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Human Recombinant Apolipoprotein E-Enriched Liposomes Can Mimic Low-Density Lipoproteins as Carriers for the Site-Specific Delivery of Antitumor Agents

PATRICK C. N. RENSEN, RAYMOND M. SCHIFFELERS, A. JENNY VERSLUIS, MARTIN K. BIJSTERBOSCH, MARLY E. M. J. VAN KUIJK-MEUWISSEN, and THEO J. C. VAN BERKEL

Division of Biopharmaceutics (P.C.N., R.M.S., A.J.V., M.K.B., T.J.C.V.B.) and Pharmaceutical Technology (M.E.M.J.M.), Leiden-Amsterdam Center for Drug Research, University of Leiden, Leiden, The Netherlands

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SUMMARY

Progressive hypocholesterolemia is a feature associated with a number of cancers of different origin, and it is caused by the high expression of low-density lipoprotein (LDL) receptors (LDLrs) on many tumor cell types. Selective delivery of chemotherapeutics using LDL as a carrier has therefore been proposed, but the endogenous nature of LDL hampers its pharmaceutical application. In the current study, we explored the possibility of synthesizing liposomes that mimic LDL from commercially available lipids and proteins. Small unilamellar liposomes were created (28.9 ± 0.9 nm) and complexed with 5.8 ± 0.7 molecules of human recombinant apolipoprotein E (apoE). On intravenous injection into rats, the liposomes retained their aqueous core, structural integrity, and the majority of the preassociated apoE. [^{3}H]Cholesteryl oleate-labeled apoE-enriched liposomes showed a relatively long serum half-life (>5 hr), and a low uptake by cells of the reticuloendothelial system was observed (<0.8% of the injected dose at 30 min after injection). Pretreatment of rats with 17α-ethinyl estradiol, which induces the expression of the LDLr on the liver and adrenals, led to a 2.5-fold accelerated serum clearance ($t_{1/2}$ = 123 ± 10 min) and a selectively increased uptake of liposomes by the liver (2.0-fold) and adrenals (3.8-fold). The liver association of the liposomes was coupled to the lysosomal uptake route, similarly as for LDL. *In vitro* studies using B16 melanoma cells showed that the liposomes bound exclusively to the LDLr via their apoE moiety (90,000 liposomes/cell), with a 14-fold higher affinity (K_d = 0.77 ± 0.09 nM) than LDL itself. Because of their favorable properties, we anticipate that these apoE-enriched liposomes are advantageous compared with native LDL in the development of a selective LDLr-targeted antitumor therapy.

Many studies have reported the J- or U-shaped relation between plasma total cholesterol levels and all-cause mortality (1). High cholesterol levels are correlated to mortality from cardiovascular diseases, but epidemiological studies have indicated that tumor-associated hypocholesterolemia is a feature associated with a number of cancers of different origin, as was recently reviewed (2). This inverse relation between plasma cholesterol levels and cancer has been found mainly in hematological malignancies such as acute myelogenous leukemia but also for adrenal adenoma and cancers of the colon, lung, breast, and prostate (2, 3).

The mechanism underlying hypocholesterolemia is postulated to be the overuse of cholesterol by cancer cells because they are characterized by accelerated growth and division (termed reverse causality), as reviewed by Markel and Brook (2). This mechanism has been underscored by the finding that a declining plasma cholesterol level, but not a stable low level, is related to subsequent mortality from cancers of the hemopoietic system, esophagus, and prostate (4, 5). Moreover, trials have correlated the normalization of the low pretreatment cholesterol levels with successful chemotherapy or curative surgery and remission of the disease (6).

Within the blood compartment, cholesterol is mainly transported by LDL, spherical lipid particles that are recognized and taken up via their exposed apoB-100 moiety by the LDLr (7). The LDLr is expressed on a variety of tissues, such as the liver and adrenals. Indirect evidence for the overuse of cholesterol by cancer cells is derived from the overexpression of the LDLr in acute myelogenous leukemia (3–100-fold), colon cancer (6-fold), adrenal adenoma (8-fold), lung carcinoma, brain tumors, and metastatic prostate tumors, as reviewed by Firestone (3).

Based on the excessive need of cancer cells for cholesterol, it has been suggested that tumor cell growth can be impeded

ABBREVIATIONS: LDL, low-density lipoprotein; apo, apolipoprotein; BSA, bovine serum albumin; CO, cholesteryl oleate; DOPC, dioleoyl phosphatidylcholine; EE, ethinyl estradiol; EYPC, egg yolk phosphatidylcholine; HDL, high-density lipoprotein; LDLr, low-density lipoprotein receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; RES, reticuloendothelial system; VLDL, very low-density lipoprotein.

by limiting the rate of utilization of cholesterol or inhibiting its synthesis (8). On the other hand, the selective uptake of LDL by tumor cells raised the possibility of targeting chemotherapeutics by using LDL as a drug carrier (3). The principle to use native LDL for drug targeting purposes has been demonstrated *in vitro* (3, 9) and *in vivo* (10, 11). The necessity to isolate LDL from human serum, however, hampers its pharmaceutical application.

As models for LDL, both 20–30-nm microemulsions (12) and 100–200-nm liposomes (13) have been synthesized. Although LDL and microemulsions consist of a hydrophobic core that is emulsified by a phospholipid monolayer, (unilamellar) liposomes contain an aqueous core that is surrounded by a phospholipid bilayer. Delipidated detergent-solubilized apoB-100 was subsequently complexed as ligand for the LDLr (13–15). These reconstituted LDL-like particles were shown to display affinity for the LDLr *in vitro* (13, 15), but *in vivo* results on these complexes have not been reported. LDL was recently shown to have an optimal size of 23–24 nm for maximum LDLr affinity (16), which is related to the conformation of apoB-100 as determined by circular dichroism (16). Concomitant with the potential loss of the structural integrity of such a large 514-kDa protein during delipidation and solubilization, it can be expected that the particle size of the reconstituted LDL is crucial for obtaining LDL-typical *in vivo* behavior when using solubilized apoB-100 as a ligand for the LDLr.

The 34-kDa glycoprotein apoE, a constituent of triglyceride-rich lipoproteins such as chylomicrons and VLDL, is also a high affinity ligand for the LDLr, although it is structurally unrelated to apoB-100. The LDLr-binding domain of apoE is a 20-amino acid region comprising residues 140–160 (17). Although apoE that is devoid of lipid lacks LDLr-binding activity (18), apoE-containing HDL binds with a very high affinity (20–25-fold higher than LDL) to the LDLr with a K_d value of ~0.12 nM (18, 19). As many as four apoE molecules assembled on a phospholipid surface can bind a single LDLr, and when more than one of the apoE molecules binds, the apparent affinity is increased 25–50-fold (18).

Recently, we observed that the apoE-mediated metabolic behavior of triglyceride-rich emulsions was dependent on the particle size. Large emulsions (>100 nm) were taken up by a liver-specific non-LDLr recognition site, but the *in vivo* clearance of small emulsions (<50 nm) by the liver and adrenals was completely mediated by the LDLr (20). In the current study, we investigated the feasibility of synthesizing small liposomes that on association with commercially available human recombinant apolipoprotein show affinity for the LDLr *in vivo*. The potential application of these carriers for tumor-directed drug targeting was assessed *in vitro* using LDLr-expressing B16 murine melanoma cells.

Materials and Methods

Chemicals. Recombinant human apoE was a generous gift from Tikva Vogel (Bio-Technology General, Rehovot, Israel) and was supplied as a lyophilized powder containing 76% apoE, 11.7% L-cysteine, and 12.0% $NaHCO_3$ (21). ApoE was dissolved in PBS, pH 7.4 (2.0 mg/ml), and stored under argon at −80°. Carrier-free ^{125}I in NaOH, [^{3}H]CO, [^{3}H]inulin, and [^{14}C]DOPC were purchased from Amersham (Buckinghamshire, UK). Triolein (99% pure) and EYPC (98%) were from Fluka (Buchs, Switzerland). L-α-Lysophosphatidylcholine (99%), cholesterol (>99%), BSA (fraction V), 17α-EE, and phospholipase A_2 (isolated from *Crotalus atrox* venom) were obtained from Sigma Chemical (St. Louis, MO). CO (97%) was from Janssen (Beersse, Belgium). Cholesterol oxidase, cholesterol esterase, peroxidase type II (200 units/mg), Precipath L, EDTA, and a solution containing 50,000 IU of penicillin and 50 mg/ml streptomycin were from Boehringer-Mannheim Biochemica (Mannheim, Germany). HEPES, 5-carboxyfluorescein, and L-glutamine were from Merck (Darmstadt, Germany), and Triton X-100 was from BDH Chemicals (Poole, UK). Dulbecco's modified Eagle medium was from GIBCO BRL (Gaithersburg, MD). Fetal calf serum was obtained from Hyclone Laboratories (Logan, UT), and a solution of 2.5% (w/v) trypsin in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} was purchased from Flow Laboratories (Irvine, UK). All other chemicals were of analytical grade.

Preparation and purification of liposomes. Liposomes were prepared by sonication according to a modification of the procedure used by Ginsburg *et al.* (12) with 50 mg of total lipid at a EYPC/CO molar ratio of 25:1. The lipids were hydrated in 11.4 ml of 0.1 M KCl and 0.01 M Tris·HCl, pH 8.0, and sonicated for 1 hr using a Soniprep 150 (MSE Scientific Instruments, Crawley, UK) at 18 μM output that is equipped with a water bath for temperature (54°) maintenance. When indicated, liposomes were synthesized in the presence of a self-quenching concentration (100 mM) of 5-carboxyfluorescein, 10–150 μCi of [^{3}H]CO, 2–3 μCi of [^{14}C]DOPC, or 50–100 μCi of [^{3}H]inulin. Liposomes were purified and concentrated by density gradient ultracentrifugation according to the method of Redgrave *et al.* (22) using NaCl/KBr/EDTA density solutions, pH 8.0, in a Beckman SW 40 Ti rotor at 40,000 rpm for 18–22 hr at 4°. Liposomes, which are visible as a narrow opalescent layer at approximately three fourths of the tube height, were routinely isolated by aspiration. Alternatively, the gradients were fractionated (0.6-ml fractions) at a flow rate of 1.2 ml/min using an LKB (Uppsala, Sweden) Bromma 2132 Microperpex peristaltic pump, starting at the bottom of the tube. The fractions were assayed for density using a DMA 40 digital density meter from Mettler (Graz, Austria). Liposomes were stored at 20° under argon and used for characterization and metabolic studies within 5 days after preparation, during which no physicochemical changes occurred, as determined on the basis of particle size analysis by photon correlation spectroscopy, agarose gel electrophoresis, and/or [^{3}H]inulin retention (described below).

Characterization of liposomes. The EYPC and CO contents of the purified liposomes and ultracentrifugation fractions were determined using the Boehringer-Mannheim enzymatic kits for phosphatidylcholine and cholesterol, respectively. Precipath L was used as an internal standard. Radioactivity was routinely counted after the addition of Emulsifier Safe (Packard, Meriden, CT). Particle size and homogeneity of the liposomes were assayed by freeze fracture electron microscopy (as described below) or photon correlation spectroscopy using a Malvern 4700 C system (Malvern Instruments, Malvern, UK). Measurements were performed at 27° and a 90-degree angle between laser and detector. The net negative charge was determined by subjecting the liposomes to 0.75% (w/v) agarose gel electrophoresis, pH 8.8, with 0.075 M Tris·HCl, 0.080 M hippuric acid, and 0.65 mM EDTA buffer. R_F values were determined relative to the front marker bromphenol blue.

Freeze fracture electron microscopy. Aliquots of liposome suspensions were positioned between copper platelets (Balzers, Fürstentum, Liechtenstein) and cryofixed by rapid freezing (10^5 K/sec) in liquid propane with the use of a KF80 plunge freezing device (Reichert-Jung, Vienna, Austria). The frozen samples were fractured in a Balzers BAF400D freeze etching device at −150° and 2×10^{-7} mm Hg. The fracture planes were replicated by shadowing with platinum/carbon (2.5 nm) at an angle of 45°, followed by evaporation of carbon at 90° (30 nm) to strengthen the replica and ensure conductance. After thawing, the replicas were cleaned overnight using 30% sodium hypochlorite, washed with demineralized water, and examined on 400-mesh grids (Balzers) using a Philips (Eind-

hoven, The Netherlands) EM 201 transmission electron microscope at an acceleration voltage of 80 kV.

Isolation of LDL. Human LDL was isolated from the blood of healthy fasted volunteers by differential ultracentrifugation ($1.019 < d < 1.063$ g/ml) as previously described (22). Subsequently, LDL was dialyzed against PBS containing 1 mM EDTA, pH 7.4, with repeated changes of buffer and sterilized by filtration through a 0.22-μm filter (Millipore, Molsheim, France). The protein concentration was determined according to the method of Lowry *et al.* (23) using BSA as a standard.

Radiolabeling of apoE. ApoE was radioiodinated at pH 10.0 with carrier-free ^{125}I according to the ICl method (24) as previously described (25). Free ^{125}I was removed by Sephadex G-25 gel filtration and extensive dialysis against PBS containing 1 mM EDTA, pH 7.4, with repeated changes of buffer. More than 98% of the radiolabel was trichloroacetic acid precipitable. The specific activity of ^{125}I-apoE was 420–475 dpm/ng.

Association of apoE with liposomes. Liposomes were incubated with ^{125}I-apoE at various ratios for 30 min at 37°, and the incubation mixtures were subjected to density gradient ultracentrifugation according to the method of Redgrave *et al.* (22) with subsequent fractionation as detailed above. For *in vivo* experiments, liposomes were incubated with ^{125}I-apoE at an EYPC/apoE ratio of 10:0.1 (w/w), allowing complete liposomal incorporation of ^{125}I-apoE.

Effect of apoE on the liposomal integrity. To determine the liposomal character and effect of apoE on particle integrity, an enzymatic method was adopted that was based on the selective digestion of phosphatidylcholine within the outer layer of phospholipid-stabilized particles (26). [^{14}C]DOPC-labeled liposomes (5 mg of phospholipid) were incubated at room temperature, with or without preincubation (30 min at 37°) with 300 μg of apoE, in 0.1 M Tris·HCl, 0.5% (w/v) BSA, and 7 mM $CaCl_2$, pH 7.4, at a total volume of 20 ml. Phospholipase A_2 (10 units) was added at t = 0. When indicated, Triton X-100 was added after 30 min of incubation at a final concentration of 1% (v/v). At the indicated times, 800-μl samples were withdrawn, and reaction was terminated by lipid extraction according to the method of Bligh and Dyer (27). [^{14}C]DOCP (R_F = 0.25), [^{14}C]oleoyl phosphatidylcholine (R_F = 0.06), and [^{14}C]oleate (R_F = 0.97) were separated by thin layer chromatography [$CHCl_3$/CH_3OH/H_2O/acetic acid 70:30:4:2 (v/v/v/v)]. Lipids were visualized with iodine vapor, scraped off, and counted in 10 ml of Emulsifier Safe. [^{14}C]DOPC-labeled emulsions of 49 ± 11 nm were prepared as previously described (20, 28) and used as a reference for particles that are emulgated by a phospholipid monolayer.

Effect of apoE and/or serum on the liposomal density and surface charge. Liposomes (0.5 mg of phospholipid) that were labeled with [^{3}H]CO, [^{14}C]DOPC, or [^{3}H]inulin were incubated (30 min at 37°) with 1 ml of rat serum, without and with prior incubation (30 min at 37°) with apoE [phospholipid/apoE 10:1 (w/w)]. Subsequently, liposomes were reisolated by density gradient ultracentrifugation according to the method of Redgrave *et al.* (22), and gradients were fractionated and assayed as described above. Proteins (10–20 μg) within the fractions were occasionally separated on 5–20% sodium dodecyl sulfate-polyacrylamide gels. The relative amounts of apolipoproteins were calculated by densitometric scanning of Coomassie blue R-250-stained gels using a GS300 Scanning Densitometer from Hoefer Scientific Instruments (San Francisco, CA). Alternatively, aliquots of incubation mixtures were subjected to agarose gel electrophoresis as detailed above, resulting gels were fractionated, and radioactivity was counted on dissolution (overnight at 37°) in 10 ml of Emulsifier Safe.

Serum decay, liver uptake, and tissue distribution of liposomes and apoE in rats. Male Wistar rats (mass, 230–280 g) that were fed *ad libitum* with regular chow were anesthetized by intraperitoneal injection of sodium pentobarbital (15 mg/kg body weight), and the abdomens were opened. ^{125}I-apoE (2.5 μg) or liposomes that were labeled with [^{3}H]CO, [^{3}H]inulin, or ^{125}I-apoE (1.0–2.5 mg of phospholipid) were injected via the inferior vena cava. At the indicated times, blood samples and tissue lobules were taken and processed as previously described in detail (28). At 30 or 120 min after injection, the rats were killed, and their organs were excised and weighed. Radioactivity in [^{3}H]inulin-containing serum, liver, and other tissue samples was counted after combustion (recovery, >97%) in a Packard Tri-Carb 306 Sample Oxidizer and corrected for the serum radioactivity in the tissues at the time of sampling (28). The ^{125}I-containing serum and tissue samples were counted in a Packard gamma counter. When indicated, serum obtained at 30 min after injection was subjected to density gradient ultracentrifugation according to the method of Redgrave *et al.* (22), and gradients were fractionated and assayed as described. In some studies, rats were pretreated with 17α-EE dissolved in propylene glycol at 5 mg/kg body weight or similar amounts of propylene glycol alone for 3 successive days (29).

Intrahepatic processing of liposomal CO in rats. To determine the rate of CO hydrolysis within the liver, [^{3}H]CO-labeled liposomes (1.0 mg of phospholipid) were injected into anesthetized rats as described. At the indicated times, liver lobules were taken and immediately frozen in liquid N_2. On homogenization in ice-cold PBS, pH 7.4, lipids were extracted according to the method of Bligh and Dyer (27) and separated using thin layer chromatography [heptane/diethyl ether/acetic acid 60:40:1 (v/v/v)]. Cholesterol (R_F = 0.23) and CO (R_F = 0.85) were visualized with iodine vapor, scraped off, and counted in 15 ml of Hionic Fluor (Packard). Occasionally, the biliary secretion of ^{3}H radioactivity was determined by insertion of a catheter in the bile duct. Bile samples were counted for radioactivity after bleaching by the addition of 10 μl of 30% (v/v) H_2O_2. Also, aliquots of bile were extracted at pH 7.0 according to the method of Bligh and Dyer (27) to distinguish between bile acid- and cholesterol (ester)-associated ^{3}H-radioactivity.

Subcellular distribution of liposomes. [^{3}H]CO-labeled liposomes (1.0 mg of phospholipid) were injected into anesthetized rats as described. At 120 min after injection, the liver was perfused with 50 ml of ice-cold 0.25 M sucrose and 10 mM Tris·HCl buffer, pH 7.4, and divided into subcellular fractions by differential centrifugation exactly according to the method of De Duve *et al.* (30). The fractions were assayed for radioactivity, protein, and the activity of several marker enzymes (acid phosphatase, glucose-6-phosphatase, and lactate dehydrogenase) as previously described (30).

Culture of B16 melanoma cells. B16 (wild-type) murine melanoma cells (provided by Dr. A. Begg, The Netherlands Cancer Institute, Amsterdam, The Netherlands) were cultured at 37° in a humidified 5% CO_2/95% air atmosphere in 500-cm^2 flasks (Costar, Cambridge, MA) containing 20 ml of Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Cells were subcultured twice a week by detachment using 0.25% trypsin in Ca^{2+}- and Mg^{2+}-deficient Hanks' buffer, followed by renewal of medium the next day. The cellular protein content was determined according to the method of Lowry *et al.* (23) using BSA as a standard.

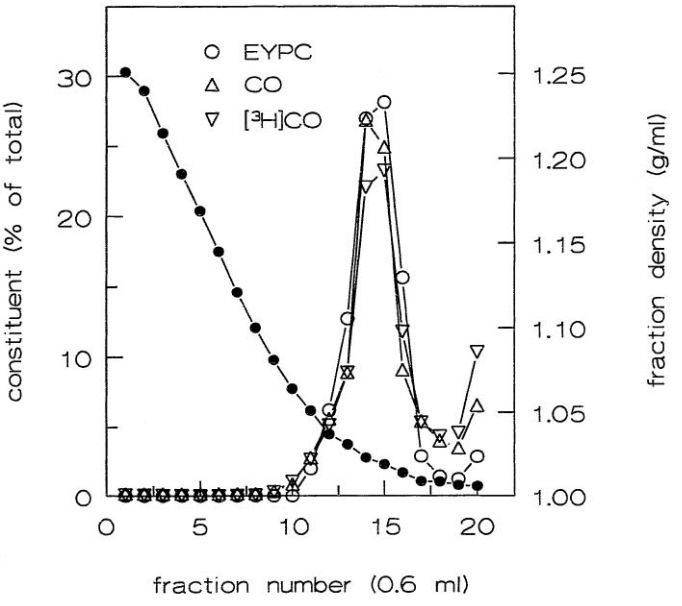
Binding and association of apoE-enriched liposomes to B16 melanoma cells in culture. At 2–3 days before the experiment, cells were transferred to 22-mm multiwell culture dishes at a density of 20,000–40,000 cells/well. When subconfluency was achieved, the culture medium was replaced by preincubation medium [including 1% (w/v) BSA instead of fetal calf serum]. The cells were washed three times (10, 10, and 30 min) and then cultured for 18 hr in this medium. After two washes with preincubation medium, experiments were started by the addition of the indicated amounts of LDL and/or liposomes in this medium. When liposomes were preincubated (30 min at 37°) with apoE [EYPC/apoE 10:0.5 (w/w)], unbound protein was removed by ultracentrifugation as detailed above. To determine the specific binding of the apoE-enriched liposomes, the cells were incubated (3 hr at 4°) with the [^{3}H]CO-labeled particles. Subsequently, the cells were washed five times with ice-cold wash buffer (0.15 M NaCl, 2.5 mM $CaCl_2$, 50 mM Tris·HCl, pH 7.4) containing

0.2% (w/v) BSA, followed by two washes with the same buffer without BSA. The cells were lysed by the addition of 1 ml of 0.1 N NaOH, and the amounts of protein as well as radioactivity in the lysate were determined. The association of apoE-enriched liposomes to the cells was assayed by incubating the cells (3 hr at 37°) with the [^{3}H]CO-labeled particles and the indicated additives. The culture plates were then placed on ice, and the cell-associated radioactivity was determined as described.

Results

Synthesis and characterization of liposomes. Particles were created by sonication of EYPC and ([^{3}H])CO at a molar ratio of 25:1, followed by concentration and purification using density gradient ultracentrifugation. A typical distribution pattern of the lipids over the gradient is depicted in Fig. 1 (*top*). The majority of constituents coincided within an opalescent layer (fractions 14 and 15) recovered at a mean density of 1.014 g/ml, which is intermediate between the range of densities of LDL (1.019–1.063 g/ml) and VLDL (0.96–1.006 g/ml). The high molar ratio of phospholipid (EYPC) versus that of neutral lipid (CO) (28.9 ± 2.5; mean ± standard error, five experiments) indicates a liposomal character of these particles, which was confirmed by the possibility of incorporating a self-quenching concentration of 5-carboxyfluorescein during particle synthesis that was retained during purification by ultracentrifugation (not shown). In addition, some emulsion particles were formed (fractions 19 and 20), as indicated by their low density (≤1.006 g/ml), a low EYPC/CO ratio of 7.6 ± 1.6 (mean ± variation, two experiments), and their inability to incorporate carboxyfluorescein. The purified liposomes were homogeneous particle populations (low polydispersity of 0.198 ± 0.001) with a mean size of only 28.9 ± 0.9 nm (mean ± standard deviation, 21 experiments) as measured by photon correlation spectroscopy, predicting a unilamellar particle structure. For comparison, under similar conditions, the size of human LDL was determined to be 20.8 ± 0.7 nm with a polydispersity of 0.192 ± 0.005 (mean ± standard deviation, three experiments). The average liposome size and polydispersity did not change during storage at room temperature for ≥4 months after preparation, indicating their long term stability. Freeze fracture electron microscopy revealed that the apparent diameters of the vast majority of particles were in the range of 15–30 nm, which confirms their small size and homogeneity (Fig. 1, *bottom*). The electrophoretic mobility of the liposomes relative to bromphenol blue was low (0.12 ± 0.01; mean ± standard deviation, seven experiments) and lower than that of LDL (0.21 ± 0.01; mean ± standard deviation, three experiments), confirming their low negative surface charge. The combination of a small size and low surface charge is beneficial with respect to avoiding phagocytotic uptake by cells of the RES (31) and scavenger receptors on endothelial cells,[1] respectively.

Association of apoE with liposomes. To mimic LDL and display a high affinity for the LDLr, at least four copies of apoE should become associated with the liposomes (18), without disruption of the liposome bilayer structure. Incubation of ^{125}I-apoE with increasing amounts of liposomes resulted in an increased recovery of apoE with the particles, as apparent from a (partial) shift in the density of apoE (>1.15 g/ml) toward the liposomal density (Fig. 2). As a consequence, the density of the liposomes increased dependent on the apoE concentration to 1.04–1.05 g/ml (fraction 11), which is in the density range of LDL (1.019–1.063 g/ml). The absence of an additional density shift at a further reduction of the liposo-



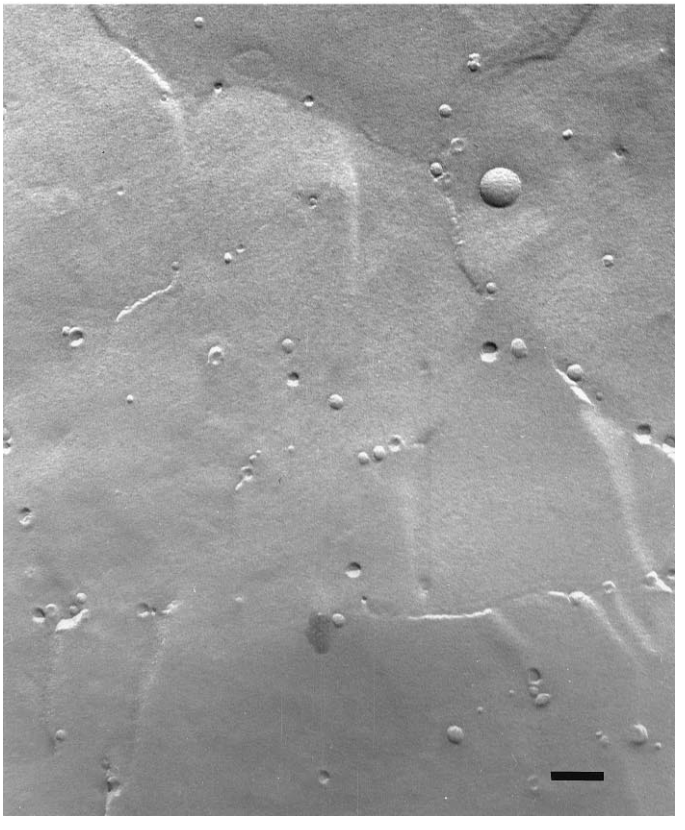


Fig. 1. Density gradient ultracentrifugation and freeze fracture electron micrograph of liposomes. *Top*, liposomes were prepared by sonication of EYPC and ([^{3}H])CO [25:1 (w/w)] and purified by density gradient ultracentrifugation (40,000 rpm for 18 hr at 4°). The gradient was subdivided, and fractions were assayed for density (●), EYPC (○), CO (△), and [^{3}H]CO (▽). *Bottom*, liposomes were isolated at a mean density of 1.014 g/ml (fractions 14 and 15) and visualized by freeze fracture electron microscopy. *Scale bar*, 100 nm.

[1] P. C. N. Rensen and T. J. C. van Berkel, unpublished observations.

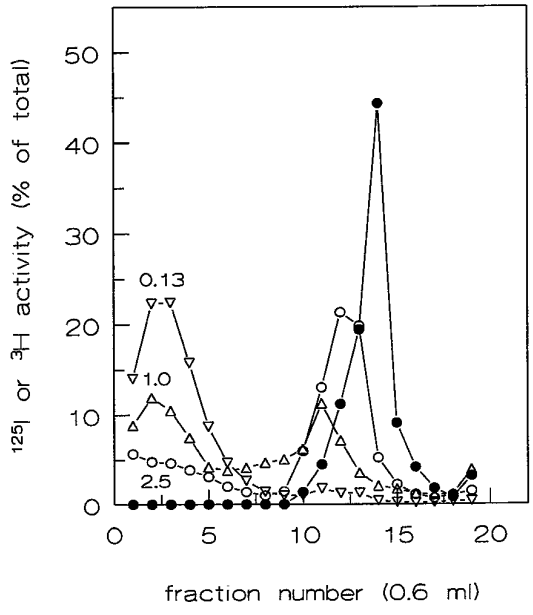


Fig. 2. Acquisition of apoE by liposomes as assessed by density gradient ultracentrifugation. ^{125}I-apoE (25 μg) was incubated (30 min at 37°) with 0.13 mg (▽), 1.0 mg (△), and 2.5 mg (○) of liposomal phospholipid. The incubations or [^{3}H]CO-labeled liposomes (●) (0.5 mg of phospholipid) were subjected to density gradient ultracentrifugation, the gradients were subdivided, and the fractions were assayed for ^{125}I (○, ▽, △) or ^{3}H-radioactivity (●).

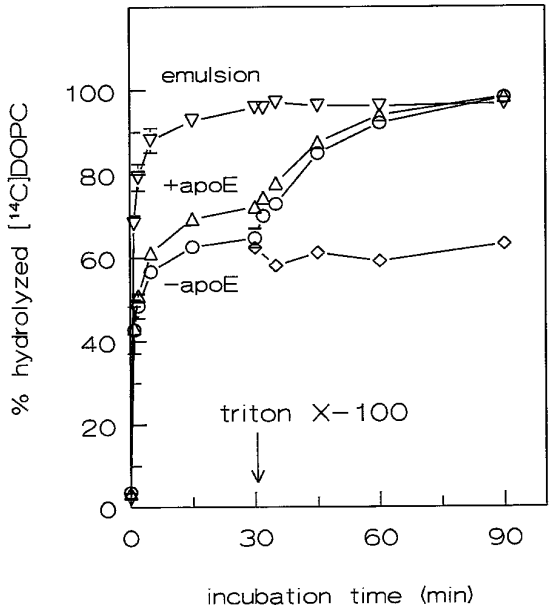


Fig. 3. Effect of apoE on liposomal integrity as assessed by phospholipase A_2 digestion. [^{14}C]DOPC-labeled liposomes (5 mg of phospholipid) were preincubated at room temperature, without (○, ◇) or with (△) preincubation (30 min at 37°) with 300 μg of apoE. At t = 0, 10 units of phospholipase A_2 was added to the liposomes or [^{14}C]DOPC-labeled emulsions (▽), followed by the addition of Triton X-100 [1% (v/v)] to all incubations at t = 30 min except for a separate incubation of control liposomes (◇). [^{14}C]DOPC, [^{14}C]oleoyl phosphatidylcholine, and [^{14}C]oleate were extracted and separated as described.

mal phospholipid amount from 1.0 to 0.13 mg indicates that saturation of the liposomal shell with apoE is achieved at an EYPC/apoE weight ratio of 10:0.25. From the established correlation between the amount of phospholipid and particle number (32), it can be calculated that 5.8 ± 0.7 molecules of apoE (mean ± standard deviation, three experiments) are acquired per liposome particle at this ratio. Acquisition of apoE did not substantially change the electrophoretic mobility of the liposomes on agarose.

Effect of apoE association on the liposomal structure. The effect of apoE insertion into the liposomal bilayer was determined by phospholipid digestion using phospholipase A_2, to which only those phospholipid molecules are accessible that are exposed in the outer layer (26). Radioactive DOPC was used as a marker of the liposomal phospholipid because oleate groups constitute 27% of the fatty acids in EYPC (32). Incubation of liposomes with phospholipase A_2 led to the hydrolysis of [^{14}C]DOPC reaching a plateau value of ~60–65%, which increased to 100% only after the addition of Triton X-100 (Fig. 3). DOPC within the inner layer apparently is not accessible to the enzyme unless the liposomal structure is disrupted. As a control, DOPC within a similarly sized emulsion, which is stabilized by a phospholipid monolayer, was quantitatively digested without the need of particle disruption. Preincubation of liposomes with sufficient apoE to saturate the particle [EYPC:apoE 10:0.6 (w/w)] did not substantially change the biphasic appearance of the DOPC digestion curve (Fig. 3), indicating that the liposomal unilamellar structure is retained on insertion of apoE.

Effect of serum on the integrity of apoE-enriched liposomes. Liposomes that are designed for intravenous administration should retain their structural integrity and preassociated apoE within the circulation; therefore, the effect of apoE and/or rat serum on the lipid constituents of liposomes was checked. The addition of shell-saturating amounts of apoE [EYPC:apoE 10:1 (w/w)] resulted in an increased particle density as judged from the shifted patterns of [^{3}H]CO and [^{14}C]DOPC over the gradients after ultracentrifugation (Fig. 4). These observations are consistent with the patterns obtained with ^{125}I-apoE (Fig. 2). Incubation of the liposomes with serum (with or without preincubation with apoE) led to an even higher increase in the density of the nonexchangable liposomal [^{3}H]CO constituent (1.05–1.06 g/ml). Under these conditions, [^{14}C]DOPC appeared as two bands. The band spanning fractions 7–10 coincided with HDL as determined by the cholesteryl ester distribution over the gradient obtained from rat serum (not shown), indicating a partial transfer of radiolabel from liposomes to HDL.

Reisolation of liposomes from incubations with serum is difficult because the resulting particles show densities overlapping those of LDL and HDL and sizes similar to LDL and VLDL (remnants). The effect of rat serum on the liposomal retention of preassociated apoE was therefore determined by subjecting fractions of subdivided gradients obtained from ultracentrifugation of incubation mixtures with serum to 5–20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Scanning of the resulting Coomassie blue-stained gels showed that within the liposomal peak fraction, the ratio of apoE to apoAI (the main protein constituent of HDL, of which the presence is explained by the overlapping distributions of liposomes and HDL) was 1.8-fold higher when liposomes were preincubated with apoE compared with incubations of apoE-deficient liposomes with serum or serum alone. In addition, no other Coomassie blue-detectable opsonins could be observed within the apoE-enriched liposomal

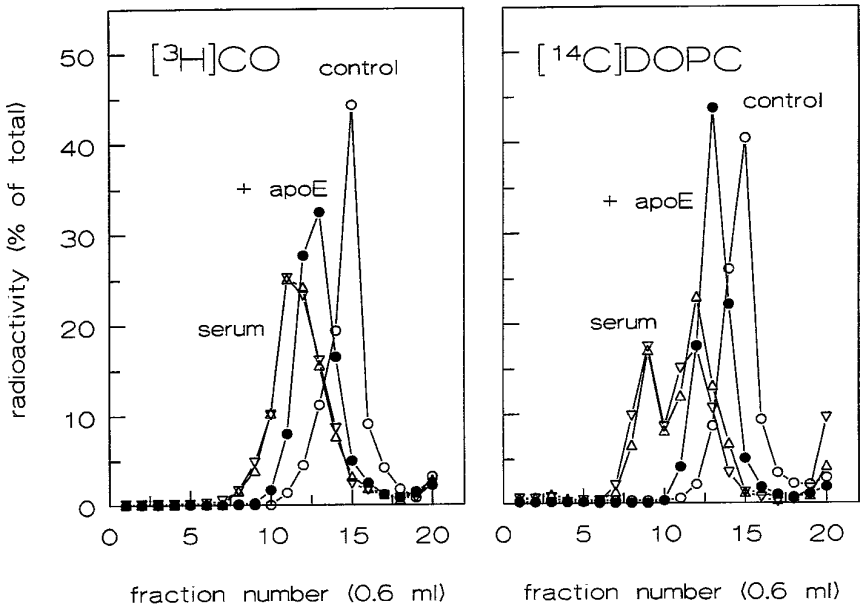


Fig. 4. Effect of apoE and serum on the density and integrity of liposomal lipids as assessed by density gradient ultracentrifugation. Liposomes (○) (0.5 mg of phospholipid) that were labeled with [^{3}H]CO (*left*) or [^{14}C]DOPC (*right*) were incubated (30 min at 37°) with 50 μg of apoE (●) or 1 ml of rat serum without (▽) or with (△) previous incubation with apoE. The incubations were subjected to density gradient ultracentrifugation, the gradients were subdivided, and the fractions were assayed for ^{3}H- or ^{14}C-radioactivity.

fractions compared with the similar fractions obtained from serum without liposomes (not shown).

Serum decay, liver uptake, and extrahepatic distribution of the apoE-enriched liposomes in rats. The serum decay and liver uptake in rats of [^{3}H]CO-labeled liposomes in the absence or presence of apoE are depicted in Fig. 5. The liposomes showed a very low uptake by the liver (2.9 ± 2.4% of the injected dose) and a high remaining fraction within the serum (81.3 ± 2.5%) at 30 min after injection. The serum clearance and liver uptake (6.8 ± 3.4%) did not significantly change ($p > 0.05$) after preincubation with apoE (Fig. 5, *left*). At 30 min after injection, the combined recovery of radiolabel in the lungs and spleen was negligible (<0.8%; not shown), indicating a low affinity of the apoE-enriched liposomes for cells of the RES. Normal rats show virtually no expression of a functional LDLr (33); the affinity for this receptor was therefore assessed in 17α-EE-treated rats. This treatment leads to a selective up-regulation of the LDLr on rat liver parenchymal cells and an increased uptake of LDL by the adrenal glands (29, 34). Placebo treatment of rats with propylene glycol did not affect the serum half-life of apoE-enriched liposomes, and the association with the liver at 30 min after injection (10.9 ± 1.3%) was not significantly changed ($p > 0.05$) (Fig. 5, *right*). In contrast, the serum clearance was substantially (2.5-fold) increased on pretreatment with 17α-EE ($t_{½}$ = 123 ± 10 min versus >5 hr). Concomitantly, a selective 2.0- and 3.8-fold increased specific uptake (i.e., per gram wet weight) was observed for the liver (3.7 ± 0.8% versus 1.9 ± 0.3%) and adrenals (8.4 ± 0.7% versus 2.2 ± 0.2%), respectively, at 120 min after injection (Fig. 6, *right*), which is indicative of an LDLr-dependent metabolic behavior.

These data imply that apoE is largely retained on the liposomes *in vivo*. To substantiate this hypothesis, the liver uptake and serum decay of free ^{125}I-apoE and ^{125}I-apoE-labeled liposomes were subsequently determined. Injection of

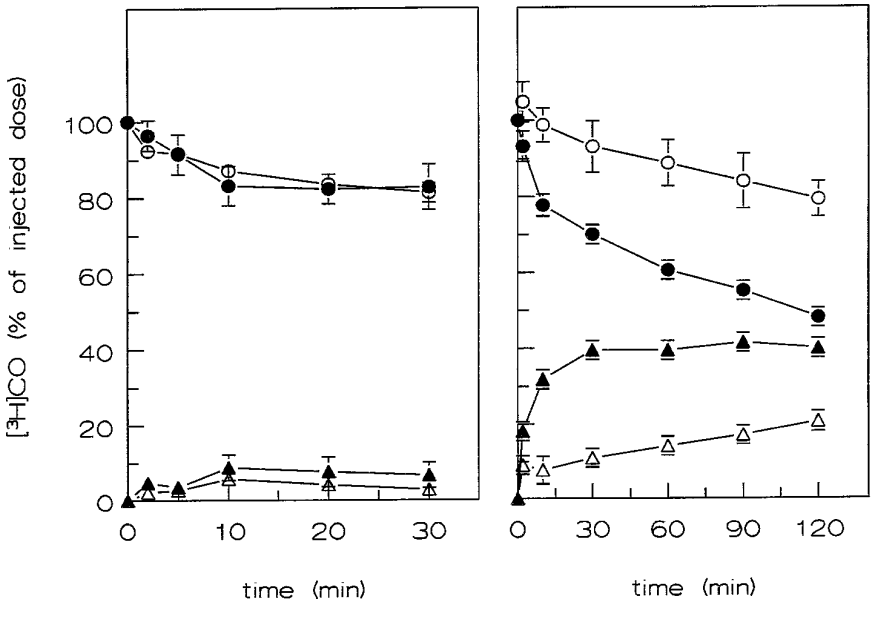


Fig. 5. Liver uptake and serum decay of apoE-enriched liposomes in control and 17α-EE-pretreated rats. *Left*, [^{3}H]CO-labeled liposomes (1.0 mg of phospholipid) were injected into anesthetized rats without (○, △) and with (●, ▲) previous incubation (30 min at 37°) with 100 μg of apoE. *Right*, [^{3}H]CO-labeled liposomes (1.0 mg of phospholipid) were injected after previous incubation (30 min at 37°) with 100 μg of apoE into anesthetized rats pretreated with polyethylene glycol (○, △) versus 17α-EE (●, ▲). At the indicated times, the serum decay (○, ●) and liver uptake (△, ▲) were determined. Liver values are corrected for serum radioactivity. Values are mean ± standard error from three experiments.

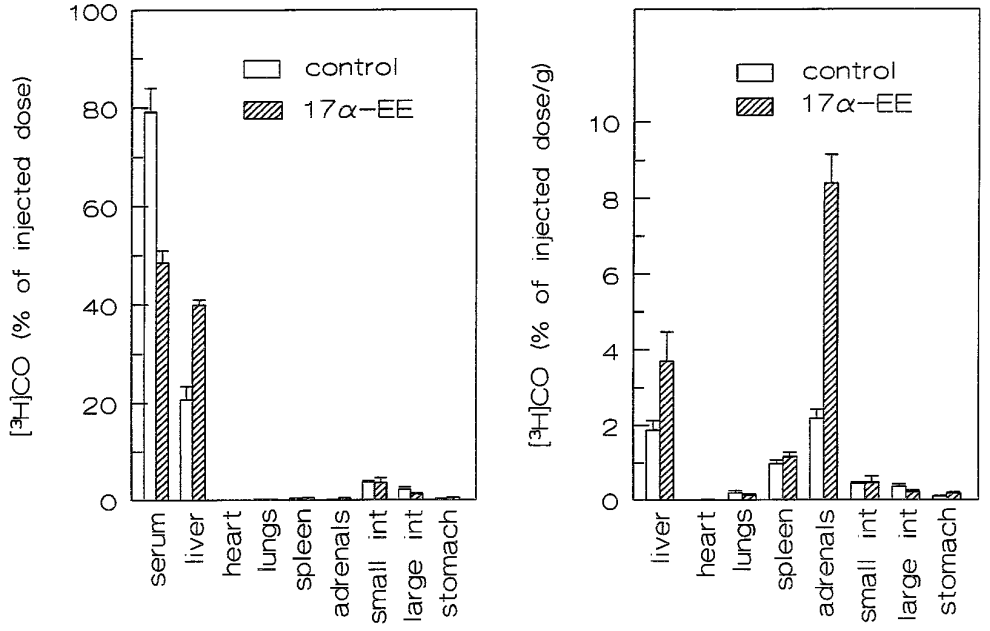


Fig. 6. Organ distribution of apoE-enriched liposomes in control and 17α-EE-pretreated rats. [^{3}H]CO-labeled liposomes (1.0 mg of phospholipid) were injected after previous incubation (30 min at 37°) with 100 μg of apoE into anesthetized propylene glycol-treated rats (□) versus 17α-EE-treated rats (▨). After 120 min of circulation, the total (*left*) and specific (i.e., per gram wet weight) (*right*) organ distributions were determined. Recoveries of ^{3}H-radioactivity in the rats exceeded 95%. Values are corrected for serum radioactivity and represent mean ± standard error from three experiments.

free ^{125}I-apoE resulted in a biphasic serum decay, with 71.4 ± 5.8% (mean ± standard deviation, three experiments) of the injected dose being cleared from the circulation within 5 min after injection. Concomitantly, 62.4 ± 5.2% (mean ± standard deviation, three experiments) of the injected dose was recovered in the liver at 5 min after injection. ^{125}I-apoE, which remains in the serum, showed a long half-life of >5 hr and was mainly recovered in the HDL fractions at 30 min after injection. Preincubation of ^{125}I-apoE with liposomes strongly decreased the clearance of ^{125}I-apoE from the serum in the first phase (26.6 ± 2.0% after 5 min after injection), as well as its association with the liver (12.5 ± 1.5% at 5 min; mean ± variation; two experiments). Accordingly, the majority of radiolabel was still associated with the liposomes at 30 min after injection.

Effect of apoE and *in vivo* circulation on the integrity of the liposomal core. To investigate the suitability of these liposomes for the targeting of hydrophilic agents, the integrity of the liposomal core was assessed *in vivo* using water-soluble [^{3}H]inulin as core marker. Sonication of liposomes in a buffer containing [^{3}H]inulin with subsequent purification by ultracentrifugation and extensive dialysis resulted in the recovery of 0.24 ± 0.02% (mean ± standard deviation, three experiments) of the added inulin within the liposomes. These data imply an internal volume of 0.55 ± 0.04 μl/mg of phospholipid, provided the incorporated inulin did not leak from the liposomes during isolation. Because this value was predicted for 30-nm particles on the nomogram relating lipid weight, diameter, and internal volume for unilamellar vesicles (32), inulin leakage apparently did not occur. Incubation of these liposomes in PBS for 0 or 60 min at 37° also did not lead to substantial leakage of entrapped inulin (5.4%) (Fig. 7, *left*). In contrast, incubation of liposomes with excess apoE [EYPC:apoE 10:1 (w/w)] resulted in

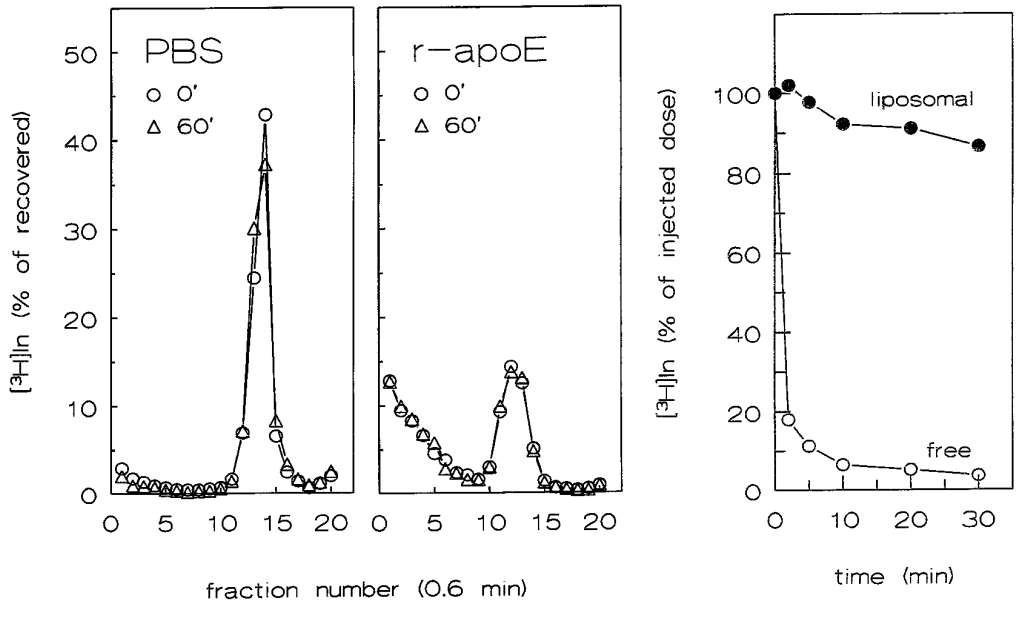


Fig. 7. Effect of apoE and *in vivo* circulation on the integrity of the liposomal aqueous core. *Left*, liposomes (0.5 mg of phospholipid) with encapsulated [^{3}H]Inulin (*In*) were incubated with PBS or 50 μg of apoE in PBS for 0 min (○) or 60 min (△) at 37°. The incubations were subjected to density gradient ultracentrifugation, the gradients were subdivided, and the fractions were assayed for ^{3}H-radioactivity. *Right*, [^{3}H]inulin (○) or [^{3}H]inulin-containing liposomes (●) (1.0 mg of phospholipid) were injected into anesthetized rats, and the serum decay was determined.

the instantaneous partial loss of [^{3}H]inulin (50.3%), which was independent of the incubation period (0–60 min). This indicates a temporary liposomal destabilization during insertion of apoE and suggests that associated apoE does not dissociate or exchange between liposomes, which would lead to a gradually increasing loss of inulin with incubation time.

Free [^{3}H]inulin was extremely rapidly cleared from the circulation ($t_{½}$ = 0.7 min) through urinary secretion (59.1% of the injected dose was recovered in the kidneys, bladder, and urine at 30 min after injection; not shown). In contrast, liposomal encapsulation of [^{3}H]inulin resulted in a ~400-fold increased serum half-life of inulin, which is comparable to that of [^{3}H]CO-labeled liposomes (Fig. 7, *right*). In addition, the urinary excretion of inulin (2.1%) showed a 28-fold reduction (not shown).

Intrahepatic processing of apoE-enriched liposomes in rats. Cellular uptake as mediated by the LDLr should be coupled to a lysosomal uptake route, which was checked by performing a subcellular distribution of the liposomal marker [^{3}H]CO within the liver at 30 min after injection of apoE-enriched liposomes into a 17α-EE-pretreated rat. The distribution pattern of [^{3}H]CO was intermediate between those of the lysosomal marker acid phosphatase and the microsomal marker glucose-6-phosphatase, whereas the distribution of the cytoplasmic marker lactate dehydrogenase was clearly different (Fig. 8). Because endosomes are recovered within the microsomal fraction (35), these data indicate that the liver association of the apoE-enriched liposomes is coupled to an endosomal uptake route toward lysosomes.

The intrahepatic processing of apoE-enriched [^{3}H]CO-labeled liposomes was subsequently analyzed on the basis of the rate of [^{3}H]CO hydrolysis after injection into control and 17α-EE-pretreated rats. Although the liver association rate of the liposomes was higher within 17α-EE-pretreated rats (Fig. 5), the rate of [^{3}H]CO hydrolysis was similar under both conditions, with 50% release of [^{3}H]cholesterol between 30 and 60 min after injection (Fig. 9).

Binding and association of apoE-enriched liposomes to B16 melanoma cells. To verify the affinity and apoE-mediated specificity of liposomes for the LDLr *in vitro*, their binding and uptake characteristics were determined on B16 melanoma cells. Recently, we reported that these tumor cells show a high expression of the LDLr (~220,000/cell) with a high affinity for LDL (11 nM) and specific uptake of LDL *in vivo* when C57/Bl6 mice were subcutaneously inoculated (36). On the basis of the binding curves of [^{3}H]CO-labeled apoE-enriched liposomes in the absence or presence of excess unlabeled particles, it is evident that the liposomes show a high affinity for a binding site on B16 cells with a calculated dissociation constant (K_d) of 0.77 ± 0.09 nM and a maximal binding (B_{max}) value of 1298 ± 20 ng of liposomal phospholipids/mg of cell protein (Fig. 10). With the assumed values of 1.09×10^6 cells/mg of cell protein and 7.62×10^{13} lipo-

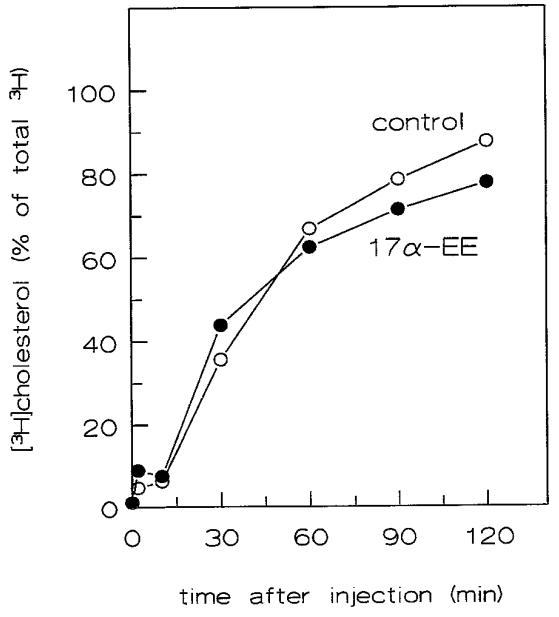


Fig. 9. Intrahepatic hydrolysis of liposomal CO in control and 17α-EE-pretreated rats. [^{3}H]CO-labeled liposomes (1.0 mg of phospholipid) were injected after previous incubation (30 min at 37°) with 100 μg of apoE into anesthetized polyethylene glycol- (○) and 17α-EE-treated (●) rats. At the indicated times, liver lobules were taken, immediately frozen in liquid N_2, and homogenized in ice-cold PBS. Lipids were extracted and [^{3}H]cholesterol was separated from [^{3}H]CO by thin layer chromatography.

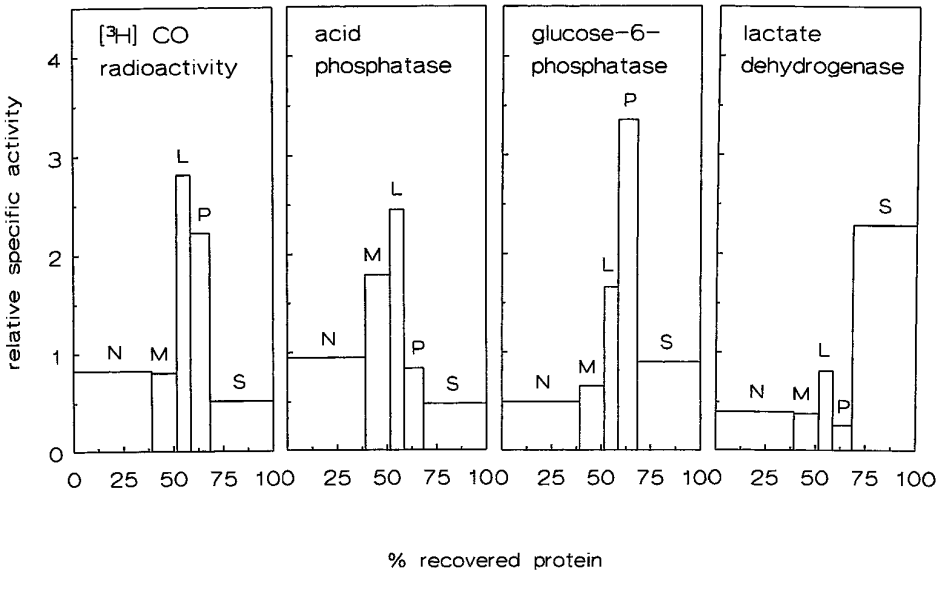


Fig. 8. Subcellular distribution of apoE-enriched liposomes in 17α-EE-pretreated rats. [^{3}H]CO-labeled liposomes (1.0 mg of phospholipid) were injected after previous incubation (30 min at 37°) with 100 μg of apoE into an anesthetized rat. After 30 min of circulation, the liver was perfused and divided into subcellular fractions as described. Recoveries of radioactivity, protein, and marker enzymes were 80–90%. *Bars* (*left* to *right*), fractions in the order in which they were isolated: nuclear (*N*), mitochondrial (*M*), lysosomal (*L*), microsomal (*P*), and final supernatant (cytosol, *S*) fractions. *% Recovered protein*, relative specific activity (percentage of total recovered activity divided by percentage of total recovered protein).

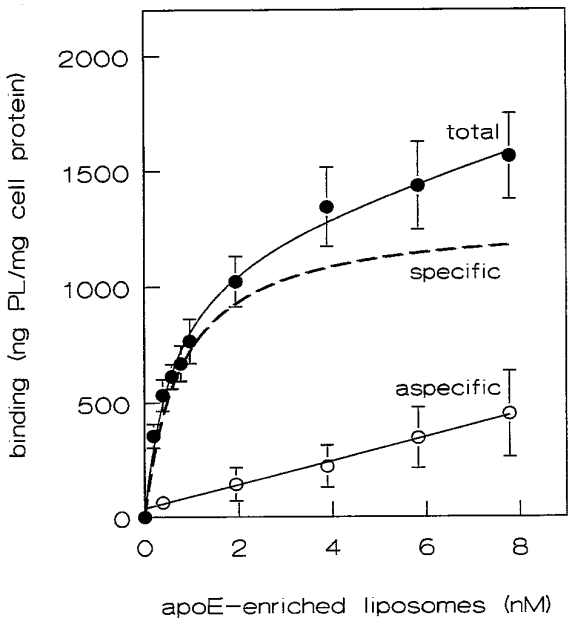


Fig. 10. Binding of apoE-enriched liposomes to B16 melanoma cells. B16 cells were incubated with increasing concentrations of [^{3}H]CO-labeled apoE-enriched liposomes for 3 hr at 4°, and the the total amount of cell-bound ^{3}H activity was determined (●). The non-specific binding, as assessed in the presence of 78 nM of unlabeled particles (○), was subtracted from the total binding to yield the receptor-specific binding (*dotted line*). Values are mean ± standard error of three separate experiments.

somes/mg of phospholipid, it can be calculated that ~90,000 liposomes/cell can be maximally bound.

To verify whether the LDLr represents the specific recognition site on B16 cells for the liposomal apoE moiety, competition studies were performed on the association of apoE-enriched liposomes (Fig. 11). ApoE-enriched liposomes, but not apoE-deficient liposomes, effectively competed for the association of [^{3}H]CO-labeled apoE-enriched liposomes (Fig. 11, *left*), indicating that apoE is crucial for the cellular interaction of liposomes. LDL also competed for the liposomal association but at much higher concentrations (on a molar basis) (Fig. 11, *right*). It can thus be concluded that LDL and apoE-enriched liposomes bind to the same recognition site on B16 cells (i.e., the LDLr) and that the apoE-enriched liposomes show a higher affinity for this site than does LDL.

Discussion

The severe adverse side effects of many cytostatics, which are a consequence of their nonspecific body distribution, can preclude the administration of these drugs at a dosage that establishes maximum efficacy. Current strategies that aim at optimization of drug delivery to cancer cells involve vehicles that selectively target to tumor cells, such as monoclonal antibodies, growth factors, and hormones. A general problem associated with these compounds, however, is their poor cellular internalization. The increased LDLr expression on certain tumors, as related to the overuse of cholesterol to meet their high rate of membrane synthesis, has stimulated research on LDL as a drug carrier. LDL is effectively bound, internalized, and processed by the LDLr, but the need for isolation from human blood precludes its large-scale pharmaceutical application. To overcome this problem, we explored the feasibility of synthesizing particles from commercially available lipids and recombinant protein, with physicochemical and biological properties similar to those of native LDL.

A sonication procedure was applied to synthesize homogeneous small unilamellar liposomes from EYPC and CO, with a size (~29 nm) and an overall negative charge (R_F = 0.12) similar to those of LDL, that can be stored for ≥4 months at room temperature. Because of its amphipathic character, apoE spontaneously associates with the liposomes, leading to an increase in liposomal density from 1.014 to 1.04–1.05 g/ml, which is similar to LDL. Insertion of apoE led to a transient liposome destabilization, as evident from partial inulin leakage from the aqueous core, but did not disrupt the liposomal structure. Approximately five or six molecules of apoE associated per particle, predicting a maximum affinity for the LDLr (18).

Liposomes that are designed for intravenous injection should retain their integrity within the circulation. Incuba-

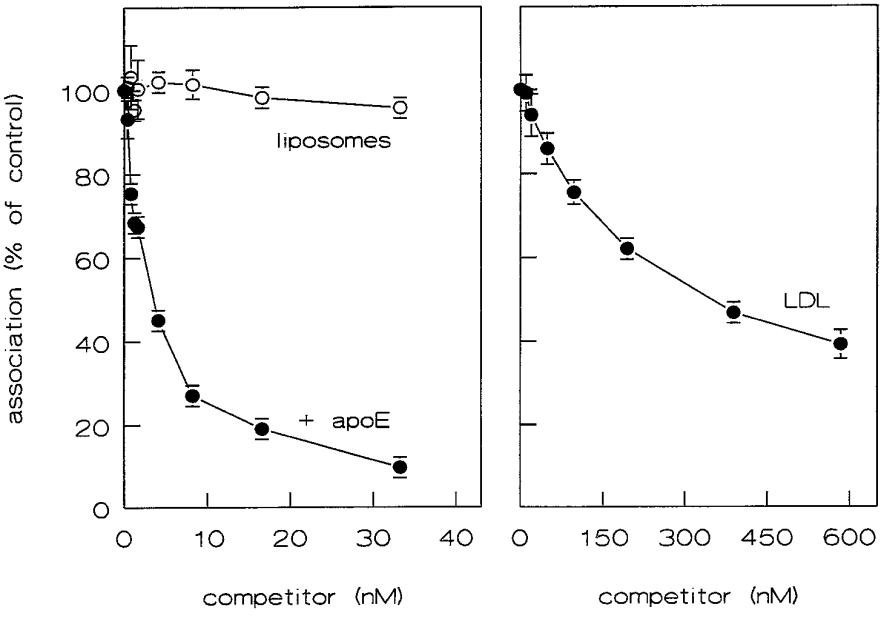


Fig. 11. Effects of apoE-enriched liposomes and LDL on the association of [^{3}H]CO-labeled apoE-enriched liposomes with B16 melanoma cells. B16 cells were incubated (3 hr at 37°) with 1.6 nM [^{3}H]CO-labeled apoE-enriched liposomes (13 μg/ml of phospholipid) in the presence of increasing amounts of unlabeled liposomes without (○) or with (●) apoE (*left*) or LDL (*right*), and the amount of cell-associated radioactivity was determined. Data are expressed as percentage of association in the absence of competitor [7.0 ± 0.6 (*left*) and 6.7 ± 0.2 (*right*) μg of phospholipid/mg of cell protein]. Values are mean ± variation of two separate experiments.

tion of apoE-enriched liposomes with serum did not have an major effect on the apoE or CO moiety. Serum opsonins, which could promote opsonophagocytosis of liposomes by cells of the RES (31), also were not detected on Coomassie blue-stained polyacrylamide gels. In serum, only a partial transfer occurred of liposomal phospholipid to HDL, which presumably indicates mutual phospholipid exchange as mediated via phospholipid transfer factors (37). Despite this phospholipid transfer, the integrity of the liposomal aqueous core was retained *in vivo* in that no leakage of inulin was observed when administered intravenously into rats. These data are in accordance with the observation that a much smaller compound, like carboxyfluorescein, is retained within phosphatidylcholine liposomes even in the presence of a purified phospholipid transfer factor (37). The preservation of the liposomal aqueous core *in vivo* also indicates that in contrast to versatile triglyceride-rich emulsions (28), endogenous apoE is not efficiently acquired from serum because apoE insertion would lead to leakage (and thus a decreased half-life) of entrapped inulin.

The liposomes showed a relatively long half-life in rats of >5 hr, comparable to LDL, as a consequence of its low affinity for the RES (Kupffer cells, lungs, and spleen). Previous association of apoE did not alter their serum kinetics, which was expected because rats show virtually no expression of a functional LDLr (33). Selective up-regulation of the LDLr on rat liver parenchymal cells by pretreatment with 17α-EE (29, 34) indeed resulted in a 2.5-fold shortened serum half-life and an increased uptake by the liver (2.0-fold) and adrenals (3.8-fold) but only when liposomes had been previously enriched with apoE. The liver uptake rate of these apoE/liposome complexes was comparable to that reported for [^{3}H]CO-labeled LDL (~40% at 60 min after injection) (38). These data indicate that a substantial amount of apoE is retained on the liposome after injection, which was confirmed by a 5-fold reduction of the liver uptake of free ^{125}I-apoE (62.4 ± 5.2%) after incubation with liposomes (12.5 ± 1.5%).

On LDLr-mediated association of the apoE-enriched liposomes with the liver of 17α-EE-pretreated rats, the liposomes were subsequently internalized via an endocytotic uptake route and degraded, as is evident from the time-dependent hydrolysis of CO. This phenomenon also occurred in control rats, which can be attributed to the low expression of the LDLr but may also result from an LDLr-independent uptake route of an as-yet-unknown nature. Preliminary data showed that in both 17α-EE and control rats, the released cholesterol was eventually secreted into the bile as bile acids after a lag time of ~20 min (not shown).

Because these liposomes are designed for tumor-directed drug delivery, their uptake characteristics were determined on LDLr-expressing B16 melanoma cells *in vitro*. Indeed, an apoE-specific binding as mediated by the LDLr was observed with an affinity (K_d = 0.77 ± 0.09 nM) that was 14.3-fold higher than that for LDL (K_d = 11 nM) (35). The number of apoE-enriched liposomes that was maximally bound per cell (B_{max}) was ~2.5-fold lower (~90,000) than that for LDL (~220,000) (36). This apparent discrepancy can be explained by the multireceptor binding model of Pitas *et al.* (20), who observed that up to four times as many LDL than apoE-containing HDL$_c$ particles were required for LDLr saturation at maximal binding to human fibroblasts. The fact that we determined a 2.5-fold (instead of a 4-fold)-reduced maximal particle binding can result from the strong dependency of the LDLr expression on the cellular confluency. Because the LDLr expression decreases with increasing confluency,[2] slight variations in the confluency will affect the calculation of B_{max}.

With respect to tumor targeting, the presented apoE-enriched liposomes possess various advantageous properties compared with LDL. First, the affinity of the LDLr for apoE is much higher than that for apoB-100 as established using human fibroblasts (20–25-fold) (19) and now demonstrated for B16 melanoma cells (14-fold). This might constitute an important observation with respect to future drug targeting application in that LDL shows a relatively high nonspecific (non-LDLr-mediated) uptake *in vivo*. Although 60–80% of the clearance is mediated by the LDLr, as described by Brown and Goldstein (7), the remainder is cleared via non-LDLr-mediated pathways (39). Down-regulation of the LDLr on nontumor tissues, as in the clinical application of cytotoxic LDL, will result in an even higher contribution of non-LDLr-mediated LDL clearance. The much higher affinity of apoE-enriched liposomes for the LDLr might thus lead to more specific drug targeting. Second, the use of commercially available lipids and, in particular, the availability of human recombinant apoE (in contrast to apoB-100) facilitates the application of these recombinant particles as pharmaceutical drug carriers. Third, the presence of an aqueous particle core allows the incorporation of hydrophilic compounds. This property could be particularly interesting with respect to the *in vivo* tumor delivery of antisense oligodeoxynucleotides. Because of their instability in serum and nonspecific body distribution, these compounds have been coupled to hydrophobic structures, such as cholesterol, and subsequently complexed with LDL (40). Although the cellular uptake and efficacy of these oligodeoxynucleotides were improved *in vitro*, they seemed to rapidly dissociate from LDL *in vivo* (40), presumably because of their bulky anionic character. Because a flip-flop mechanism did not occur for liposomal phospholipid (i.e., exchange of phospholipid between the inner and outer layers) and is certainly not expected for polyanionic oligodeoxynucleotides, leakage of derivatized oligodeoxynucleotides would be prevented on liposomal incorporation. Fourth, the liposomes showed a low tendency to aggregate because their size was stable for ≥4 months at room temperature, and they could therefore be used for *in vivo* application for extended periods without the need of particle size preservation, which is in contrast to LDL or emulsions (15, 28). Finally, the higher affinity for the LDLr of the apoE-enriched liposomes compared with LDL allows a more efficient tumor localization of the liposomes compared with LDL/drug complexes by virtue of a more efficient competition with endogenous LDL for LDLr-mediated tumor uptake.

In conclusion, we demonstrated the feasibility of using an endogenous receptor-mediated uptake route for liposome delivery. We anticipate that these apoE-enriched liposomes represent a conceptual advance in the development of a selective LDLr-targeted antitumor therapy compared with LDL.

[2] Versluis, A.J., M.K. Bysterbosch, and T.J.C. van Berkel, unpublished observations.

Acknowledgments

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Send reprint requests to: Dr. Patrick C. N. Rensen, Division of Biopharmaceutics, Leiden-Amsterdam Center for Drug Research, University of Leiden, Sylvius Laboratories, P.O. Box 9503, 2300 RA Leiden, The Netherlands. E-mail: p.rensen@lacdr.leidenuniv.nl