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Separation of large unilamellar liposomes from blood components by a spin column procedure: towards identifying plasma proteins which mediate liposome clearance in vivo

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In order to facilitate the isolation of liposomes from blood components, we have developed a simple and rapid procedure combining chromatographic and centrifugal methods. This 'spin column' procedure was used to isolate liposomes from incubation mixtures with human serum or from the blood of CD1 mice after intravenous administration of liposomes. An advantage of this procedure is that processing times are fast (typically minutes) such that the isolation procedure can be done in the absence of chelators or other coagulation inhibitors which may affect protein / liposome interactions. Furthermore, several samples can be analyzed together and small sample volumes can be processed. In addition, we show that this spin column procedure can be employed to isolate large unilamellar vesicles averaging 100 nm in diameter from lipoproteins and plasma proteins. The applicability of this spin column procedure in studying protein / liposome interactions is demonstrated by quantitating the amount of human complement component C3 bound per liposome using a C3 competitive ELISA assay after incubation with human serum. The proteins associated with the recovered liposomes were further analyzed by conventional SDS-polyacrylamide gel electrophoresis. We show that egg phosphatidylcholine / cholesterol (55 : 45, mol / mol) or egg phosphatidylcholine / cholesterol / dioleoylphosphatidylserine (35 : 45 : 20, mol / mol) liposomes isolated from the circulation of CD1 mice within minutes of administration have distinct, complex profiles of associated proteins. By isolating circulating large unilamellar liposomes using the spin column method and characterizing the proteins associated with their membranes, this protein fingerprinting approach will expedite identifying protein interactions which affect liposome stability and clearance in vivo.

Introduction

Large unilamellar vesicles (LUVs) are widely used for liposome-based carrier systems [1,2]. As with all liposome systems, however, LUVs are rapidly cleared from the circulation, having half-lives ranging from minutes to hours. The interactions liposomes encounter in the circulation are believed to significantly influence their clearance and inherent stability in blood via at least two mechanisms. First, the interactions with lipoproteins, complement and serum albumin have been shown to result in liposome membrane destabilization leading to leakage of contents (for reviews, see Refs. 3–6). This has been attributed to a net transfer of lipid to lipoproteins or to the formation of pores in the membrane in the case of complement proteins. Second, liposome interactions with complement, fibronectin and immunoglobulins have been suggested to mediate liposome clearance by the fixed and free macrophages of the reticuloendothelial system, as liposomes coated with these proteins exhibit enhanced uptake by cultured macrophages [7–9].

The factors which mediate liposome opsonization in vivo are poorly understood, particularly for large uni-

Abbreviations: C3, complement component C3; ELISA, enzyme-linked immunosorbent assay; CH, cholesterol; CL, bovine heart cardiolipin; DOPA, dioleoylphosphatidic acid; DOPS, dioleoylphosphatidylserine; EPC, egg phosphatidylcholine; EPG, egg phosphatidylglycerol; PI, plant phosphatidylinositol; LDL, low density lipoproteins; LUV, large unilamellar vesicle; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; VLDL, very low density lipoproteins.

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lamellar liposomal systems. This is because of the difficulty in isolating LUVs from blood components such as lipoproteins and recovering sufficient amounts to analyze by conventional SDS-polyacrylamide gel electrophoresis. Much early work involved multilamellar vesicles or sonicated vesicles which were recovered from plasma mixtures by ultracentrifugation followed by multiple washes or by gel filtration chromatography, respectively [3,4,10,11]. No convenient procedure for isolating LUVs from plasma components has been available. Such a technique is important to study the protein interactions LUVs experience in the circulation. These interactions are likely to differ from those observed for multilamellar and sonicated vesicles as vesicle size affects liposome stability [12,13].

In this paper we describe a method for the rapid isolation of LUVs from plasma components. We have applied this method to isolate liposomes from incubation mixtures with human serum and more significantly, from the blood of mice injected intravenously with liposomes. In the latter case the liposomes are exposed to the entire biological milieu, i.e. they are in contact with the immune system, blood cells, coagulation proteins, endothelial cells and physiological ion (notably Ca^{2+} and Mg^{2+}) concentrations. The applicability of this isolation procedure for studying protein/liposome interactions is demonstrated by quantitating the amount of C3 bound to various anionic or neutral LUVs after incubation with human serum. Further, as liposomes bearing a net surface charge are rapidly cleared from the circulation [12] and are potent activators of the complement system [14], we had previously proposed that liposome-associated C3b may play a significant role in receptor-mediated liposomal uptake. We have therefore determined here whether liposome compositions which exhibited rapid clearance kinetics bound greater amounts of C3. In addition, the proteins associated with the recovered liposomes were analyzed by conventional SDS-polyacrylamide gel electrophoresis and visualized by a sensitive silver stain protocol. Our findings indicate that liposomes isolated from the circulation of CD1 mice within minutes of intravenous administration have a complex profile of proteins associated with their membranes which is dependent on the lipid composition, and that liposomes which are cleared more rapidly bind significantly more C3b.

Methods and Materials

Preparation of liposomes. Large unilamellar vesicles (LUVs) were prepared by extrusion of freeze-thawed multilamellar vesicles through two stacked 100 nm polycarbonate filters (Nuclepore, Pleasanton, CA) using an extrusion device (Lipex Biomembranes, Vancouver, Canada) as described in detail elsewhere [15]. Liposome suspensions were 20 mM total lipid in isotonic Hepes-buffered saline (HBS; 20 mM Hepes (pH 7.4), 145 mM NaCl) sterilized using Syrfil 0.22 μm filters (Nuclepore). The size of the extruded vesicles was determined by quasi-elastic light scattering analysis on a NICOMP Model 270 Submicron Particle Sizer (NICOMP Instruments, Santa Barbara, CA) to be 100 $\pm$ 30 nm. The liposomes were radiolabelled by incorporating the non-exchangeable, non-metabolizeable marker [^{3}H]cholesterylhexadecyl ether (10 μCi/30 μmol lipid) to follow the biodistribution of the liposomes in mice and to quantitate the concentration of the recovered liposome suspensions [16]. The specific activity of the liposomes was determined by measuring the radioactivity content using standard liquid scintillation counting methods on a Hewlett Packard Tri-Carb 2000CA liquid scintillation analyzer and the phospholipid content using a colorimetric (absorbance recorded at 815 nm) phosphorus assay [17]. Lipids were purchased from the following companies: egg phosphatidylcholine (EPC), egg phosphatidylglycerol (EPG), dioleoylphosphatidylserine (DOPS), dioleoylphosphatidic acid (DOPA), plant phosphatidylinositol (PI) and bovine heart cardiolipin (CL) from Avanti Polar Lipids, Pelham, AL; cholesterol (CH) from Sigma; and [^{3}H]cholesterylhexadecyl ether from Amersham. These lipids were used without further purification. Liposome compositions are expressed as molar ratios.

Serum and lipoproteins. Human serum was prepared from venous blood pooled from 20 healthy individuals (10 males, 10 females) and stored at -70°C. Purified very low density lipoproteins (VLDL) and low density lipoproteins (LDL) were provided by Dr. H.P. Pritchard, Lipoprotein Research Group, Vancouver, Canada.

Preparation of spin columns. The procedure for packing the spin columns is as follows. 1 ml tuberculin syringes plugged with glass wool were filled with Bio-Gel A-15m, 200–400 mesh size, chromatographic gel (Bio-Rad, Mississauga, Canada) equilibrated with isotonic veronal-buffered saline (VBS, 10 mM sodium barbital (pH 7.4), 145 mM NaCl) and centrifuged (Silencer H-103N Series bench top centrifuge or Jouan centrifuge G4.11; 2000 rpm, 2 min, 4°C) in 13 × 100 mm glass culture tubes. A series of fills and centrifugations were done until the bed volume approximated 1.0 ml. A final spin at 2000 rpm for 5 min assured that the Bio-Gel A-15m gel was uniformly packed and that excess buffer was removed.

Spin column profiles of liposomes incubated with human serum. 100 μl of the liposome suspension was incubated with 400 μl of pooled normal human serum at 37°C for 30 min. Aliquots of the liposome/serum incubation mixtures (50 μl) were applied to spin columns and immediately centrifuged (1000 rpm, 1 min, 4°C). Column fractions were collected in glass culture tubes by applying 50 μl of VBS to the spin

columns and centrifuging (1000 rpm, 1 min, 4°C). The liposome content of the column fractions were assayed by determining the ^3H radioactivity content of the fractions using standard liquid scintillation counting methods. Protein content of the column fractions was determined using the BCA protein assay (Pierce Chemical Co., Rockford, IL). Briefly, 10 μl of column fractions were incubated with 200 μl BCA protein assay reagent in microtiter plates. After an overnight incubation at room temperature, the absorbance (540 nm) of the solutions was read using an SLT-Labinstruments Austria EAR400AT microtiter plate reader. As controls in all our experiments, 50 μl of 80% human serum without liposomes was chromatographed using similar spin columns under identical conditions and the column fractions analyzed for protein content.

Conventional Bio-Gel A-15m chromatography of liposome/human serum incubation mixtures. Two ml of 100 mM EPC/CH/CL (35:45:10) LUVs in VBS was incubated with 8 ml of normal human serum at 37°C for 30 min. To isolate the liposomes from human serum components, the incubation mixture was chromatographed on a 2.5 × 90 cm Bio-Gel A-15m, 100–200 mesh, column pre-equilibrated with VBS buffer at 4°C. Fractions (120 drops/fraction) were collected at a flow rate of 30 ml/h. The column fractions were analyzed for phospholipid content using a colorimetric phosphorus assay [17] and for protein content using the BCA protein assay. Fractions 25–32 were pooled.

Recovery of liposomes from circulation of CD1 mice. 200 μl of the liposome suspension was administered intravenously via the dorsal tail vein of CD1 mice (4 mice/time point). After 2–3 min, 30 min, or 1 h post-injection, the mice were anesthetized with ether and blood withdrawn via cardiac puncture and collected in ice cold 1.5 ml polypropylene micro test tubes (Eppendorf). The blood was immediately cooled to 0°C using an ice-water bath to prevent coagulation and centrifuged (12000 rpm, 2 min, 4°C) to pellet the blood cells. Aliquots of the plasma (50 μl) were applied to spin columns (5 columns/mouse) and immediately centrifuged (Jouan Centrifuge G4.11; 1000 rpm, 1 min, 4°C). Column fractions were collected in glass culture tubes by applying 50 μl of VBS to the spin columns and centrifuging (1000 rpm, 1 min, 4°C). The first two column fractions containing radioactivity (typically fractions 5 and 6, or 6 and 7) were collected, pooled and concentrated using Centricon 30 microconcentrators (Amicon, Danvers, MA) at 4°C. The radioactivity recoveries from the Centricon 30 microconcentrators were typically greater than 95%. The samples were stored at −20°C.

Competitive ELISA for C3. C3 competitive ELISA was performed on serially diluted isolated liposomes using the procedure described by Mold [18]. Human C3 was purified according to the method of Tack and Prahl [19]. Horse radish peroxidase-conjugated goat anti-human C3 (CalBiochem) was used at a 1/10000 dilution.

SDS-polyacrilamide gel electrophoretic analysis of proteins associated with liposomes. Protein separation was performed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini Protean-II electrophoretic apparatus (Bio-Rad) on precast 4–20% gradient Mini Protean-II gels (Bio-Rad) under nonreducing conditions. Prestained SDS-PAGE molecular weight standards (Diversified Biotech, Newton, MA) were used to estimate the molecular weights of the proteins. Proteins were detected using an optimized silver stain procedure [20].

Results

Isolation of large unilamellar liposomes from plasma using Bio-Gel A-15m spin columns

Initial studies indicated that coventional Bio-Gel A-15m, 100–200 mesh, gel filtration chromatography was effective in isolating LUVs (extruded through 100 nm pore sized filters) from human serum components (Fig. 1). However, this procedure, requiring lengthy processing times (typically 3–4 h per separation) and relatively large sample volumes, was not practical for our studies. Therefore, several factors including use of a finer mesh (200–400 mesh) to increase the number of theoretical plates; use of shorter columns, thereby reducing the sample volume requirement; and increasing operating pressures, to reduce the separation time were considered in order to improve this isolation procedure. A rapid method for the isolation of LUVs

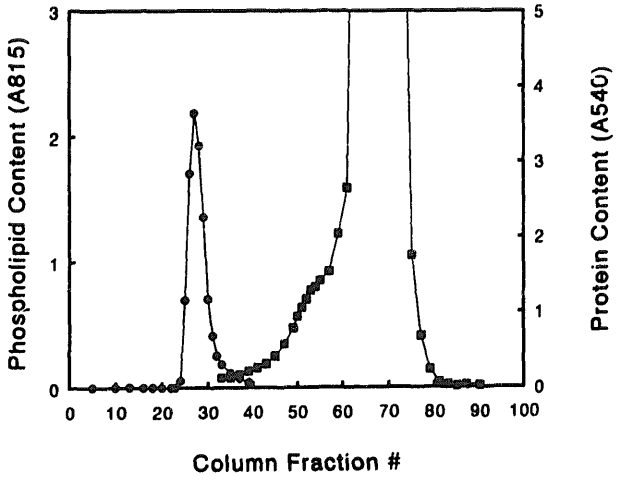

Fig. 1. Elution profile of EPC/CH/CL LUV/human serum incubation mixtures chromatographed on conventional columns. EPC/CH/CL (35:45:10) LUV/human serum incubation mixtures were chromatographed on a 2.5×90 cm Bio-Gel A-15m, 100–200 mesh, column as described in Methods and Materials. The phosphorus content (●) or the protein content (■) of the column fractions was determined as in Methods and Materials.

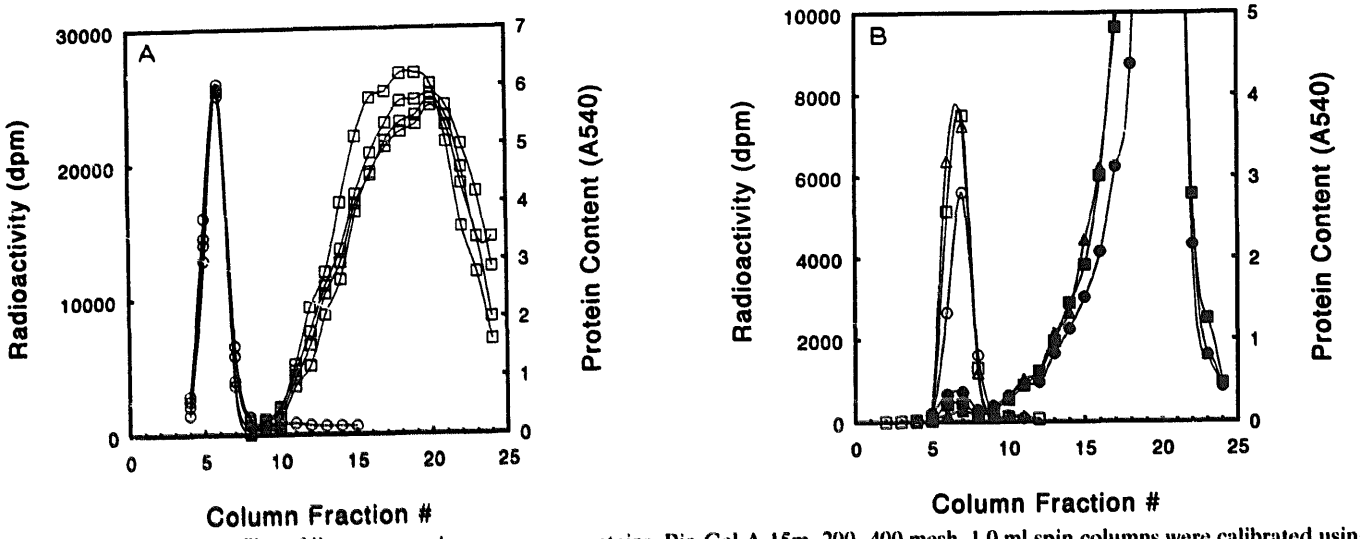

Fig. 2. (A) Spin column profiles of liposomes or human serum proteins. Bio-Gel A-15m, 200–400 mesh, 1.0 ml spin columns were calibrated using 50 μl 20 mM EPC/CH (55:45) LUVs radiolabelled with [^{3}H]cholesterylhexadecyl ether (○, 4 columns) or 50 μl 80% human serum (□, 4 columns) chromatographed separately under identical conditions. Column fractions represent the eluant recovered from one centrifugation (1000 rpm, 1 min). (B) Spin column profiles of liposome/human serum incubations. EPC/CH/DOPA (35:45:20) (□), EPC/CH/DOPS (35:45:20) (△), or EPC/CH/PI (35:45:20) (○) LUVs containing trace amounts of [^{3}H]cholesterylhexadecyl ether were incubated with human serum at 37 °C for 30 min. The incubation mixtures were then chromatographed using the Bio-Gel A-15m spin columns as described in Methods and Materials. The open symbols are the radioactive content and the filled symbols are the protein content of the column fractions.

from plasma components was thus developed using a 'spin column' procedure described in Methods.

The efficiency of separation using this technique is depicted in Fig. 2. Fig. 2A shows the column profiles of EPC/CH (55:45) LUVs or of 80% human serum chromatographed on Bio-Gel A-15m 1.0 ml spin columns. Fig. 2B shows the column profiles of various LUV/80% human serum incubation mixtures. As shown in Fig. 2B, the liposome composition does not affect the elution profile of the liposomes. These profiles are representative of hundreds of columns using LUVs composed of net neutral or anionic lipids. By centrifuging the column in a 13 × 100 mm glass culture tube carrier, the flow rate through the column was considerably increased. The profiles shown were obtained using the optimized spin conditions of 1000 rpm for 1 min. These optimal conditions were determined by varying the rate and duration of centrifugation. The conditions of 1000 rpm for 1 min gave the most consistent elution profiles and fraction volumes (typically

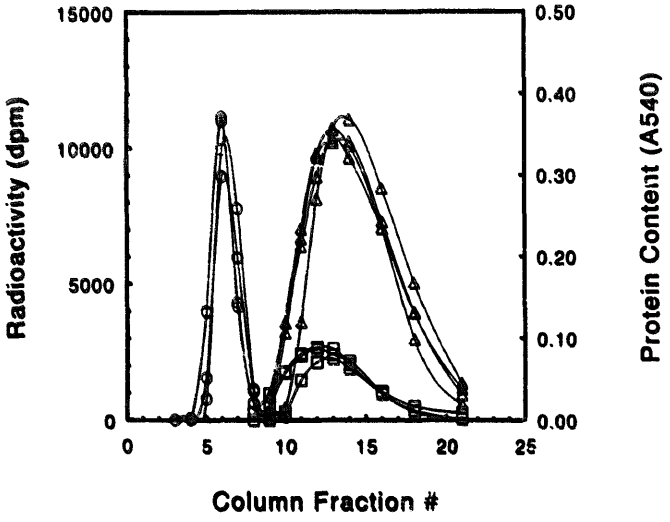

Fig. 3. Separation of 100 nm LUVs from VLDL and LDL. Bio-Gel A-15m, 200–400 mesh, 1.0 ml spin columns were calibrated using 50 μl 20 mM EPC/CH (55:45) LUVs radiolabelled with [^{3}H]cholesterylhexadecyl ether (○, 4 columns), 50 μl purified VLDL (□, 4 columns) or 50 μl purified LDL (△, 4 columns). VLDL and LDL content were detected using the BCA protein assay. These were chromatographed separately on similar spin columns under identical conditions.

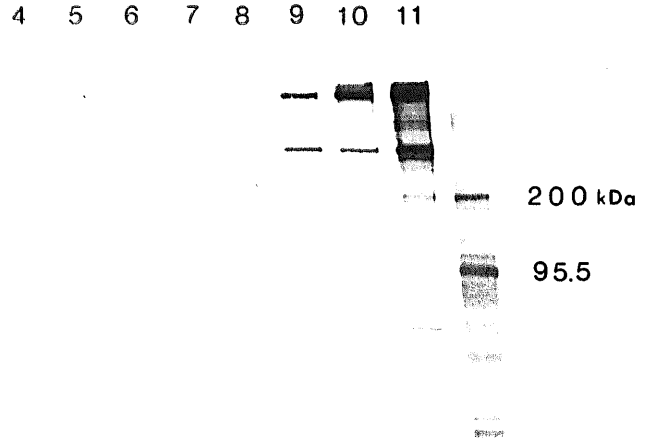

Fig. 4. SDS-polyacrylamide gel electrophoretic analysis of the protein content of the spin column fractions. The column fractions of chromatographed 50 ml 80% human serum were analyzed for their protein content by SDS-polyacrylamide gel electrophoresis followed by silver staining as described in Methods and Materials.

30–40 μl). Spin times longer than 2 min resulted in channel formation at the top of the column. Column bed volumes averaged 1.00 ± 0.15 ml. The mean liposome recovery in the first 9 fractions was 70%.

Elution profiles obtained with purified lipoproteins showed that the liposomes were effectively resolved from the very low density and low density lipoproteins (Fig. 3) using the spin column procedure.

The column fractions were analyzed for their protein content by SDS-PAGE followed by silver staining (Fig. 4). When 80% serum in VBS was chromatographed, the fractions where the liposomes would elute (fractions 5–7) did not contain any detectable protein. The fraction where the serum proteins started eluting (fraction 9) contained very high molecular weight proteins having mobilities corresponding to molecular masses greater than 200 kDa.

The protein profile of the liposomes isolated using the spin column procedure was compared to that obtained using the conventional gel filtration column procedure by analyzing equal amounts of lipid on 4–20% gradient SDS-polyacrylamide gels (Fig. 5). Fig. 5 shows that the protein profiles are very similar in type and amount of proteins associated with EPC/CH/CL (35:45:10) LUVs. EPC/CH/CL LUVs were used because this composition bound the most complex protein profile of all the anionic liposomes investigated.

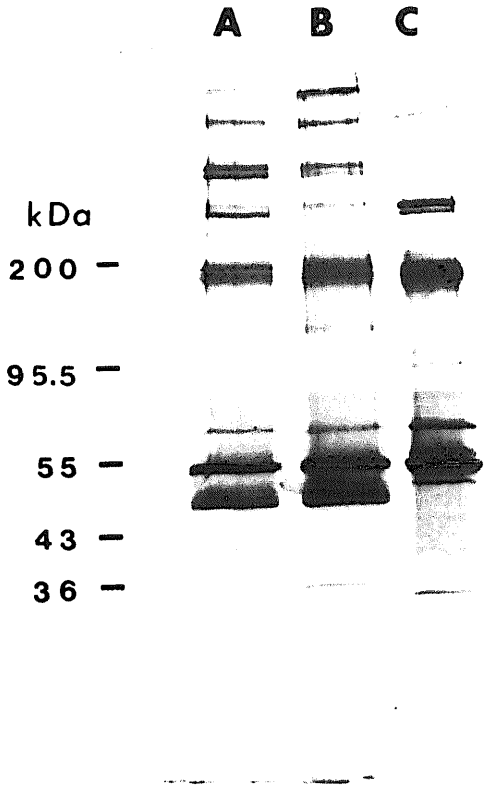

Fig. 5. Comparison of the protein profile associated with EPC/CH/CL LUVs isolated using the spin column procedure or conventional chromatographic procedures. EPC/CH/CL (35:45:10) LUVs recovered using the Bio-Gel A-15m, 100–200 mesh, conventional column (Lane A) or the Bio-Gel A15m, 200–400 mesh, spin column (Lane B) procedures were analyzed for the proteins associated with their membranes by SDS-PAGE analysis as in Methods and Materials. Equal amounts of lipid (30 nmol total lipid) were applied to each lane. Lane C is 25 μl of 1/750 dilution of pooled normal human serum.

TABLE I

Amount of C3 associated with various LUVs

The LUVs recovered from human serum incubations using the spin column procedure were analyzed for C3 content using a C3 competitive ELISA (see Methods and Materials). The results from two separate experiments are given.

LUV composition	Amount of C3 bound [a] (nmol C3/mmol total lipid)
EPC/CH (55:45)	3.15, 4.61
EPC/CH/PI (35:45:20)	8.76, 6.66
EPC/CH/PG (35:45:20)	13.7 , 16.6
EPC/CH/DOPS (35:45:20)	31.5 , 19.8
EPC/CH/DOPA (35:45:20)	36.9 , 33.1
EPC/CH/CL (35:45:10)	46.2 , 33.9

Measurement of C3 bound per liposome recovered from human serum incubation mixtures using the spin columns

Previous studies have demonstrated that anionic liposomes activate the complement system via the classical pathway leading to the liposomal association of C3b [14]. It was suggested that C3b was one of the plasma proteins which marked the liposomes as foreign particles because C3b is a potent opsonin. As the population of liposomes is essentially unilamellar for all lipid compositions employed here, we can estimate the amount of C3b bound per lipid as a function of lipid composition. Using a human C3 competitive ELISA, the amount of C3 associated with the various liposomes was quantitated (Table I). The amount of C3 associated with EPC/CH/CL (35 : 45 : 10) or EPC/CH/DOPA (35:45:20) LUVs is approximately 4–10-times greater than for EPC/CH (55 : 45), EPC/CH/EPG (35 : 45 : 20) or EPC/CH/PI (35:45:20) LUVs.

In vivo characterization of murine plasma proteins associated with EPC / CH or EPC / CH / DOPS LUVs over time

The utility of this spin column method in recovering LUVs from blood of mice administered intravenously with EPC/CH (55:45) or EPC/CH/DOPS (35:45:20) liposomes was investigated. Fig. 6 shows the recovery of LUVs from the plasma of CD1 mice over a 1 h period. Whereas EPC/CH/DOPS LUVs are cleared rapidly from the plasma, EPC/CH LUVs are relatively long-lived in the circulation. The liposomes were isolated from the blood samples in the absence of chelators or other coagulation inhibitors by cooling the

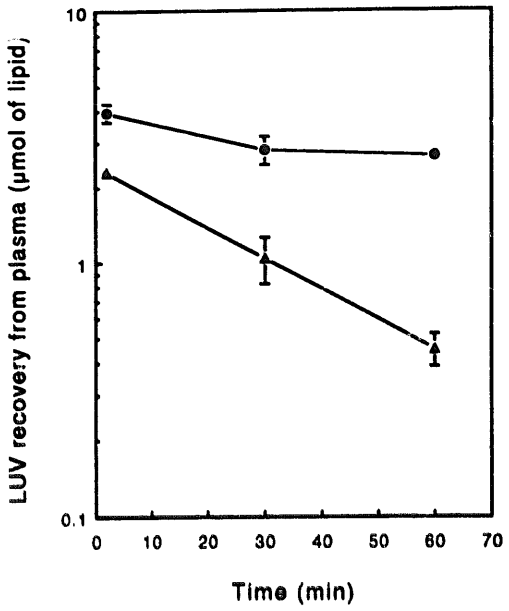

Fig. 6. Recoveries of EPC/CH and EPC/CH/DOPS LUVs over time from mouse plasma. EPC/CH (55:45) (●) or EPC/CH: DOPS (35:45:20) (▲) liposomes were administered intravenously into CD1 mice and over time aliquots of plasma were counted for ^{3}H radioactivity to follow the clearance of liposomes from the circulation. Each mouse received a dose of approx. 4 μmol total lipid in a volume of 200 ml HBS. Plasma volume of a mouse was taken to be 5% of body weight.

blood samples to 0°C to retard the coagulation process, centrifuging to pellet the blood cells, and separated from the plasma using the Bio-Gel A-15m spin columns. The protein profiles were characterized using SDS-PAGE analysis (Fig. 7). Within a few minutes after administration, EPC/CH LUVs are shown to have an associated protein composition consisting mainly of albumin and very high molecular weight proteins. Qualitatively, the overall protein profile associated with circulating EPC/CH LUVs is not markedly altered over a 1 h period. The rapidly cleared EPC/CH/DOPS LUVs, on the other hand, have a more complex protein profile, both in amount and type of associated proteins, than the EPC/CH LUVs. No protein was detected in these column fractions when mouse serum alone was chromatographed.

Discussion

The development of methods to rapidly isolate LUVs from blood components is important to understanding the protein/liposome interactions which mediate liposome leakage and clearance from the circulation. Until now, there has been no satisfactory procedure for isolating LUVs from plasma. In this report, we describe a procedure which is very convenient for the study of protein/liposome interactions which occur in vivo. Compared to conventional column chromatographic methods which take about 3–4 h per analysis (Fig. 1 in this report; Refs. 21 and 22), spin column processing times are extremely rapid. From the point of sample application to the collection of the liposome fractions, the isolation procedure takes approx. 6–8 min. Other advantages of this spin column procedure are that many columns can be processed at the same time (up to 96 depending on the capacity of the centrifuge) and that small sample volumes can be analyzed. The major advantage in particular to the study of protein-mediated liposome clearance mechanisms, however, is that because processing times are rapid and the procedure can be readily performed at 4°C, the isolation procedure can be done in the absence of coagulation inhibitors which may affect protein–liposome interactions.

Good reproducibility in isolating LUVs from blood components using the spin column method is obviously necessary for detecting the proteins associated with circulating liposomes. The results presented here are representative of hundreds of spin columns; however, we have encountered some variation in the performance of different batches of Bio-Gel A-15m gel. These variations result in different flow rates and col-

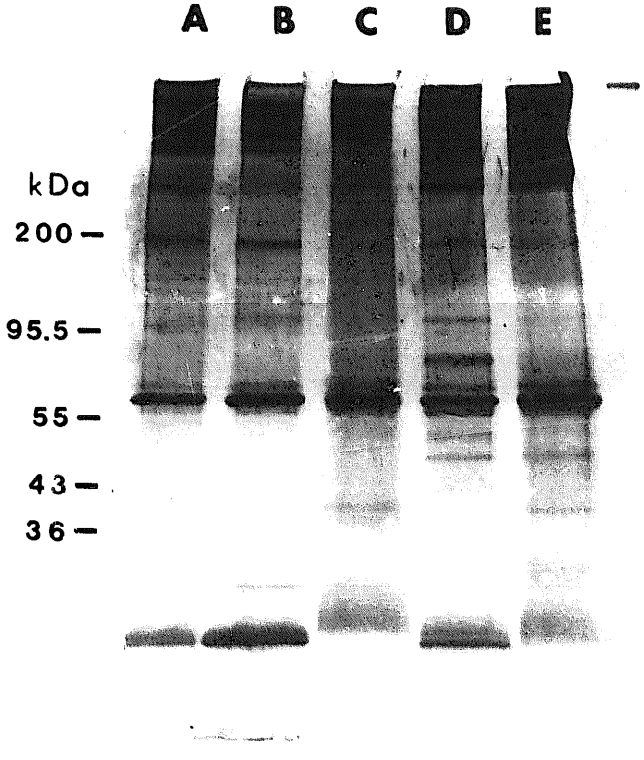

Fig. 7. Protein profiles of EPC/CH (55:45) and EPC/CH/DOPS (35:45:20) LUVs recovered from mice over time. The proteins associated with the recovered liposomes were analyzed using 4–20% gradient SDS-polyacrylamide gel electrophoresis and silver stained as in Methods and Materials. Lane A, EPC/CH, 2 min; Lane B, EPC/CH, 30 min; Lane C, EPC/CH, 1 h; Lane D, EPC/CH/DOPS, 2 min; EPC/CH/DOPS, 30 min. Lanes A–D represent the proteins associated with 40 nmol total lipid; Lane E represents the proteins associated with 20 nmol total lipid.

umn fraction volumes. The elution profiles are therefore sometimes different and on one occasion resulted in serious tailing in the liposome elution profile. This potential problem can be overcome by careful characterization of the elution profiles of the spin columns using different batches of Bio-Gel A-15m gel. Within a particular batch, the elution profiles are very reproducible.

Using this spin column procedure a population of EPC/CH (55:45) LUVs which remained circulating over a 1 h period has been separated and the associated proteins analyzed. We find that these net neutral liposomes bound a number of high molecular weight (> 200000) proteins which is similar to the in vitro findings of Juliano and Lin [23] using human plasma. This similarity is noteworthy since the earlier study involved multilamellar vesicles isolated by ultracentrifugation followed by multiple washes with buffer. This indicates that proteins which are associated with the spin column-isolated LUVs are tightly bound. The high molecular weight proteins as well as albumin are associated with EPC/CH LUVs within minutes of intravenous administration, and do not appear to enhance the clearance of liposomes from the circulation over a 1 h period because the population of liposomes bearing these proteins remain in the circulation over this period.

In contrast to the EPC/CH (55:45) protein profiles, the profile of proteins associated with rapidly cleared EPC/CH/DOPS (35:45:20) LUVs is more complex. The same proteins which are associated with EPC/CH LUVs are also found associated with EPC/CH/DOPS LUVs; however, more proteins, both in terms of amount and type, are associated with the anionic EPC/CH/DOPS LUVs. Some of these proteins may include clotting proteins and complement proteins as it has been reported that PS-containing liposomes activate the coagulation [23–26] and complement systems [14,27]. The identification of the proteins associated with these liposomes, especially those proteins which are uniquely associated with rapidly cleared liposomes, should elucidate proteins which affect liposome clearance from the circulation.

Changes in the protein fingerprints of the liposomes over time may provide insight into the proteins affecting liposome stability in the circulation. The finding that specific associated proteins disappear from the protein profiles over time suggest that liposomes bearing these proteins are cleared more readily. For example, EPC/CH LUVs at 2 min and 30 min have a pronounced band, migrating similarly to the 200 kDa molecular mass standard, associated with their membranes. This band notably disappears at the 1 h time point. Similarly, a prominent band, migrating with a molecular weight corresponding roughly to 80000, is associated with the EPC/CH/DOPS LUVs at the 2 min time point but is less intense at 30 min, although it should be noted that Lane D in Fig. 7 has twice the amount of total lipid loaded as in Lane E. Similar studies that characterize the protein fingerprint of liposomes over time, involving more quantitative methods, should be very useful in elucidating the proteins involved in liposome clearance.

The EPC/CH/DOPS (35:45:20) LUVs recovered from the circulation of mice had a more extensive protein fingerprint (Fig. 7) than the EPC/CH/CL LUVs recovered from in vitro incubations with human serum (Fig. 5), especially with regard to the very high molecular weight proteins (> 200000). This underlines the necessity to study the in vivo plasma protein interactions liposomes experience in order to fully understand the protein-mediated clearance behavior of liposomes.

One of the plasma proteins which has been implicated in mediating liposome uptake is complement component C3, specifically the opsonic form C3b. We had previously demonstrated that liposomes bearing a net surface charge are potent activators of the complement system resulting in the deposition of C3b molecules onto the liposome membranes [14]. The results presented here clearly show that liposomes containing DOPA or CL exhibit considerably more associated C3. If liposome clearance behavior is similar in mice and humans, these results suggest a correlation between the amount of C3 bound per liposome and liposome clearance behavior. CL- and DOPA-containing systems, which bind the most C3 (Table I), are cleared very rapidly from the circulation [12,28]. EPC/CH, and EPG- and PI-containing systems are cleared more slowly [29,30] and bind much less C3.

In summary, the spin column procedure is a rapid and effective method for recovering LUVs from incubation mixtures with blood components. This procedure requires little manipulation of the system; no coagulation inhibitors are necessary and no multiple washes with buffer are required. Thus, the liposomes can be readily recovered from the blood of mice after intravenous administration of liposomes. This was demonstrated in the recovery of EPC/CH (55:45) LUVs over a period of 1 hr (Fig. 7). The amount of liposomes recovered from this procedure is adequate to analyze the protein content of the liposomes by SDS-polyacrylamide gel electrophoresis followed by silver staining. As well, the liposome proteins are readily analyzed using immunological methods such as immunosorbent assays. Further, this spin column isolation procedure allows the use of large unilamellar liposomal systems as opposed to multilamellar liposomal systems in studies involving protein/membrane interactions. The advantage of using LUVs, where all the lamellae are exposed to the extravesicular environment, as opposed to MLVs, where only the outermost

lamellae is exposed to the extravesicular environment, is clearly demonstrated in the studies quantitating the amount of liposome-associated C3 (Table I). The relation between the amount of associated C3 (Table I) and liposome clearance from mouse blood strongly suggests that C3 fragments play a role in liposome clearance. This protein fingerprinting approach has great potential in elucidating the protein/liposome interactions which occur in vivo and in identifying protein interactions which may affect liposome clearance from the circulation. A detailed in vivo study of the proteins that interact with various liposomes exhibiting markedly different clearance pharmacokinetics, and identification of some of the proteins mediating liposome clearance are in progress in this laboratory. This spin column procedure could also have a general utility in studies involving protein–membrane interactions.

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