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Recognition and clearance of liposomes containing phosphatidylserine are mediated by serum opsonin

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Abstract

Liver uptake of liposomes containing phosphatidylserine was studied in a single pass liver perfusion system and found to be serum dependent. The effectiveness of serum in mediating liposome uptake by the liver depends on liposome size. Large liposomes appeared to be opsonized more efficiently and, therefore, taken up more by the liver than the smaller ones. The effects of liposome size on liver uptake did not occur in the absence of serum. Treatment of serum at 56° C for 30 min abolished the serum activity, suggesting the involvement of complement components. Inhibition of the hemolytic activity of complement through the alternative pathway by PS-containing liposomes suggests that components in this pathway are responsible for liposome opsonization. Liposomes containing phosphatidic acid, phosphatidylglycerol, and dicetyl phosphate compete in different degrees for serum components which mediate the liver uptake of PS-containing liposomes. These results suggest that the opsonization of liposomes by serum opsonins are the determining factors for the recognition and clearance of liposomes by the RES. Complement components are most likely involved in this process. The results presented here are relevant to the use of liposomes as drug delivery vehicle in vivo and to the PS-mediated clearance of red blood cells from the blood circulation.

Keywords: Liposome; Phosphatidylserine; Opsonin; Complement; Erythrocyte; Reticuloendothelial system

1. Introduction

Liposomes, serving as both a model membrane and a drug carrier, have been intensively studied in the past thirty years ([1,2] for review). Among the many aspects of liposome research, interaction of liposomes with macrophages of the reticuloendothelial system (RES), particularly those in the liver and spleen have drawn much attention [3–8]. Generally, systematically administered liposomes are rapidly taken up by the RES. The rate of clearance of injected liposomes from the blood circulation depends on liposome composition and size [6–8]. It has been found that presence of phosphatidylserine (PS) in the liposome membrane, regardless of the matrix lipids [7,9,10], results in a rapid removal of the liposomes from the blood and their accumulation in the liver and spleen. The effect of PS on liposome uptake by the RES can not be overcome by inclusion of ganglioside GM_1 into liposomes. GM_1 has been shown to possess the activity in decreasing liposome affinity for the RES and prolonging their circulation time in mice [9,10]. This observation is related to the physiological function of PS as a signal for triggering the recognition of sickled red blood cells (RBC) by macrophages [11]. Under physiological conditions, PS is localized exclusively in the inner leaflet of the cell membrane [12]. The exposure of PS as occurs in activated platelets and sickled RBC leads to their recognition and removal from the blood circulation by mononuclear phagocytes [11–13].

Clearly, PS plays a pivotal role in directing vesicles or cells to the RES. However, the mechanism responsible for such activity remains unknown. Evidence suggests that PS may serve as a signal that is directly recognized by macrophages. For example, it has been shown that insertion of a PS analog (NBD-PS) into mouse or human RBC membranes stimulates their binding and concomitant phagocytosis by cultured syngenic macrophages and allo-

Abbreviations: Chol, cholesterol; DCP, dicetyl phosphate; DTPA-SA, diethylenetriamine pentaacetic acid distearylamine complex; GM_1, monosialoganglioside; PA, phosphatidic acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PS, phosphatidylserine; RBC, red blood cells; RES, reticuloendothelial system.

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genic monocytes [14]. Similar results have also been reported for PS-containing liposomes in studies using purified macrophages [15] and established cell lines [16]. However, it is not clear if this is the case in vivo, because the macrophages used in vitro may not have the same functional activity as those in liver and spleen. More importantly, the role of blood components in PS-mediated liposome or cell uptake by the RES has not been carefully examined.

In this study, PS-containing liposomes were used as a model to demonstrate that the recognition of PS by the liver macrophages is mediated by serum components. It was shown that PS-containing liposomes can not be recognized by liver macrophages in the absence of serum. Complement components involved in the alternative pathway appeared to serve as opsonins that mediate the recognition and phagocytosis of PS-containing membrane vesicles by macrophages.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (PC), bovine brain phosphatidylserine (PS), dipalmitoylphosphatidylglycerol (DPPG), and egg phosphatidic acid (PA) were purchased from Avanti Polar Lipids (Birmingham, AL); dicetyl phosphate (DCP), and cholesterol (Chol) were purchased from Sigma (St. Louis, MO). ^{111}In-diethylenetriamine pentaacetic acid distearylamine complex (^{111}In-DTPA-SA) was prepared as described previously [7]. Rabbit whole blood in Alsever's solution was purchased from Pel Freez Biologicals (Rogers, AR). Animals used were from Harlan Sprague Dawley (Indianapolis, IN).

2.2. Liposome preparations

Liposomes composed of PC/Chol/X (X represents the negatively charged phospholipids) with a molar ratio of 10:5:1 were prepared by extrusion [7]. Briefly, lipids with a trace amount of ^{111}In-DTPA-SA were mixed and dried under N_2 gas. The dried lipid film was then vacuum desiccated for at least 30 min to remove the residual organic solvent before it was hydrated in PBS (pH 7.4) overnight at room temperature. The lipid suspension was then extruded 20 times through two layers of polycarbonate filters (Nucleopore) with defined pore sizes by using LiposoFast extruder (Avestin, Ottawa, Canada)). The average diameter of liposomes were measured by a submicron particle analyzer (Coulter N4SD).

2.3. In situ liver perfusion

Liposomes (0.6 μmol total lipids in 60 μl) were added to 24 ml of saline or saline containing different amounts of serum and incubated for 10 minutes at 37° C. 20 ml of the mixture was then perfused via the portal vein through the liver of an anaesthetized rat (using diethyl ether vapor) at a rate of 7 ml/min. The inferior vena cava was cut at the beginning of perfusion to allow the perfusion solution to drain. The perfused liver was then washed with 50 ml of saline at the same perfusion rate. The amount of liposomes taken up by the liver was determined by the ^{111}In radioactivity in the liver and was expressed as the percentage of perfused dose.

2.4. Inhibition of hemolytic activity of complement by PS-containing liposomes

Liposomes (0.1 ml containing different amounts of lipids) were incubated with 0.2 ml of freshly collected rat serum for 30 min at 37° C in the presence of EGTA and Mg^{2+} which were kept at a final concentration of 7.5 mM, respectively. Liposome-treated serum (0.15 ml) was then diluted with PBS to 0.3 ml. Rabbit erythrocytes (0.1 ml, $2 \cdot 10^8$ cells/ml) were then added and the mixture was further incubated for 30 min at 37° C. The reaction was stopped by the addition of 1.6 ml of cold PBS containing EDTA (10 mM) and the mixture was then centrifuged at $2500 \times g$ for 10 min to remove the cells. The extent of hemolysis was measured spectrophotometrically at a wavelength of 413 nm.

3. Results

3.1. Uptake of PS-containing liposomes by perfused liver

To examine the uptake of PS-containing liposomes by the RES, we used a well developed rat liver perfusion model system [17,18]. PS-containing liposomes with different diameters were incubated with saline, saline containing 33% freshly collected serum or saline containing 66% blood (the final concentration of serum is about 33% assuming blood cells contribute 50% of the blood volume). After 10 min, the mixture was perfused through the liver via the portal vein. At the beginning of the perfusion, blood in liver circulation was released by cutting the inferior vena cava. Liposome uptake by the perfused liver in the presence or absence of blood components was compared. As shown in Fig. 1, there is a minimal level of liposome uptake by the liver in the absence of blood components. An approximate 5-fold increase in liposome uptake in comparison to the basal level obtained in the absence of blood components was observed for liposomes (d = 110 nm) preincubated with either 33% serum or 66% whole blood, indicating that serum components, but not the blood cells, are responsible for the increased liposome uptake by the liver. It was also noted that serum component-enhanced liposome uptake by the liver was further enhanced for liposomes with larger diameters. For example, about a 10-fold increase in uptake by the liver was obtained for liposomes with an average diameter of 410

nm as compared to that of basal level. Again, liposome uptake in the presence of 66% blood and 33% serum in saline showed little difference. Saline or saline presaturated with oxygen (O_2, 95%, CO_2, 5%) or Krebs-Henseleit buffer (pH 7.4) solution gave similar results (data not shown).

To confirm our observation that the serum enhanced uptake of liposomes is not due to artifacts introduced in our perfusion system, we infused the same amount of liposomes via the portal vein (0.6 μmol in 1 ml) into live animals in a period of 3 min (the same time period used for the perfusion study). After infusion, an incision was made at the inferior vena cava and the liver was washed with 50 ml of saline via portal vein to remove unbound liposomes in the liver. Liposome uptake by the liver was then quantitated by ^{111}In radioactivity detected in the liver. It is obvious from Fig. 1 that the amount of liposomes taken up by the liver under these conditions is also dependent on liposome size. Thus, the larger the diameter of liposomes, the more liposomes were taken up by the liver.

3.2. Effect of serum concentration on liposome uptake

To examine the effect of serum concentration on the amount of liposome uptake by the perfused liver, a fixed amount of liposomes (0.6 μmol total lipids) was incubated with different amounts of serum and the total liposome uptake examined. Fig. 2A shows that liver uptake of liposomes increased with increasing amounts of serum (up to 33%). Further increase of the serum concentration resulted in a decrease of liposome uptake by the perfused liver.

In an separate set of experiments, liposomes were first incubated with serum and then diluted to the final volume of perfusate. As is shown in Fig. 2B, direct incubation of liposomes with serum results in more efficient liposome opsonization. For example, incubation of liposomes with 0.5 ml of serum directly is sufficient to produce about a 40% uptake while it requires about 8 ml of serum to reach

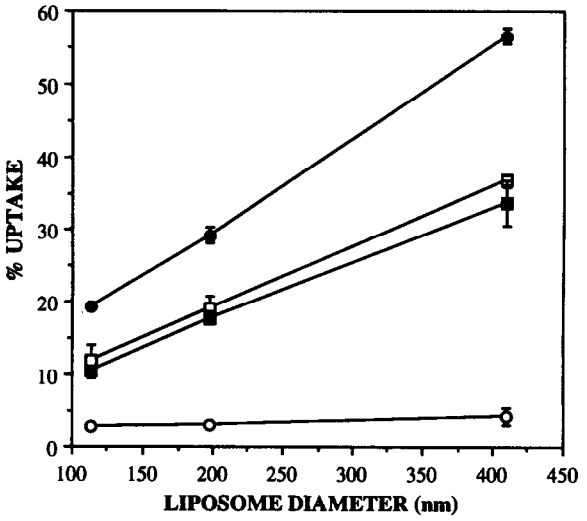

Fig. 1. Effect of blood components on liver uptake of PS-containing liposomes with different diameters. Liposomes (PC/Chol/PS = 10:5:1, molar ratio) containing 0.6 μmol total lipids were incubated with 24 ml of saline (○), saline containing 33% serum (■) or containing 66% whole blood (□) (heparin as anticoagulant) for 10 min at 37° C, respectively. 20 ml of mixed solution (0.5 μmol total lipids) was then perfused via the portal vein through the liver of a rat under anesthetization. The perfusion rate was about 7 ml/min. For infusion experiments (●), liposomes (0.5 μmol total lipids in 1 ml of saline) were directly infused to the anesthetized animals via the portal vein at the rate of 0.33 ml/min. At the end of the infusion, the blood circulation was broken by breaking the inferior vena cava. The liver was then washed with 50 ml of saline to remove unbound liposomes. The percentage of liposome uptake by the liver was analyzed by the amount of liposome accumulated in the liver divided by the total amount either perfused or infused.

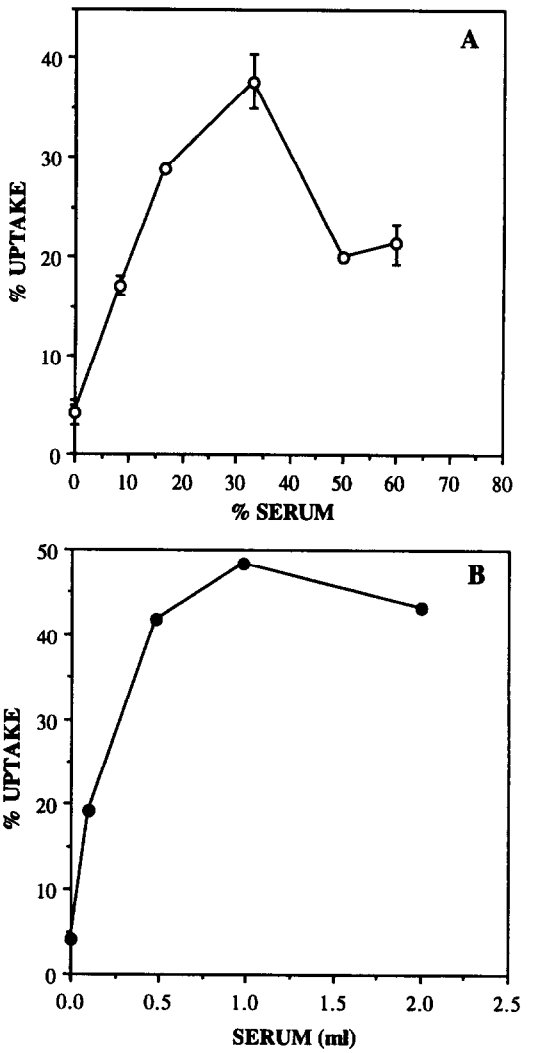

Fig. 2. Effect of the amount of serum on liposome uptake by the perfused liver. (A) Liposomes (0.6 μmol total lipids in 60 μl, $d = 410$ nm) were added to and then incubated with saline (24 ml total volume) or saline containing different amounts of serum for 10 min at 37° C before perfusion. (B) Same amount of liposomes as in A were directly added to different amounts of serum and incubated for 10 min at 37° C. The mixture was then diluted to a total volume of 24 ml by saline followed by perfusion. The conditions for perfusion and the data analysis are the same as those described in Fig. 1.

the similar level of uptake if incubation was carried out with diluted serum (0.6 μmol lipids, 16 ml saline and 8 ml serum). Again, with higher amounts of serum, liposome uptake by the perfused liver decreased. Human and bovine serum showed similar activity in mediating liver uptake of PS-containing liposomes (data not shown).

3.3. Liposome uptake is mediated by complement

Based on the results shown in Figs. 1 and 2, the obvious question one would ask is that what are the specific serum components (opsonins) responsible for the liposome uptake? Among the many possibilities, we tested the possible involvement of complement in liposome uptake. As shown in Table 1, treatment of serum at 56° C for 30 min to inactivate complement abolished the serum activity in mediating the liver uptake of liposomes. Dialysis of serum against saline (molecular mass cut off of the membrane is 12–14 kDa) removes about 40% of the serum activity, suggesting that small molecules in serum with molecular mass less than 12–14 kDa play some role in mediating liposome uptake by the liver.

If complement components serve as opsonins for PS-containing liposomes, we reasoned that serum complement activity should be consumed upon incubation with liposomes. To test this possibility, we examined the inhibitory effect of PS-containing liposomes on the hemolysis of erythrocytes by the alternative complement pathway. The results of this experiment (Fig. 3) showed that larger liposomes are more active than smaller ones in reducing the complement activity and, therefore, are more effective in inhibiting complement-mediated hemolysis. For example, about 95% of the lytic activity was inhibited at a liposome concentration of 0.5 mM for liposomes in a diameter of 570 nm in comparison to less than 20% inhibition for those with an average diameter of 103 nm. These results agree well with the conclusion drawn from the data in Table 1 and suggest that complement components serve as opsonins for the uptake of PS-containing liposomes. Also, opsonization activity of complement varies with the diameter of liposomes.

Table 1
Effect of serum treatments on serum mediated uptake of liposomes by liver

Treatment	% Uptake	% Inhibition
No treatment	32.9 (1.2)	0
56° C, 30 min	3.6 (1.2)	98.0
Dialysis	21.8 (1.7)	37.1

8 ml of rat serum was treated at 56° C for 30 min or dialyzed against saline (3×4 l of saline at 4° C for 12 h). PS-containing liposomes (0.6 μmol total lipids in 60 μl, $d = 410$ nm) were then incubated with 24 ml of saline containing 8 ml of serum with or without treatment for 10 min at 37° C. Perfusion was then performed as described in Materials and methods. The percent of inhibition by the various treatments was calculated by dividing the total uptake of liposomes using treated serum by the total liposome uptake using serum without treatment. Data represent an average (S.D.) of 2–4 individual experiments.

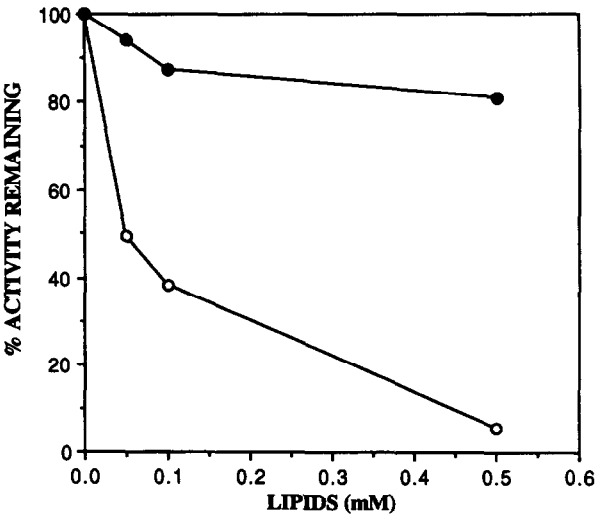

Fig. 3. Liposome size dependent inhibition of hemolytic activity of complement. PS-containing liposomes with an average diameter of 103 nm or 570 nm were incubated with rat serum for 30 min at 37° C. The hemolytic activity of rat serum preincubated with different amounts of liposomes against rabbit red blood cells was measured as described in Materials and methods. Liposomes with diameter of 103 nm (●) and 570 nm (○).

3.4. Effect of preincubation of other negatively charged liposomes with serum on serum-mediated liver uptake of PS-containing liposomes

It is well known that liposomes containing negatively charged lipids, such as PS, phosphatidic acid (PA), phosphatidylglycerol (PG) and DCP show high affinity for the RES [5]. To examine whether such high affinity for the RES is mediated by the same opsonins in all cases. we preincubated serum with liposomes containing various negatively charged lipids and then determined the liver uptake of PS-containing liposomes (testing liposomes) in the presence of the treated-serum. Thus, 1 ml of bovine serum was incubated with different types of liposomes (2 μmol total lipids in 0.2 ml) at 37° C for 10 min and liposomes removed by centrifugation (microfuge, max. speed, 10 min). To ensure that the liposomes were completely removed from the preincubation mixture, [^{3}H]cholestanyl ether (a lipid marker) was incorporated into the liposomes. A fraction of serum after centrifugation was counted in a scintillation counter to determine the total liposome removal by centrifugation. Over 95% of the total liposomes were removed by this method. The centrifuged serum fraction was then incubated with PS-containing liposomes labeled with ^{111}In-DTPA-SA (testing liposomes). After a 10 min incubation at 37° C, the mixture was then diluted by adding 23 ml of saline prewarmed to 37° C. Liver perfusion was then performed as described

Table 2
Effect of preincubation of liposomes on the serum-mediated liver uptake of liposomes containing PS [a]

Liposomes [b]	Diameter [c] (nm)	% Activity remaining
Saline		100
PC/Chol/PS	526	10
PC/Chol/DPPG	509	20
PC/Chol/PA	461	30
PC/Chol/DCP	481	35

[a] 1 ml of freshly collected bovine serum was incubated with either saline (200 μl) or saline containing liposomes with different negatively charged phospholipids (2 μmol total lipids) for 10 min at 37° C. Liposomes in the mixture were removed by centrifugation. Liposome-treated serum (1 ml) was then incubated with PS-containing liposomes (0.6 μmol lipids in 60 μl, ^{111}In-labeled) for 10 min at 37° C before dilution to a final volume of 24 ml with saline. 20 ml of the mixture was then perfused through the liver as described in Materials and methods. The remaining activity in liposome-treated serum for PS-containing liposomes was measured and expressed as a percentage of liposome uptake for serum treated with saline. Data are presented as an average of two individual experiments.
[b] The molar ratio of lipids in liposomes was 10:5:1.
[c] Average size of liposomes represents the results of unimodal analysis using submicron particle analyzer (Coulter N4SD).

Materials and methods. As is shown in Table 2, over 60% of the serum activity for the testing liposomes was inhibited by each of the negatively charged liposomes tested. PS-containing liposomes (the same as the testing liposomes) showed the highest inhibition (90%) followed by liposomes containing PG (80% inhibition). Liposomes containing PA and DCP have a similar inhibition activity (~ 65–70%). These results suggest that complement components may serve as opsonins for all negatively charged liposomes. In contrast, it also suggests that other types of negatively charged lipids, such as ganglioside GM_1 and phosphatidylinositol, which increases the liposome circulation time in mice may decrease the binding of components to the liposome surface. This prediction agrees well with the previous report that the GM_1 inhibits the activation of alternative pathway of the complement system [19].

4. Discussion

The results presented here clearly demonstrate that the uptake of liposomes containing PS by the RES is mediated by serum opsonins, most likely by complement components. This conclusion was supported by several experimental results. First, minimum uptake of liposomes by the liver was observed in the absence of serum. An approx. 5 to 10-fold increase was obtained when liposomes were preincubated with either blood or serum (Fig. 1). Second, the amount of liposomes taken up by the liver is related to the amount of serum present: high concentrations of serum generally gave higher uptake of liposomes (up to 33% serum in the perfusate, Fig. 2). Third, heat-treatment of serum at 56° C for 30 min to inactivate complement system, abolished the activity of serum in enhancing liposome uptake (Table 1). Finally, PS-containing liposomes inhibit the hemolysis of erythrocytes. These results suggest that PS on the surface of liposomes, sickled red blood cells or platelets serves as a signal to activate the complement system. It is the complement-derived opsonin bound to the membrane surface that triggers the recognition and clearance of these membrane vesicles by the RES.

An important observation described in this report is the effect of liposome size on serum mediated liposome uptake by the liver. The larger the liposome diameter, the greater the serum effect on liposome uptake. One explanation for this result is that uptake depends on the density of opsonins on the liposome surface. For a given amount of serum, liposomes with small diameters and thus, larger surface area will have a lower density of opsonin on the membrane surface than those with large diameters. Therefore, the total amount of liposomes that have recognizable amounts of opsonin by the RES is lower for liposomes with small diameters than those with large diameters, resulting in a lower liposome uptake by the liver macrophages. The large liposomes which have relatively smaller surface areas will have more opsonins on the surface per liposome, resulting in a higher liver uptake. This explanation, although reasonable, can not explain the results in Fig. 3. If surface area is the key factor for opsonin binding, one would expect that small liposomes should inhibit the hemolysis of erythrocytes in the hemolysis assay more efficiently than the larger ones. In fact, larger liposomes inhibit the hemolysis of erythrocytes better than the small ones at any given concentration of liposomes. Thus, the possible mechanism for the size effect on serum mediated liposome uptake may relate to the efficiency of opsonization. The opsonization or binding of opsonins to liposomes may depend on the liposome diameter and the binding of opsonins to small liposomes may be less efficient than that of the large ones. Therefore, the number of opsonin molecules bound to the large liposomes will be greater than the number bound to small ones under experimental conditions. The work of Harashima et al. [20] support this hypothesis. They demonstrated that small liposomes composed of hydrogenated-PC/Chol/DCP failed to activate the alternative pathway of the complement. The mechanism and the physiological importance of size dependent activation of complement are yet to be elucidated. It will be of interest to elucidate how liposome sizes are discriminated by the complement system.

Comparison of liposome uptake by the liver in live animals versus a perfused liver preparation showed that the total liver uptake of liposomes was higher in live animals than in the perfused liver in the presence of either 66% whole blood or 33% serum (Fig. 1). This result is likely due to multiple passes of liposomes through the liver in the case of the live animals. In these experiments, blood circulation was continuous until the end of the 3 min. Liposomes entering the bloodstream at the beginning of

infusion would have multiple chances to be taken up by the liver had they escaped liver uptake on the first pass. Conversely in the perfusion experiments, blood circulation was terminated at the beginning of the perfusion, and therefore, liposomes passed through liver only once.

While the evidence for the involvement of opsonins in liposome uptake is strong, the molecular identity of the opsonin(s) is not clear. As is shown in Tables 1 and 2, it seems that complement components may bind to all negatively charged liposomes. The binding affinity depends on the type of negative charge on the liposome surface. This conclusion agrees well with the reports by Harashima et al. [20] and others [18] that showed that liver uptake of liposomes composed of hydrogenated-PC/Chol/DCP is mediated by components in the alternative pathway of complement. It has also been reported that insertion of NBD-PG to the outer leaflet of red blood cell membrane resulted in a rapid removal of the cells from the circulation, but at a rate lower than that of RBC containing NBD-PS [21]. From the results presented and from others [20,22–24], it is reasonable to conclude that the clearance of liposomes by the RES is determined by liposome opsonization by complement components. The opsonin could bind to liposomes directly or be mediated by other serum factors such as antibodies. Opsonized liposomes could then trigger the binding and subsequent phagocytosis of liposomes by the macrophages in the RES. Liposomes containing glycolipids, such as ganglioside GM_1, that inhibits the binding of complement components to the liposome surface will have low affinity to the RES, and therefore have a prolonged circulation time.

It has been reported that C3b became associated with liposomes when they were administered into animals [25] and that removal of C3 from the serum by anti-C3 antibody eliminates serum mediated liposome uptake by the perfused liver [20]. Furthermore, the known function of C3b in eliminating the blood born pathogens, such as bacteria and viruses [25], through C3 receptors makes it a strong candidate for opsonin. The role of C3b in mediating the liver uptake of PS-containing liposomes is under investigation in our laboratory.

The fact that 40% of the serum activity could be removed by dialysis with a molecular mass cut off of 12–14 kDa suggests the involvement of multiple serum factors in liposome opsonization (Table 1). This conclusion agrees well with the earlier work of Kiwada et al. [18] where liposomes composed of hydrogenated egg PC, cholesterol and DCP (4:4:1, molar ratio) were used in liver uptake analysis. It is unknown at present what the factor(s) is and how it works in increasing liposome affinity for the liver. It is possible that the factor(s) with molecular mass of less than 12–14 kDa serves as a cofactor(s) for the activity of the opsonin. Removal of this factor(s) by dialysis may change the conformation of the opsonin such that binding to the liposomes is no longer possible. Alternatively, the factor(s) may involve in the process of liposome opsonization. It could be involved in the activation, binding (K_{on}) or dissociation (K_{off}) of the opsonin to liposomes. Furthermore, the dialyzable factor(s) may work independently from the higher molecular weights serum components. In this case, the factor(s) must be heat labile since heat treatment (56° C, 30 min) of serum abolishes the total activity (Table 1). These possibilities plus many more are yet to be tested.

It is interesting to point out that higher concentrations of serum actually decrease the liver uptake of PS-containing liposomes (Fig. 2). Although it is not clear at the moment what the mechanism is, it seems that there are two systems in the serum working against each other in regard to liposome uptake by the liver. Such protection activity of serum against the activity of opsonin may suggest the existence of dysopsonins in the serum. Dysopsonin activity in serum has been demonstrated by Moghimi et al. [26] in a polystyrene micro sphere system. A possible explanation for the observation that the dysopsonin activity for PS-containing liposomes only appears at high concentrations of serum is that the concentration of dysopsonins in serum is low or alternatively, the binding affinity of dysopsonins for liposomes is low in comparison to that of opsonins. At low serum concentration, opsonin dominates the affinity of liposomes for the RES either because of higher concentration or higher affinity to liposomes. At high concentrations of serum however, dysopsonin activity appears, resulting in a decrease of liposome affinity to the RES. It is worth noting that the dysopsonin activity can not surpass the activity of opsonin in the case of PS-containing liposomes. For example, increase of serum concentration from 50% to 60% did not result in a further decrease of liposome uptake by the liver (Fig. 2A). This suggests that the affinity of liposomes to the RES in vivo is likely determined by two opposite processes. The mechanism of this process awaits elucidation.

In conclusion, PS-mediated liposome uptake by the macrophages in the liver is a result of opsonization by complement components. PS, itself, does not serve as a direct signal to trigger the recognition and phagocytosis by the macrophages in the RES in rats. Opsonization efficiency depends on the liposome diameter. Although we have not determined the detailed mechanism of the opsonization and the factors involved in the process, it does appear that the complement system that is involved in the fight against invasion of microbes by our body may also have the same function in removal of liposomes, microspheres, emulsion particles and aged red blood cells.

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