrophages[a]

	- Heparin	+ Heparin
Baseline[b]	0.25 ± 0.03	1.55 ± 0.20
Type IV VLDL[c]	0.23 ± 0.07	2.33 ± 0.30
1 M NaCl[d]	0.01 ± 0.01	0.01 ± 0.01

[a]Aliquots of media (200 μl) conditioned by J774 macrophages, incubated in the absence or presence of heparin (10 I.U./ml of media) for 24 h, were assayed for lipoprotein lipase activity as described in Materials and Methods. Values are the results of duplicate determinations for two experiments expressed as means ± SEM in units of enzyme activity/mg of cell protein, where 1 unit is defined as 1 μmol of free fatty acid released per ml of media per h.

[b]J774 macrophages incubated with lipoprotein-deficient media.

[c]J774 macrophages incubated in the presence of Type IV, S_f 60-400 VLDL (50 μg cholesterol/ml of media).

[d]Aliquots of conditioned media were assayed in the presence of 1 M NaCl.

cumulation in J774 cells ($P < 0.005$ versus control cells for all VLDL samples).

We investigated the role of extracellular lipolysis of VLDL by macrophage-secreted LPL in the cellular lipid accumulation induced by hyperlipidemic human VLDL. **Table 3** shows that LPL activity was detectable in media conditioned by J774 cells for 24 h under standard conditions. The activity of this enzyme was completely abolished during assays in the presence of 1 M NaCl. The amount of LPL activity in the media after incubation for 24 h was not affected by the addition of Type IV VLDL, but was enhanced 6-fold by the addition of heparin alone to the media (10 I.U./ml of media) during the incubation. The amount of LPL activity in the presence of heparin was enhanced a further 67% upon co-incubation with Type IV VLDL over the 24-h period ($P < 0.05$). **Fig. 4A** shows that addition of heparin to the media resulted in a significant enhancement of the cellular triglyceride accumulation induced by large Type IV VLDL ($P < 0.01$ relative to the absence of heparin). It is also of note that $VLDL_1$ and $VLDL_2$ from typical Type III subjects responded in an identical manner to Type IV VLDL in the presence of heparin, in terms of the effect on J774 cell triglyceride content. The linear relationship between the cellular triglyceride mass and the concentration of VLDL added to the cells was unaffected by the addition of heparin (Fig. 4B). Fig. 4C shows that, in contrast to its enhancing effect on VLDL-induced triglyceride accumulation, heparin addition was associated with a significant 67% decrease in the extent of esterified cholesterol accumulation caused by Type IV VLDL ($P < 0.025$).

The effect of the anti-apoE monoclonal antibody, 1D7, and heparin on Type IV VLDL-induced lipid accumulation in J774 cells was assessed in short term (2–6 h) cultures and compared to longer term (16–24 h) cultures (**Fig. 5**). Cellular cholesteryl ester and triglyceride increased with time with increases being observed at 2 h. Figs. 5A and 5B show that monoclonal antibody 1D7 in-

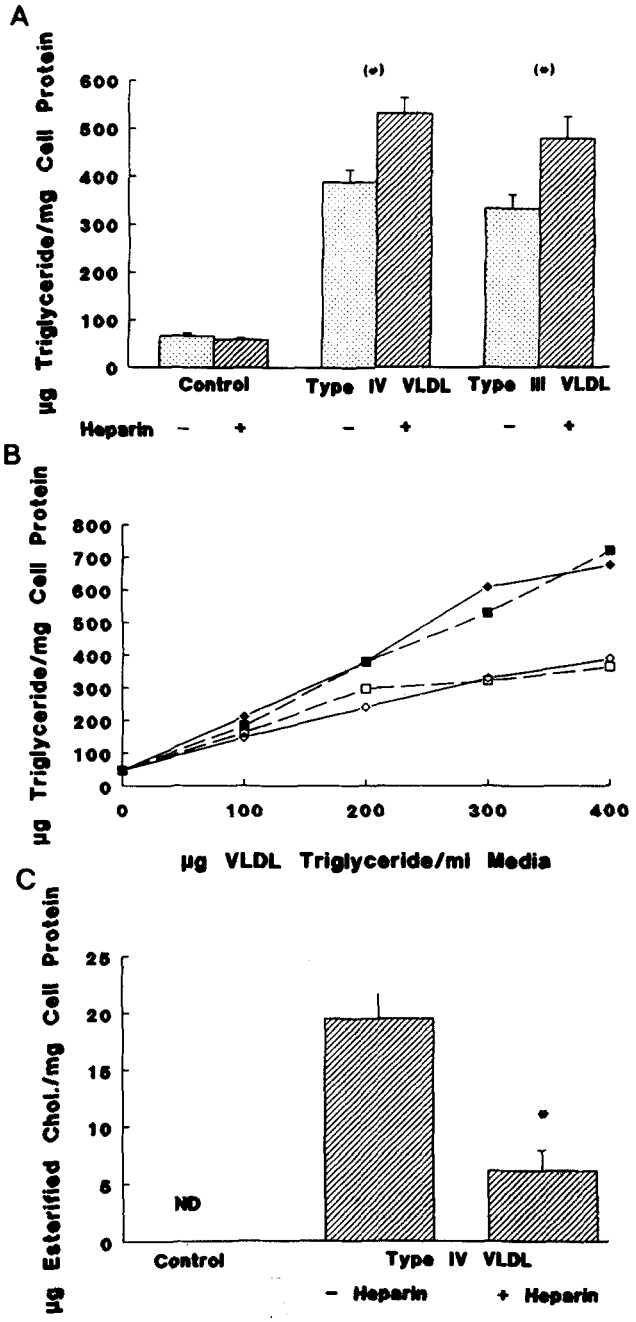

Fig. 4. Lipid content of J774 macrophages incubated with S_f 60-400 VLDL from Type IV subjects and Type III subjects in the absence or presence of heparin. (A) fifty μg cholesterol/ml of media of S_f 60-400 VLDL from three Type IV and three Type III subjects was incubated with J774 cells for 24 h in the absence or presence of heparin (10 I.U./ml of media). Values are the results of duplicate determinations for three experiments expressed as means ± SEM. *, $P < 0.01$ relative to cells not treated with heparin. (B) Various concentrations of S_f 60-400 VLDL from one Type IV subject (□, ■) and one Type III subject (◇, ◆) were incubated with J774 cells in the absence (open symbols) or presence (closed symbols) of heparin as in (A). The values are the results of duplicate determinations for one experiment. (C) fifty μg cholesterol/ml of media of S_f 60-400 VLDL from five Type IV subjects was incubated with J774 cells as in (A). The values are the results of duplicate determinations for five experiments expressed as means ± SEM. ND, not detectable; *, $P < 0.025$ relative to cells not treated with heparin. Cellular triglyceride (A and B) and esterified cholesterol (C) contents were determined as described in Materials and Methods.

hibits cholesteryl ester but not triglyceride accumulation throughout the time course studied. Heparin stimulated VLDL-induced triglyceride accumulation, but decreased the extent of cholesteryl ester accumulation at all time points. Figs. 5C and 5D demonstrate that Type III VLDL fails to augment cellular cholesteryl ester to the same extent as Type IV VLDL, even though equivalent concentrations of lipoprotein cholesterol were added to cells. In short-term incubations (2-6 h) Type III VLDL causes 35-50% of the cholesteryl ester increase observed for Type IV VLDL. At 16 h, Type III-induced cholesteryl ester accumulation was only 15% of that observed for Type IV

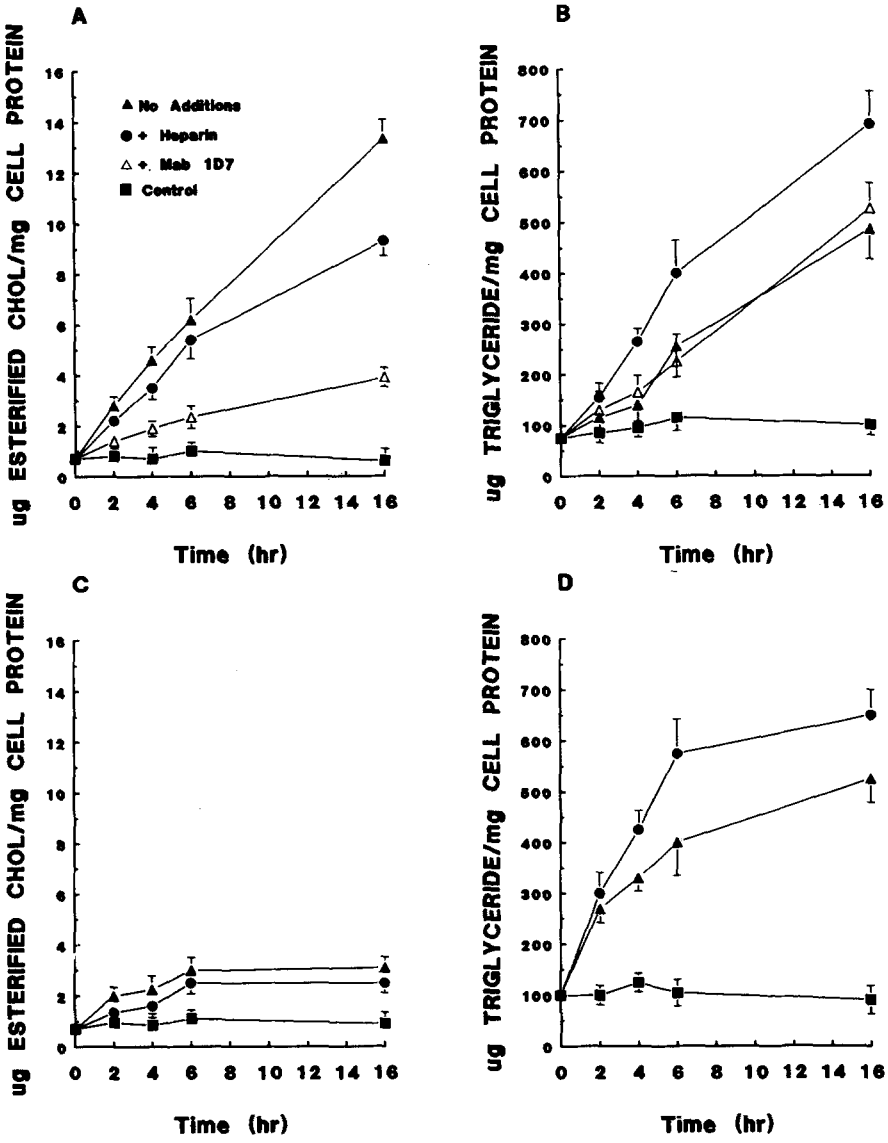

Fig. 5. Lipid content of J774 macrophages incubated with S_f 60-400 VLDL from Type IV subjects and Type III subjects in the absence (▲) or presence (●) of heparin (10 I.U./ml of media) or in the absence (▲) or presence (△) of the monoclonal antibody, 1D7, (50 μg of 1D7 Fab fragment/ml of media). Fifty μg cholesterol/ml of media of S_f 60-400 VLDL from three Type IV (A and B) and three Type III subjects (C and D) was incubated with J774 cells for 2, 4, 6, and 16 h. Values are the mean of duplicate determinations for three experiments. Cellular cholesteryl ester (A and C) and triglyceride (B and D) contents were determined as described in Materials and Methods. The cholesteryl ester values for Type IV VLDL are significantly higher, at all time points, than control, Type IV VLDL + 1D7, and Type III VLDL ($P < 0.04$). The Type IV + 1D7 and Type III values are not different from each other at any time point (with the exception of 16 h). The Type IV + 1D7 and Type III values were statistically different from control at 4, 6, and 16 h ($P < 0.035$), but not at 2 h. Type IV plus heparin values were higher than control at all time points and significantly less than Type IV VLDL alone only at 16 h ($P < 0.02$). The triglyceride values for Type IV VLDL were significantly greater than control at all time points ($P < 0.04$). Values for Type IV VLDL + 1D7 were not different from Type IV VLDL alone at any time point. Values for Type IV VLDL + heparin were higher than those for Type IV VLDL alone at 4, 6, and 16 h ($P < 0.03$). Type III VLDL and Type III VLDL + heparin triglyceride values are greater than control at all time points ($P < 0.01$) and the values for Type III + heparin are higher than Type III alone at 4, 6, and 16 h.

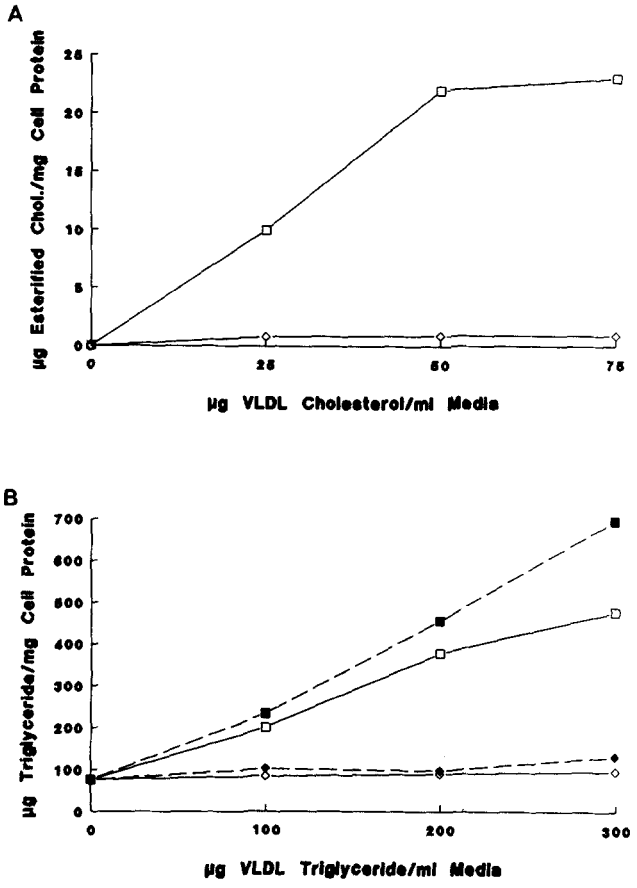

Fig. 6. Effects of S_f 60-400 VLDL from two Type IV subjects and two subjects homozygous for apoC-II deficiency on the lipid content of J774 macrophages. (A) Various concentrations of VLDL from two Type IV subjects (□) and two apoC-II-deficient subjects (apoE3E3 phenotype) (◇) were incubated with J774 cells for 24 h. (B) VLDL from the Type IV subjects (□, ■) and the apoC-II-deficient subjects (◇, ◆) were incubated with J774 cells as in (A) only in the absence (□, ◇) or presence (■, ◆) of heparin (10 I.U./ml of media). The values are the results of duplicate determinations for two experiments. Cellular esterified cholesterol (A) and triglyceride (B) contents were determined as described in Materials and Methods.

VLDL. However, addition of heparin to Type III VLDL enhanced triglyceride accumulation at each time point studied, similar to that observed with Type IV VLDL.

Finally, we assessed the ability of S_f 60-400 VLDL from two different subjects homozygous for apoC-II deficiency to induce lipid accumulation in J774 cells. These subjects were also homozygous for apoE3 and Table 2 shows that their VLDL is compositionally similar to the Type IV VLDL used these studies. VLDL from this C-II-deficient kindred have been demonstrated to be poor substrates for LPL in the absence of exogenous apoC-II (31). **Fig. 6A** demonstrates that the apoC-II-deficient VLDL did not induce any increases in cellular esterified cholesterol or triglyceride when compared to Type IV VLDL. **Fig. 7** shows that the lack of lipid uptake was observed in both short-term cultures (2-6 h) as well as longer term cultures (16 h). In addition, the presence of heparin in the media did not stimulate greater cellular triglyceride mass accumulation in the presence of apoC-II-deficient VLDL (Fig. 6B) as was shown for Type IV VLDL (Figs. 4 and 5). The effect of adding apoC-II to the apoC-II-deficient VLDL on the lipid content of J774 cells is shown in **Fig. 8A and B.** Pre-incubation of apoC-II-deficient VLDL with functional apoC-II (added at 10% of total VLDL protein) was associated with a marked stimulation of cellular esterified cholesterol (Fig. 8A) and triglyceride (Fig. 8B). ApoC-II-stimulated cellular lipid accumulation was continuous over time with increases being observed at 2 h of culture (Fig. 7). In the presence of apoC-II, anti-apoE monoclonal antibody 1D7, but not antibody 6C5, blocked cholesteryl ester accumulation by apoC-II-deficient VLDL; neither antibody has any effect on the increase in cellular triglyceride (Fig. 7).

DISCUSSION

It has been suggested that the mechanism of uptake of triglyceride-rich lipoproteins by macrophages and subsequent lipid accumulation involves several steps. First, VLDL particles can be taken up as such which results in both cholesterol and triglyceride accumulation (3, 48). Second, macrophage-secreted LPL mediates the extracellular hydrolysis of triglyceride, releasing free fatty acids which are then taken up by the cells and re-esterified into triglycerides (3, 6, 49). Subsequently, the cholesterol-carrying remnant particle created by the lipolysis step is then internalized by the macrophage (3). In this report, we have directly assessed the relative importance of each of these steps in the catabolism of "hypertriglyceridemic" VLDL by J774 cells using several approaches.

Our observations provide five lines of evidence that the cholesterol and triglyceride components of S_f 60-400 VLDL particles from hypertriglyceridemic subjects are internalized by J774 cells largely by different mechanisms and that VLDL do not contribute to lipid accumulation without prior lipolysis. *1)* The apoE-rich VLDL fraction isolated by heparin-Sepharose chromatography had a significantly greater ability to induce cellular cholesterol accumulation than the apoE-poor fraction, yet there was no difference between the two subfractions in terms of their effects on cellular triglyceride. *2)* The presence of heparin in the media, which increased media LPL activity, significantly increased the extent of cellular triglyceride content but decreased cellular cholesteryl ester content during incubation with Type IV VLDL. *3)* The monoclonal antibody 1D7 that specifically inhibits binding by apoE to the LDL receptor, blocked cholesteryl ester accumulation but had no effect on the increase in cellular triglyceride. *4)* VLDL from Type III subjects caused the same degree of cellular triglyceride accumulation as Type IV VLDL, yet Type III VLDL caused only a modest increase in the esterified cholesterol content of J774 cells.

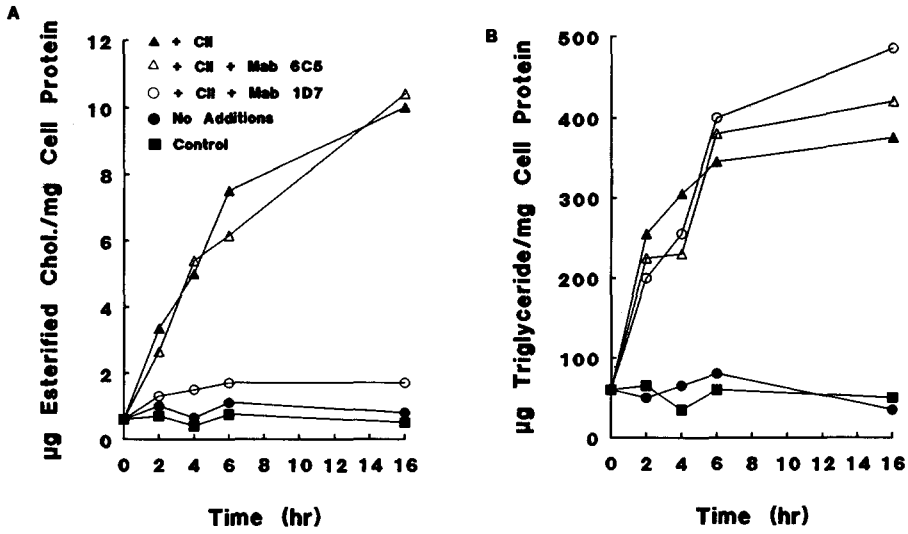

Fig. 7. Lipid content of J774 macrophages incubated with S_f 60–400 VLDL from one apoC-II-deficient subject in the absence (●) or presence of exogenous apoC-II (▲) (10% of total VLDL protein, wt/wt) or in the presence of exogenous apoC-II without (▲) or with monoclonal antibodies, 1D7 (blocking) (○) and 6C5 (nonblocking) (△), at 50 μg of 1D7 Fab fragment/ml of media. Fifty μg cholesterol/ml of media of S_f 60–400 VLDL from the apoC-II-deficient subject was incubated with J774 cells for 2, 4, 6, and 16 h. Values are the mean of duplicate determinations for one experiment. Cellular cholesteryl ester (A) and triglyceride (B) contents were determined as described in Materials and Methods.

5) VLDL from subjects deficient in apoC-II did not cause any significant increases in cellular triglyceride or cholesteryl ester unless exogenous apoC-II was added. In the presence of apoC-II, anti-apoE monoclonal antibody 1D7 blocked cholesteryl ester accumulation by apoC-II-deficient VLDL but had no effect on the increase in cellular triglyceride. Collectively, these findings suggest that extracellular lipolysis of VLDL by macrophage-derived LPL, followed by uptake and re-esterification of the free fatty acids, is the major route by which Type IV VLDL caused the triglyceride accumulation in J774 cells, whereas, uptake of the triglyceride-depleted remnant is the main mechanism for cholesteryl ester accumulation. Evidence to support this mechanism was provided by experiments carried out in both short term (2–6 h) as well as longer term cultures (16–24 h).

Incubation of J774 cells with Type IV VLDL apoE-poor and apoE-rich subfractions, separated by heparin-Sepharose chromatography (34, 35), increased cellular total and esterified cholesterol content and ACAT activity; however, the apoE-rich fraction did so to a significantly greater extent (Fig. 1). In contrast, the apoE-rich and apoE-poor fractions induced similar increases in cellular triglyceride. These observations demonstrate that the amount of apoE (relative to apoC) on large Type IV VLDL is an important determinant of the extent of cholesterol accumulation by J774 cells but not of triglyceride. Previous studies have provided evidence that the amount of apoE on VLDL particles determines their interaction with cultured macrophages with respect to cholesterol accumulation; however, none of these studies explored the role of apoE on triglyceride accumulation. Bates et al. (19) and Soltys et al. (50) demonstrated a strong positive correlation between the number of apoE molecules on VLDL from cholesterol-fed animals (primates) and the extent of ACAT stimulation in J774 cells. Ishibashi et al. (20) and Granot and Eisenberg (21) demonstrated that addition of exogenous apoE to radiolabeled human VLDL resulted in more avid uptake by macrophages; however, cellular lipid concentrations were not measured. Our use of heparin-Sepharose VLDL subfractions represents the first demonstration of the relationship between the amount of apoE on human VLDL, as isolated from plasma, and the ability to load cultured macrophages with cholesterol and triglyceride.

We have recently reported that blocking the receptor-binding domain of apoE on Type IV VLDL with a monoclonal antibody markedly decreased its ability to load cholesterol into J774 cells (5). This report extends these findings and shows that the blocking effect of antibody 1D7 is present in both short and longer term cultures. Although 1D7 is effective in blocking cellular cholesteryl ester accumulation, this antibody has no effect on the increase in cellular triglyceride content (Fig. 2).

The importance of functional isoforms of apoE on VLDL-induced esterified cholesterol and triglyceride accumulation in J774 macrophages, cells that do not secrete apoE (40, 41) but are known to express the LDL receptor (51), was assessed using VLDL isolated from Type III subjects homozygous for apoE2. Both $VLDL_1$ (S_f 60–400) and $VLDL_2$ (S_f 20–60) from typical Type III subjects caused only modest increases in cellular esterified cholesterol (< 20% of that induced by Type IV VLDL) in J774 cells. This observation was not dependent on the length

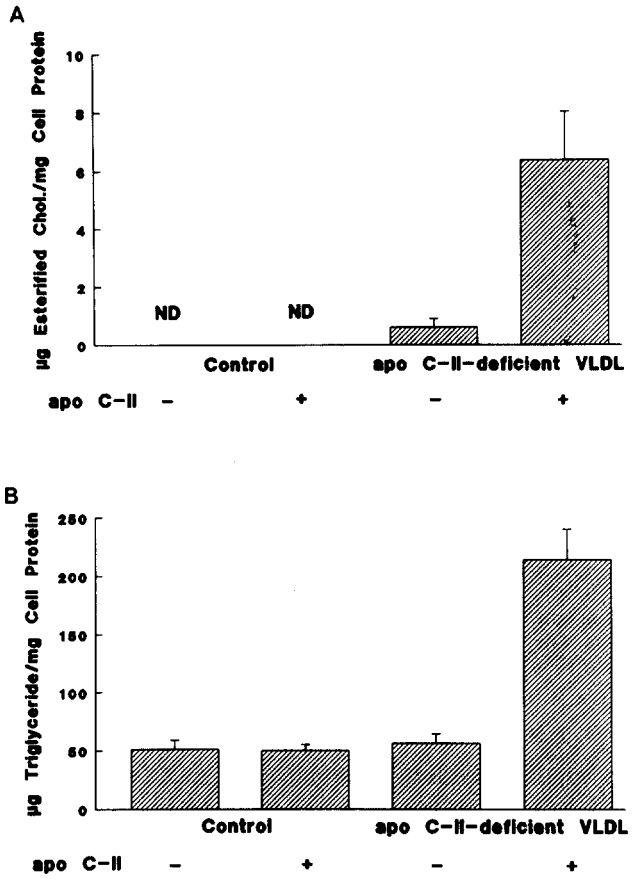

Fig. 8. Lipid content of J774 macrophages incubated with S_f 60-400 VLDL from apoC-II-deficient subjects in the absence or presence of exogenous apoC-II. Fifty μg cholesterol/ml of media of large VLDL from two apoC-II-deficient subjects (both with the apoE3E3 phenotype) was incubated with J774 cells for 14 h after being pre-incubated for 6 h at 37°C in the absence or presence of purified apoC-II (10% of total VLDL protein, wt/wt). Cellular esterified cholesterol (A) and triglyceride (B) contents were determined as described in Materials and Methods. The values, expressed as the mean ± SEM, are the results of duplicate determinations for three experiments using VLDL from two apoC-II-deficient subjects.

of the incubation (Fig. 4C).

In contrast to the failure of Type III VLDL subfractions to cause any increase in cellular cholesteryl ester, the present studies provide the first demonstration that Type III VLDL particles were equally as effective at inducing triglyceride accumulation in J774 macrophages as Type IV VLDL (Figs. 3, 4 and 5). These observations provide further evidence that most of the VLDL-induced triglyceride accumulation occurred by a mechanism independent of apoE-mediated uptake of intact VLDL particles.

Our results differ from those of Fainaru et al. (26) and Rall et al. (27), who have reported that β-VLDL from typical Type III subjects enhanced the cholesteryl ester content of mouse peritoneal macrophages, cells that are known to secrete functional apoE (28, 29). The macrophage-secreted apoE may have facilitated the uptake of the Type III VLDL in these studies. This point is supported by recent studies demonstrating that the addition of functional apoE to radioiodinated human VLDL greatly enhanced their uptake by human monocyte-derived (20) and J774 macrophages (21). Also, apoE2 secreted by macrophages from Type III subjects retarded the degradation of VLDL containing only apoE3 (20). These findings are consistent with our results obtained using J774 cells which do not secrete apoE (40, 41) and demonstrate that functional apoE is required for cholesteryl ester accumulation induced by VLDL (as isolated from plasma) by J774 macrophages in culture.

In agreement with a previous report, we found that LPL is constitutively secreted by J774 cells (52) (Table 3). In addition, we found that the amount of LPL activity in the media increased 6-fold or 10-fold in the presence of heparin or heparin plus large Type IV VLDL, respectively. The potentiating effect of Type IV VLDL on media LPL activity in the presence of heparin likely reflects stabilization of the enzyme-VLDL complex (6). The increase in LPL activity in the presence of heparin enhanced the ability of Type IV VLDL and Type III VLDL to cause cellular triglyceride accumulation (Fig. 4). This was observed in both short and longer term cultures (Figs. 5C and 5D). This finding agrees with the results of Bates et al. (49) who demonstrated that heparin stimulated triglyceride accumulation in mouse peritoneal macrophages induced by normal rat and monkey VLDL. Conversely, Gianturco et al. (48) reported that heparin did not enhance the ability of hypertriglyceridemic VLDL to load triglyceride into $P388D_1$ macrophages; however, these cells do not appear to secrete LPL.

In contrast to enhancing cellular triglyceride accumulation, heparin significantly decreased VLDL-induced esterified cholesterol accumulation in J774 cells (Figs. 4 and 5). This observation is likely explained by heparin binding to the apoE and/or apoB on the VLDL remnants thus preventing their uptake. This has been demonstrated using triglyceride emulsions (53) and human LDL (54) in other cell types. An alternate interpretation is that the increased triglyceride accumulation causes an increase in cholesteryl ester hydrolysis and clearance of cellular cholesteryl ester to VLDL remnants in the media. This phenomenon has been demonstrated in the rat hepatoma cell line Fu5AH by Adelman et al. (55), using HDL_3 as a cholesterol acceptor. However, Bernard et al. (56) reported that cholesteryl esters in cholesterol-loaded J774 cells are very resistant to clearance by media cholesterol acceptors, even when cellular triglyceride is increased by co-incubation with excess oleic acid.

Although significantly reduced compared to Type IV VLDL, cholesteryl ester accumulation did occur with Type III VLDL. Taken together with the finding that the anti-apoE 1D7 antibody did not fully block cholesteryl ester accumulation induced by Type IV VLDL, this suggests that low residual binding activity of apoE2 (24, 57) allowed for the uptake of Type III particles and that 1D7

is not a complete blocking antibody. Alternately, other uptake mechanisms, not requiring apoE, may be responsible for some of the cholesteryl ester accumulation. Gianturco et al. (18) have provided evidence for a binding site (MBP 190) in P388D macrophages, distinct from the LDL receptor, that mediates binding and triglyceride accumulation. This binding site does not require prior lipolysis of the VLDL particle and does not require apoE as a ligand, suggesting intact particle uptake. If present, the role of this binding site in J774 macrophages is likely small. ApoC-II-deficient VLDL caused no lipid accumulation in the absence of lipolysis, and the amount of cholesteryl ester accumulation not dependent on apoE (not blocked by anti-apoE antibody 1D7) is less than 20% (Fig. 7).

We found that S_f 60–400 VLDL from two subjects homozygous for apoC-II deficiency did not cause any increase in either cellular esterified cholesterol or triglyceride content, despite the fact these VLDL particles contained only apoE3 (Fig. 6). This was observed in both short and longer term cultures (Fig. 7). In addition, heparin had no effect on the ability of apoC-II-deficient VLDL to enhance cellular triglyceride accumulation (Fig. 8B). In contrast, after the addition of exogenous apoC-II, cellular cholesteryl ester and triglyceride increased significantly. These results demonstrate that extracellular lipolysis of VLDL is required prior to triglyceride and cholesteryl ester accumulation in J774 cells. The finding that anti-apoE antibody, 1D7, could selectively block cholesteryl ester accumulation in the presence of apoC-II further supports our conclusion that VLDL-induced triglyceride and cholesteryl ester accumulation by J774 cells occurs mainly by different mechanisms. These findings are consistent with the concept that, after interaction with cellular LPL, VLDL triglycerides are hydrolyzed. The resulting free fatty acids are readily taken up by the macrophage, and re-esterified into triglyceride. Lipolysis proceeds until apoE epitopes are exposed, allowing the remnant, containing all the cholesteryl ester and remaining unhydrolyzed triglyceride, to be taken up via an apoE-mediated process.

Lindqvist et al. (3) have reported that apoC-II-deficient VLDL from the same kindred was able to induce both cholesteryl ester and triglyceride accumulation in mouse peritoneal macrophages, and that exogenous apoC-II only modestly enhanced (approximately 20%) the lipid accumulation observed in the absence of apoC-II. These investigators concluded that macrophages could internalize intact VLDL particles and that extracellular lipolysis only enhanced the process. These results differ from those in this paper. One possible explanation is the presence of BSA in the media that may have been contaminated with bovine apoC-II, as commercially available, fatty acid-poor albumins are not apolipoprotein-free (58). In addition, the 2.5% BSA concentration used may have been sufficient, on the one hand, to prevent uptake of LPL-mediated free fatty acids and, on the other hand, to promote cellular triglyceride efflux (59), explaining the lack of effect of exogenous apoC-II on cellular triglyceride accumulation. Our results with J774 cells are consistent with those of Ishibashi et al. (20), who studied the uptake of apoC-II-deficient VLDL (labeled with [^{3}H]triolein) by human monocyte-derived macrophages. These investigators found VLDL uptake (as assessed by cellular radioactivity) was minimal in the absence of lipolysis and was markedly enhanced by the addition of exogenous apoC-II. However, these investigators did not measure cellular triglyceride or cholesterol mass. It has been shown that J774 cells secrete a neutral triglyceride hydrolase (42). It is unlikely that this enzyme plays a role in extracellular lipolysis, thereby mediating Type IV-induced lipid accumulation, as this enzyme does not require apoC-II for full activity. Our observation of the complete lack of uptake of C-II-deficient VLDL triglyceride or cholesteryl ester in the absence of apoC-II indicates that lipoprotein lipase, secreted by J774 cells, is primarily responsible for extracellular lipolysis.

In summary, we have demonstrated that extracellular lipolysis of Type IV S_f 60–400 VLDL by lipoprotein lipase is required for these lipoproteins to induce triglyceride and cholesteryl ester accumulation in J774 macrophages. Lipolysis and free fatty acid uptake and triglyceride formation is independent of apoE; however, after lipolysis, the subsequent internalization of the cholesterol-carrying VLDL remnant is mediated by VLDL-associated apoE.

These studies indicate that large VLDL contain sufficient cholesteryl esters to cause significant macrophage cholesteryl ester deposition in vivo. However, it is unlikely that they are able to induce foam cell development as such, but first must be acted upon by lipoprotein lipase-forming remnants. By virtue of the increased concentration and large flux of S_f 60–400 VLDL in hypertriglyceridemia (15–17), it is possible that large VLDL enter the artery wall as such or after hydrolysis by endothelium-bound lipoprotein lipase (60). These lipoproteins may be hydrolyzed further by macrophage lipoprotein lipase resulting in triglyceride and cholesteryl ester accumulation. Whether lipolysis by macrophage lipoprotein lipase is required for uptake would depend on the extent to which these particles had been exposed to lipoprotein lipase. Uptake of the remnant would be mediated by the apoE associated with the lipoprotein in plasma or after addition of receptor-competent apoE secreted from the macrophage (20). As pointed out by Lindqvist et al. (3) and von Hodenberg et al. (59), the relatively high concentration of albumin, an acceptor of free fatty acids, in cellular fluid may induce preferential triglyceride mobilization, leaving cholesteryl ester-rich foam cells, characteristic of atherosclerotic lesions.

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