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 GM₁, which has been previously shown to effectively limit liposomal uptake by the fixed macrophages of the reticuloendothelial system. The protein binding ability (Pₙ, g of protein/mol of lipid) of the liposomes was quantitated and related to the circulation half-life (τ₁/₂) of the liposomes. Liposomes having similar membrane surface charge imparted by different anionic phospholipids were found to exhibit markedly different protein binding potentials. Furthermore, Pₙ values determined from the in vivo experiments were found to be inversely related to circulation half-lives. Pₙ 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 (τ₁/₂ < 2 min). Pₙ values for ganglioside GM₁-containing liposomes (τ₁/₂ > 2 h) were significantly less (Pₙ < 15 g of total protein/mol of total lipid). Pₙ values were also determined for liposomes recovered from in vitro incubations with isolated human serum; relative Pₙ values obtained from these in vitro experiments were in agreement with relative Pₙ values measured from in vivo experiments. Pₙ values, therefore, could be a useful parameter for predicting the clearance behavior of liposomes in the circulation. Liposomes exhibiting increased Pₙ 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 GM₁ 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; Roerink 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 GM₁ (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988), or amphiphatic 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 α₂-macroglobulin or rat α₂-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:CH₁ multilamellar systems (Juliano and Lin, 1980).

The abbreviations used are: PC, egg phosphatidylcholine; CH₁, cholesterol; CL, bovine heart cardiolipin; DOPS, dioleoylphosphatidylserine; PA, egg phosphatidic acid; PG, egg phosphatidylglycerol; PI, phosphatidylinositol; PS, bovine liver phosphatidylserine; VBS, veronal-buffered saline; Pₙ, protein binding ability; τ₁/₂, 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 vitro 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 relationship between the amount of total blood protein binding to liposomes (FP) 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 SyrG10.22-pm filters (Nuclepore). The preparation of liposomes containing gangliosides was facilitated by extrusion at 65 °C. A radiolabeled lipid marker, [1H]cholesterolhexadecyl ether (10 μCi/30 μmol of total lipid), was incorporated to follow the biodistribution of the liposomes in mouse and to quantitate the concentration of the recovered liposome suspensions. This lipid marker has previously been shown to be nonexchangeable and nonmetabolizable 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., 1988). 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 GM1, and ganglioside GD1a, from Sigma; and [1H]cholesterolhexadecyl 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 Laboratories, Hornby, Ontario, Canada). After various times, the mice were anesthetized with ether or killed by an overdose 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 4.4, 2.5% 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 columns and immediately centrifuged (18,000 g, 110 rpm, 1 min). Column fractions were collected in glass culture tubes by applying 50 μl of VBS to the spin columns and centrifuging at 1000 rpm, 1 min. Column fractions were analyzed for radioactivity and fractions containing liposomes, typically fractions 5 and 6 from each column, were 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.

Quantification 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 Panesiti, 1986). The extracted proteins were resuspended in 1 ml of 0.5% SDS in Milli-Q water. Then, 1 ml of normal bicinchoninic acid protein determination (Pierce Chemical Co.) was added and the mixture incubated at 60 °C for 30 min. After cooling the mixture to room temperature, the absorbance of the solution was measured spectrophotometrically at a wavelength of 562 nm. The A595 was compared to a standard curve derived from known amounts of bovine serum albumin and bovine serum albumin standard curve was linear in the range of 0.15-15 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 separations were performed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini Protein-II electrophoretic apparatus (Bio-Rad) on precast 4-20% gradient Mini Protein-II gels (Bio-Rad) under nonreducing conditions (Laemmli, 1970). Prestained SDS-PAGE molecular weight standards (Bio-Rad and Biochem, North Ryde, Australia) and silver stain 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 electrochemically transferred onto nitrocellulose (Nitropius 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 antiserum 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 DGBV2 (VBS containing 2.5% glucose, 0.5 mM MgCl2, 0.15 mM CaCl2, and 0.1% gelatin) was incubated with an equal volume of 30 mM liposomes for 30 min at 37 °C. After the incubation period, the mixtures were diluted with 150 μl of DGBV2 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 levels of plasma proteins and an equal volume of DGBV2 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 vivo upon exposure to human plasma or serum (Bonte and Julianas, 1986).
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 GM1 (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. 1A), intermediate lifetimes (PG or plant PI-containing LUVs, Fig. 1B), and relatively long lifetimes (ganglioside GM1-containing LUVs, Fig. 1C). 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.

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 proteins (Fig. 2A). 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 (Pb, 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 Pb 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 Pb 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 Pb values obtained from in vitro incubations are given in Table I. The Pb 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,
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 (55:45:10; 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 (65:45; lane 7), SM:PC:GM (72:18:10; lane 8), and SM:PG:GM (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:GM (72:18:10; lane 2). Lane M contains silver-stained SDS-PAGE molecular weight standards from Bio-Rad (myosin, 200,000; α-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).

Table I

| Composition of LUVs | In vivo \( P_s \) | In vitro \( P_s \) |
|--------------------|----------------|----------------|
| PC:CH (55:45)      | 28 ± 5         | 21 ± 2         |
| PC:CH:PG (35:45:20)| 23 ± 3         | 23 ± 3         |
| PC:CH:DOPS (35:45:20)| 41 ± 3       | 41 ± 3         |
| PC:CH:DOPA (35:45:20)| 46 ± 3       | 46 ± 3         |
| PC:CH:CL (35:45:10)| 61 ± 1         | 101 ± 7        |

*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 human 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 vivo 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). (I) 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:GM (72:18:10; lane 7), and SM:PG:GM (72:18:10; lane 8).
formed. As shown qualitatively in Fig. 5, higher levels of these opossums could be detected among the more rapidly cleared liposomes such as those containing 10 mol % CL or 20 mol % DOPA.

**Influence of Ganglioside GM1 Content on the Binding of Blood Proteins to LUVs**—Incorporation of ganglioside GM1 into SM:PC:PG:GM1 (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. 2B and 3, the amount of protein associated with SM:PC:PG:GM1 liposomes in vivo is significantly reduced by inclusion of 10 mol % ganglioside GM1, with a corresponding increase in circulation lifetime. A particular question concerns whether ganglioside GM1 acts to specifically decrease binding of opossums such as IgG or C3 fragments, or whether the ganglioside GM1 effect arises from a nonspecific decrease in the binding of all blood proteins. Using the in vitro system involving incubation of LUVs with serum, it was found that increasing the ganglioside GM1 content of PC:CH LUVs progressively reduced the amount of total protein bound to PC:CH LUVs (Fig. 6A). The PS values were 36.4, 19.6, 15.4, 11.6, and 13.0 for 2, 4, 6, 8, and 10 mol % ganglioside GM1 containing PC:CH liposomes. It is interesting to note that the PC:CH LUVs containing 2 mol % ganglioside GM1 had a higher P50 value than PC:CH (55:45) LUVs (PS 5 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. 6B where it is shown that the protein profile for PC:CH LUVs containing 2 or 10 mol % ganglioside GM1 are virtually identical when equal amounts of protein are loaded in each lane.

Previous studies have established that other gangliosides, such as ganglioside GD1a, are not effective in prolonging the circulation lifetime of liposomes (Allen and Chonn, 1987). As shown in Fig. 6C, increasing the ganglioside GD1a content of PC:CH (55:45) LUVs promoted the lipo-protein 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 GM1 and GD1a on human serum protein association with liposomes.](image)

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 GM1 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 (55:45:20), were capable of reducing the complement hemolytic levels of human or guinea pig serum; however, if 10 mol % ganglioside GM1 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 (τ1/2 < 2 min) have the greatest ability to bind blood proteins (P50 > 50 g of protein/mol of lipid). On the other hand, liposomes exhibiting extended circulation residence times (τ1/2 > 2 h) have markedly reduced amounts of associated blood proteins (P50 < 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 vitro
Liposome/Blood Protein Interactions in Vivo

The in 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 vitro 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) 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 GM1, also reduce or inhibit immune reactions such as activation of the complement system. Ganglioside GM1 appears to inhibit this protein association in a relatively nonspecific manner, inasmuch as increasing the ganglioside GM1 content of PC:CH liposomes progressively reduces the binding of all detected proteins. This reduction in protein binding results in LUVs 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 amphiphatic phosphatidylethanolamine derivatives of polyethylene glycols, prolong the circulation half-life of liposomes by similarly reducing the amount of proteins associated with the liposomes.

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 amount of surface proteins 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 $P_n$ 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 $P_n$ 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.
