THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1992 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 267, No. 26, Issue of September 15, pp. 18759-18765, 1992
Printed in U.S.A.

Association of Blood Proteins with Large Unilamellar Liposomes *in Vivo*

RELATION TO CIRCULATION LIFETIMES*

(Received for publication, February 20, 1992)

Arcadio Chonn‡, Sean C. Semple, and Pieter R. Cullis

From the Department of Biochemistry, The University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

The proteins associated with liposomes in the circulation of mice were analyzed in order to determine whether bound proteins significantly influence the fate of liposomes *in vivo*. Liposomes were administered intravenously via the dorsal tail vein of CD1 mice and were isolated from blood after 2 min in the absence of coagulation inhibitors using a rapid "spin column" procedure. Various negatively charged liposomes exhibiting markedly different clearance properties were studied; notably, these included liposomes containing 10 mol % ganglioside G_{M1} which has been previously shown to effectively limit liposomal uptake by the fixed macrophages of the reticuloendothelial system. The protein binding ability (P_B; g of protein/mol of lipid) of the liposomes was quantitated and related to the circulation half-life ($\tau_{1/2}$) of the liposomes. Liposomes having similar membrane surface charge imparted by different anionic phospholipids were found to exhibit markedly different protein binding potentials. Furthermore, P_B values determined from the *in vivo* experiments were found to be inversely related to circulation half-lives. P_B values in excess of 50 g of protein/mol of lipid were observed for rapidly cleared liposomes such as those containing cardiolipin or phosphatidic acid ($\tau_{1/2} < 2$ min). P_B values for ganglioside G_{M1}-containing liposomes ($\tau_{1/2} > 2$ h) were significantly less ($P_B < 15$ g of total protein/mol of total lipid). P_B values were also determined for liposomes recovered from *in vitro* incubations with isolated human serum; relative P_B values obtained from these *in vitro* experiments were in agreement with relative P_B values measured from *in vivo* experiments. P_B values, therefore, could be a useful parameter for predicting the clearance behavior of liposomes in the circulation. Liposomes exhibiting increased P_B values *in vivo* were shown by immunoblot analysis to bind more immune opsonins, leading to a higher probability of phagocytic uptake. Finally, based on results obtained using the *in vitro* system, it is suggested that the mechanism by which ganglioside G_{M1} prolongs the murine circulation half-life of liposomes is by reducing the total amount of blood protein bound to the liposomes in a relatively nonspecific manner.

A central problem to the use of liposomes and other carriers for drug delivery is their rapid clearance from the circulation (reviewed by Gregoriadis (1988) and Senior (1987)). Although the mechanisms involved in the *in vivo* clearance of liposomes from the circulation are poorly understood, various aspects of liposome design are known to strongly influence liposome clearance behavior. For example, negatively charged liposomes are cleared more rapidly than net neutral or positively charged systems (Juliano and Stamp, 1975). The presence of saturated phospholipids (Gregoriadis and Senior, 1980) or equimolar amounts of cholesterol (Kirby *et al.*, 1980; Patel *et al.*, 1983; Roerdink *et al.*, 1989) stabilize liposomes in the circulation and also reduce their uptake by the phagocytic cells of the reticuloendothelial system. Liposomes containing hydrogenated plant phosphatidylinositol (Papahadjopoulos and Gabizon, 1987), ganglioside G_{M1} (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988), or amphipathic phosphatidylethanolamine derivatives of polyethylene glycols (Klibanov *et al.*, 1990; Blume and Cevc, 1990) have been shown to exhibit extended circulation lifetimes. The basis of the effect of lipid composition on the clearance rate of liposomes is not known.

It is widely believed that blood proteins mediate the increased liposome permeability and rapid liposome uptake by the reticuloendothelial system that liposomes experience *in vivo*. This potential role of blood proteins in liposome clearance has been studied extensively using *in vitro* systems employing liposome incubations with isolated plasma or serum. From these *in vitro* studies, however, there appears to be no unambiguous relation between the amount and type of protein bound and liposome clearance behavior. For example, Black and Gregoriadis (1976) reported that human α_2-macroglobulin or rat α_1-macroglobulin was the only protein associated with liposomes exposed to plasma, and that this protein imparted a net negative liposome surface charge regardless of the inherent charge of the membrane. In contrast, Juliano and Lin (1980) reported that neutral or positively charged liposomes bound several plasma proteins including albumin, apolipoprotein A1, IgG, and a group of high molecular weight (>200, 000) proteins. Negatively charged liposomes failed to bind these high molecular weight components. The amount of protein associated with phosphatidylserine-containing liposomes did not significantly differ from that associated with PC:CH[1] multilamellar systems (Juliano and Lin, 1980).

* This study was supported by a grant from the National Cancer Institute of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be sent: The University of British Columbia, Dept. of Biochemistry, Faculty of Medicine, 2146 Health Sciences Mall, Vancouver, British Columbia V6T 1Z3, Canada. Tel: 604-822-4955; Fax: 604-822-4843.

[1] The abbreviations used are: PC, egg phosphatidylcholine; CH, cholesterol; CL, bovine heart cardiolipin; DOPA, dioleoylphosphatidic acid; DOPS, dioleoylphosphatidylserine; PA, egg phosphatidic acid; PG, egg phosphatidylglycerol; PI, phosphatidylinositol; PS, bovine liver phosphatidylserine; VBS, veronal-buffered saline; P_B, protein binding ability; $\tau_{1/2}$, circulation half-life; LUVs, large unilamellar vesicles; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

To date, there have been no studies demonstrating that liposome clearance *in vivo* correlates with the amount and type of associated blood protein. There are two main reasons for this. First, the large majority of studies on the association of plasma proteins with liposomes *in vitro* have been performed employing multilamellar systems. Due to the variable lamellarity of liposomes of different lipid compositions, quantification of the amount of various proteins associated per liposome has not been possible. Second, and more importantly, techniques have not been available for the isolation of liposomes, particularly large unilamellar vesicles (LUVs), from blood components recovered in the absence of coagulation inhibitors following the *in vivo* administration of liposomes. In this study, we employed a recently developed spin column procedure (Chonn *et al.*, 1991b) to isolate LUVs of variable lipid compositions from the blood of CD1 mice following intravenous administration. These *in vivo* findings suggest that there is an inverse relation between the amount of total blood protein binding to liposomes (P_B) and the clearance rate of the liposomes from the circulation.

MATERIALS AND METHODS

Preparation of Liposomes—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 previously (Hope *et al.*, 1985). The 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 preparation of liposomes containing gangliosides was facilitated by extrusion at 65 °C. A radiolabeled lipid marker, [^{3}H] cholesterylhexadecyl ether (10 μCi/30 μmol of total lipid), was incorporated to follow the biodistribution of the liposomes in mice and to quantitate the concentration of the recovered liposome suspensions. This lipid marker has previously been shown to be nonexchangeable and nonmetabolizeable in animal systems with low levels of cholesterol ester transfer protein activity, such as the mouse or rat system (Stein *et al.*, 1980; Halperin *et al.*, 1986; Derksen *et al.*, 1987; Green *et al.*, 1989).[2] The specific radioactivity of the liposome suspensions was determined by measuring the radioactivity content using standard liquid scintillation counting methods, and the phospholipid content using a colorimetric phosphorus assay (Fiske and Subbarow, 1924). All phospholipids were purchased from Avanti Polar Lipids; cholesterol, ganglioside G_{M1}, and ganglioside G_{D1a}, from Sigma; and [^{3}H]cholesterylhexadecyl ether from Amersham Corp. These lipids were used without further purification. Liposome compositions are expressed in molar ratios.

In Vivo Mouse Plasma Distribution of Liposomes—Two hundred μl of the liposome suspension was administered intravenously via the dorsal tail vein of CD1 mice (female, 6–8-week-old, Jackson Laboratory Animals). After various times, the mice were anesthesized with ether or killed by an overexposure to carbon dioxide, and blood withdrawn via cardiac puncture and collected in 1.5-ml microcentrifuge tubes (Eppendorf). The blood was immediately cooled to 0 °C using an ice-water bath to prevent coagulation and centrifuged (12,000 rpm, 2 min, 4 °C) to pellet the blood cells. Aliquots of plasma were measured for radioactivity content using standard liquid scintillation methods. Plasma volume was assumed to be 5% of total body weight. For the 2-min time point, experiments were repeated at least twice, with a sample size of 4 mice. The values reported for the 30-min and 2-h time points are from a single determination with a sample size of four mice.

In Vitro Serum/Liposome Incubations—Liposomes were prepared as above except that the liposome suspensions were 50 mM total lipid in isotonic veronal-buffered saline (VBS: 10 mM sodium barbital, pH 7.4, 145 mM NaCl). To 120 μl of LUVs, 480 μl of normal human or mouse serum was added and the LUVs/serum mixture incubated for 30 min at 37 °C. Normal human serum was prepared from venous blood pooled from 20 healthy individuals (10 males, 10 females) and stored at −70 °C. Normal mouse serum was purchased from Cedarlane Laboratories, Hornby, Ontario, Canada.

Isolation of Liposomes from Blood Components—A simple and rapid "spin column" procedure employing Bio-Gel A-15m, 200–400 mesh size (Bio-Rad), 1.0-ml chromatography columns was used to isolate liposomes from blood components, including very low density and low density lipoproteins, as previously described in detail (Chonn *et al.*, 1991b). Briefly, aliquots of the plasma (50 μl) were applied to spin columns and immediately centrifuged (Jouan Centrifuge G4.11; 1000 rpm, 1 min). Column fractions were collected in glass culture tubes by applying 50 μl of VBS to the spin columns and centrifuging (1000 rpm, 1 min). Column fractions were analyzed for radioactivity and fractions containing liposomes, typically fractions 5 and 6 from each column, were collected, pooled, and concentrated using Centricon 30 microconcentrators (Amicon, Danvers, MA) at 4 °C. For *in vivo* experiments, 5 columns/mouse were used and the LUVs recovered from four mice were pooled. For *in vitro* experiments, LUVs recovered from 10 columns were pooled. The samples were stored at −20 °C.

Quantitation of Amount of Total Protein Associated with Recovered Liposomes/Amount of Total Lipid—The liposome-associated proteins were efficiently extracted and delipidated using a procedure described by Wessel and Flugge (1984). This delipidation step was required because lipids interfere with most protein assays (Kessler and Fanestil, 1986). The extracted proteins were resuspended in 1 ml of 0.5% SDS in Milli-Q water. Then, 1 ml of micro bicinchoninic acid protein assay reagent (Pierce Chemical Co.) was added and the mixture incubated at 60 °C for 60 min. After cooling the mixture to room temperature, the absorbance of the solution was measured spectrophotometrically at a wavelength of 562 nm. The A_{562} was compared to a standard curve derived from known amounts of bovine serum albumin (Pierce). The standard curve was linear in the range of 0–16 μg/ml. The amount of lipid was calculated from the specific activity of the liposome suspensions and the volume of liposomes used to extract the proteins. For *in vivo* experiments, at least 2 determinations for each liposome composition were done.

SDS-Polyacrylamide 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 (Laemmli, 1970). Prestained SDS-PAGE molecular weight standards (Diversified Biotech, Newton, MA) or silver stain SDS-PAGE molecular weight standards (Bio-Rad) were used to estimate the molecular weights of the proteins. Detection of the proteins was performed by a silver-stain procedure (Rabilloud *et al.*, 1988). The silver-stained SDS-PAGE results are representative of 3 or 4 analyses. For immunodetection of the opsonins, C3, and IgG, the SDS-PAGE separated proteins were electrophoretically transferred onto nitrocellulose (Nitroplus 2000; Micron Separations, Westboro, MA) using the Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad) at a constant current of 300 mA for 60 min followed by immunoblot analysis using the Enhanced Chemiluminescence Western blotting detection system (Amersham). The blocking buffer consisted of 10 mM Tris, pH 7.6, 150 mM NaCl, 5% dried skim milk powder, and 1% Tween 20 detergent (Sigma). Goat antisera to mouse C3 (Organon Teknika Inc., Scarborough, Ontario, Canada) was used at a 1/1000 dilution; peroxidase-conjugated goat anti-mouse IgG and peroxidase-conjugated rabbit anti-goat IgG (both from The Jackson Immunoresearch Laboratories, Bar Harbor, ME) were used at a 1/5000 dilution. Blocking buffer was used as the diluent. The immunoblot analyses were repeated twice.

Complement Hemolytic Assays—Fifty μl of 25% normal human serum or 5% guinea pig serum in $DGVB^{2+}$ (VBS containing 2.5% glucose, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, and 0.1% gelatin) was incubated with an equal volume of 20 mM liposomes at 37 °C for 30 min. After the incubation period, the mixtures were diluted with 150 μl of $DGVB^{2+}$ and kept on ice. Complement hemolytic assays were performed to determine the level of complement activation that occurred after incubating normal human serum with liposomes as previously described (Chonn *et al.*, 1991a). The complement hemolytic level of incubations of serum and an equal volume of $DGVB^{2+}$ was used as a measure of 100% residual complement activity.

RESULTS

In Vivo Association of Murine Blood Proteins with Liposomes Exhibiting Markedly Different Clearance Properties—It has previously been demonstrated that liposomes rapidly bind a complex profile of plasma proteins *in vitro* upon exposure to human plasma or serum (Bonte and Juliano,

[2] A. Chonn, S. C. Semple, and P. R. Cullis, unpublished results.

1986; Sommerman, 1986; Juliano and Lin, 1980). It has also previously been observed that liposomes composed of different acidic phospholipids have markedly different clearance properties. In particular, liposomes containing PA (Abra and Hunt, 1981) or PS (Chonn *et al.*, 1991b) have been shown to have rapid clearance kinetics; whereas liposomes containing hydrogenated plant PI (Papahadjopoulos and Gabizon, 1987) or ganglioside G_{M1} (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988) were shown to be relatively long-lived in the circulation of mice. A primary objective of this study was to determine whether the apparent differences in liposome clearance behavior observed *in vivo* was related to the amount and type of protein associated with the negatively charged liposomes *in vivo*.

Fig. 1 depicts the clearance of liposomes composed of various lipid compositions from the circulation of CD1 mice over a 2-h period, following intravenous administration of LUVs at a dose level of 200 μmol/kg (approximately 120 mg/kg). Three types of behavior in the circulation are evident: short lifetimes (CL-, DOPA-, or DOPS-containing LUVs, Fig. 1*A*), intermediate lifetimes (PG or plant PI-containing LUVs, Fig. 1*B*), and relatively long lifetimes (ganglioside G_{M1}-containing LUVs, Fig. 1*B*). At 2 min post-injection, the liposomes were recovered from the blood of liposome-treated CD1 mice using the spin column procedure (see "Materials and Methods"). The proteins associated with the recovered liposomes were then analyzed by SDS-PAGE under nonreducing conditions.

Fig. 2 shows the silver-stained gels of the proteins associated with liposomes recovered at 2 min post-injection. Each lane represents an equivalent amount of recovered lipid. It is immediately apparent that the amount of protein associated with 25 nmol of lipid differs dramatically for different lipid species, with the rapidly cleared liposomes having the most associated protein (Fig. 2*A*). Furthermore, liposomes having similar membrane surface charge imparted by different anionic phospholipids can exhibit significantly different protein binding abilities. This was quantified by measuring the protein binding ability (P_B: grams of total protein/mol of total lipid) which, in turn, was related to the clearance behavior of the liposomes in the circulation (Fig. 3). It may be observed that P_B is inversely related to the half-life of the liposomes in the circulation.

A striking result demonstrating the correlation between clearance rates and the amount of bound protein, as well as the lack of correlation between protein binding and surface charge is illustrated by the behavior of liposomes containing 20 mol % plant PI or bovine liver PI. As shown in Fig. 1, liposomes containing plant PI exhibit a circulation half-life of approximately 110 min, whereas liposomes containing the same amount of bovine liver PI have a half-life of less than 2 min, with only 19.6 ± 2.0% of injected dose ($n = 8$) recovered in the blood after 2 min. This correlates with marked differences in protein binding, where the rapidly cleared bovine liver PI liposomes exhibit P_B values of 158 g of protein/mol of lipid as compared to 26 g of protein/mol of lipid for liposomes containing plant PI.

Comparison of in Vivo and in Vitro Systems—The results to this stage clearly indicate that the amount of blood protein associated with liposomes in the circulation dramatically affects liposome clearance behavior *in vivo*. Liposomes exhibiting very rapid clearance kinetics have the greatest ability to bind blood proteins. In contrast, liposomes exhibiting extended circulation residence times have markedly reduced amounts of associated blood proteins. It should be noted, however, that the *in vivo* analysis is limited by the amount of liposomes recovered, especially for rapidly cleared liposomes. In order to further characterize the surface properties of LUV systems in relation to protein binding, and known clearance properties, it would be useful to develop an *in vitro* assay. In this regard, it is first important to show that the amount of protein bound to various species of LUVs *in vitro* correlates with the amount of protein bound *in vivo*. Second, it is of interest to compare the protein profile associated with the LUVs obtained *in vivo* with that obtained *in vitro*. The P_B values obtained from *in vitro* incubations are given in Table I. The P_B values obtained using both the *in vivo* and *in vitro* assays for various species of LUVs are similar in that LUVs exhibiting very rapid clearance *in vivo* bind higher levels of proteins *in vitro* than do LUVs having longer circulation lifetimes (see Table I and Fig. 3). It is of interest to note,

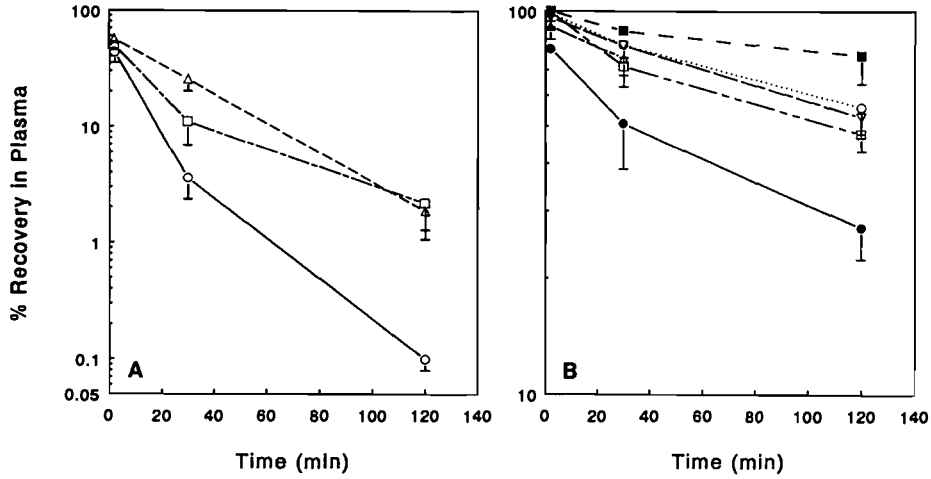

FIG. 1. **Plasma clearance of liposomes.** Large unilamellar liposomes containing trace amounts of [^{3}H]cholesterylhexadecyl ether were administered intravenously via the dorsal tail vein of CD1 mice at an approximate dose of 20 μmol of total lipid per 100 g of mouse weight. After various times, the recovery of liposomes in plasma was measured by counting aliquots of the plasma using standard scintillation methods as detailed under "Methods and Materials." *Panel A*, liposome compositions which are rapidly cleared: (○) PC:CH:CL (35:45:10), (□) PC:CH:DOPA (35:45:20), and (△) PC:CH:DOPS (35:45:20). *Panel B* include the following liposome compositions: (○) PC:CH (55:45), (●) PC:CH:PG (35:45:20), (□) PC:CH:plant PI (35:45:20), (▽) SM:PC (4:1), (■) SM:PC:G_{M1} (72:18:10), and (△) SM:PG:G_{M1} (72:18:10). Liposome compositions are expressed in molar ratios. The data points represent the average plasma recovery and sample standard deviation from four mice.

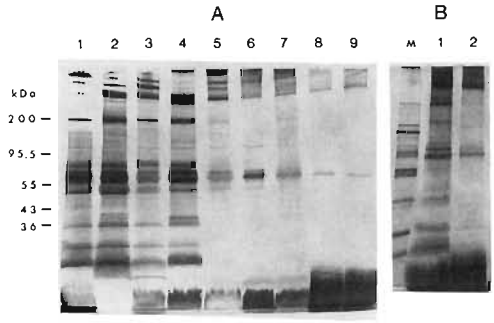

FIG. 2. **Silver-stained nonreducing SDS-PAGE gels of proteins associated with liposomes recovered from the circulation of mice after 2 min post-injection.** The proteins associated with the liposomes were separated electrophoretically on 4–20% SDS-polyacrylamide gels and visualized by silver stain. *Panel A* consists of proteins associated with 25 nmol of total lipid of liposomes composed of the following: PC:CH:CL (35:45:10; *lane 1*), PC:CH:DOPA (35:45:20; *lane 2*), PC:CH:DOPS (35:45:20; *lane 3*), PC:CH:PI (bovine liver; 35:45:20; *lane 4*), PC:CH:PI (plant; 35:45:20; *lane 5*), PC:CH:PG (35:45:20; *lane 6*), PC:CH (55:45; *lane 7*), SM:PC:G_{M1} (72:18:10; *lane 8*), and SM:PG:G_{M1} (72:18:10; *lane 9*). *Panel B* consists of proteins associated with 50 nmol of total lipid of SM:PC (4:1; *lane 1*) or SM:PC:GM1 (72:18:10; *lane 2*). *Lane M* contains silver-stained SDS-PAGE molecular weight standards from Bio-Rad (myosin, 200,000; β-galactosidase, 116,250; phosphorylase *b*, 97,400; serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; trypsin inhibitor, 21,500; and lysozyme, 14,400).

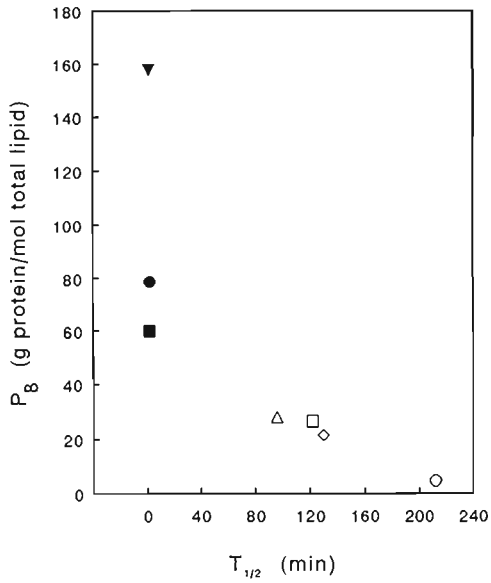

FIG. 3. **Relation of total amount of protein bound to liposomes and circulation half-life.** Aliquots of the recovered liposomes were delipidated, and the extracted proteins quantitated using the micro bicinchoninic acid protein assay as described under "Methods and Materials." The liposomes were composed of (▼) PC:CH:PI (bovine liver, 35:45:20), (●) PC:CH:DOPA (35:45:20), (■) PC:CH:CL (35:45:10), (□) PC:CH (55:45), (△) PC:CH:PI (plant; 35:45:20), (◇) SM:PC (4:1), and (○) SM:PC:G_{M1} (72:18:10).

however, that the profile of bound proteins attained employing the *in vitro* assay differs from that observed *in vivo* (Fig. 4).

Association of Blood Opsonins with Rapidly Cleared Liposomes—The rapid clearance of liposomes containing high levels of adsorbed protein would suggest that these adsorbed proteins include substantial levels of opsonins, leading to rapid uptake by phagocytic cells of the reticuloendothelial system. In order to demonstrate this, immunoblot analysis specific for the immune opsonins, C3 and IgG, were per-

TABLE I

P_B values for LUVs recovered from in vitro incubations with isolated human serum or in vivo incubations in mice

Composition of LUVs	*In vivo* P_B	*In vitro* P_B
	g protein/mol lipid[a]	
PC:CH (55:45)	28 ± 5	21 ± 2
PC:CH:PG (35:45:20)		23 ± 3
PC:CH:DOPS (35:45:20)		41 ± 3
PC:CH:DOPA (35:45:20)		46 ± 3
PC:CH:CL (35:45:10)	61 ± 1	101 ± 7

[a] Values represent average and standard deviation from 3 independent vesicle preparations as described under "Materials and Methods."

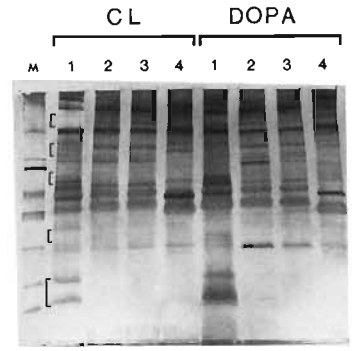

FIG. 4. **Comparison of protein profiles of LUVs recovered from the circulation of mice and from *in vitro* incubations with isolated serum.** Using the spin column method, LUVs were isolated from: *lane 1*, 2-min *in vivo* incubation in mice; *lane 2*, 2-min *in vitro* incubation with isolated mouse serum; *lane 3*, 30-min *in vitro* incubation with isolated mouse serum; or *lane 4*, 30-min *in vitro* incubation with isolated human serum. The proteins associated with LUVs (25 nmol of total lipid) were electrophoresed on a 4–20% gradient SDS-polyacrylamide gel and detected by silver stain. *Lane M* represents silver-stained SDS-PAGE molecular weight standards from Bio-Rad (refer to Fig. 2 for molecular weights). ([) highlights regions of the gel where the proteins associated with LUVs recovered from the circulation of mice differ from those associated with LUVs recovered from *in vitro* incubations with isolated mouse serum.

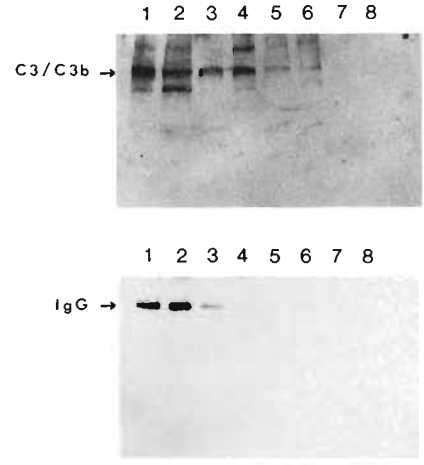

FIG. 5. **Immunoblot analysis of murine opsonins associated with LUVs.** The proteins associated with LUVs (25 nmol of total lipid) were separated electrophoretically on 4–20% SDS-PAGE gels under nonreducing conditions and analyzed by immunoblot analysis specific for mouse C3 or mouse IgG. The lanes contain the following liposome compositions: PC:CH:CL (35:45:10; *lane 1*), PC:CH:DOPA (35:45:20; *lane 2*), PC:CH:DOPS (35:45:20; *lane 3*), PC:CH:PI (plant; 35:45:20; *lane 4*), PC:CH:PG (35:45:20; *lane 5*), PC:CH (55:45; *lane 6*), SM:PC:G_{M1} (72:18:10; *lane 7*), and SM:PG:G_{M1} (72:18:10; *lane 8*).

formed. As shown qualitatively in Fig. 5, higher levels of these opsonins could be detected among the more rapidly cleared liposomes such as those containing 10 mol % CL or 20 mol % DOPA.

Influence of Ganglioside G_{M1} Content on the Binding of Blood Proteins to LUVs—Incorporation of ganglioside G_{M1} into SM:PC (4:1) liposomes has been shown to result in extended circulation lifetimes (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988), and it is of interest to examine the mechanism involved. As shown in Figs. 2*B* and 3, the amount of protein associated with SM:PC (4:1) LUVs *in vivo* is significantly reduced by inclusion of 10 mol % ganglioside G_{M1}, with a corresponding increase in circulation lifetime. A particular question concerns whether ganglioside G_{M1} acts to specifically decrease binding of opsonins such as IgG or C3 fragments, or whether the ganglioside G_{M1} effect arises from a nonspecific decrease in the binding of all blood proteins. Using the *in vitro* system involving incubation of LUVs with human serum, it was found that increasing the ganglioside G_{M1} content of PC:CH LUVs progressively reduced the amount of total protein bound to PC:CH LUVs (Fig. 6*A*). The P_B values were 36.4, 19.6, 15.4, 11.6, and 13.0 for 2, 4, 6, 8, and 10 mol % ganglioside G_{M1} containing PC:CH liposomes. It is interesting to note that the PC:CH LUVs containing 2 mol % ganglioside G_{M1} had a higher P_B value than PC:CH (55:45) LUVs (P_B = 23.0). Furthermore, the decrease in protein binding was apparent for all blood serum proteins, suggesting a nonspecific effect. This is illustrated more clearly in Fig. 6*B* where it is shown that the protein profile for PC:CH LUVs containing 2 or 10 mol % ganglioside G_{M1} are virtually identical when equal amounts of protein are loaded in each lane.

Previous studies have established that other gangliosides, such as ganglioside G_{D1a}, are not effective in prolonging the circulation lifetime of liposomes (Allen and Chonn, 1987). As shown in Fig. 6*C*, increasing the ganglioside G_{D1a} content of PC:CH (55:45) LUVs promoted the liposome association of serum proteins. This further supports a significant role of proteins in determining the fate of liposomes in the circulation.

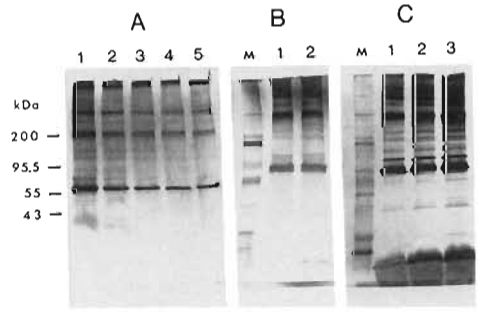

FIG. 6. **Effect of ganglioside G_{M1} and G_{D1a} on human serum protein association with liposomes.** The proteins associated with the liposomes were separated electrophoretically on 4–20% SDS-polyacrylamide gels and visualized by silver stain as described under "Methods and Materials." In *panel A*, equivalent amounts of lipid (25 nmol of total lipid) were loaded per lane. The liposome compositions consisted of PC:CH:G_{M1} (53:45:2; *lane 1*), PC:CH:G_{M1} (51:45:4; *lane 2*), PC:CH:G_{M1} (49:45:6; *lane 3*), PC:CH:G_{M1} (47:45:8; *lane 4*), and PC:CH:G_{M1} (45:45:10; *lane 5*). In *panel B*, equal amounts of protein (0.5 mg) associated with PC:CH:G_{M1} (53:45:2; *lane 1*) and PC:CH:G_{M1} (45:45:10; *lane 2*) were loaded per lane. In *panel C*, equal amounts of lipid (25 nmol of total lipid) were loaded per lane. The liposome compositions consisted of PC:CH:G_{D1a} (53:45:2; *lane 1*), PC:CH:G_{D1a} (51:45:4; *lane 2*), and PC:CH:G_{D1a} (49:45:6; *lane 3*). *Lane M* contains silver-stained SDS-PAGE molecular weight standards from Bio-Rad (refer to Fig. 2 for molecular weights).

The reduction in associated blood proteins has other consequences. For example, inhibition of binding of C3 or other complement proteins may result in an inhibition of complement activation, a membrane-requiring process. We have previously shown *in vitro* using guinea pig and human systems (Chonn *et al.*, 1991a) that liposomes containing anionic phospholipids activate the complement system via the classical pathway, resulting in C3b deposition onto these liposome membranes. Using a similar *in vitro* human or guinea pig system, the ability of ganglioside G_{M1} to inhibit complement activation was demonstrated (Fig. 7). This assay measures the total functional complement levels of human or guinea pig serum after exposure to liposomes (Chonn *et al.*, 1991a). A reduction in the complement hemolytic activity of the serum after incubation with liposomes implies complement consumption and activation by liposomes had occurred. As shown in Fig. 7, complement-activating liposomes, PC:CH:PG (35:45:20), were capable of reducing the complement hemolytic levels of human or guinea pig serum; however, if 10 mol % ganglioside G_{M1} was included in 20 mol % PG-containing vesicles, the liposomes were no longer complement activating.

DISCUSSION

One aspect of liposomes that is known to affect liposome clearance is surface charge (reviewed extensively by Senior (1987)). However, LUVs composed of different negatively charged lipids have been shown to exhibit markedly different clearance kinetics. The findings of this report indicate that this may be due to the dramatically different abilities of LUVs, which are composed of different anionic phospholipids but exhibit similar membrane surface charge, to bind blood proteins. Using the *in vivo* mouse animal model, which has very low cholesterylhexadecyl ether transfer activity, and the "spin column" method for isolating LUVs from triglyceride-rich lipoproteins and blood proteins, we further demonstrate that the amount of protein associated with LUVs in blood is inversely related to liposome circulation half-life. In general, liposomes exhibiting very rapid clearance kinetics ($\tau_{1/2} < 2$ min) have the greatest ability to bind blood proteins ($P_B > 50$ g of protein/mol of lipid). On the other hand, liposomes exhibiting extended circulation residence times ($\tau_{1/2} > 2$ h) have markedly reduced amounts of associated blood proteins ($P_B < 15$ g of protein/mol of lipid). This relationship strongly indicates that blood proteins play a dominant role in determining the circulation lifetimes of liposomes *in vivo*.

The markedly different ability of various anionic LUVs to interact with blood proteins was also observed using an *in*

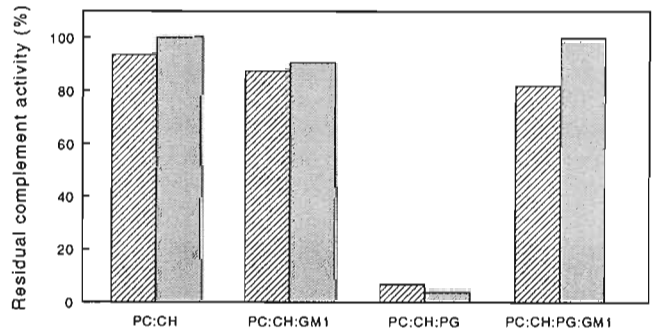

FIG. 7. **Inhibition of complement activation by liposomes containing ganglioside G_{M1}.** Complement hemolytic assays were used to determine the residual complement hemolytic activity of the serum after incubation of the serum with 1 μmol of total lipid of liposomes composed of PC:CH (55:45), PC:CH:G_{M1} (45:45:10), PC:CH:PG (35:45:20), or PC:CH:PG:G_{M1} (25:45:20:10). The *bars* represent human (*striped*) and guinea pig (*shaded*) serum.

vitro system involving liposome incubations with isolated serum. The *in vitro* findings strongly correlate with the *in vivo* findings in terms of total amount of protein bound, indicating that assays for the total amount of serum protein binding to LUVs *in vitro* should be predictive of their clearance behavior *in vivo*. Differences between the protein profiles observed when employing the *in vitro* assay as compared to the *in vivo* assay are noteworthy, however. The profiles of the rapidly cleared LUVs recovered from the *in vivo* assays are more complex and likely reflect the more complex nature of the *in vivo* system. In particular, the proteins that associate with LUVs *in vivo* and not in isolated serum may involve activation fragments resulting from proteolytic activation of blood pathways, such as the complement or coagulation systems. These proteins may also include extracellular matrix proteins, cell-derived proteins, or cell-mediated cleavage products of blood proteins. This finding underlines the importance of characterizing the blood protein interactions that liposomes experience *in vivo* in order to best resolve the role of specific proteins in mediating factors such as enhanced liposome permeability and liposome uptake by the reticuloendothelial system.

The rapid clearance of liposomes containing high amounts of adsorbed surface protein would suggest that these adsorbed proteins include substantial levels of opsonins, leading to rapid uptake by the phagocytic cells of the reticuloendothelial system. Consistent with this hypothesis is the finding that PC:CH:CL (35:45:10) or PC:CH:DOPA (35:45:20) LUVs bind the most C3, as demonstrated by specific immunoblot analysis of the proteins associated with LUVs *in vivo* (see Fig. 5). This is in agreement with our previous observation that the amount of C3 associated with LUVs is 8–10 times greater than for PC:CH (55:45) LUVs after a 30-min incubation of the LUVs with isolated human serum (Chonn *et al.*, 1991b). By further analyzing the protein profiles of the various LUVs recovered from the circulation of mice, it is clear that IgG is also associated predominantly with rapidly cleared liposomes (Fig. 5). This interaction may be specific for the phospholipids inasmuch as antiphospholipid antibodies have been shown to be present in normal human serum (Alving, 1984), or they may be nonspecific as demonstrated by Senior *et al.* (1986). These findings indicate that a determining property of foreign or "non-self" membranes is a greater ability to interact with blood protein components, resulting in higher levels of opsonin association.

As demonstrated in this study, the inclusion of agents which reduce the association of proteins, such as ganglioside G_{M1}, also reduce or inhibit immune reactions such as activation of the complement system. Ganglioside G_{M1} appears to inhibit this protein association in a relatively nonspecific manner, inasmuch as increasing the ganglioside G_{M1} content of PC:CH liposomes progressively reduces the binding of all detected proteins. This reduction in protein binding results in a surface that cannot support the membrane-dependent assembly of multimeric complexes, such as the C3 or C5 convertases of the complement system. This finding is significant because it predicts that any molecule that is capable of reducing protein binding to liposomes, even in a relatively nonspecific manner, will prolong the circulation half-life of the liposomes. Preliminary results from this laboratory indicate that other molecules, such as the recently described amphipathic phosphatidylethanolamine derivatives of polyethylene glycols, prolong the circulation half-life of liposomes by similarly reducing the amount of proteins associated with the liposomes.[2]

It is interesting to relate the findings of this study to our current understanding of how and why proteins interact with inert polymeric surfaces. This understanding stems mainly from studies involving one or two protein component systems that do not accurately reflect the interactions that occur in the complex biological milieu (reviewed by Horbett and Brash (1987)). The surface properties of the liposomes clearly influence the interactions of liposomes with blood proteins, as liposomes containing equivalent amounts of net negatively charged phospholipids have different capacities to interact with blood proteins. These interactions, however, are not simply electrostatic because liposomes composed of equivalent amounts of the various anionic phospholipids have the same surface potentials. Furthermore, as dramatically illustrated by the case of the two different species of PI, the fatty acyl composition of the phospholipid can markedly influence the blood protein/membrane interactions. In this regard, plant PI is composed of more saturated (mainly 16:0 and 18:0) fatty acyl groups than bovine liver PI (mainly 18:0, 18:2, and 20:4). This suggests that the ability of the proteins to insert into the liposome membrane may be an important determinant. Our findings therefore suggest that the presence of CL or DOPA increases the likelihood of protein insertion rather than electrostatic association.

Several molecular properties of blood proteins that are considered to have a major influence on their surface adsorption properties include size (proteins and other macromolecules are thought to form multiple contact points when adsorbed to a surface), surface charge (most charged residues reside on external surfaces of proteins), stability of the proteins in plasma (unfolding of proteins at the surface would increase the number of adsorption sites), and carbohydrate content (Horbett and Brash, 1987). As well, the relative concentration of the proteins in plasma should affect the distribution of proteins adsorbed on surfaces (Andrade and Hlady, 1987). However, from our findings on the proteins associated with LUVs in the circulation of mice, it does not appear that size is an overriding factor determining the surface activity differences among proteins; a complex profile of proteins of varying molecular sizes is associated with rapidly cleared LUVs (see Figs. 2, 4, and 6). Furthermore, certain proteins are associated with rapidly cleared LUVs in relatively greater concentration than found in plasma. A striking example here is a protein that migrates with a molecular weight corresponding to approximately 53,000 (see Figs. 2 and 4; and also Sommerman (1987)). Further studies that identify and quantitate the various proteins associated with circulating LUVs are clearly necessary to precisely assess the role of various factors in surface/protein interactions that influence biocompatibility.

In summary, the studies described here demonstrate the usefulness of liposome systems in characterizing the membrane properties that distinguish "self" from "non-self." The relation between P_B values and half-life observed *in vivo* strongly suggest that blood proteins play a significant role in determining the *in vivo* fate of liposomes. Furthermore, this relationship suggests that the amount of protein associated with the vesicles can be used as an indicator of how long-lived the liposomes will be in the circulation. The P_B values obtained from LUVs recovered from *in vitro* incubations with isolated serum exhibit similar trends to those from LUVs recovered *in vivo*. The *in vitro* determinations, being simpler and allowing for greater recoveries of LUVs, should therefore be a useful assay for predicting the clearance behavior of liposomes of novel liposome compositions.

Acknowledgment—We thank Dana Masin for invaluable assistance with the animal work.

REFERENCES

Abra, R. M., and Hunt, C. A. (1981) *Biochim. Biophys. Acta* **666,** 493–503

Allen, T. M., and Chonn, A. (1987) *FEBS Lett.* **223,** 42–46

Alving, C. R. (1984) *Biochem. Soc. Trans.* **12,** 342–344

Andrade, J. D., and Hlady, V. (1987) *Ann. N. Y. Acad. Sci.* **516,** 158–172

Black, C. D. V., and Gregoriadis, G. (1976) *Biochem. Soc. Trans.* **4,** 253–256

Blume, G., and Cevc, G. (1990) *Biochim. Biophys. Acta* **1029,** 91–97

Bonte, F., and Juliano, R. L. (1986) *Chem. Phys. Lipids* **40,** 359–372

Chonn, A., Cullis, P. R., and Devine, D. V. (1991a) *J. Immunol.* **146,** 4234–4241

Chonn, A., Semple, S. C., and Cullis, P. R. (1991b) *Biochim. Biophys. Acta.* **1070,** 215–222

Derksen, J. T. P., Morselt, H. W. M., and Scherphof, G. L. (1987) *Biochim. Biophys. Acta.* **931,** 33–40

Fiske, C. H., and Subbarow, Y. (1925) *J. Biol. Chem.* **66,** 375–400

Gabizon, A., and Papahadjopoulos, D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 6949–6953

Green, S. R., Beltz, W. F., Goldberg, D. I., and Pittman, R. C. (1989) *J. Lipid Res.* **30,** 1405–1410

Gregoriadis, G. (1988) in *Liposomes as Drug Carriers: Recent Trends and Progress* (Gregoriadis, G., ed) pp. 3–18, John Wiley and Sons Ltd., Chichester, United Kingdom

Gregoriadis, G., and Senior, J. (1980) *FEBS Lett.* **119,** 43–46

Halperin, G., Stein, O., and Stein, Y. (1986) *Methods Enzymol.* **129,** 816–848

Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985) *Biochim. Biophys. Acta* **812,** 55–65

Horbett, T. A., and Brash, J. L. (1987) in *Proteins at Interfaces: Physicochemical and Biochemical Studies* (Brash, J. L., and Horbett, T. A., eds) pp. 1–33, American Chemical Society, Washington, D. C.

Juliano, R. L., and Lin, G. (1980) in *Liposomes and Immunobiology* (Six, H., and Tom, B., eds) pp. 49–66, Elsevier Science Publishers B. V., Amsterdam

Juliano, R. L., and Stamp, D. (1975) *Biochem. Biophys. Res. Commun.* **63,** 651–658

Kessler, R., and Fanestil, D. (1986) *Anal. Biochem.* **159,** 138–142

Kirby, C., Clarke, J., and Gregoriadis, G. (1980) *Biochem. J.* **186,** 591–598

Klibanov, A. L., Maruyama, K., Torchilin, V. P., and Huang, L. (1990) *FEBS Lett.* **268,** 235–237

Laemmli, U. K. (1970) *Nature* **227,** 680–685

Papahadjopoulos, D., and Gabizon, A. (1987) *Ann. N. Y. Acad. Sci.* **507,** 64–74

Patel, H. M., Tuzel, N. S., and Ryman, B. E. (1983) *Biochim. Biophys. Acta* **761,** 142–151

Rabilloud, T., Capentier, G., and Tarroux, P. (1988) *Electrophoresis* **9,** 288–291

Roerdink, F. H., Regts, J., Handel, T., Sullivan, S. M., Baldeschwieler, J. D., and Sherphof, G. L. (1989) *Biochim. Biophys. Acta* **980,** 234–240

Senior, J. (1987) *Crit. Rev. Ther. Drug Carrier Syst.* **3,** 123–193

Senior, J., Waters, J. A., and Gregoriadis, G. (1986) *FEBS Lett.* **196,** 54–58

Sommerman, E. F. (1986) *Factors Influencing the Biodistribution of Liposomal Systems,* Ph. D. Thesis, The University of British Columbia, Vancouver, Canada

Stein, Y., Halperin, G., and Stein, O. (1980) *FEBS Lett.* **111,** 104–106

Wessel, D., and Flugge, U. I. (1984) *Anal. Biochem.* **138,** 141–143