Effects of Particle Size on Cell Uptake of Model Triglyceride-Rich Particles with and without Apoprotein E†

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Received July 15, 1994; Revised Manuscript Received October 3, 1994[⊗]

ABSTRACT: The effect of apoprotein E on cellular uptake of "VLDL-size" and "IDL-size" triacylglycerol−phospholipid emulsion particles was studied in J-774 macrophages and fibroblasts. In the absence of apoprotein E (apo E), uptake of the smaller IDL-size particles was up to 2-fold higher by mass and 100-fold higher as calculated by particle number. Apo E enhanced the uptake of both VLDL-size and IDL-size emulsion particles, but the effect was greater on the uptake of larger particles (4−5-fold) as compared to up to a 2-fold increase in the uptake of IDL-size particles. In fibroblasts, particle uptake was less than in macrophages (30−50%), but preferential uptake of smaller particles was similarly observed. Particle internalization was demonstrated by ^{125}I-apo E degradation and resistance to particle release by heparin−suramin. In the absence of apo E, cholesteryl ester of emulsion particles (prepared with trace amounts of [^{3}H]cholesteryl ester) was hydrolyzed to free cholesterol, proving internalization and intracellular metabolism. Double-label experiments using DiI-labeled emulsion particles, in the absence and presence of apo E, showed that emulsion particles are rapidly targeted to perinuclear lysosomes. Thus, at physiological concentrations of triglyceride-rich particles, non-receptor-mediated uptake is a mechanism for the uptake of VLDL-size and IDL-size particles into cells.

Triglyceride-rich particles, such as very low density lipoproteins, chylomicron remnants, and synthetic lipid emulsions, have the ability to convert macrophages into foam cells *in vitro*, and may play a role in atherogenesis *in vivo* (Zilversmit, 1979); Gianturco et al., 1982, 1985). Cellular uptake of these particles has been shown to be enhanced by apoprotein E (apo E).[1] For example, internalization of large triglyceride-rich very low density lipoproteins from hypertriglyceridemic patients by human fibroblasts is mediated through apo E binding (Bradley & Gianturco, 1986). In fibroblasts, HepG2 cells, and J-774 macrophages, exogenous apo E enhances the cellular metabolism of VLDL from both normolipemic and hypertriglyceridemic subjects (Eisenberg et al., 1988; Eisenberg & Sehayek, 1989; Granot & Eisenberg, 1990).

Triacylglycerol−phospholipid emulsions also rapidly acquire apo E in plasma (Granot et al., 1985). The apo E−emulsion complex effectively binds to the LDL receptor and competes with endogenous lipoproteins for cellular uptake (Bradley & Gianturco, 1986; Aviram et al., 1989). Similarly, in perfused rat liver, remnant-like emulsion particles are capable of imitating the metabolism of natural triglyceride-rich lipoprotein remnants only when sufficient apo E is present (Redgrave et al., 1988). Triolein emulsions with apo E were more rapidly metabolized by rat hepatocytes in culture than unsupplemented emulsions (Oswald & Quarfordt, 1987).

Previous studies have shown that for both native triglyceride-rich lipoproteins and emulsion particles, the larger particles are more rapidly metabolized than the smaller particles (Quarfordt & Goodman, 1967; Redgrave & Maranhao, 1985). The cellular binding of larger VLDL particles has been shown to be mediated by apo E in preference to apo B (Krul et al., 1985). Indeed, particle size has been previously shown to be an important factor in regulating the distribution of apoproteins A and C among lipoprotein classes (Tajima et al., 1983), and apo E has been noted to transfer more effectively to large rather than small diameter particles (Connelly & Kuksis, 1981). Since the plasma triglyceride-rich particles vary not only in size but also in composition, specifically their apoprotein and cholesterol content, observed differences in the effects of apo E on their metabolism may relate to differences in either particle size or particle composition.

† This work was supported by NIH Grants HL40404, HL21006, and HL39703.

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⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1994.

[1] Abbreviations: apo, apoprotein; *d*, density; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LPDS, lipoprotein-deficient serum; DiI, dioctadecyltetramethylindocarbocyanine perchlorate.

0006-2960/94/0433-15190$04.50/0

As compositional variation can be minimized in artificially produced triglyceride–phospholipid emulsions, we used emulsion particles varying in size rather than composition to determine the role of particle size in the apo E-mediated cellular uptake of triglyceride-rich particles. To better understand the contribution of apo E to triglyceride-rich particle metabolism at "physiological" triglyceride concentrations, we compared the uptake of particles in the absence and presence of apo E. We found that at physiological concentrations, even emulsions without apo E show high cellular uptake and in the absence of apo E uptake of smaller IDL-size particles is greater than that of larger VLDL-size particles. In the presence of apo E, uptake of smaller particles is enhanced less than that of larger particles. Our findings suggest that at physiologic concentrations, non-receptor-mediated uptake of triglyceride particles can be substantial in some cells, and this uptake may be important to the development of atherosclerotic lesions in apo E deficiency states in animals and humans.

EXPERIMENTAL PROCEDURES

Materials. Triolein was purchased from NuChek Prep Inc. (Elysian, MN). Egg yolk phosphatidylcholine was purchased from Avanti Polar Lipids. Cholesteryl hexadecyl ether, [*cholesterol*-1,2-^{3}H(N)], and [^{3}H]sucrose, [*fructose*-1-^{3}H(N)], were purchased from New England Nuclear, Boston, MA. DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) and fluorescein were obtained from Molecular Probes Inc. (Junction City, OR), suramin was from Miles (West Haven, CT), and [1*x*,2*x*(N)-^{3}H]cholesteryl linoleate and $^{125}I_2$ were from Amersham (Arlington Heights, IL). Recombinant apoprotein E-3 prepared in Bacteria was kindly provided by Biotechnology General, Rehovot, Israel, as previously detailed (Vogel et al., 1985).

Preparation of Emulsions. Triolein and phosphatidylcholine were combined in chloroform at mass ratios of 4:1. Trace mass amounts of [^{3}H]cholesteryl hexadecyl ether were added. Since [^{3}H]cholesteryl ether is not degradable by mammalian cells (Stein et al., 1984), it can be used as a marker for particle uptake. In a typical preparation, 160 mg of triolein, 40 mg of egg yolk phosphatidylcholine, and 75 μL (0.01 mg/mL, 1000 μCi/mL) of [^{3}H]cholesteryl ether were combined. The mixture was dried under nitrogen in a glass vial and vacuum-desiccated overnight.

The dried lipids were resuspended in 10 mL of 0.15 M NaCl/0.24 mM EDTA, pH 7.4, and sonicated under N_2 using a Branson sonifier with a 1 cm probe, set at 90 W continuous power, with the temperature controlled at 40 ± 5 °C by immersion in an ice/water bath. Following 1 h of sonication, 4 mL of the sonicate was removed and the remaining 6 mL sonicated further for 5 h. Sonicated emulsions were transferred into polyallomer tubes, overlaid with 0.15 M NaCl, and centrifuged for 10 min at 28 000 rpm in a Beckman SW 41 rotor, at a temperature of 4 °C. The top cream layer was removed—fraction A of the 1 and 6 h sonicates. The infranatants were then overlaid with 0.15 M NaCl and centrifuged in an SW 41 rotor for another 20 min at 40 000 rpm. The top layer was removed, and the infranatants were transferred to fixed-angle tubes and centrifuged in a Beckman 50.3 rotor at 28 000 rpm for 18 h. The resulting top layer—fraction B of the 6 h sonicates—and fraction A of the 1 h sonicates were again overlayered and washed with 0.15 M NaCl (d = 1.006 g/mL) in a Beckman 50.3 rotor at 28 000 rpm for 18 h to prevent contamination with phospholipid-rich liposomes.

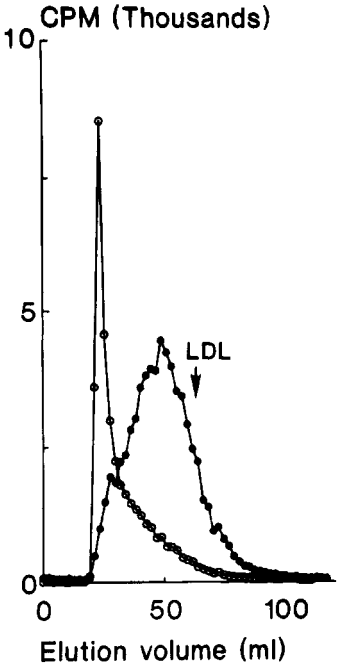

FIGURE 1: Elution of "large" and "small" emulsion particles on a Sepharose CL-2B column. For each emulsion, a volume containing 0.5 mg of triglyceride was placed on a 1.5 × 50 cm column. Triglyceride:phospholipid ratios were 3.9:1 for larger particles and 1.5:1 for small particles, respectively, and large and small particles were radiolabeled with [^{3}H]cholesteryl ether with 501 and 560 cpm/ng of triglyceride, respectively. "Large" particles (○) eluted in the void similar to VLDL. "Small" particles (●) eluted slightly earlier than LDL, Similar to IDL particles.

This method resulted in production of "large" and "small" size particles as judged by the triglyceride:phospholipid mass ratio and gel filtration elution profiles on Sepharose CL-2B columns. Emulsions consisting of "large" particles were isolated after washing of the first spin of the 1 h sonicate (fraction A). The triglyceride:phospholipid mass ratio was (3–5):1, and, on a Sepharose CL-2B column, they eluted in the void, similar to very low density lipoproteins (VLDL). "Small" particles were isolated after washing of fraction B of the 6 h sonicate. Their triglyceride:phospholipid mass ratio was (1–2):1, and on a Sepharose CL-2B column, they eluted slightly earlier than LDL with an elution profile similar to that of intermediate density lipoprotein (IDL) particles (Figure 1). The diameter of the "large" and "small" emulsion particles was estimated from their surface:core lipid ratios and column elution profiles in comparison with subfractions of native VLDL, IDL, and LDL, and of previously published model emulsion particles with similar lipid ratios (Mims et al., 1990).

Emulsion particles were also prepared from lipids extracted from human VLDL obtained from a fasting hypertriglyceridemic donor (plasma triglyceride level, 2300 mg/dL). The d < 1.006 g/mL fraction was isolated. Extracted lipids were mixed with a trace amount of radiolabeled cholesterol ether, dried under N_2, and sonicated as described for the triolein phosphatidylcholine emulsions, and sonicated fractions were similarly sequentially ultracentrifuged. All emulsions were used within 1 week of preparation, during which time no changes were detectable either in the purity of lipid components (assayed by thin-layer chromatography) or in particle size and stability (assayed by gel column chromatography).

Lipid Analyses. The triglyceride and phospholipid composition of the isolated emulsion fractions was determined using the triacylglycerol GPO-PAP kit (Boehringer Mannheim GmbH) and the method of Bartlett (1959), respectively.

Cell Cultures. Monolayer cultures of J-774 AI cells, a murine macrophage-like cell line, and in some experiments human skin fibroblasts, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, 100 ng/mL streptomycin, and 29.2 ng/mL glutamine. As well, in one experiment HepG2 cells were utilized, and these were grown similarly but in unmodified Eagle's medium on a collagen matrix. Cells were routinely split 1:3 every 5−6 days. For each experiment, the cells were plated in 16 × 35 mm plastic dishes and incubated for 24 h in medium containing 10% human lipoprotein-deficient serum (LPDS). Mouse peritoneal macrophages were also used for microscopy experiments and were obtained as detailed elsewhere (Tabas et al., 1985). Cells were incubated with emulsions of either "large" VLDL-size particles or "small" IDL-like particles at varying triglyceride concentrations, in DMEM and 1.0% BSA, on a shaker, for 5−6 h, at 37 or 4 °C.

Incubations were performed in the presence or absence of bacterial recombinant apo E3. Apo E at varying apo E to triglyceride concentration ratios was added to the emulsions at room temperature in the incubation media 20 min prior to addition to the cells to allow equilibrium binding of apo E to the particles (Oswald & Quarfordt, 1987; Tajima et al., 1983). Apo E−emulsion complexes in tissue culture incubation media showed no significant difference in size, as judged by Sepharose CL-2B chromatography as compared to emulsions without E. In particular, there was no evidence for particle aggregation or formation of separate discoidal apo E−lipid complexes. At the end of the incubation period, medium was removed, and the cells were chilled on ice and washed twice with ice-cold PBS buffer containing 0.2% BSA (5 and 1 min washes) and twice with PBS buffer alone. Cells were then incubated with 1 mL of 10 mg/mL heparin in DMEM for 1 h.

At the end of the 1 h incubation period, medium was removed, and cells were washed twice again in PBS buffer. Cells were then dissolved in 1 mL of 0.1 N NaOH. Cell-associated ^{3}H radioactivity was measured, and a 100 μL aliquot was used for protein concentration determination by the Lowry method (Lowry et al., 1952). Cellular triglyceride uptake was calculated from the specific activity of [^{3}H]-cholesteryl ether to triglyceride in the emulsion used for each experiment.

In experiments designed to demonstrate internalization and metabolism of emulsion particle lipids, emulsions were prepared using trace mass amounts of [^{3}H]cholesteryl linoleate rather than radiolabeled cholesteryl ether. After incubation, cell lipids were extracted in hexane/2-propanol (3:2) v/v), and hydrolysis of the radiolabeled cholesteryl ester to ^{3}H-free cholesterol and remaining [^{3}H]cholesteryl linoleate was assayed after separation of free from esterified cholesterol by thin-layer chromatography in the solvent system hexane/diethyl ether/acetic acid (70:30:1, v/v).

^{125}I-labeled LDL and ^{125}I-labeled apo E were prepared by the iodine monochloride method as described (McFarlane, 1958; Bilheimer et al., 1972). For competition experiments with ^{125}I-LDL and apo E−emulsion complexes, apo E was equilibrated with either "large" VLDL-size emulsion particles or "small" IDL-size emulsion particles at a 1:10 protein: triglyceride ratio. Up-regulated macrophages (grown for 24 h in lipoprotein-deficient serum) were incubated for 2.5 h with ^{125}-I-labeled LDL (5 μg of protein/mL) in the presence of increasing concentrations of apo E−"large" emulsion particles, apo E−"small" emulsion particles, free apo E, and LDL.

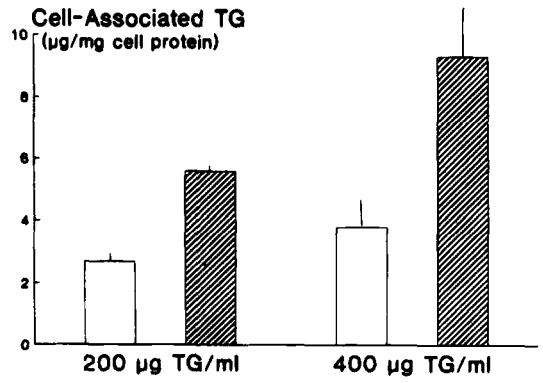

FIGURE 2: Emulsion triglyceride mass uptake by J-774 macrophages. Cells were incubated for 6 h at 37 °C with 1 mL of DMEM/1% BSA containing either 200 μg of TG/mL or 400 μg of TG/mL of "large" VLDL-size particles. Open bars: triglyceride: phospholipid ratio of 2.7:1, with trace amounts of [^{3}H]cholesteryl ether; specific radioactivity, 598 cpm/μg of TG. Hatched bars: "small" IDL-size particles (triglyceride:phospholipid ratio of 1.0: 1) with trace amounts of [^{3}H]cholesteryl ether; specific radioactivity, 576 cpm/μg of TG. Cellular uptake was expressed as micrograms of TG per milligram of cell protein. Results are the mean ± SD of eight parallel experiments.

Video intensification fluorescence microscopy was performed as previously described (Tabas et al., 1990). In brief, cells were incubated at 37 °C in a heating box (no CO_2) with the DiI-labeled emulsions and fluorescein-labeled α_2-macroglobulin (Tabas et al., 1991) in DMEM without bicarbonate containing 0.2% (w/v) fatty acid-free BSA (Sigma Chemical Co., St. Louis, MO) and 10 mM Hepes, pH 7.4. At the end of the incubation, the cells were quickly washed 3 times with PBS and either viewed immediately or fixed with 2% formaldehyde in PBS, and then placed in PBS for viewing. Fluorescence images were obtained using a Leitz Diavert microscope with a 63, NA 1.4 objective. For observation of DiI fluorescence, a "rhodamine" filter set was used (530−560 nm excitation filter, 580 nm dichroic, and 580 nm long-pass filter). For fluorescein fluorescence, the filter set was 450−490 nm excitation, 510 nm dichroic, and 515 nm long-pass filter. Images were recorded with a Videoscope (Washington, D.C.) KS 1381 image intensifier, a VS 2000N video camera, and a JVC 6650U videocassette recorder. Images were digitized with a Gould IP 8400 image processor, and photographs were produced with a Polaroid Freeze Frame.

RESULTS

In the absence of apo E, triglyceride uptake of the smaller IDL-size emulsion particles in J-774 macrophages [which do not synthesize apo E (Werb & Chin, 1983)] was consistently higher than that of the larger VLDL-size emulsion particles (Figure 2). At triglyceride concentrations of 200 and 400 μg/mL, triglyceride mass uptake was about 2-fold higher in incubations with smaller particles. Assuming that the cell-associated triglyceride shown in Figure 2 resulted from cellular uptake of the emulsion particles (see below), the number of internalized smaller particles was much greater

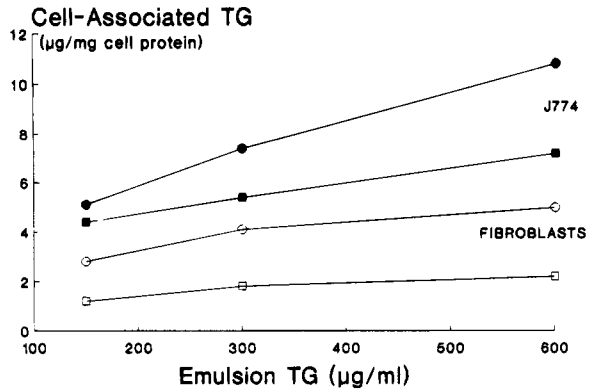

FIGURE 3: Triglyceride mass uptake of "large" and "small" emulsion particles, at varying triglyceride concentrations, by J-774 macrophages and fibroblasts. Macrophages (closed symbols) or fibroblasts (open symbols) were incubated for 6 h at 37 °C with 1 mL of DMEM/1% BSA containing either "large" VLDL-size emulsion particles (■, □) (triglyceride:phospholipid ratio of 2.7:1, with trace amounts of [^{3}H]cholesteryl ether; specific radioactivity, 446 cpm/μg of TG) or "small" IDL-size emulsion particles (●, ○) (triglyceride:phospholipid ratio of 1.1:1; specific radioactivity, 407 cpm/μg of TG), at varying TG concentrations. Each point represents the mean of two parallel experiments. Results varied by not more than 5%.

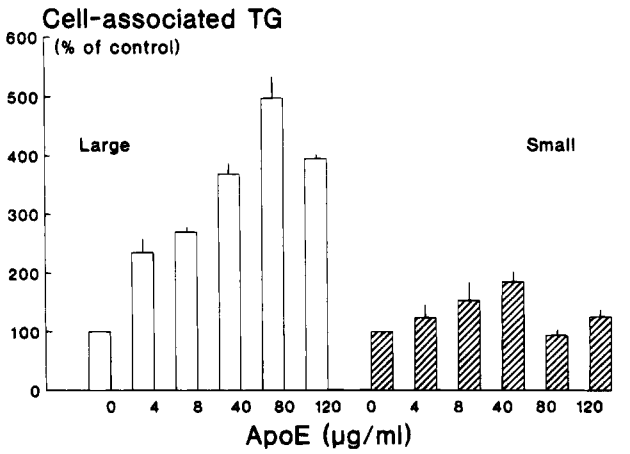

FIGURE 4: Effect of apo E on emulsion triglyceride mass uptake by J-774 macrophages. Cells were incubated for 6 h at 37 °C with 1 mL of DMEM/1% BSA containing either "large" VLDL-size particles (open bars, triglyceride:phospholipid ratio of 3.9:1, with trace amounts of [^{3}H]cholesteryl ether; specific radioactivity, 340 cpm/μg of TG) or "small" IDL-size particles (hatched bars, triglyceride:phospholipid ratio of 2.0:1, with trace amounts of [^{3}H]-cholesteryl ether; specific radioactivity, 280 cpm/μg of TG) at a concentration of 400 μg of TG/mL in the presence of varying concentrations of apoprotein E. Cellular uptake is expressed as percent of control (i.e., cellular triglyceride uptake in the absence of apo E is regarded as 100%). Control values were 4.4 ± 0.9 μg of triglyceride/mg of cell protein for the large particles and 8.0 ± 1.2 μg of triglyceride/mg of cell protein for the small particles. Results are the mean ± SD of three parallel experiments.

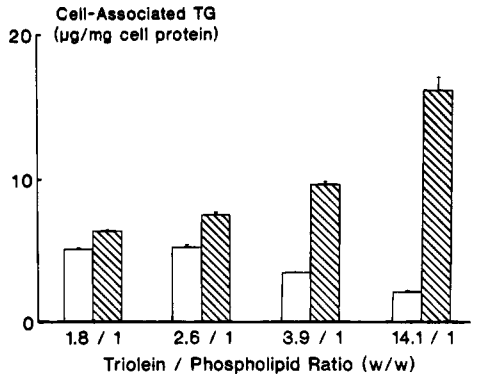

FIGURE 5: Effects of incremental changes of particle size on emulsion triglyceride mass uptake by HepG2 cells in the absence and presence of apo E. A series of different size emulsions were prepared by mixing varying triglyceride to phospholipid ratios and adding trace amounts of [^{3}H]cholesteryl ether (specific radioactivities, 430−535 cpm/μg of TG). HepG2 cells were incubated for 4 h at 37 °C with 1 mL of DMEM 1% BSA containing emulsion particles (100 μg of TG/mL) in the absence (open bars) or presence (hatched bars) of 0.15 μg of apo E. Smaller particles have the lower triolein:phospholipid ratios, and larger particles, higher ratios. Results are the mean ± SD of three parallel experiments.

than that of the larger particles. On the basis of the estimated diameter of VLDL-size (~60 nm) and IDL-size (~28 nm) particles, and triglyceride molecular weight and partial specific volume (1.093 mL/g), the number of particles required to carry this triglyceride mass into the cells was calculated.[2] Uptake of small particles was 20−100-fold greater than uptake of the large particles.

To determine that uptake of particles, in the absence of apo E, is not due to a property of the synthetic emulsion lipids, we studied the uptake of emulsions prepared from extracted "native" VLDL lipids. Cellular uptake of VLDL lipid prepared emulsions (at 300 μg of TG/mL) in the absence of apo E ranged from 4.1 to 7.7 μg of TG/mg of cell protein with uptake of larger particles at the lower uptake levels. This is in the range of uptake of tiolein−phosphatidylcholine emulsion particles, in the absence of apo E (cf. Figures 2 and 3). We, conclude, therefore, that our observations on cellular uptake of model emulsion particles and uptake of particles with lipid composition of native VLDL are similar.

In order to determine whether the observed differences in mass uptake of different size particles are also apparent in another cell line, we examined triglyceride mass uptake in fibroblasts and compared it to uptake into J-774 macrophages. In both cell lines, at varying triglyceride concentrations, triglyceride uptake in the absence of apo E was higher for the small particles (Figure 3). Although in fibroblasts triglyceride mass uptake was only 30−50% that of uptake into macrophages, the preferential uptake of the smaller particles was also observed in this cell line.

The effect of apo E on the cellular uptake of the different size particles was studied. Apo E enhanced the cellular uptake of both the larger VLDL-size and smaller IDL-size particles, but the effect was greater on uptake of VLDL-size particles. Uptake of VLDL-size particles increased up to 4−5-fold compared to an up to 2-fold increase in IDL-size particle uptake (Figure 4).

We next questioned whether the different effects of particle size and exogenous apo E would also be present in a cell line that does synthesize apo E, e.g., HepG2 cells. Figure 5 demonstrates that with a series of emulsions with graded increases in size cellular uptake of smaller particles is greater than that of larger particles, in the absence of apo E. Similar to results in J-774 cells, exogenous apo E enhanced the uptake of larger particles more than smaller particles, and did so in a graded manner as particle size increased.

[2] The calculated volume of particles divided by the volume of one triolein molecule [calculated by triglyceride partial specific volume (1.093)/number of molecules per gram of triolein] yielded the number of triolein molecules per particle. The number of molecules in 1 μg of triolein (calculated based on the triolein MW, 884, and Avogadro's number) divided by the number of triolein molecules per particle is the number of particles needed to carry 1 μg of TG.

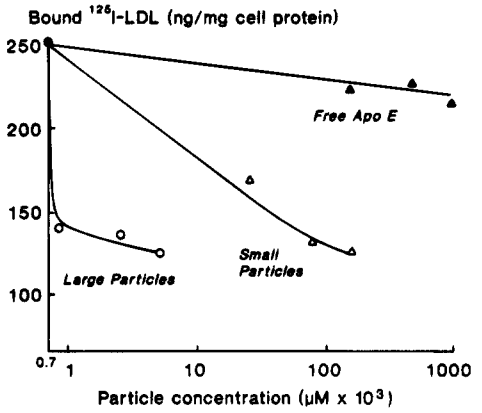

FIGURE 6: Competitive displacement of ^{125}I-LDL by free apo E, LDL, and apo E−emulsion particles in J-774 macrophages. Apo E was equilibrated with either "large" VLDL-size emulsion particles or "small" IDL-size emulsion particles at a 1:10 protein:triglyceride ratio. ("Large" particles, triglyceride:phospholipid ratio of 4.2:1; "small" particles, triglyceride:phospholipid ratio of 1.7:1). Up-regulated macrophages were incubated for 2.5 h with ^{125}I-labeled LDL (5 μg of protein/mL) in the presence of increasing concentrations of apo E−large emulsion particles (○), apo E−small emulsion particles (△), and free apo E (▲). Results on the *x*-axis are expressed in terms of relative particle numbers (μM × 10^3) for LDL and apo E−emulsion particles or in the case of free apo E as the number of apo E molecules. Small particles and large particles were calculated to bind respectively 4 and 40 apo E molecules each, as described under Results. Each point represents the mean value of two parallel experiments which did not vary by more than 5%.

The proportion of apo E that bound to the emulsion particles in the system was calculated after gel filtration. ^{125}I-labeled apo E was equilibrated with the emulsions (at a 1:10 protein:triglyceride ratio), and the ^{125}I-apo E−emulsion particle complexes were loaded onto a Sepharose CL-2B column. Fractions were collected, and radioactivity was monitored for the presence of ^{125}I-apo E. Apo E−[^{3}H]-cholesteryl ether labeled emulsion particle complexes were similarly loaded onto an identical column and eluted fractions monitored for the presence of [^{3}H]cholesteryl ether. Fractions which overlapped in ^{3}H and ^{125}I radioactivity represented the bound apo E−emulsion particle complex. At an apo E:triglyceride emulsion weight ratio of 1:10, VLDL-size emulsion particles bound 23% of the apo E, whereas IDL-size emulsion particles bound 88% of exogenous apo E. Calculating from the concentration of apo E in our media (and hence the number of apo E molecules), the percent of total apo E which bound to emulsion particles, and the number of emulsion particles, we estimated the number of apo E molecules associated with each emulsion particle. At a concentration of 40 μg/mL apo E and 400 μg/mL emulsion triglyceride, the VLDL-size particles bound approximately 40 apo E molecules per particle, and IDL-size particles bound approximately 4 apo E molecules per particle.

We then questioned at these apo E:emulsion ratios that produced maximal uptake, if the relative affinities of large vs small particles for cells were similar or different. The data in Figure 6 show that both larger VLDL-size emulsion particles and the smaller IDL-size particles, in the presence of apo E, competed with LDL for binding and uptake by the LDL receptor. Consistent with previous results (Bradley & Gianturco, 1986), free apo E, over a protein concentration range equivalent to the apo E concentration associated with the particle complexes, did not displace bound ^{125}I-LDL. Like LDL, apo E−emulsion particle complexes compete for cell binding of ^{125}I-labeled LDL to both J-774 macrophages and fibroblasts. Moreover, when expressed in terms of the number of particles required to displace LDL, apo E on larger particles showed much greater cell affinity then did apo E on smaller particles. This explains, in part, the greater ability of apo E to increase uptake of larger as compared to smaller particles.

We explored the mechanism of cellular uptake of emulsion particles in the absence of apo E and whether fluid-phase pinocytosis could be responsible for emulsion particle uptake. Cellular uptake of ^{3}H-labeled sucrose was used as a marker of fluid-phase pinocytosis. J-774 macrophages and fibroblasts were incubated for 6 h with either large-size emulsion particles, small-size emulsion particles, or ^{3}H-labeled sucrose. Cellular uptake was expressed as percent removal of initial cpm per well from the media. In J-774 macrophages, ^{3}H-labeled sucrose uptake constituted ~0.05% of total media counts, whereas the percent of larger-size emulsion particle removal was 3.5−12-fold higher and small-size particle uptake 5−15-fold higher than that of sucrose. In fibroblasts, sucrose clearance was 0.014% of total media counts, and emulsion particle clearance (percent of total media counts internalized) was 4−6-fold higher. In the presence of apo E, particle cell removal of both large and small particles reached as high as 2−5% of the total particles initially placed in the media. These results show that fluid-phase pinocytosis, per se, can only account for a very small portion of emulsion particle uptake. Since adsorptive endocytosis is a likely mechanism whereby particles are interiorized at rates considerably above those of fluid-phase reactants (Silverstein et al., 1977), this process probably accounts for the uptake of the emulsion particles without apo E.

In order to verify that the disproportionate cellular uptake of small-size emulsion particles vs large-size particles is not, in fact, a result of exocytosis of the larger particles after their initial internalization, the following experiments were designed: Incubations were performed as previously described in the absence or presence of apo E for 2 h, but at the end of the 2 h incubation, the medium was removed and replaced every 30 min with fresh emulsion-free DMEM. Remaining cell triglyceride uptake was determined at sequential time points and expressed relative to uptake at the end of the 2 h incubation, which was considered as 100% uptake. Three hours after removal of emulsion-containing medium, cell-associated radioactivity for both large and small emulsion particles remained constant, and essentially no radioactivity was released into the medium. Thus, increased cellular uptake of the smaller, IDL-size emulsion particles cannot be attributed to a preferential exocytosis of the larger, VLDL-size particles.

To determine that our results reflect true cellular particle uptake and not merely adhesion of the apo E−emulsion particle complex to the surface cell membrane, a number of approaches were used. In the first approach, cell-associated triglyceride, after incubation of the cells with emulsion plus apo E, was shown to be resistant to release by heparin or suramin. Following 5 h incubation with the emulsion plus apo E-containing medium, the medium was removed, and cells were incubated for 1 h in 10 mg/mL heparin or 10 mM suramin at 37 °C. Cell triglyceride uptake did not change significantly following incubations with either heparin or suramin. Similarly, in experiments with ^{125}I-apo E emulsions, after a 5 h incubation, only 0.2% and 0.9% of cell ^{125}I-apo E remained cell surface bound with small and large particles, respectively, while 22.4% and 19.7% of ^{125}I-apo

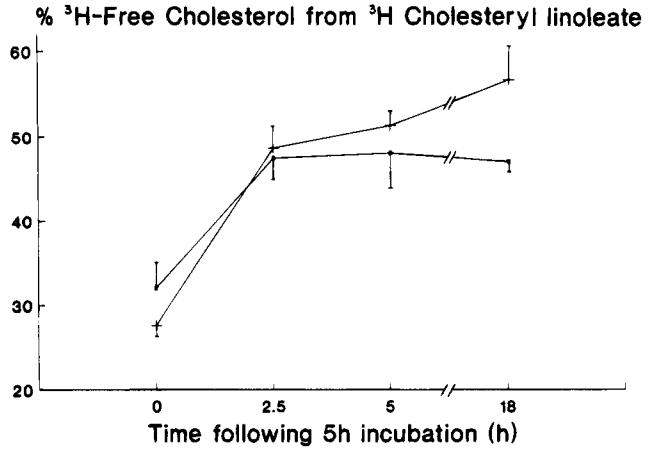

FIGURE 7: Hydrolysis of emulsion cholesteryl ester to free cholesterol in J-774 macrophages. Large (■) and small (+) emulsion particles, 300 μg of TG/mL, were prepared containing trace mass amounts of [^{3}H]cholesteryl linoleate and incubated for 5 h at 37 °C. After 5 h incubation (time 0), the medium containing emulsions was replaced by emulsion-free medium (DMEM/1.0% BSA), and cells were analyzed for ^{3}H-free cholesterol and [^{3}H]-cholesteryl linoleate at the times indicated after emulsion incubation. Cholesteryl ester hydrolysis is expressed as the percent of ^{3}H-free cholesterol formed from initial [^{3}H]cholesteryl linoleate placed in each emulsion (specific radioactivity was 1454 cpm/μg of TG for the large and 1293 cpm/μg of TG for the small emulsions). Results are expressed as the mean ± SD (n = 3) at each time point.

E was internalized intact, and 77.3% and 79.4% of ^{125}I-apo E was degraded, again with small and large particles, respectively. Therefore, in the presence of apo E, cell association can almost totally be accounted for by particle internalization.

To determine if emulsions without apo E were also internalized, we labeled emulsions with radiolabeled cholesteryl ester (rather than cholesteryl ether) and assayed cholesteryl ester hydrolysis. In incubations performed with emulsions prepared with trace amounts of [^{3}H]cholesteryl linoleate, the cholesteryl ester was efficiently hydrolyzed to free [^{3}H]cholesterol (Figure 7). Hydrolysis of [^{3}H]cholesteryl linoleate to ^{3}H-free cholesterol accounted for about one-third of the cell-associated radiolabeled cholesterol after 5 h incubation, with net hydrolysis increasing further over time even after the emulsion was removed from the media. Conditioned media from these cells led to no degradation of emulsion cholesteryl ester to free cholesterol after 5 h incubation. Also, in the presence of chloroquine (which inhibits cell lysosomal hydrolases), almost no hydrolysis of [^{3}H]cholesteryl ester occurs. Chloroquine had no effect on uptake of particles (data not shown). These results clearly demonstrate that particles even in the absence of apo E were efficiently internalized and underwent intracellular metabolism.

The internalization of emulsions with and without apo E was examined by fluorescence microscopy. In double-label experiments, J-774 or mouse peritoneal macrophages were incubated for 10 min with DiI-labeled small and large emulsion particles, in the absence or presence of apoprotein E, plus fluorescein-labeled α_2-macroglobulin, a ligand which is targeted rapidly to lysosomes (Ward et al., 1989). The data in Figure 8 show that after this 10 min incubation with mouse peritoneal macrophages the DiI label in small emulsion particles both in the absence (Figure 8b) and in the presence (Figure 8E) of apo E largely colocalized with the fluorescein label in perinuclear compartments. Data (not shown) with large emulsions without apo E also showed almost complete colocalization with fluorescein-labeled α_2-macroglobulin, while those with apo E also showed colocalization with α_2-macroglobulin, as well as some nonoverlapping peripheral localization (Tabas et al., 1991). (In the figure, results are shown for mouse peritoneal macrophages because the perinuclear location of the fluorescein is illustrated better than in the more rounded J-774 cells with relatively larger nuclei and smaller cytoplasmic compartments. Microscopy experiments with J-774 macrophages showed the same patterns as shown in Figure 8 for the mouse peritoneal macrophages.) These data are consistent with the [^{3}H]cholesteryl ester hydrolysis data (Figure 7), and indicate that the emulsions with and without apo E are rapidly targeted to perinuclear lysosomes.

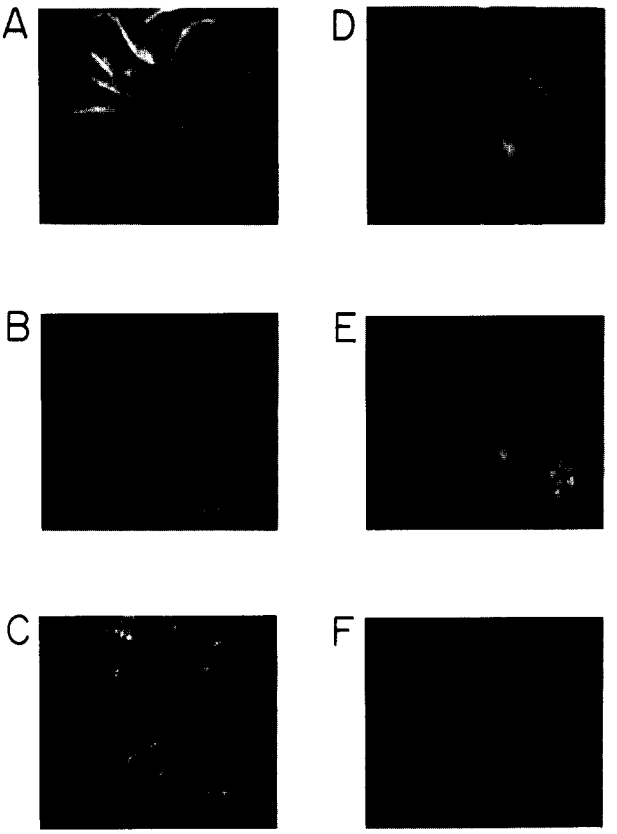

FIGURE 8: Intracellular localization of DiI-labeled emulsion particles. Emulsions were labeled with the fluorescent probe DiI by cosonication of 4 mg of DiI added to the 200 mg of triolein–phospholipid mixture. DiI small emulsion particles (400 μg of TG/mL) without (A–C) and with (D–F) added apo E (40 μg of apo E/mL) were incubated with macrophages in DMEM/1.0% BSA medium containing added fluorescein-labeled α_2-macroglobulin (4 mg/mL) for 10 min. The medium was removed, and the cells were fixed with 2% formaldehyde in PBS and studied by phase and fluorescent microscopy as detailed under Experimental Procedures. Phase contrast microscopy (A, D), DiI fluorescence (B, E), and fluorescein fluorescence (C, F) are shown.

DISCUSSION

Particle size modulates the cellular uptake of triolein–phosphatidylcholine emulsion particles both in the absence and in the presence of apo E. In the absence of apo E, the level of uptake of smaller IDL-size particles was approximately 2-fold that of larger VLDL-size particles when expressed as mass triglyceride uptake and up to 100-fold higher in terms of particle number. Thus, for emulsion particles of both VLDL and IDL size, non-receptor-mediated

uptake constitutes an important pathway for uptake into J-774 macrophages and to a lesser degree also into fibroblasts.

The substantial cellular uptake demonstrated in our study, even in the absence of apo E, may be due to the higher and more physiologic concentrations of triglyceride-rich particles compared to those used in other studies. In a previous study which examined uptake of triglyceride-rich particles into hepatocytes, only minimal triglyceride uptake of apo E-free emulsions was observed when compared to the apo E-containing emulsions at emulsion triglyceride concentrations of 25 μg/mL (Oswald & Quarfordt, 1987). Similarly, uptake of large triglyceride-rich VLDL into fibroblasts was suggested to be absolutely dependent on apo E (Gianturco et al., 1982b). It is noteworthy that in previous studies on the effects of apo E on receptor-mediated pathways, the concentrations of triglyceride-rich particles were lower, in keeping with conditions recommended for saturation receptor binding assays (Gianturco et al., 1982b); Granot & Eisenberg, 1990). It may be that at more physiologic particle concentrations, low-affinity uptake pathways via nonreceptor mechanisms can account for a relatively high degree of particle uptake. Indeed, studies on the metabolism of chylomicrons in rabbits have established that, whereas at low concentrations of triglyceride-rich lipoproteins particle uptake is principally by the liver, at higher concentrations bone marrow constituted a major site of uptake (Hussain et al., 1989). Electron microscopic examinations of the bone marrow established that perisinusoidal macrophages uniquely accounted for the uptake of chylomicrons, chylomicron remnants, and very low density lipoproteins. Uptake into bone marrow macrophages was suggested to be non-apo E-specific (Hussain et al., 1989).

The non-receptor-mediated cellular uptake of emulsion particles may likely be attributed to adsorptive endocytosis by which solutes that bind to the cell membrane are interiorized at rates significantly above those of fluid-phase reactants (Ward et al., 1989). Since, at similar triglyceride concentrations, more particle surface area is exposed on the smaller particles to interact with cells, adsorptive endocytosis can thus account for the greater cell uptake of smaller emulsion particles as compared to larger particles.

Apo E enhanced cellular uptake of the larger VLDL-size emulsion particles to a greater extent than that of the smaller IDL-size particles. A possible explanation for this may relate to the number of apo E molecules which can bind to each emulsion particle. At the physiologic concentrations used by us, VLDL-size particles bind approximately 40 apo E molecules per particle, whereas IDL-size particles bind approximately 4 apo E molecules per particle. The absolute number of apo E molecules which can be accommodated on the particle surface may determine the binding affinity of the particle to the receptor. In a study of apo E cooperativity on lipid microemulsion particles of LDL size, the affinity of these microemulsion particles to the LDL receptor was of the same order as that of LDL but increased substantially when at least four apo E molecules were associated per particle (Funahashi et al., 1989). Studies of the apo B, E receptor indicate that the receptor possesses multiple binding sites (Innerarity et al., 1981). Multivalent receptor binding (either to multiple receptors and/or to multiple apo E−receptor interactions) may facilitate higher affinity binding of lipid particles which have more apo E molecules per particle. This may explain the higher receptor affinity of the large emulsion particles (with about 40 apo E molecules per particle) compared to the smaller particles (with 4 apo E molecules per particle) (Figure 6). Another potential mechanism for different effects of apo E on different size particles is that the apo E−lipid interaction and conformational changes in apo E prior to recognition of the receptor binding domain may be dependent on the surface size and curvature of the lipid particle (Mims et al., 1990). The apparent discrepancy between our results and those of Mims et al. (1990), who found that smaller emulsion particles were better ligands for the LDL receptor than larger particles, may be due to the fact that those emulsion particles were composed of dimyristoylphosphatidylcholine and cholesteryl oleate and thus their metabolism may not be analogous to that of triolein−phosphatidylcholine particles (Mims et al., 1990). Also, while our results on apo E binding are expressed per particle number, those of Mims et al. were expressed as a function of apo E mass. Since in both our present study and that of Mims et al. (1990) large particles bound 7−10 times more apo E molecules per particle than small particles, the data of Mims et al. also indicate higher affinity of large particles, when expressed as particle number.

Are these in vitro observations relevant to the *in vivo* metabolism of triglyceride-rich particles? The fact that we studied artificial emulsion particles containing only two lipids, triolein and egg yolk lecithin, in a system in which only one apoprotein, E, is present may limit the degree to which our results pertain to native triglyceride-rich particles. Previous studies have noted that the metabolism of native triglyceride-rich lipoproteins and lipid emulsions is dependent not only on particle size but also on the ratios of A, C, and E apoproteins and the amount of cholesteryl ester and free cholesterol in the particle. Changes in the binding affinity of A, C, and E apoproteins to triolein−phospholipid particles can also be associated with varying lipid composition and particle size (Tajima et al., 1983; Miller & Small, 1983; Vessby & Ontko, 1983; Redgrave & Maranhao, 1985; Maranhao et al., 1986; Redgrave et al., 1987, 1988; Al-Haideri et al., 1993). Nevertheless, it is noteworthy that the cellular uptake of emulsion particles prepared from a more "physiologic" lipid mixture, i.e., extracted human VLDL lipid, was in a range similar to that observed with triolein−phospholipid emulsion particles. Of particular interest, Krul et al. (1985) previously reported that with native VLDL subfractions (with varying relative lipid compositions) the relative importance of apo E on particle uptake increased with increasing particle size.

Our study was designed to address the effect of size on particle metabolism by restricting compositional changes solely to differences in the triglyceride to phospholipid ratio in the particle and by conducting the experiments of a model system devoid of endogenous apoproteins. Our results demonstrate that the cellular uptake of triglyceride−phospholipid emulsion particles in J-774 macrophages and fibroblasts is indeed related to particle size and can occur by two mechanisms: non-receptor-mediated uptake and apo E-dependent uptake. Clearly, in the absence of apo E, particle uptake must occur by non-receptor-mediated uptake pathways, mainly as our results suggest, by adsorptive endocytosis.

Physiologically, triglyceride-rich particles likely never exist without apo E. Nevertheless, in many of our experiments, we calculated that as much as 2−5% of apo E−emulsion particles in the media were internalized by cells over a 5 h incubation, a degree of uptake likely greater than can be

accounted for by receptor-mediated pathways over this time period. This suggests that at physiologic particle concentrations, non-receptor-mediated uptake mechanism pathways may contribute to uptake of triglyceride-rich particles containing apo E. Support for such a concept is provided in ongoing experiments showing that at high "physiologic" triglyceride concentrations (200−1000 μg/mL) removal of cell surface heparan sulfate proteoglycans by heparinase and heparitinase treatment substantially decreases cell uptake of apo E−emulsion particles by one-half to two-thirds. However, at the relatively low particle concentration required to saturate receptor-mediated uptake (20−50 μg of triglyceride/mL), removal of cell surface proteoglycans does not diminish apo E−emulsion particle uptake (Al-Haideri et al., 1993). This suggests that at high particle concentrations critical particle binding to heparan sulfate proteoglycans may be important for either direct internalization of particles or efficient transfer to nearby receptors (Ghiselli et al., 1981; Ji et al., 1993).

Since in our studies apo E had a substantially lesser effect on enhancing the uptake of IDL-size emulsions as compared to larger VLDL-size emulsions, particle size is likely one factor that modulates the relative degree of receptor vs nonreceptor pathways contributing to particle uptake. If these observations in cultured cells *in vitro* also occur *in vivo*, then nonreceptor pathways may be an important contributor for particle uptake at physiological triglyceride concentrations, not only for apoprotein-free model lipid emulsions but also for lipoproteins containing apo E, such as triglyceride-rich particle remnants. Indeed, in type III hyperlipidemia and in the rare condition of apo E deficiency (Mabuchi et al., 1989; Plump et al., 1992), despite defective or deficient apo E, severe atheromatous lesions and xanthomas develop. Furthermore, apo E-deficient mice, recently created by gene targeting in embryonic stem cells, show a marked elevation of very low and intermediate density lipoproteins and extensive atherosclerotic lesions (Plump et al., 1992). Since, in mice, receptor-mediated clearance of triglyceride-rich particles depends almost entirely on apo E, then in the absence of apo E uptake of these particles almost certainly occurs via non-receptor-mediated pathways, at least in some tissues.

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