Biochimica et Biophysica Acta, 1153 (1993) 9–19

BBAMEM 76112

The influence of size and composition on the cholesterol mobilizing properties of liposomes in vivo

Wendi V. Rodrigueza [a,c,*], P. Haydn Pritchard [b] and Michael J. Hope [a,c]

[a] *Liposome Research Unit, 2176 Health Sciences Mall, Vancouver, British Columbia V6T 1W5 (Canada),* [b] *Department of Pathology, University of British Columbia, 950 West 28th Avenue, R. 375, Vancouver, British Columbia V5Z 4H4 (Canada) and* [c] *Skin Barrier Research Laboratory, Department of Medicine, Koerner Pavilion, 2211 Wesbrook Mall Vancouver, British Columbia V6T 2B5 (Canada)*

(Received 26 April 1993)
(Revised manuscript received 15 July 1993)

Key words: Liposome; Cholesterol; Reverse cholesterol transport; Atherosclerosis

As part of a study into the antiatherogenic properties of phospholipid liposomes we have investigated the capacity of a variety of preparations to increase plasma cholesterol concentrations in mice. Large unilamellar vesicles, composed of egg phosphatidylcholine, were found to be approximately twice as effective at mobilizing cholesterol than sonicated vesicles of the same composition. For egg phosphatidylcholine liposomes the change in plasma cholesterol profile is proportional to the residence time of vesicles in the circulation. Large unilamellar vesicles with a diameter of approx. 100 nm accumulate the most sterol in the animal model tested here, reaching equimolar concentrations with phospholipid after 24 h. Gel-state vesicles gave rise to a smaller increase in plasma cholesterol compared to liquid-crystalline vesicles. Our data indicate that, in vivo, net transfer of cholesterol into liposomes occurs more extensively from the lipoprotein cholesterol pool than from the erythrocyte cell membrane pool. This is consistent with the hypothesis (Williams, K.J., Werth, V.P. and Wolff, J.A. (1984) Perspect. Biol. Med. 27, 417–431) that liposomes enhance reverse cholesterol transport by generating cholesterol-poor HDL particles that can extravasate and promote more sterol efflux from peripheral tissues.

Introduction

Many studies have demonstrated that experimentally induced atherosclerosis, in a variety of animal models, can be significantly reversed following the intravenous administration of liposomes (for a review, see Ref. 1). As a result of this work and other observations on the interaction between liposomes, cholesterol and plasma lipoproteins, Williams et al. [1] proposed that the infusion of phospholipids might be a viable means of inducing the rapid regression of atherosclerotic plaque in humans.

The development of atherosclerosis involves the accumulation of cholesterol, cholesterol esters and phospholipids in the intima of the major arteries. As the lesion increases in size, blood flow through the vessel is restricted and can eventually result in clinical symptoms associated with tissue ischemia. It has been noted [2] that the core of atherosclerotic plaques can contain 30–65% (dry weight) lipid which, therefore, contributes a major part to the plaque volume. If this lipid could be removed significant regression would occur.

Cholesterol metabolism and homeostasis is the result of a complex equilibrium between free sterol in the cell and in plasma [3]. Delivery of cholesterol to cells occurs via the receptor-mediated LDL pathway and by passive exchange of sterol between plasma membranes and lipoproteins. Only tissues that produce steroid hormones and bile acids can metabolize cholesterol and in order to prevent accumulation of excess free sterol in remaining peripheral tissues there is a reverse transport of cholesterol from plasma membranes into high-density lipoproteins (HDL) and lipoprotein-like particles. HDL transports excess cholesterol to the liver where it can either be processed into bile salts for excretion or incorporated into very low density lipoproteins (VLDL) and so re-enter the lipoprotein pool [3].

The passive exchange of cholesterol between cells and lipoproteins occurs via the diffusion of sterol molecules across the aqueous space (for review, see Refs. 3 and 4). Net cellular efflux occurs if the chemical potential of free cholesterol is lower in the plasma such that sterol moves out of the membrane down its

* Corresponding author. Correspondence to: Skin Barrier Research Laboratory, Room F254, Koerner Pavilion, Department of Medicine, 2211 Wesbrook Mall, Vancouver, British Columbia, Canada V6T 1Z3. Fax: +1 (604) 8227950.

activity gradient. Under these conditions, it has been shown that cholesterol-ester-loaded cells, which are morphologically characteristic of early atherosclerotic lesions, not only loose cholesterol, but ester hydrolysis is promoted, resulting in the reduction of intracellular deposits of this lipid [2,3]. Moreover, there is epidemiological evidence that conditions which might be expected to enhance reverse cholesterol transport (low plasma cholesterol concentrations, or increased HDL concentrations) are correlated with reduced risk of premature atherosclerosis and can give rise to plaque regression [2,3].

Williams et al. [1] suggest that phospholipid infusion promotes reverse cholesterol transport and the mobilization of peripheral cell cholesterol for several reasons. First, circulating phospholipid liposomes act as a 'sink' for cholesterol reducing the effective chemical potential of plasma cholesterol, so therefore promoting cellular efflux. Secondly, vesicles deplete lipoproteins of cholesterol and the resulting sterol-poor, phospholipid-rich HDL particles are able to scavenge more cholesterol from extravascular tissue. Finally, a proportion of vesicles also bind apolipoproteins and assimilate into the lipoprotein pool as lipoprotein-like particles, which are also capable of entering the interstitial space, promoting cholesterol efflux from cells. The volume of atherosclerotic lesions is reduced due to the mobilization of cholesterol from the arterial wall which promotes the depletion of cholesterol and cholesteryl esters deposited within the tissue [2]. The normal clearance route of liposomes through the reticuloendothelial system (RES) delivers the majority of excess cholesterol to the liver for excretion and re-distribution.

In this study we examine some of the physical characteristics of liposomal systems that would be expected to influence cholesterol mobilization given the mechanisms discussed above. In particular, we would anticipate that the vesicle preparation most likely to exhibit the greatest antiatherogenic effect is one which can accumulate the maximum amount of cholesterol whilst circulating and is cleared via a route that maximizes excretion. It is well established that liposome clearance rates are dependent upon size and composition [5], physical characteristics which will also influence the extent of cholesterol equilibration [3]. Consequently, we have determined the rate and extent of cholesterol accumulation into a variety of liposomes that range in size from 30–250 nm diameter.

Materials and Methods

Materials

Cholesterol and [4-(2-hydroxyethyl)]-piperazine-ethanesulfonic acid (Hepes) were obtained from Sigma. [^{14}C]cholesteryl hexadecyl ether and [^{3}H]cholesterol were purchased from New England Nuclear. Egg phosphatidylcholine (EPC), distearoylphosphatidylcholine (DSPC), distearoylphosphatidylglycerol (DSPG) and egg phosphatidylglycerol (EPG) were supplied by Avanti Polar Lipids. Bio-Gel A-15m medium was purchased from Bio-Rad. All chemicals, thin layer chromatography plates and solvents were of analytical grade and purchased from BDH Chemicals.

Preparation of vesicles

All liposome preparations were labelled using trace amounts of [^{14}C]cholesteryl hexadecyl ether (CHE) for the following reasons; (1), it does not undergo passive exchange between membranes; (2), the mouse does not exhibit cholesterol ester exchange protein activity and (3), the ether-linked fatty acid is not cleaved in the plasma. Consequently, in this model system CHE is an excellent liposome marker and vesicle concentrations in the plasma were estimated from the specific activity of this label. A chloroform solution of EPC and [^{14}C]CHE was vortexed and solvent was removed under a stream of N_2. The sample was subsequently dried under high vacuum for 2 h. The dry lipid film was hydrated in 150 mM NaCl, 20 mM Hepes (pH 7.4) to generate multilamellar vesicles (MLV). Vesicles were prepared from MLV either by sonication, to generate small unilamellar vesicles (SUV) or extrusion to produce large unilamellar vesicles (LUV). Sonication was performed using a Branson tip sonifier, following standard protocols. The MLV suspension was diluted to 30 mg/ml, immersed in an ice bath and subjected to 3 cycles of sonication each of 10-min duration after which time the initial milky suspension became clear and the vesicle size was 30 nm, as determined by quasi-elastic light scattering (QELS). The SUV were centrifuged at $10\,000 \times g$ for 30 min to remove titanium fragments originating from the sonicator tip. Extrusion was carried out using a 10 ml Lipex Biomembranes Extruder equipped with a water jacketed thermobarrel [6]. MLV were sized through two stacked polycarbonate filters of defined pore size to generate a variety of LUV and homogeneous MLV as described previously [6,7].

Vesicle diameters

The size of vesicles generated by sonication and extrusion procedures was determined by QELS analysis utilizing a Nicomp Model 370 submicron laser particle sizer equipped with a 5-mW He-Ne Laser. The Nicomp QELS analyzes fluctuations in light-scattering intensities due to vesicle diffusion in solution. The measured diffusion coefficient is used to obtain the average hydrodynamic radius and thus the mean diameter of vesicles. The following diameters are expressed as the mean $\pm$ S.D. of vesicle preparations prior to injection. Vesicles prepared by sonication were 30 ± 7

nm in diameter (SUV_{30}). Vesicles prepared by extrusion through filters with a pore size of 0.05 μm were 70 ± 19 nm, 0.1 μm pore size 125 ± 30, and 0.4 μm pore size 237 ± 90 nm. In most cases the vesicles prepared by extrusion are referred to by the filter pore size used in their preparation, i.e., LUV_{50}, LUV_{100} and MLV_{400}.

Vesicle infusions

Female BDF-1 or CD-1 mice, weighing 20–22 g (Sprague-Dawley), were used throughout this study. Vesicles were injected via the tail vein at a dose of 300 mg/kg, which was typically 6 mg of lipid in 200 ml of buffer injected for each animal. Control mice were injected with an equal volume of buffer and both groups were sacrificed at specified times and blood collected in EDTA microtainer tubes by heart puncture. Plasma was obtained following centrifugation at $2000 \times g$ for 10 min, and an aliquot removed for scintillation analysis using a Beckman LS 3801 liquid scintillation counter. Each time point is the average of data from 16 mice (from four separate experiments), unless indicated otherwise.

Gel filtration

A 27×1.5 cm Bio-Gel A-15m gel filtration column, equilibrated with 150 mM NaCl, 10 mM Tris, 0.1% EDTA, 0.3% NaN_3 (pH 7.4) was used to fractionate plasma samples. Columns were eluted at a flow rate of 1 ml/min and 1-ml fractions were collected for radioactivity and lipid analyses. Data on the cholesterol:phospholipid (C/P) ratio of vesicles and lipoproteins after infusion was obtained from pooled fractions corresponding to the liposomal and lipoprotein peaks. The Bio-Gel columns were calibrated with respect to lipoprotein elution by preparing purified human lipoprotein fractions using standard ultracentrifugation procedures [8] labelling each with [^{3}H]cholesterol and monitoring the elution profile for radioactivity.

Lipid analysis

Pooled column fractions and plasma samples were extracted employing the Bligh and Dyer procedure [9]. The lipid extracts were analyzed for total cholesterol using the assay method of Rudell and Morris [10], free and esterified cholesterol concentrations were determined following separation by TLC using hexane/ether/acetic acid (70:30:1, v/v). Standards were used to identify the area of the plate corresponding to these two lipids, the silica was aspirated and the lipid eluted for assay using chloroform/methanol (2:1, v/v). Plasma vesicle phospholipid content was determined by dividing [^{14}C]CHE radioactivity by liposomal specific activity and phospholipid concentrations were determined by the method of Fiske and SubbaRow [11]. Erythrocytes were extracted using the method of Rose and Oklander [12], followed by a Bligh and Dyer wash to remove residual salts. An aliquot of red blood cells was retained for cell number determination using a Coulter cell counter in order to express cholesterol and phospholipid concentrations as nmol/10^9 cells.

Erythrocyte cholesterol specific activity

Blood was pooled from a group of mice and red cells packed by low-speed centrifugation. The serum was labelled with [^{3}H]cholesterol by incubation for 10 min at 37°C with 100 μCi of radioisotope dried from ethanol. The labelled serum was added to the packed cells and the mixture incubated at room temperature for 30 min. The cells were washed and approx. 10^6 dpm of [^{3}H]cholesterol-labelled cells injected into the experimental groups via the tail vein. Approx. 1 min after the injection of cells, saline or liposomes were administered.

Cholesterol exchange in vitro

Donor and acceptor vesicles were separated employing ion exchange chromatography. A 10-fold excess of donor vesicles (100 nm diameter) composed of EPC/EPG/Chol (40:15:45 molar ratio) were incubated with 100-nm or 400-nm EPC acceptors. Donor vesicles were labelled with [^{3}H]cholesterol at 5 μCi/100 mg total lipid and acceptors were labelled with [^{14}C]CHE at 0.5 μCi/100 mg lipid. At the specified time intervals, 50 μl of the incubation mixture (1 mg acceptor + 10 mg donor/ml) were removed and passed down a DEAE-Sepharose 6B-CL column prepared in a 1-ml tuberculin syringe equilibrated with 30 mM NaCl, 20 mM Hepes (pH 8.0). Columns were spun at $1000 \times g$ for 1 min prior to applying aliquots of the incubation mixture. The vesicle mixture was spun through the column and the eluant (acceptors) eluted with two subsequent wash/spin cycles with 500-ml aliquots of buffer. Recovery of ^{14}C-labelled vesicles (acceptors) was typically > 90%. Control experiments in which donors were labelled with a non-exchangeable marker indicated that all of the donor vesicles bound to the ion exchange column under the conditions of the experiment. Cholesterol accumulation by acceptors was determined using an LS 3801 Beckman scintillation counter equipped with a ^{14}C/^{3}H dual-label program.

Cholesterol excretion

Two groups of mice ($n = 4$) were maintained in metabolic cages and faeces collected daily. After 3 days one group was injected with 200 μl of saline and the second group with approx. 6 mg of EPC LUV_{100} (dose 300 mg/kg). Faecal material was collected for a further 7 days. Samples were extracted using an isopropanol/chloroform extraction procedure [10] and

subsequently assayed for total cholesterol, free cholesterol and cholesterol esters, as described earlier.

Experiments were carried out on mice maintained on regular, laboratory food for rodents (cholesterol excretion rate 10–12 μmol/g faeces) and on Teklad low cholesterol, casein-based diet which resulted in an excretion rate of approx. 0.8 μmol cholesterol/g faeces).

Results

Liposome clearance in vivo is chiefly dictated by liposome size, composition and lipid dose (wt lipid/body wt); liposomal half-life in the circulation, for example, increases as the dose increases [13]. In the studies reported here the lipid dose was not varied but maintained at 300 mg/kg in order to ascertain the importance of size and composition on cholesterol mobilization. This dose is high with respect to traditional intravenous drug-delivery studies using liposomes which are typically in the range of 50–100 mg/kg in mice. However, it falls well within the tolerated dose range for this type of particle and it approximates the dose that is likely to be practicable for human administration [1,14].

Cholesterol mobilization

An example of cholesterol mobilization by liposomes is shown in Fig. 1. Extrusion [6] was used to prepare a homogeneous population of LUV with a mean diameter of 125 nm as determined by QELS (referred to as LUV_{100}). A dramatic increase in plasma cholesterol is observed for animals receiving vesicles (Fig. 1A). Sterol levels peak 4–8 h after injection at a concentration that is nearly double that measured in the control mice injected with an equivalent volume of saline. Plasma cholesterol concentrations gradually return to normal levels after 48 h, which correlates well with the vesicle clearance profile shown in Fig. 1B. Liposomes were labelled with trace amounts of [^{14}C]CHE which is a non-exchangeable, non-metabolizable marker frequently used to monitor liposome clearance and distribution in vivo.

Using gel filtration (see Materials and Methods) mouse plasma was fractionated and the cholesterol profile determined using a chemical assay procedure [10]. Plasma from control and liposome-treated animals were compared and the results are shown in Fig. 2A,B. Fig. 2A shows a normal cholesterol distribution with the majority of cholesterol associated with combined LDL and HDL peaks (fractions 22–50). The elution volumes of VLDL, LDL and HDL were determined as described in Materials and Methods. A minor quantity of sterol is detected in the void volume, corresponding to the larger chylomicron and VLDL lipoprotein particles, but quantitatively these fractions represent < 5%

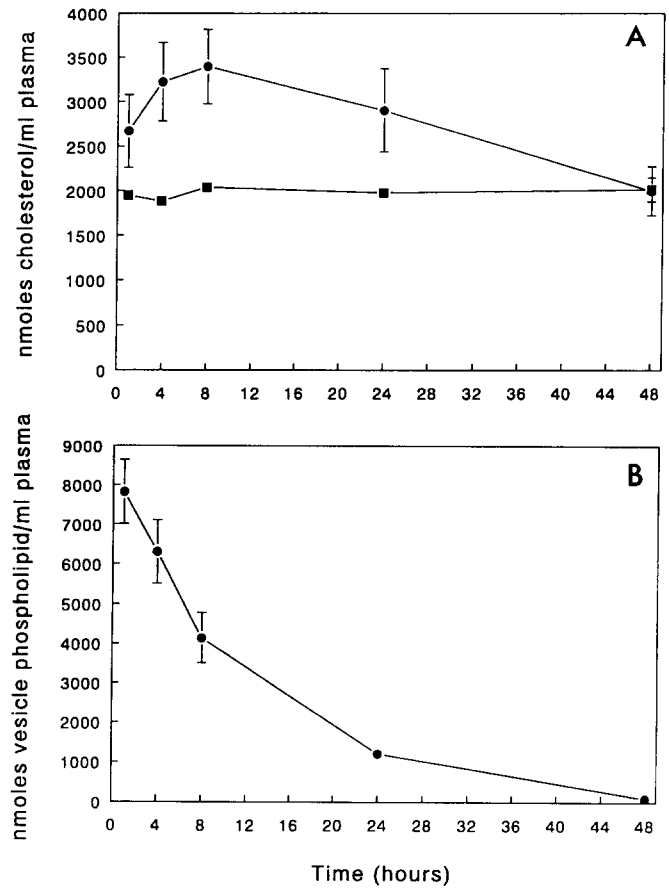

Fig. 1. (A) Total cholesterol concentration in the plasma of mice treated with saline (■) or EPC LUV_{100} (●) at a dose of 300 mg/kg body wt. Note that the first time point is at $t = 1$ h. Inital plasma cholesterol concentrations at $t = 0$ for both groups were the same. Each time point is the mean ± S.D. of a minimum of four experiments. (B) Clearance of [^{14}C]cholesteryl hexadecyl ether labelled LUV_{100} expressed as nmol vesicle phospholipid/ml plasma. Saline and vesicles were administered as a single, 200-μl bolus injection into the tail vein. Whole blood was collected via heart puncture. Lipid analysis was as described in Materials and Methods. Each time point represents the mean ± S.D. of four separate experiments.

of the total cholesterol content of the plasma. The elution profile of plasma from liposome-treated animals (4 h time point) is shown in Fig. 2B. The [^{14}C]CHE liposome marker is almost exclusively detected in the void volume, indicating that the LUV_{100} are well separated from the fractions containing LDL and HDL (vesicles smaller than 100 nm diameter are included in the gel and cannot be separated from LDL). The absence of radioactivity in the remaining fractions also indicates that little, if any, assimilation of vesicles into the lipoprotein pool has occurred. However, it is possible that small quantities of vesicles have undergone structural transitions to lipoprotein-like particles but are removed rapidly from the circulation and cannot be detected. It should be noted that assimilation is distinct from phospholipid exchange. During assimilation it is postulated that whole vesicles are structurally re-

organized, primarily as a result of binding apolipoproteins, to form smaller, lipoprotein-like particles. Phospholipid exchange with lipoproteins is known to occur, but would not be detected in these experiments using the non-exchangeable lipid marker described. The cholesterol content of column fractions shown in Fig. 2B clearly shows that the excess sterol in the plasma of treated mice is associated with LUV. The slight frame shift of peaks between 2A and 2B is the result of differences in elution rate and not due to changes in lipoprotein size. Using TLC analysis we determined that > 90% of the liposomal cholesterol was free cholesterol, the remainder being cholesterol ester.

The excellent separation of LUV_{100} from the quantitatively most abundant lipoproteins enabled the straightforward isolation and subsequent analysis of the vesicle lipids. Cholesterol accumulation is shown by

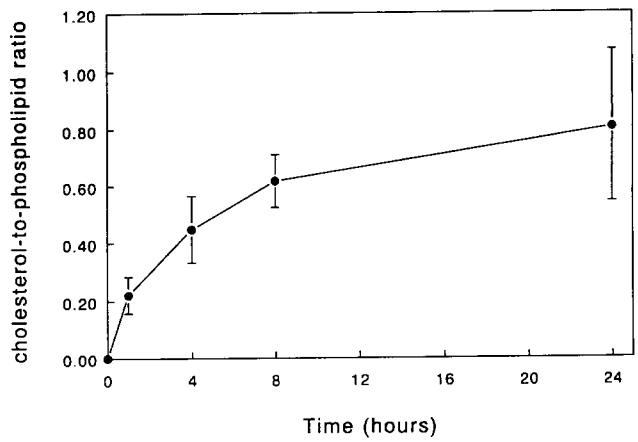

Fig. 3. Cholesterol/phospholipid molar ratio of EPC LUV_{100} isolated from mouse plasma. Vesicles were separated from lipoproteins by gel filtration, the cholesterol and phospholipid content of lipid extracts were determined by chemical assay. Each point represents the mean ± S.D. of four separate experiments.

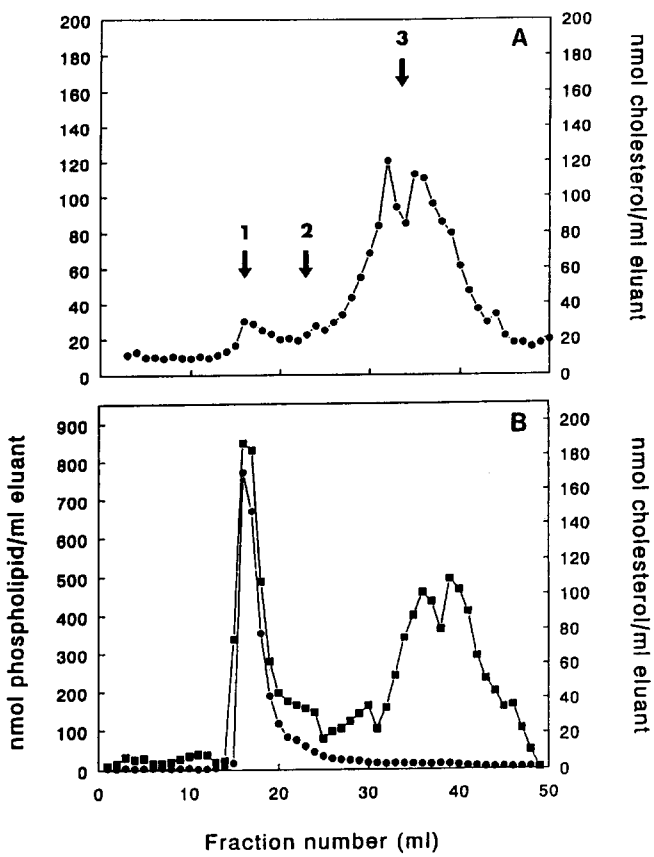

Fig. 2. (A) The elution profile of normal mouse plasma (●) from a Bio-Gel A-15m gel filtration column. Fractions were extracted and subjected to a total cholesterol assay as described in Materials and Methods. Previous calibration of this gel filtration material, using purified human lipoprotein fractions labelled with [^{3}H]cholesterol are identified with arrows illustrating (1) VLDL (void volume) (2) LDL and (3) HDL peaks. (B) A 4-h plasma time point from mice treated with EPC LUV_{100}. (●) cholesterol content of each fraction and (■) indicates the concentration of vesicle phospholipid measured from the specific activity of [^{14}C]CHE as described in Materials and Methods.

the increasing C/P ratio of vesicles over a 24-h timecourse in vivo, presented in Fig. 3. The rate of sterol accumulation by the liposomes is consistent with previously published rates of exchange and transfer for membrane cholesterol [3,4]. The apparent equilibration at an approximately equimolar C/P ratio also fits with our knowledge of cholesterol dynamics between phospholipid bilayers and plasma membranes [3], and represents the maximum amount of liposome associated sterol expected under these conditions. Consequently, after 24 h the liposomes that remain in the circulation (approx. 10–15% of the initial dose) are in equilibrium with respect to cholesterol and net sterol movement is negligible.

Liposome size and cholesterol mobilization

Previous studies investigating phospholipid-induced mobilization of cholesterol in vivo have employed multilamellar or sonicated vesicles [1,15,16,29,30]. Liposome size is a key characteristic in clearance kinetics and is one of several reasons why sonicated vesicles might be expected to represent the bilayer structure best suited to enhance reverse cholesterol transport. Sonication reduces multilamellar vesicles (MLV) to 'limit size' vesicles. These systems exhibit the minimum radius of curvature that can be adopted by the bilayer configuration without disruption [17] and for EPC this is typically a 30-nm diameter vesicle often classified as a small unilamellar vesicle (SUV). For a given liposome composition, it is assumed that the smaller the particle diameter the greater the circulation half-life [5], consequently we would expect SUV composed of EPC to circulate longer than other types of liposome, and therefore mobilize more cholesterol. Furthermore, the packing constraints experienced by phospholipids in SUV, (due to the acute radius of curvature) gives rise

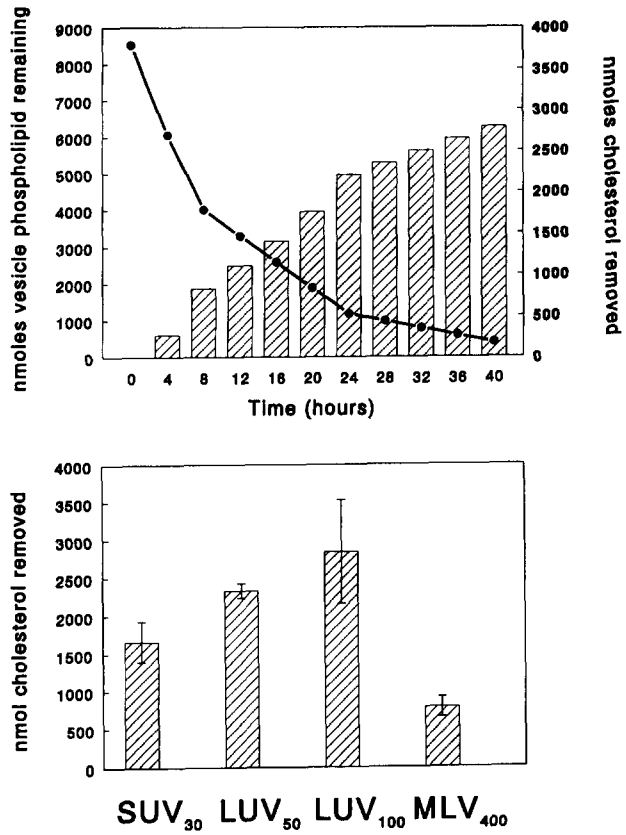

Fig. 4. (A) Cholesterol mobilization by LUV_{100} expressed as the cummulative nmol of cholesterol (bar graph) removed with vesicle phospholipid (●). The clearance kinetics of the liposomes was followed by [^{14}C]CHE label. (B) Cholesterol mobilization by SUV_{30}, LUV_{50}, LUV_{100} and MLV_{400}. All vesicles were prepared from EPC and administered at 300 mg/kg body weight. Total cholesterol removed by liposomes ± S.D. was calculated for four separate experiments as described in the text. Significance levels determined were: SUV_{30} vs. LUV_{50} ($P < 0.001$), SUV_{30} vs. LUV_{100} ($P < 0.025$), SUV_{30} vs. MLV_{400} ($P < 0.001$), LUV_{50} vs. LUV_{100} (n.s.), LUV_{50} vs. MLV_{400} ($P < 0.001$), LUV_{100} vs. MLV_{400} ($P < 0.001$).

to an instability that can result in fusion [17], as well as an increased tendency to assimilate with lipoproteins [18,19]. Therefore, given the mechanism by which liposomes are thought to enhance reverse cholesterol transport [1] it might be expected that SUV would produce a greater number of HDL-like particles, thus promoting efflux of sterol from peripheral tissues.

We measured plasma cholesterol concentrations over a 48-h period in animals treated with a variety of liposomal preparations varying in diameter from 30–250 nm. Sonicated vesicles were prepared as described in Materials and Methods, the remaining vesicles were produced by extrusion of MLV through filters with defined pore sizes to give vesicle populations with the mean diameters given in Materials and Methods. In the text, and in Fig. 4, vesicles are referred to by the filter pore size.

The amount of cholesterol accumulated and removed by liposomes in vivo is a function of both the rate of cholesterol uptake and the rate of liposome clearance. An estimate of the mass of cholesterol removed from the circulation (mostly by the RES) can be made by calculating the C/P ratio of vesicles in vivo from plasma concentration of vesicle phospholipid and cholesterol as the excess plasma concentration above the control at the various experimental time points. From our data we know that all the cholesterol above control levels is associated with circulating liposomes. It should be noted that we have not taken into account the likelihood that control cholesterol levels in treated animals is lower than saline treated but this point is addressed in the discussion. The plasma volume of mice used in these studies was approx. 1 ml, consequently the total amount of phospholipid cleared from the circulation between time points was known. Using the average C/P ratio measured for vesicles between each assay interval an estimate of the cholesterol removed was obtained. The analysis was not continued beyond the point where less than 5% of the initial phospholipid dose remains in the circulation, because below this level the measurement error was too large to determine accurate C/P ratios. In Fig. 4A we show the cummulative level of cholesterol removed by LUV_{100} up to the time when approx. 5% of the dose remains. After 40 h we estimate that 2800 nmol of cholesterol have been removed from the circulation by the RES, which represents 33 mol% of the injected phospholipid dose. We have used this analysis to compare the various liposomal preparations tested. For each preparation the plasma cholesterol and phospholipid clearance profiles were determined and analysed as described above. The results in Fig. 4B show that LUV mobilize the most cholesterol, and the reasons for this are discussed later.

We have shown previously, using freeze-fracture electron microscopy and NMR analysis, that MLV sized through 400-nm pores retain a number of internal lamellae and therefore cannot be classified as LUV [17]. It was not clear from the literature how much of a kinetic barrier to complete cholesterol equilibrium would be presented by a multibilayer system. Despite some discrepancies [3,4], it appears generally accepted that the transbilayer movement (flip-flop) of cholesterol is rapid, on the order of seconds to minutes in a liquid crystalline bilayer under conditions that promote net sterol flux. Consequently, it might be expected that multilamellar systems would still act as a good sink for cholesterol as sterol should rapidly disperse through the internal lamellae. However, as shown in Fig. 5 this is not the case. Using an in vitro model in which LUV_{100} or MLV_{400} were incubated with a 10-fold excess of donor vesicles containing tritiated cholesterol (see Materials and Methods) the net transfer of sterol

from donor to acceptor was monitored. The rate of cholesterol accumulation in the unilamellar preparation was greater than that observed for the oligolamellar vesicles. It is interesting to note that in the presence of a 10-fold excess of donor vesicles the equilibrium C/P ratio of the acceptor should be approx. 0.9 : 1. The data in Fig. 5 show that the 100-nm acceptors only achieve a ratio of 0.35 : 1 after 8 h at 37°C. This is approximately half the rate of accumulation observed for the same vesicles in vivo (Fig. 3).

Liposome composition

Another physical characteristic known to influence the circulation time of liposomes in vivo is lipid composition. The addition of cholesterol to liposomes has the most dramatic effect on circulation half-life, increasing it by an order of magnitude in some cases. However, in the presence of equimolar cholesterol there is a further increase in circulation half-life for long-chain, saturated phospholipids compared to liquid crystalline systems. It has also been demonstrated that vesicles, composed of saturated lipids in the gel state are still capable of acting as acceptors of cholesterol [3]. Consequently, we compared the cholesterol mobilizing properties of two types of LUV_{100} composed of EPC/EPG (95 : 5 mol ratio) which is liquid-crystalline at 37°C and DSPC/DSPG (95 : 5) a gel-state lipid matrix at the body temperature of the mouse. Phosphatidylglycerol (PG) was incorporated to impart a surface negative charge, necessary to prevent the gel-state vesicles from aggregating in the absence of cholesterol [20]. In order to properly compare the two systems a negative charge was also added to EPC vesicles. The results, presented in Fig. 6A, reveal that the gel-state vesicles produce a

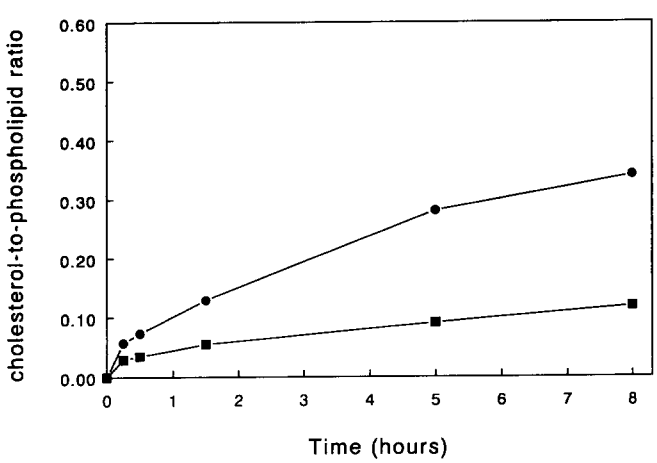

Fig. 5. The capacity of EPC LUV_{100} (●) and EPC MLV_{400} (■) to accumulate cholesterol in vitro. Cholesterol containing donor vesicles were incubated with acceptors at 37°C as described in Materials and Methods. Donors and acceptors were separated at the indicated times by ion-exchange column chromatography.

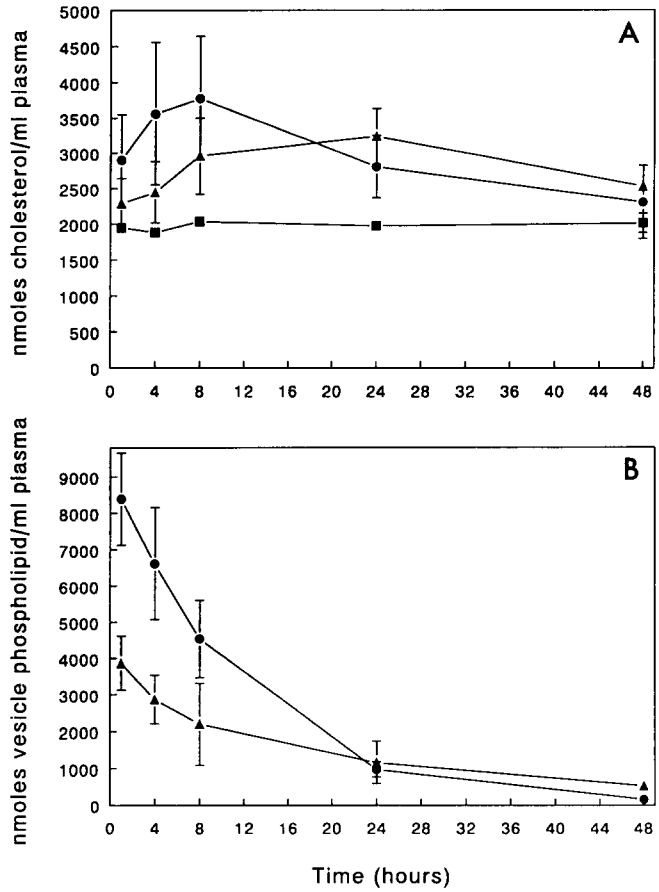

Fig. 6. (A) A comparison of the plasma cholesterol profiles of mice following injections of saline (■), liquid-crystalline EPC/EPG (95 : 5) LUV_{100} (●) and crystalline DSPC/DSPG (95 : 5) LUV_{100} (▲). (B) Clearance kinetics of EPC/EPG (●) and DSPC/DSPG (▲) labelled with [^{14}C]CHE (each time point is the mean of four experiments).

delayed increase in plasma cholesterol which does not peak until after 24 h, whereas EPC/EPG vesicles give rise to a cholesterol profile similar to that observed for EPC alone.

From the data in Fig. 6A it can be shown that the rate of cholesterol accumulation for these two types of vesicle is the same. The different plasma cholesterol profiles result from the fact that approx. 70% of the DSPC/DSPG vesicles are cleared within 4 h compared to less than 30% of the EPC/EPG LUV_{100} (Fig. 6B). The bulk of cholesterol mobilization occurs in the first 24 h, consequently liquid crystalline EPC/EPG removed more than 3000 nmol to the RES, whereas DSPC/DSPG vesicles removed 1700 nmol. It has been suggested that gel-state vesicles are not as good an acceptor of cholesterol as liquid crystalline vesicles [21]. However, our data obtained in vivo are supported by observations in vitro (data not shown) and those of Phillips et al. [3] who do not detect significant differences in the rate of cholesterol accumulation between the two.

Source of liposomal cholesterol

The final series of experiments presented were designed to provide some information as to the source of the liposomal cholesterol and its fate. Ultimately, cholesterol efflux must occur from atherosclerotic plaque to achieve regression. However, it is known that the cholesterol within cells and atherosclerotic lesions equilibrates more slowly than sterol present in plasma membranes directly exposed to acceptor particles [2,3]. Movement of this cholesterol will be a secondary event initiated by the primary efflux of outermembrane cholesterol. In a 20-g mouse approx. 35% of the circulating sterol is associated with lipoproteins and about 65% with the plasma membranes of erythrocytes. However, all of the sterol associated with erythrocytes is free cholesterol, whereas a large proportion of lipoprotein sterol is esterified. Consequently, the largest pool of free cholesterol in the circulation is in the red blood cell plasma membrane but this source of cholesterol does not change significantly in the presence of liposomes, despite a two fold increase in plasma sterol concentration. This result is shown in Fig. 7A and confirms a recent observation made by Mendez et al. in rabbits [16].

It is well known that erythrocyte membrane cholesterol can be depleted by liposomes in vitro [3,22], consequently it is of interest to determine if erythrocytes act as the primary sterol donor but their cholesterol content is rapidly replenished by lipoproteins which are in turn able to extravasate and scavenge more sterol from peripheral tissues. We isolated erythrocytes from the mouse and labelled them with [^{3}H]cholesterol in vitro. The labelled cells were injected into a group of mice, half of which were subsequently treated with saline and half with 300 mg/kg of EPC LUV_{100}. The specific activity of red blood cell cholesterol was determined over an 8-h time-course and the two groups compared. As can be seen from Fig. 7B the decrease in cholesterol specific activity is the same for both the control and experimental group. Interpretation of these data is limited by the fact that cells labelled in vitro are also removed from the circulation over a similar time-course (determined by chromium labelling, data not shown). However, we can estimate that at least 50% of cell sterol would have to efflux to account for the rise in plasma cholesterol observed after 8 h, which would result in a considerable dilution of erythrocyte cholesterol if this sterol pool were continuously replenished. Clearly this is not the case, suggesting that red blood cell cholesterol is not the primary source of the liposomal sterol accumulated in vivo, and extends previous observations that liposomal infusion causes an increase in cholesterol concentration for whole blood and not simply a redistribution between components of the blood [29,30].

C/P ratios of lipoproteins showed a significant decrease over control values in the first 8 h (Fig. 7C), however, we did not distinguish between increased phospholipid content of lipoproteins due to net transfer from the liposomes or net transfer of cholesterol from lipoproteins to vesicles. The ratio returns to nor-

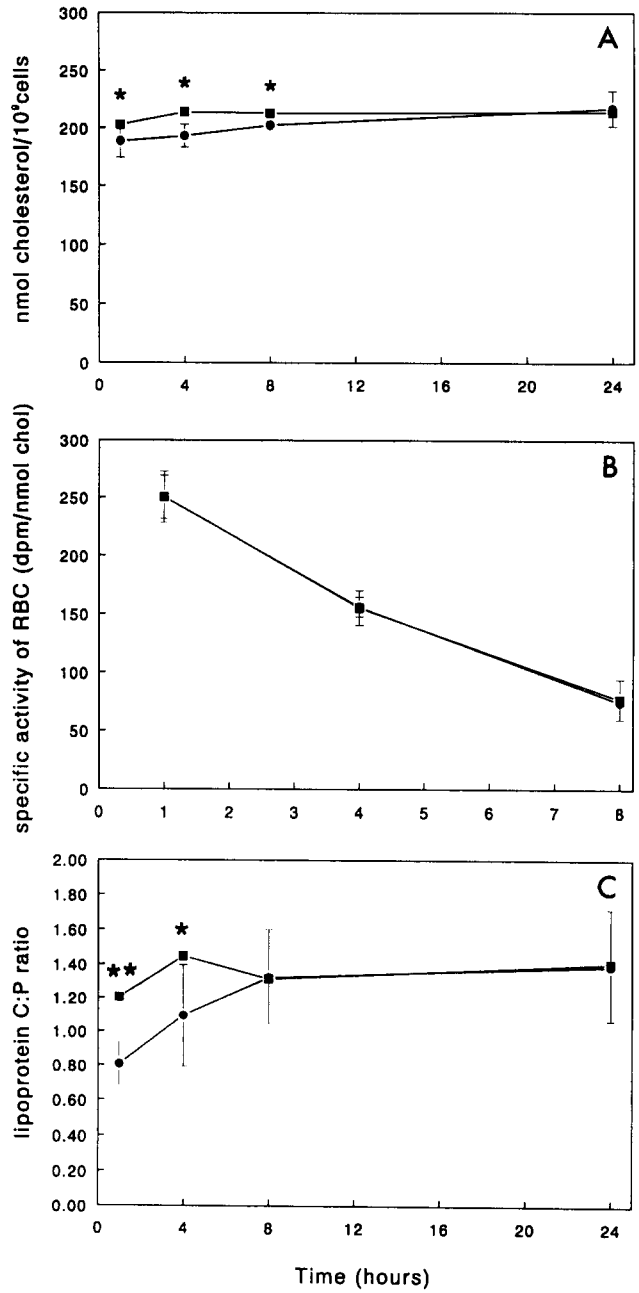

Fig. 7. (A) The cholesterol content of erythrocytes from mice treated with EPC LUV_{100} (●) and saline (■). Cells were isolated, washed and counted prior to extraction for lipid analysis (Materials and Methods). (B) The specific activity of erythrocyte cholesterol from saline (■) and liposome (●) treated mice (data points are superimposable). Erythrocytes were labelled in vitro by incubation with serum lipoproteins equilibrated with [^{3}H]cholesterol (see Materials and Methods). (C) The change in C/P ratio of the pooled lipoproteins obtained by combining fractions 22–50 from the gel-filtration column of saline (■) and liposome treated (●) groups. Significance levels, $P < 0.05$ (*) and $P < 0.01$ (**), are as indicated.

mal values after 8 h which mirrors the time-course of cholesterol accumulation by vesicles. This supports the hypothesis that it is primarily lipoprotein cholesterol in equilibrium with circulating vesicles, and that lipoproteins mediate the transfer of cholesterol from peripheral tissues to liposomes. The results are also consistent with observations in vitro that indicate cholesterol can undergo desorption from lipoproteins more readily than from erythrocytes [3]. Finally, we noted above that the rate of cholesterol accumulation by LUV_{100} in vivo (Fig. 3) is considerably faster than that observed in vitro (Fig. 5), which indicates that the rate of cholesterol desorption from sources in vivo is greater than from the 100 nm vesicle donors used to obtain the data in Fig. 5.

Cholesterol excretion

At a dose of 300 mg/kg > 70% of the liposomes are removed by the liver, the remaining vesicles are phagocytosed by fixed macrophages in the spleen and bone marrow with a small percentage distributed in skin and lung tissue (data not shown). This biodistribution follows the pattern observed in many studies on LUV in vivo [22]. The hypothesis of Williams et al. [1] proposes that vesicles removed by the liver will deliver cholesterol into the bile synthetic pathway for eventual excretion as a mixture of bile salts and free cholesterol. In order to see if we could detect an increase in the excretion of free cholesterol we maintained two groups of mice (saline-treated and liposome-treated) in metabolic cages for ten days and assayed faeces for free cholesterol. On a normal laboratory diet for rodents, we found that mice excrete 10–12 μmol of total cholesterol per gram of faeces. Following the administration of saline or liposomes we were unable to detect a significant change in this level over a 7-day period. This was not unexpected, since the dietary sterol was so much greater than the amounts being mobilized by vesicles. However, employing a low cholesterol diet, which reduced background excretion to approx. 1 mmol cholesterol/g faeces we were still unable to detect a difference between saline and liposome-treated groups. Both groups produced approx. 1 g of faeces/day; at the end of the experimental period (1 week) the accumulated excretion rates for total faecal cholesterol in control and treated mice were 0.795 μmol/g and 0.777 μmol/g, respectively.

Discussion

The potential use of phospholipid vesicles to manage atherosclerosis and other membrane-associated, degenerative processes of the cardiovascular system is gaining interest [1–3,14–16,22–24]. Although it has been recognized for many years that liposomal infusion could promote the regression of atherosclerotic plaque [1], it is only recently that their use is being considered seriously as a mode of clinical therapy. In part this is due to the increasing use of liposomes in the pharmaceutical industry as topical [25] and intravenous [26] drug-delivery vehicles. As a result of this activity many problems associated with the cost and manufacture of liposomes on a large scale have been overcome. For example, there are now many suppliers, worldwide, of injection grade phospholipids in kilogram quantities, that use manufacturing processes that conform to the regulatory requirements for clinical use in humans.

Experience in using vesicle systems for intravenous drug delivery indicate the importance of vesicle size and composition on the circulation half-life and in vivo stability of liposomes in blood. From the proposed mechanism of phospholipid-induced cholesterol mobilization it is clear that the mass of sterol removed to the liver will be proportional to the concentration of liposomes that can be maintained for the first 24 h, during which time cholesterol equilibrates to an equimolar concentration with phospholipid of unilamellar vesicles (Fig. 3). The finding that SUV_{30} were less effective than LUV_{100} in the mass of cholesterol moved from peripheral tissues to plasma, is consistent with our observation that EPC SUV_{30} are cleared more rapidly than EPC LUV (data not shown). Although previous studies indicate that smaller particles circulate longer than large particles [5,13], we are not aware of studies comparing cholesterol-free SUV_{30} with LUV_{100}. It is likely that the relatively fast clearance of SUV_{30} from the circulation is due to several factors. First, with a diameter of 30 nm these vesicles can extravasate from the sinusoidal tissues of liver and spleen and, as a consequence, will experience a greater volume of distribution than the LUV preparations, which cannot leave the vasculature as readily [13,27]. This is supported by our observations (not shown) that SUV accumulated in the liver to a greater extent than MLV_{400} liposomes despite the fact that the latter were cleared more rapidly. Scherphof and colleagues have elegantly shown that SUV can access liver hepatocytes, whereas larger liposomes are confined to the Kupffer cell population lining sinusoid surfaces [27]. Secondly, SUV are more unstable in plasma than large liposomes [27], and it is likely that a proportion of the small vesicles are assimilated into the lipoprotein pool and cleared rapidly by receptor mediated pathways (as well as phagocytosis) in the liver.

We did not find any advantage to using vesicles composed of saturated lipids or using multilamellar systems. The latter observation is to be expected considering the decreased surface area of bilayer available for cholesterol absorption and the inability of cholesterol to rapidly equilibrate across the internal lamellae. Using freeze-fracture analysis we have determined that for the same lipid concentration there are four times as

many particles present in a LUV_{100} preparation than in a MLV_{400} preparation (unpublished observations).

The primary source of liposome associated cholesterol is probably lipoproteins rather than circulating erythrocytes. Not only does total red blood cell cholesterol remain constant throughout the experiment but the rate of cholesterol turnover does not significantly change in the presence of liposomes, indicating that net sterol movement is occurring from other sources. The fact that the C/P ratio of lipoproteins from treated plasma drops sharply at the first time point (1 h) and then climbs back to normal values over the first 8 h suggests that this cholesterol pool equilibrates rapidly with vesicles. After 8 h the circulating liposomes are approaching cholesterol equilibrium with their surroundings and net flux is reduced. It is known that cholesterol desorption from lipoproteins occurs more readily than from erythrocytes [3], consequently, our data support the hypothesis that liposomes generate cholesterol poor lipoprotein particles (particularly HDL) that can access extravascular tissue and promote the efflux of cellular cholesterol. In this way sterol is shuttled from peripheral tissues to liposomes and reverse cholesterol transport is enhanced. Furthermore, several groups have demonstrated that there is a rapid exchange of cholesterol between arterial tissue and lipoproteins (see Ref. 3 and references therein), and recently it has been demonstrated that, in vivo, rat HDL can take up cholesterol from endothelial cells and transport it to liver parenchymal cells [28] which is the proposed route of reverse cholesterol transport. Consequently, it seems reasonable to predict that there is a net efflux of arterial wall cholesterol into plasma during liposome infusion.

Although we estimate that the EPC LUV_{100}, used in these studies, mobilize approx. 2800 nmol of cholesterol, it is likely to be greater than this. The rate of increase in C/P ratio for vesicles calculated from the LUV_{100} and cholesterol plasma concentrations (Fig. 1) is less than that measured in vesicles recovered from plasma (Fig. 3). This apparent discrepancy can be explained if lipoprotein cholesterol decreases (as indicated by the data shown in Fig. 7C) so that the 'control' plasma cholesterol level (i.e., cholesterol not associated with vesicles) is lower than the 2000 nmol/ml plasma found in saline-treated mice. The C/P ratios shown in Fig. 3 would result in cholesterol mobilization as high as 4600 nmol, approx. 50 mol% of the injected phospholipid.

Our studies have been unable to detect the appearance of free cholesterol in the faeces of mice treated with liposomes. Using [^{3}H]cholesterol loaded liposomes we demonstrated that excretion of radioactivity occurs at a constant rate over a 10-day period (data not shown). We have estimated that vesicles mobilize approx. 3 μmol of cholesterol in a typical EPC experiment. Less than half of this would be expected to appear in bile as free cholesterol, even if all liposomal sterol entered the bile pathway, consequently a maximum 1.5 μmol of cholesterol might be excreted over a 10-day period (less if re-absorption is taken into account) a level that is difficult to measure above background. We did attempt to extract and quantitate bile salts, however, our results were too varied to detect differences between control and treated mice.

In summary, from the observations reported here we conclude that liquid crystalline 100-nm LUV systems are the most effective liposomal preparation for cholesterol mobilization. We are currently testing the antiatherogenic properties of these vesicles in rabbits.

Acknowledgement

We wish to thank the Canadian Heart Foundation for supporting this research.

References

1 Williams, K.J., Werth, V.P. and Wolff, J.A. (1984) Perspect. Biol. Med. 27, 417–431.
2 Small, D.M. (1988) Arteriosclerosis 8, 103–129.
3 Phillips, M.C., Johnson, W.J. and Rothblat, G.H. (1987) Biochim. Biophys. Acta 906, 223–276.
4 Schroeder, F., Jefferson, J.R., Kier, A.B., Knittel, J., Scallen, T.J., Wood, W.G. and Hapala, I. (1991) Exp. Biol. Med. 196, 235–252.
5 Gregoriadis, G. and Senior, J. (1986) Life Sci. 113, 183–192.
6 Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1985) Biochim. Biophys. Acta 812, 55–65.
7 Mayer, L.D., Hope, M.J. and Cullis, P.R. (1986) Biochim. Biophys. Acta 858, 161–168.
8 Schumaker, V.N. and Puppione, D.L. (1986) Methods Enzymol. 128, 155–181.
9 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
10 Rudel, L. and Morris, M.D. (1973) J. Lipid Res. 14, 364–366.
11 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400.
12 Rose, G.H. and Oklander M. (1965) J. Lipid Res. 6, 428–431.
13 Hwang, K.J. (1987) in Liposomes: From Biophysics to Therapeutics (Ostro, M.J., ed.), pp. 109–156, Marcel Dekker, New York.
14 Aviram, M., Williams, K.J., McIntosh, R.A., Carpentier, Y.A., Tall, A.R. and Deckelbaum, R.J. (1989) Arteriosclerosis 9, 67–75.
15 Williams, K.J., Vallabhajosula, S., Rahman, I.U., Donnelly, T.M., Parker, T.S., Weinrauch, M. and Goldsmith, S.J. (1988) Proc. Natl. Acad. Sci. USA 85, 242–246.
16 Mendez, A.J., He, J.L., Huang, H.S., Wen, S.R. and Hsia, S.L. (1988) Lipids 23, 961–967.
17 Hope, M.J., Bally, M.B., Mayer, L.D., Janoff, A.S and Cullis, P.R. (1986) Chem. Phys. Lipids 40, 89–107.
18 Scherphof, G., Roerdink, F., Waite, M. and Parks, J. (1978) Biochim. Biophys. Acta 542, 296–307.
19 Krupp, L., Chobanian, A.V. and Brecker, P.I. (1976) Biochem. Biophys. Res. Commun. 72, 1251–1258.
20 Nayar, R., Hope, M.J. and Cullis, P.R. (1989) Biochim. Biophys. Acta 986, 200–206.
21 Yeagle, P.L. and Young, J.E. (1986) J. Biol. Chem. 261, 8175–8181.
22 Mayer, L.D., Tai, L.C., Ko, D.S.C., Masin, D., Ginsberg, R.S., Cullis, P.R. and Bally, M.B. (1989) Cancer Res. 49, 5922–5930.

23 Stein, O. Oette, K., Haratz, D., Halperin, G. and Stein, Y. (1988) Biochim. Biophys. Acta 960, 322–333.
24 Barenholz, Y. and Yechiel, E. (1989) U.S. Patent 4.812.314.
25 Mezei, M. (1988) in Liposomes and Drug Carriers (Gregoriadis, G., ed.), pp. 663–677, Wiley, New York.
26 Cullis, P.R., Mayer, L.D., Bally, M.B., Madden, T.D. and Hope, M.J. (1989) Adv. Drug Deliv. Rev. 3, 267–282.
27 Scherphof, G., Damen, J., Dijkstra, J., Roerdink, F. and Spanjer, H. (1984) Life Sci. 82, 267–295.
28 Bakkeren, H.F., Kuipers, F., Vonk, R.J. and Van Berkel, T.J.C. (1990) Biochem. J. 268, 685–691.
29 Friedman, M. and Byers, S.O. (1958) Am. J. Physiol. 195, 185–188.
30 Byers, S.O., Friedman, M. and Sugiyama, T. (1962) J. Biol. Chem. 237, 3375–3380.