Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 242–246, January 1988
Medical Sciences

Low density lipoprotein receptor-independent hepatic uptake of a synthetic, cholesterol-scavenging lipoprotein: Implications for the treatment of receptor-deficient atherosclerosis

(liposome/radiopharmaceutical/intravenous fat emulsion/Watanabe heritable hyperlipidemic rabbits/familial hypercholesterolemia)

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Communicated by Rosalyn S. Yalow, September 17, 1987 (received for review June 15, 1987)

ABSTRACT **The metabolism of infused ^{111}In-labeled phospholipid liposomes was examined in Watanabe heritable hyperlipidemic (WHHL) rabbits, which lack low density lipoprotein (LDL) receptors, and in normal control rabbits. The half-times ($t_{1/2}$) for clearance of ^{111}In and excess phospholipid from plasma were 20.8 ± 0.9 hr and 20.3 ± 4.6 hr in WHHL and 20.0 ± 0.8 hr and 19.6 ± 2.2 hr in the normal rabbits (means ± SEM; n = 4). By 6 hr postinfusion, the plasma concentration of unesterified cholesterol increased by 2.2 ± 0.23 mmol/liter in WHHL and 2.1 ± 0.04 mmol/liter in normal rabbits, presumably reflecting mobilization of tissue stores. Disappearance of excess plasma cholesterol was >90% complete in both groups of rabbits by 70 hr postinfusion. By quantitative γ camera imaging, hepatic trapping of ^{111}In-labeled liposomes over time was indistinguishable between the two groups. At autopsy, the liver was the major organ of clearance, acquiring 22.0% ± 1.7% (WHHL) and 16.8% ± 1.0% (normal) of total ^{111}In. Aortic uptake of ^{111}In was <0.02%. Thus, mobilization of cholesterol and hepatic uptake of phospholipid liposomes do not require LDL receptors. Because phospholipid infusions produce rapid substantial regression of atherosclerosis in genetically normal animals, our results suggest that phospholipid liposomes or triglyceride phospholipid emulsions (e.g., Intralipid) might reduce atherosclerosis in WHHL rabbits and in humans with familial hypercholesterolemia.**

Thirty years ago, intravenous administration of dispersed phospholipid was shown to produce rapid substantial shrinkage of atherosclerotic lesions in experimental animals (1). The finding was confirmed by several groups (2–8), but, lacking an explanation (4, 5, 8, 9), it has remained largely ignored in the scientific literature.

Infusions of cholesterol-free phospholipid liposomes were recently shown to result in the appearance of vesicular lipoproteins in the plasma (10). These vesicular particles acquire unesterified cholesterol from native lipoproteins and from tissues, thereby causing a transient increase in the plasma concentration of this sterol (10, 11). Similar particles used in liposomal drug delivery systems have been shown to be cleared primarily by the liver (12–14). These observations support the hypothesis that the antiatherogenic effects of phospholipid infusions result in part from the scavenging of tissue cholesterol by synthetic vesicular particles, followed by delivery of this cholesterol to the liver (15).

The actual mechanism responsible for hepatic uptake of vesicular particles is unknown. Phagocytosis by the reticuloendothelial system has been hypothesized, but the evidence has been inconclusive. The relative contribution of hepatic reticuloendothelial (Kupffer) cells and hepatic parenchymal cells is in dispute (13, 16–19), and blockade of the reticuloendothelial system has been variably reported to enhance (20), slightly inhibit (21), and substantially inhibit (22) hepatic uptake.

It was recently proposed that hepatic apoprotein receptors may be involved in hepatic uptake of vesicular lipoproteins (15). Infused phospholipid liposomes have been shown to acquire apolipoproteins in the circulation, including apoE, apoA-I, and possibly apoA-IV (10, 23). Thus, receptors potentially involved in hepatic clearance of vesicular lipoproteins would include the low density lipoprotein (LDL) receptor (24) and the apoE receptor (25), both of which recognize apoE, and the putative apoA-I (26) and apoA-IV (27) receptors.

The possibility that hepatic clearance may be mediated by LDL receptors is of particular interest. LDL receptors were recently shown to mediate uptake of vesicular lipoproteins by cultured cells, which suggests a role for this receptor in catabolism in the whole animal (28). Concerning atherosclerosis, hepatic LDL receptors are impaired in several well-described atherogenic conditions, including cholesterol feeding (29) and genetic deficiencies (24, 30). Involvement of hepatic LDL receptors in the clearance of vesicular particles could limit the ability of phospholipid infusions to produce reverse cholesterol transport and thereby shrink atherosclerotic lesions in these conditions.

To determine the role of LDL receptors in the metabolism of vesicular particles, we infused labeled phospholipid vesicles into normal rabbits and into homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits, which have a near-total lack of functional LDL receptors (24, 30). Cholesterol mobilization, plasma clearance, and organ uptakes were compared in the two groups of animals. Our results suggest an antiatherogenic role for infused phospholipid in LDL receptor deficiency.

MATERIALS AND METHODS

Rabbits. Homozygous WHHL rabbits and control New Zealand White rabbits were raised in the colony established by the Rogosin Institute at The Rockefeller University. All rabbits were 2.5- to 3.2-kg males.

Labeled Phospholipid Liposomes and Labeled LDL. To trace the metabolism of infused liposomes, label entrapped within their aqueous cores is preferred. Surface labels, such

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Abbreviations: WHHL, Watanabe heritable hyperlipidemic; LDL, low density lipoprotein; NTA, nitrilotriacetic acid.

as radioactive cholesterol, phospholipid, or apoproteins, are unreliable, because of exchange with other lipoproteins and cell membranes (31, 32).

To achieve the high specific activities required for our studies, core-labeled liposomes were prepared by a modification (33) of the chelation technique of Mauk and Gamble (34). One gram of 97% pure soybean phosphatidylcholine (Calbiochem, San Diego, CA, catalogue no. 429415) was added to 10 ml of an aqueous solution of 0.9% NaCl/1 mM nitrilotriacetic acid (NTA). The mixture was sonicated at 0°C under N_2 for a total of 40 min (35). Unencapsulated NTA was separated from liposome-entrapped NTA by chromatography on Sephadex G-25; the liposomes eluted at the void volume. ^{111}In–tropolone (36) was then added to the NTA-containing liposomes. During a 30-min incubation at 60°C, ^{111}In–tropolone diffuses into the aqueous cores of the particles, where the ^{111}In is trapped by chelation to NTA. The process was stopped by adding ethylenediaminetetraacetic acid to the reaction mixture to achieve a concentration of 1 mM. Encapsulated ^{111}In–NTA was then separated from unencapsulated ^{111}In by a second gel filtration on Sephadex G-25. This method reliably entrapped 50–70% of added ^{111}In, as had been described (33). In contrast, direct sonication of phosphatidylcholine into an aqueous solution of ^{111}In–NTA entrapped only 2–5% of the label.

To achieve sufficient phospholipid mass for our experiments, unlabeled liposomes were prepared by sonicating mixtures of 1 g of soybean phosphatidylcholine with 10 ml of saline. Labeled and unlabeled liposomes were mixed together, sterilized by passage through a 0.45-μm filter (Millex-GS, Millipore), and then immediately injected into the animals. By gas–liquid chromatography (37), there was no detectable cholesterol in these preparations.

LDL was isolated from rabbit plasma by ultracentrifugation (38), washed by reflotation, and shown by gel electrophoresis to contain no contaminating albumin. This LDL was labeled with ^{99m}Tc by reductive coupling to [^{99m}Tc]pertechnetate (39, 40).

Clearance Experiments. Four WHHL and four control rabbits were each injected with a bolus of 325 mg of soybean phosphatidylcholine liposomes per kg of body weight. Total administered ^{111}In radioactivity was 80–100 μCi per rabbit (1 Ci = 37 GBq).

Plasma was obtained just before the injection (t = 0 hr) and at 10 min, 1 hr, 3 hr, 6 hr, 22 hr, 46 hr, 70 hr, and 116 hr after the injection. Concentrations of phospholipid (41), unesterified cholesterol (42), cholesteryl ester (42), and radioactivity were determined in each plasma sample. Radioactivity determinations were corrected for the physical half-life of ^{111}In. The biological half-times ($t_{1/2}$s) for clearance of radioactivity and excess phospholipid were determined for each rabbit by linear regression of semilogarithmically transformed data (43).

Simultaneous with each blood draw, each animal was imaged in an anterior view by a γ camera (General Electric, model GE400T) fitted with a medium energy collimator. Each image was acquired over 5–10 min using both photopeaks of ^{111}In (window, 156–190 keV and 222–272 keV). Each image contained >100,000 counts and was digitally stored in a 128 × 128 matrix. Images were analyzed by manually assigning areas of interest over the liver, heart, kidneys, bladder, and other organs. Counts in these assigned areas and total body counts were quantified by computer. The ratio of hepatic to cardiac blood pools was calculated for each rabbit based on its first postinjection scan. Trapped hepatic radioactivity was then computed from the later scans as the difference between total hepatic radioactivity and the contribution from the hepatic blood pool (hepatic blood pool radioactivity = ratio × cardiac radioactivity at that time).

After the 116-hr sample and scan were obtained, animals were injected with 3 mCi of ^{99m}Tc-labeled LDL. Clearance of ^{99m}Tc-labeled LDL was followed over the next 24 hr by γ camera with a high-resolution collimator (window, 126–154 keV) (39, 40).

Animals were then sacrificed and organs were obtained for direct measurement of radioactivity. ^{99m}Tc radioactivity was measured immediately. Because the γ counter uses no collimator, a narrow window (133–147 keV) was used to reduce ^{111}In downscatter to <1%. Organ samples were then stored at 4°C for 2 days to allow ^{99m}Tc radioactivity (physical $t_{1/2}$ = 6 hr) to decay to negligible levels compared to ^{111}In radioactivity ($t_{1/2}$ = 67 hr). Organ contents of ^{111}In liposome radioactivity were then determined (window, 150–300 keV).

In a separate experiment, a normal rabbit was injected with unencapsulated ^{111}In–NTA and imaged by γ camera.

Statistical Analyses. Unless otherwise stated, values for which errors are given are means ± SEM (n = 4). Comparisons between parameters measured at different times within one group of rabbits were performed using the paired two-tailed t test. Comparisons between parameters measured in the WHHL and control rabbits were performed using the unpaired two-tailed t test (43).

RESULTS

Stability of Liposomes in the Circulation. Liposomal stability was assessed by the persistence of label encapsulation (16, 33). After injection of unencapsulated ^{111}In–NTA, 52% of the radioactivity appeared in the bladder within 1 hr, and 78% was excreted within 4 hr. In contrast, after injection of ^{111}In–NTA-labeled liposomes into the WHHL and control rabbits, no counts appeared in the bladder, and the total counts in each animal, when corrected for the physical decay of ^{111}In, varied by <12% during the experimental period (116 hr). In addition, gel filtration of selected plasma samples from these animals revealed no low molecular weight (i.e., unencapsulated) radioactivity. Thus, the infused liposomes remained intact while in the circulation, and no ^{111}In–NTA leaked into the circulation after cellular uptake of the liposomes (cf. ref. 44).

Clearance Data. After bolus injection of ^{111}In–NTA-labeled liposomes, plasma ^{111}In radioactivity and plasma phospholipid concentration showed nearly identical patterns of clearance in WHHL and normal rabbits (Fig. 1). In all cases, clearance was monoexponential, as evidenced by the high degree of linearity on semilogarithmic plot (Fig. 1C). In the WHHL and normal rabbits, the half-times ($t_{1/2}$s) for clearance of plasma counts were 20.8 ± 0.9 hr and 20.0 ± 0.8 hr, respectively. The $t_{1/2}$s for clearance of excess plasma phospholipid (i.e., phospholipid above what was present at 0 hr) were 20.3 ± 4.6 hr and 19.6 ± 2.2 hr, respectively. These four mean $t_{1/2}$ values are statistically indistinguishable, thereby also indicating that radioactivity and phospholipid mass were cleared as a unit.

Based on the mean y intercepts in Fig. 1C, the volumes of distribution for administered liposomes were smaller in the WHHL rabbits (V_d = 31.1 ± 1.5 ml/kg based on counts, V_d = 31.8 ± 3.0 ml/kg based on phospholipid) than in the normal rabbits (V_d = 43.6 ± 1.1 ml/kg and 45.0 ± 1.2 ml/kg; $P < 0.01$). In addition, between 10 min and 1 hr after the injection, plasma radioactivity and phospholipid concentration did not decrease in the WHHL rabbits, but did in the controls (Fig. 1).

Mobilization of unesterified cholesterol after phospholipid infusion was unimpeded in the receptor-deficient rabbits (Fig. 2A). Plasma unesterified cholesterol increased by a maximum of 2.2 ± 0.23 mmol/liter in the WHHL rabbits and by 2.1 ± 0.04 mmol/liter in the normal rabbits by 6 hr after the injection. The disappearance of this excess cholesterol was not monoexponential, as evidenced by increasingly

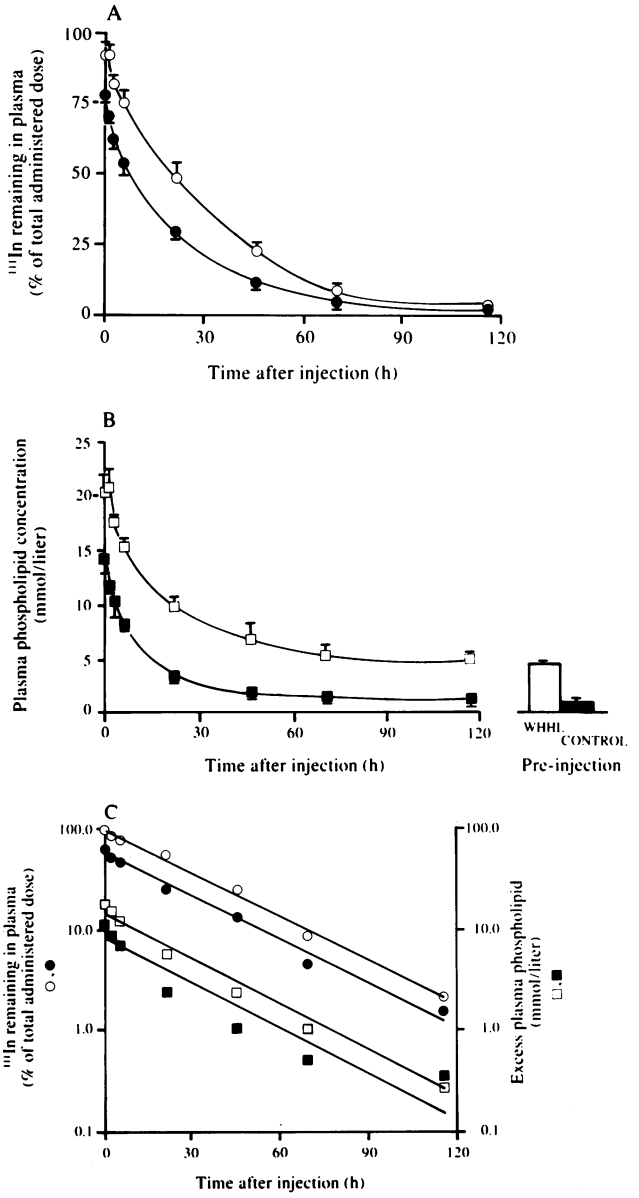

FIG. 1. (*A*) Total administered ^{111}In remaining in the plasma of rabbits injected with ^{111}In-labeled phospholipid liposomes. Plasma (29 ml per kg body weight) was assumed (45). Displayed are means ± SEM for the four WHHL (○) and four control (●) rabbits. (*B*) Plasma phospholipid concentrations postinjection (line graph) and preinjection (column graph). □ and open column, WHHL; ■ and solid column, control rabbits. Means ± SEM are shown. (*C*) Semilogarithmic plot of plasma ^{111}In, excess plasma phospholipid, and average regression lines. Plasma ^{111}In data (○, WHHL; ●, control) are described in *A*. Excess plasma phospholipid concentrations (□, WHHL; ■, control) were calculated for each rabbit as the increase above the phospholipid concentration before injection. Because of overlap with nearby points, data from 1 hr postinjection are not shown. The lines were derived by performing linear regression analysis on semilogarithmically transformed data from each individual rabbit and then averaging the *y* intercepts and $t_{1/2}$s. Correlation coefficients for the radioactivity data ranged from −0.98 to −0.999 in WHHL and control rabbits. Coefficients for excess plasma phospholipid were −0.99, −0.98, −0.97, and −0.83 in the WHHL rabbits, and −0.997, −0.97, −0.96, and −0.92 in the controls.

negative slopes on a semilogarithmic plot (not shown). The nonmonoexponential disappearance of excess unesterified cholesterol was slower in the WHHL rabbits than in the controls. For example, between 6 and 22 hr after the injection, the plasma concentration of unesterified cholesterol decreased by only 0.318 ± 0.165 mmol/liter in the WHHL rabbits, compared to 0.903 ± 0.106 mmol/liter in the controls ($P < 0.05$). Nevertheless, in both groups of rabbits, disappearance of excess plasma unesterified cholesterol was >90% complete by 70 hr postinjection (Fig. 2*A*).

Plasma concentration of cholesteryl ester in the WHHL rabbits decreased 22–70 hr after the phospholipid infusion, although the changes were not statistically significant (Fig. 2*B*). In contrast, plasma concentration of cholesteryl ester in the normal rabbits increased by an average of 0.44 ± 0.2 mmol/liter by 22–46 hr ($P < 0.05$), then returned to within 0.09 mmol/liter of its initial value by 116 hr (Fig. 2*B*).

By quantitative γ camera imaging, hepatic trapping of ^{111}In-labeled liposomes *in vivo* was statistically indistinguishable in the two groups of animals (Fig. 3). In contrast, hepatic trapping of ^{99m}Tc-labeled LDL *in vivo* was significantly reduced in the WHHL rabbits (Fig. 3), as described (46).

At autopsy, organ distributions of ^{111}In-labeled liposomes were similar in the WHHL and normal rabbits, although uptake by the liver, small bowel, and adrenals were statistically higher in the WHHL rabbits (Fig. 4*A*). In contrast, the amounts of ^{99m}Tc-labeled LDL in the liver and adrenals at autopsy were markedly and significantly reduced in the WHHL rabbits (Fig. 4*B*). Overall clearance of ^{99m}Tc-labeled LDL was reduced in the WHHL rabbits, based on the amount of ^{99m}Tc radioactivity remaining in whole blood (Fig. 4*B*). Uptake of ^{99m}Tc-labeled LDL by bone marrow, muscle, small bowel, and lungs was enhanced in the WHHL rabbits, similar to previous findings (40, 48). Aortic uptake of ^{111}In-labeled liposomes in WHHL and control rabbits was <0.02% of the total administered dose (Fig. 4*A*).

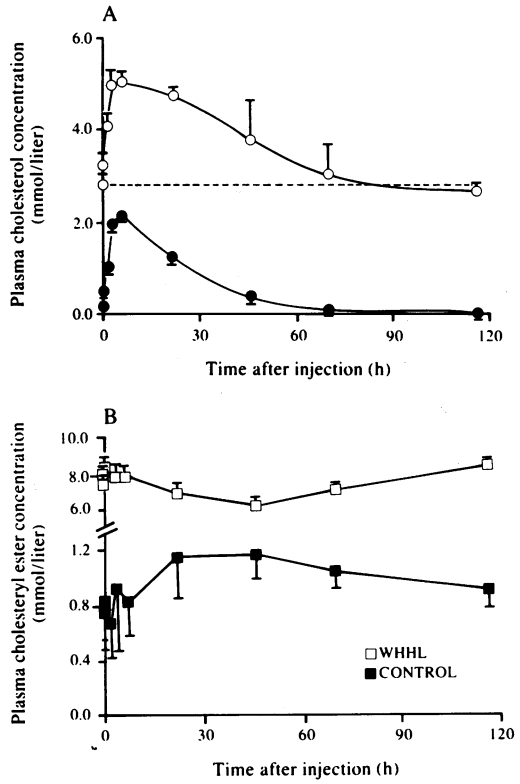

FIG. 2. (*A*) Plasma unesterified cholesterol concentrations after injection of phospholipid liposomes. ○, WHHL; ●, control. The mean preinfusion plasma unesterified cholesterol concentration in the WHHL rabbits is also shown by the horizontal dotted line. (*B*) Plasma cholesteryl ester concentrations after injection of phospholipid liposomes. □, WHHL; ■, control. Vertical bars denote SEM.

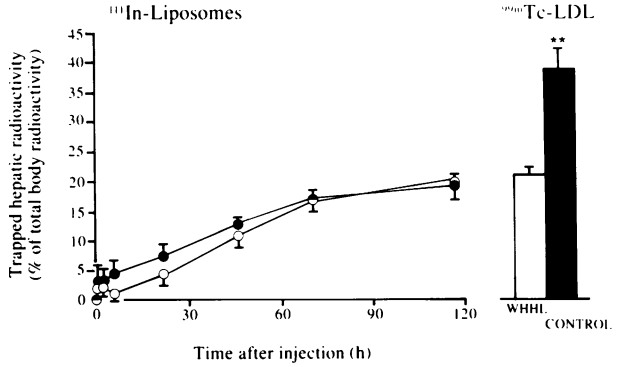

FIG. 3. Hepatic trapping of ^{111}In-labeled liposomes and ^{99m}Tc-labeled LDL *in vivo*. Animals were injected with ^{111}In-labeled liposomes. Over the next 116 hr, hepatic, cardiac, and total body radioactivity were serially quantitated by γ camera. Animals were then injected with ^{99m}Tc-labeled LDL. Twenty-four hours after this second injection, hepatic, cardiac, and total body ^{99m}Tc radioactivity were quantitated by γ camera. Trapped hepatic ^{111}In (line graph) and ^{99m}Tc (column graph) radioactivity were calculated from each γ camera image by subtracting the contribution of the hepatic blood pool from the total hepatic radioactivity, as described in the text. Values are percentages of total body ^{111}In or ^{99m}Tc radioactivity. ○ and open column, WHHL; ● and solid column, controls. Vertical bars denote SEM. Hepatic trapping of ^{99m}Tc-labeled LDL was significantly greater in the control rabbits than in the WHHL rabbits (**, $P < 0.01$). No other values were statistically distinguishable between the two groups of rabbits.

DISCUSSION

Our results indicate that the metabolism of infused phospholipid liposomes does not require LDL receptors. Infused liposomes mobilize endogenous cholesterol and then undergo primarily hepatic clearance in normal and receptor-deficient animals. Our results are consistent with prior demonstrations that phospholipid infusions reduce experimental atherosclerosis even during continued feeding of high cholesterol diets (4, 6–8), which would have caused suppression and saturation of hepatic LDL receptors (29).

Despite similar clearance kinetics and organ distributions, we observed four major differences between WHHL and normal rabbits after phospholipid infusion. First, the volume of distribution of infused liposomes was smaller in the WHHL rabbits and closely matched the plasma volume. In WHHL rabbits, liposomes possibly have impaired access to extravascular sites or impaired margination to vessel walls (49, 50). Second, there was no detectable clearance of liposomes between 10 min and 1 hr after injection in the WHHL rabbits. This result might suggest that WHHL rabbits lack a rapid initial clearance process that is present in normal rabbits and may be LDL receptor mediated. A distinct initial component of liposome clearance in genetically normal animals has been described (14–16) but was not discernable in our control rabbits.

The third difference was that the disappearance of excess plasma unesterified cholesterol was slower in the WHHL rabbits than in the controls. In both groups of rabbits, the nonmonoexponential pattern of cholesterol disappearance presumably resulted from two competing processes: monoexponential clearance of liposomes, and continued cholesterol uptake by liposomes remaining in the circulation. Because the monoexponential clearance of liposomes was not different in the two groups of rabbits during the period of declining plasma cholesterol concentration, our results suggest that liposomal uptake of unesterified cholesterol may have been enhanced in the WHHL rabbits, perhaps as a

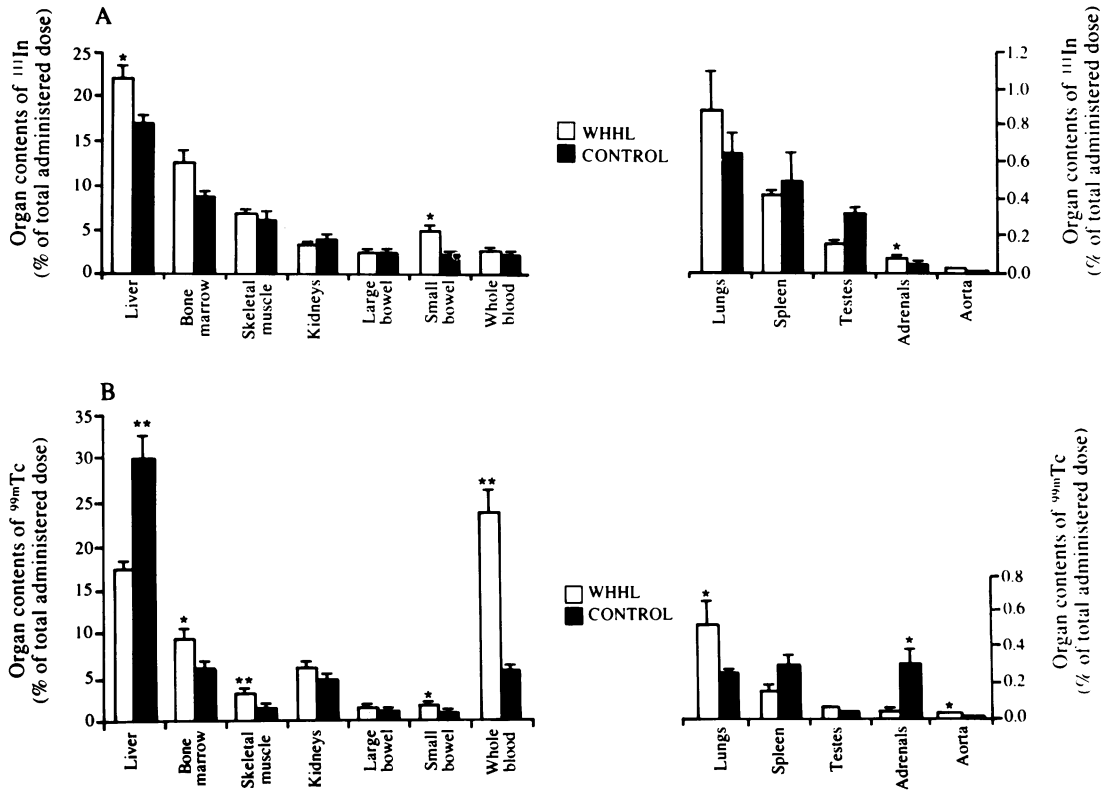

FIG. 4. Organ contents of ^{111}In-labeled liposomes and ^{99m}Tc-labeled LDL at autopsy. ^{111}In-labeled liposomes and ^{99m}Tc-labeled LDL were injected into rabbits as described in Fig. 3 and in the text. At autopsy, organ contents of ^{111}In (*A*) and ^{99m}Tc (*B*) were separately determined. Total radioactivity in skeletal muscle, small bowel, and large bowel was calculated based on reported organ weights per kg body weight (47). Whole blood and bone marrow were assumed to be 60 mg/kg and 20 mg/kg, respectively. Means ± SEM for WHHL (open columns) and control (solid columns) rabbits are shown. Values significantly higher in one group of rabbits than the other are marked as follows: *, $P < 0.05$; **, $P < 0.01$. Data for the lungs, spleen, testes, adrenals, and aorta are shown on an expanded scale.

consequence of their greater tissue stores (cf. refs. 24 and 51) and greater absolute concentration of excess plasma phospholipid (see Fig. 1*C* and V_d calculations).

Finally, the plasma concentration of cholesteryl ester increased in the normal but not in the WHHL rabbits. This increase in cholesteryl ester concentration has been observed following phospholipid infusion into normal dogs (10). It may have resulted from increased phospholipid substrate for lecithin–cholesterol acyltransferase (15) or enhanced hepatic secretion of cholesteryl ester-rich lipoproteins following delivery of liposomal cholesterol to the liver (ref. 15; cf. refs. 52 and 53). The increase might also reflect impaired catabolism of cholesteryl ester-rich lipoproteins, owing to competition by liposomes for clearance (10, 23) or transient suppression of hepatic LDL receptors following delivery of liposomal cholesterol. Elevation of plasma cholesteryl ester in WHHL rabbits might have been obscured by the large initial concentration. Alternatively, it may have been absent in WHHL rabbits, particularly if the increase depended on a transient change in hepatic LDL receptors, which these rabbits lack.

We have shown that LDL receptors are not required for liposome metabolism *in vivo*. The mechanisms, however, for uptake of liposomes by the liver and other organs remain to be elucidated. Possibilities include other receptors, such as the apoE receptor (25), and receptor-independent processes (48, 54).

Our results, together with the previous regression studies (1–8), suggest that infused phospholipid might be a rational, effective treatment for receptor-deficient atherosclerosis. Although there is no generally available preparation of liposomes approved for human use, vesicular particles that acquire endogenous cholesterol have been shown to appear in plasma following infusion of clinically available triglyceride phospholipid emulsions, such as Intralipid, which consist of liposomes and related particles (15, 28, 55–58). Infusion of liposomes or Intralipid may offer the possibility to treat familial hypercholesterolemia in humans.

The authors thank Helena Lipszyc, Christine Brown, and Dr. Debora Fineman of Mt. Sinai Medical Center for their assistance with γ camera imaging and Dr. Robert Sciacca of Columbia University for assistance with statistical analysis of data. K.J.W. is a recipient of a Clinician Scientist Award from the American Heart Association and E. R. Squibb & Sons Company, with funds contributed in part by the American Heart Association New York City Affiliate. This work was supported by National Institutes of Health Grants RR01180 and HL38956.

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