ELSEVIER

Biochimica et Biophysica Acta 1240 (1995) 277–284

BBA Biochimica et Biophysica Acta

Liposome clearance from blood: different animal species have different mechanisms

Dexi Liu *, Qingru Hu, Young K. Song

Department of Pharmaceutical Sciences, School of Pharmacy, 709 Salk Hall, University of Pittsburgh, Pittsburgh, PA 15261, USA

Received 15 March 1995; revised 5 July 1995; accepted 28 July 1995

Abstract

The kinetics of blood clearance and the mechanisms of liposome uptake by the reticuloendothelial system (RES) were compared in two animal species (mice and rats). By employing an in situ liver perfusion technique with selected liposome compositions (PC/Chol, PC/Chol/PS, PC/Chol/GM_1 and PC/Chol/PEG5000-PE), we demonstrated that liposomes with same lipid composition exhibited different blood circulation half-lives in different animal species. Although liver is the major organ responsible for the clearance of liposomes from blood in both animal species, the specific mechanisms differ. In mice, liposome uptake by the liver did not involve specific serum opsonins. In contrast, liposome uptake by the rat liver was strongly dependent on serum opsonins. Further, the activity of serum opsonins for a given liposome composition differed among animal species. Human serum exhibited higher opsonin activities for PC/Chol and PC/Chol/GM_1 liposomes than bovine sera, while rat serum displayed a high opsonizing activity for GM_1 liposomes and none for liposomes composed of PC and Chol. The opsonin activity of human serum could be removed or decreased by treatment with EGTA/Mg^{2+}, EDTA or cobra venom factor, suggesting that the activity is likely due to complement components. It is likely that C3 of the human complement system plays an important role in mediating the uptake of liposomes by the liver.

Keywords: Drug delivery; Liposome; Liposome clearance; Liver perfusion; Kupffer cell

1. Introduction

The blood clearance and tissue distribution of various types of liposome has been extensively studied in the past [1,2]. Similar to other types of drug carriers including polymer microspheres and lipid emulsions, injected liposomes are cleared by the phagocytic cells in the reticuloendothelial system (RES) of the liver, spleen and to lesser extent, bone marrow and lymphoid organs [1,2]. Liposomes appear to be recognized as 'foreign bodies' by the immune defense system and removed from blood as potential pathogens. Although much progress has been made in manipulating their clearance kinetics by either varying the size or surface characteristics, the mechanisms underlying liposome clearance by the RES remain to be elucidated.

It has been known for some time that size and specific lipid composition are critical factors in determining the rate of liposome clearance. Large liposomes are cleared more rapidly than small ones. Liposomes with negatively charged surfaces are generally cleared more rapidly than those that are neutral or positively charged. For example, inclusion of either phosphatidylserine or dicetylphosphate into liposomes composed of phosphatidylcholine (PC) and cholesterol (Chol) greatly increases their uptake by the RES and reduces their liposome circulation time in the blood [1–5]. However, the conclusion that liposomes with negatively charged surface are cleared faster than those of neutral or positively charged have been challenged by Allen et al. [6] and Gabizon and Papahadojoupolos [3], who demonstrated that inclusion of monosialoganglioside GM_1 (which is also negatively charged at the physiological

Abbreviations: Chol, cholesterol; DCP, dicetyl phosphate; DTPA-SA, diethylenetriamine pentaacetic acid distearylamine complex; GM_1, monosialoganglioside; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PEG-PE, poly(ethylene glycol) conjugated to dioleoylphosphatidylethanolamine; PS, phosphatidylserine; RES, reticuloendothelial system.

* Corresponding author. Fax: +1 (412) 6482116.

SSDI 0005-2736(95)00184-0

pH) into liposomes composed of PC and Chol actually decreases liposome uptake by the RES and prolongs their circulation time in the blood. Amphipathic polyethylene glycols have also been demonstrated to have similar activity in prolonging blood circulation time [7–9]. Liposomes that exhibit a prolonged blood circulation time have been called Stealth liposomes [10], sterically stabilized liposomes [11], Ninja liposomes or cryptosomes (for review, see [12]). With their demonstrated ability in animal models to extravasate from the leaky vessels in tumors and accumulate at the tumor site, the newly discovered long-circulating liposomes have spurred interest in the application of liposomes as drug carriers in cancer chemotherapy [3,5,11].

Although it has been amply demonstrated that long-circulating liposomes are much more efficacious in delivering drugs and in reducing tumor growth than conventional liposomes [13], the mechanism by which GM_1 or amphipathic PEG affects the affinity of the RES for these liposomes in prolonging circulation time remains unclear. Indeed, there is not much available information to explain explicitly why subtle differences in liposome composition cause the dramatic differences observed in the rate of blood clearance by the RES. The critical issue that needs to be addressed is how liposomes are recognized by the macrophages of the RES.

To elucidate the mechanism of liposome clearance by the RES and thus to develop better liposome formulations for drug delivery, we have employed a liver perfusion system to examine the effect of serum on liposome uptake. Using two different animal species (mice and rats), we have examined the relationship between liposome clearance in live animals and in vitro by the perfused liver. Results obtained from these experiments suggest that blood clearance and the mechanism by which the clearance rate is regulated differs among animal species.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine (PC), bovine brain phosphatidylserine (PS) and dioleoylphosphatidylethanolamine-*N*-(poly(ethylene glycol) 5000) (PEG5000-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol (Chol) was purchased from Sigma Chemical (St. Louis, MO). Cobra venom factor was from Quidel (San Diego, CA). Monosialoganglioside (GM_1) was purchased from Matreya (Pleasant Gap, PA). Diethylenetriaminepentaacetic acid stearylamide (DTPA-SA) [14] was a kind gift from Dr. Leaf Huang (Department of Pharmacology, University of Pittsburgh). $^{111}InCl_3$ (carrier-free) was from New England Nuclear (Wilmington, DE). Sera used were freshly collected from animals except bovine serum, which was freshly collected from a local slaughter house (Rendulec Packing, McKeesport, PA), stored immediately at −80°C freezer and used within two months. The frozen serum was thawed at 37°C and used immediately. The mice and rats used were purchased from Harlan Sprague Dawley (Indianapolis, IN).

2.2. Preparation of liposomes

Large multilamellar liposomes were prepared by the extrusion method [5]. Briefly, lipids of desired composition were mixed with a trace amount of ^{111}In-DTPA-SA (lipid marker). The mixture was then dried under a stream of N_2 gas and in a vacuum desiccator for at least 2 h to remove organic solvent. Lipid film obtained was hydrated (10 μmol lipids/ml) in PBS (pH 7.4) overnight at room temperature. The resulting lipid suspension was extruded 20 times through two layers of membrane filter with pore size of 100 nm using a LiposoFast extruder (Ottawa, Canada). To obtain liposomes smaller than 100 nm, extruded liposomes were sonicated for 5 min (bath sonicator, Hicksville, NY) followed by additional extrusion through two layers of a membrane filter with pore size of 50 nm. For liposomes with larger diameters, lipid suspension was extruded 10 times through membrane filters with pore size of either 400 nm or 800 nm. Average diameter of liposomes used in most of the experiments was 100 ± 20 nm as measured by dynamic light scattering using a Coulter N4SD submicron particle analyzer (Coulter Electronic, Hiateah, FL).

2.3. Kinetics of blood clearance and biodistribution of liposomes

Liposomes (4 μmol lipids/kg) were injected via the tail vein into either mice (NIH Swiss, male, 25–30 g) or rats (Sprague Dawley, male 250–300 g) in a volume of 100 μl for mice or 200 μl for rats. At different time intervals after injection, blood samples (approx. 200 μl) were collected from the rats via the tail vein. Mice were bled at the retro orbital sinus and killed by cervical dislocation at the desired time after liposome injection. In both the rats and mice, the exact volume of each blood sample was determined by weighing. The blood concentration of liposomes at the various time intervals was calculated based on the assumption that the blood is 7.4% of the body weight for mice [15] and 5.4% for rats [16].

Distribution of liposomes into organs other than blood was determined 4 h after administration. Liver, spleen, kidney, heart and lung were collected and the radioactivity in each organ was counted in a gamma counter. Contamination from blood in each organ was corrected for by means of correction factors as described previously [5]. Tissue distribution studies in rats were performed in the same way except that blood was collected via the portal vein to insure maximum removal.

2.4. Analysis of the effect of serum components on liposome uptake by the liver using a single pass liver perfusion system

The amount of liposomes and the volume of the perfusates for individual animals were based on the body weight and the blood volume of the animals. For rat liver perfusion studies, liposomes (1.2 μmol total lipids in 120 μl) were incubated with 1 ml of either Krebs Henseleit buffer (pH 7.4) or serum for 10 min at 37°C and then diluted to 24 ml with Krebs Henseleit buffer. 20 ml of this mixture was perfused via the portal vein through the rat liver which had previously been washed with 20 ml of buffer to remove contaminating blood. For mouse liver perfusion studies, liposomes (0.12 μmol total lipids in 12 μl) from the same batch used for rats was incubated with 0.2 ml of either buffer or serum under the same conditions outlined previously. The mixture was then diluted to 2.4 ml with buffer and 2 ml of the diluted mixture was perfused via the portal vein through the mouse liver that had been pre-washed with 3 ml of buffer. Animals were anesthetized with ether vapor during cannulation. The inferior vena cava was cut to drain blood after the catheter had been inserted into the portal vein. This was immediately followed by washing with buffer and subsequent perfusion with liposomes. Liposomes were passed through the liver only once and any unbound liposomes were removed by washing the perfused liver with 50 ml (for rats) and 5 ml (for mice) of buffer. The perfusion rate used was 7 ml/min for rat liver and 2 ml/min for mouse liver. The uptake of liposomes was determined by measuring the ^{111}In radioactivity retained in the perfused liver.

3. Results

3.1. Circulation time of liposomes in blood is both composition and species dependent

Based on the known pharmacokinetics of liposomes in vivo [1–10], four different liposome compositions were selected for this study. For all preparations, liposome size was kept approximately 100 nm to eliminate the effect of size on clearance and tissue distribution. The amount of liposomes injected to the animals was 4 μmol total lipids/kg. The average dose injected was 0.1 μmol for mice (25–30 g) and 1 μmol for rats (250–300 g).

As shown in Fig. 1A, liposomes containing 10% of either GM_1 or PEG5000-PE exhibited very long circulation time in mice. However, inclusion of the same molar percentage of PS into liposomes resulted in rapid blood clearance with a half-life of less than 10 min. Liposomes of PC/Chol without additional lipid components exhibited a circulation time shorter than those containing GM_1 or PEG5000-PE but longer than PS containing liposomes. The order of longevity of blood circulation time in mice for the various liposome types was PC/Chol/GM_1 > PC/Chol/PEG5000-PE > PC/Chol > PC/Chol/PS. In contrast, GM_1 containing liposomes showed a very short circulation time in rats (Fig. 1B) with an estimated half-life in blood of less than 10 min; over 100-fold less than that observed in mice. Liposomes with or without PEG-PE displayed almost identical clearance kinetics in rats. 4 h after injection, about 57% of the injected dose of liposomes composed of PC/Chol remained in the blood compared to about 52% for PEG-containing liposomes. The effect of PEG5000-PE in prolonging liposome circulation time as was observed in mice was not obvious in rats within the time period of our experiments (4 h).

One common feature observed in both animal species was that blood circulation time of liposomes was inversely proportional to liposome accumulation in the RES (liver

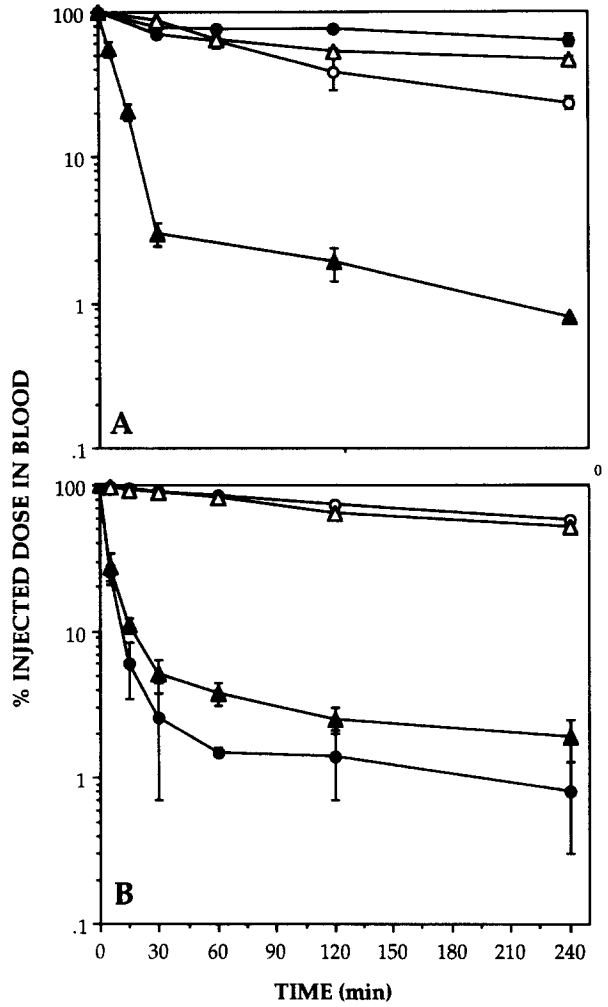

Fig. 1. Blood clearance of liposomes composed of PC/Chol (○), PC/Chol/GM_1 (●), PC/Chol/PS (▲) and PC/Chol/PEG-PE (△) in mice (A) and rats (B). ^{111}In-labeled liposomes with an average diameter of 100 nm were intravenously injected to animals at a dose of 4 μmol/kg. Blood concentration of liposomes at different time intervals was analyzed by measurement of the radioactivity of ^{111}In in blood samples (see Section 2 for detail).

Table 1
Tissue distribution of liposomes

Liposome composition (molar ratio)	Animal species	% Injected dose		
		liver	spleen	other [a]
PC/Chol (6:3)	rat	16.0 ± 1.1	1.8 ± 0.1	0.4 ± 0.3
	mouse	43.4 ± 2.6	8.9 ± 1.7	0.9 ± 0.3
PC/Chol/PS (6:3:1)	rat	79.0 ± 1.1	4.4 ± 0.8	0.1 ± 0.0
	mouse	67.5 ± 4.5	18.4 ± 3.5	0.3 ± 0.1
PC/Chol/GM_1 (6:3:1)	rat	80.3 ± 4.1	10.5 ± 2.6	0.1 ± 0.0
	mouse	7.4 ± 1.2	0.3 ± 0.1	1.9 ± 0.4
PC/Chol/PEG5000-PE (6:3:1)	rat	27.3 ± 1.9	0.1 ± 0.1	0
	mouse	22.4 ± 2.1	2.5 ± 1.0	1.3 ± 0.3

[111]In-DTPA-SA labeled liposomes with diameter of 100 nm were intravenously injected to animals at a dose of 4 μmol/kg. 4 h after injection, animals were killed and organs were collected. The amount of liposomes in each organ was determined by measuring the total radioactivity in each organ using a gamma counter. Data represent average ± S.D. (n = 3–5).
[a] Including lung, heart and kidney.

and spleen). As is shown in Table 1, over 80% of the total recovered dose of liposomes containing PS (in mice and rats) and GM_1 (in rats) was retained in the liver 4 h after administration. Minimal liposome accumulation was found in kidney, heart and lung.

3.2. Mechanism of liposome uptake by the liver

It is apparent from Fig. 1 that liposomes of the same composition can have very different blood circulation times in different animal species. The blood circulation time of liposomes is seen to be inversely proportional to the level of liposome accumulation in the liver (Table 1), suggesting that the circulation time is determined by the affinity of liver macrophages for the liposomes. For example, liposomes with low affinity (or low uptake) for the RES (PC/Chol/GM_1 and PC/Chol/PEG in mice, and PC/Chol, PC/Chol/PEG in rats) will have a relatively long circulation time in blood, while those with higher affinity for (or higher uptake by) the RES will exhibit a short circulation time (PS liposomes in mice, PS and GM_1 liposomes in rats). There are two possibilities to account for the liposome affinity for the RES. First, liposomes may be directly recognized by liver macrophages. For example, rat liver Kupffer cells may have high affinity receptors for both GM_1 and PS liposomes, permitting their capture as they pass through the liver, resulting in a short blood circulation time. On the other hand, receptors for GM_1 liposomes may be absent on mouse Kupffer cells and thus these liposomes display a long blood circulation time. The second possibility is that the affinity of the RES for liposomes may be dependent on the effect of serum components. Upon exposure to blood, liposomes may become coated with serum proteins. Depending on the properties of the liposomes, different amounts and/or types of serum proteins could coat the surface. It could be either the amount or type of proteins bound to the surface of the liposomes that determines the affinity of the RES for the liposomes and thus, modulates the blood circulation time.

To test these possibilities, we have developed a simple liver perfusion system. Under the experimental conditions, liposomes can be perfused through the prewashed liver either in the presence or absence of serum. All four compositions tested in vivo were studied in both perfused mouse and rat liver. The results of these perfusion studies are summarized in Table 2. For liposomes exhibiting a

Table 2
Effect of serum on liver uptake of liposomes

Liposome composition (molar ratio)	Uptake (%)			
	mouse liver		rat liver	
	buffer	mouse serum	buffer	rat serum
PC/Chol (6:3)	1.2 ± 0.1	1.9 ± 0.3	1.3 ± 0.4	1.6 ± 0.6
PC/Chol/PS (6:3:1)	19.5 ± 2.6	10.3 ± 1.5	2.1 ± 0.1	4.6 ± 1.1 [a]
PC/Chol/GM_1 (6:3:1)	1.9 ± 0.5	1.3 ± 0.1	4.3 ± 0.4	8.1 ± 0.5 [b]
PC/Chol/PEG5000-PE (6:3:1)	2.6 ± 0.1	2.6 ± 0.1	1.5 ± 0.1	1.0 ± 0.1

[111]In-labeled liposomes were incubated with either buffer or serum at 37°C for 10 min and diluted with prewarmed buffer. The mixture was then perfused through the liver (see Section 2 for detail). The total liposome uptake by the perfused liver was presented as the percentage of the total perfused liposomes. Data represent the average ± S.D. (n = 3–5).
[a] $P < 0.05$ (Student's t-test) compared with that of the same liposomes without serum incubation.
[b] $P < 0.001$ (Student's t-test) compared with that of the same liposomes without serum incubation.

fairly long circulation time in either mice (PC/Chol/GM_1, PC/Chol/PEG5000-PE and PC/Chol) or rats (PC/Chol/PEG-5000-PE and PC/Chol), a very low uptake (~ 2%) by the perfused liver was observed. Perfusions with or without serum gave essentially the same results. However, for liposomes exhibiting short circulation time in vivo, an apparent difference in liposome uptake between mice and rats by the perfused liver was observed. In mice, approx. 20% of the total perfused PS containing liposomes was taken up by the perfused liver. Pre-incubating these liposomes with freshly collected mouse serum decreased the total liver uptake to about 10%. These results suggest that PS liposomes are directly recognized by the mouse liver macrophages, and that this direct recognition can be partially suppressed by serum. Compared to the hepatic uptake of PS liposomes in mice, serum caused an opposite effect on uptake by the rat liver. In the absence of serum, only the basal level of PS liposomes were taken up by the rat liver. However, the total liver uptake of these same liposomes was doubled when serum was included, suggesting that it is the serum components, and not the liposomes themselves, that determines the affinity for the RES. A similar observation was also made for liposomes containing GM_1 (Table 2).

3.3. Effect of human and bovine serum on liposome uptake by the liver

From the preceding, it was obvious that serum components are involved in mediating the liver uptake of PS and GM_1 containing liposomes in rats. We wondered if the sera of other animal species would have similar activity. To test this possibility, human and bovine serum were selected as examples and tested their effects on liposome uptake by the liver using our perfusion system. As is summarized in Table 3, different degrees of serum effects on liposome uptake by the perfused liver were observed. For instance, when mouse liver was used as a model, the total liver uptake of liposomes composed of either PC/Chol or PC/Chol/GM_1 was greatly increased in the presence of both human and bovine serum. The total uptake for PC/Chol liposomes by the perfused liver in the absence of serum is less than 2% (Table 2), and is increased to about 20% or 5% in the presence of human or bovine serum respectively. A similar increase in liposome uptake was also obtained for GM_1-containing liposomes. Interestingly, both human and bovine serum decreased the total mouse liver uptake of liposomes containing PS (from 20% in buffer to about 14 and 13% in human and bovine serum, respectively). The level of this decrease is about the same as that caused by the mouse serum (Table 2). When rat liver was used, both human and bovine serum increased the total uptake of liposomes composed of either PC and Chol or similar liposomes containing additional PS or GM_1. In comparison to liposomes not exposed to serum, a more than three fold increase in the total liposome uptake was observed in perfused rat liver in the presence of either human or bovine serum. It is important to note that neither human nor bovine serum has a noticeable effect on the uptake of PEG-PE containing liposomes regardless of the tested animal species (Tables 2 and 3).

Table 3
Effect of human and bovine serum on liposome uptake by liver

Liposome composition (molar ratio)	Uptake (%)			
	mouse liver		rat liver	
	human	bovine	human	bovine
PC/Chol (6:3)	20.0±1.3	5.4±0.6	8.1±3.1	7.9±1.3
PC/Chol/PS (6:3:1)	13.9±2.4	12.6±0.7	7.3±1.1	6.0±2.0
PC/Chol/GM_1 (6:3:1)	11.6±0.3	4.8±0.3	14.2±1.3	8.4±0.6
PC/Chol/PEG5000-PE (6:3:1)	2.0±0.1	2.9±1.4	1.3±0.1	1.0±0.1

[1] ^{111}In-labeled liposomes incubated with either human or bovine serum were perfused through liver under the conditions described in Section 2. Data analysis and presentation are the same as in Table 2 ($n = 3$–5).

3.4. The suppressive activity of serum on the uptake of PS-containing liposomes by mouse liver depends on liposome size

It is interesting to note that serum had a negative effect on the total liposome uptake by the perfused mouse liver when PS was included as part of the lipid composition. About a 50% reduction in the uptake of PS containing liposomes was observed when serum was present (Table 2). Also, the negative effect seems to be independent of the serum source. All the serum tested showed a similar activity (Table 3). We wondered if the serum effect on liver uptake of PS containing liposomes was size dependent since a serum effect on liposome uptake by the rat liver has recently been reported by us [17]. To test this possibility, liposomes of various diameters ranging from 60 to 600 nm were prepared and perfused through the mouse liver either in the presence or absence of freshly collected mouse serum. As is presented in Table 4, the overall liver uptake of liposomes was similar for all sizes tested. However, a clear difference in total liposome uptake by the perfused liver was observed in the presence of serum. Serum reduced liver uptake of liposomes of average diameter 60 to 100 nm by about 50–60%. The activity of serum in reducing the total liposome uptake was dramatically decreased with increasing liposome diameter.

3.5. The complement system is involved in the serum enhanced liver uptake of liposomes

It is apparent from the data presented in Table 3 that human serum affects the liver uptake of liposomes composed of PC/Chol, PC/Chol/PS and PC/Chol/GM_1. The complement system in rats has been suspected of being involved in the opsonization of liposomes [17–19]. To demonstrate whether this is true for human serum, we selected three different means of blocking complement

Table 4
Liposome size dependent serum effect on liver uptake of liposomes containing PS

Liposome diameter (nm)	Uptake (%)		Serum inhibition (%) [a]
	in buffer	in serum	
66 (broad)	23.8±1.7	9.6±0.3	60.0
101 (21)	19.5±2.6	10.3±1.5	47.2
216 (68)	22.3±0.9	17.0±1.3	23.7
395 (broad)	22.0±2.5	20.2±2.0	8.2
602 (broad)	28.1±4.5	24.2±4.5	13.8

[111]In-labeled, PS-containing liposomes with different diameters were perfused through the mouse liver after incubation with either buffer or freshly collected mouse serum. The diameter (SD) of liposomes was analyzed by the unimodal analysis in a Coulter N4SD submicron particle analyzer. Percent inhibition by the serum was calculated by dividing the difference in liposome uptake in the absence and presence of serum by the total liver uptake in the absence of serum ($n = 3$–5).

[a]

$$\text{Inhibition}(\%) = \frac{\text{uptake in buffer}(\%) - \text{uptake in serum}(\%)}{\text{uptake in buffer}(\%)} \times 100$$

activation and tested their effect on liposome uptake by perfused mouse liver. Human serum was treated with either EDTA, EGTA/Mg^{2+} or cobra venom factor to block complement activation [20,21]. The treated serum was then incubated with liposomes followed by perfusion. The results of these experiments are presented in Fig. 2. For PC/Chol liposomes, treatment of human serum with either EDTA (blocking both the classical and alternative pathway), EGTA/Mg^{2+} (blocking the classic pathway only) or cobra venom factor (eliminating C3) inhibits at least 80% of the serum activity. More dramatic was the

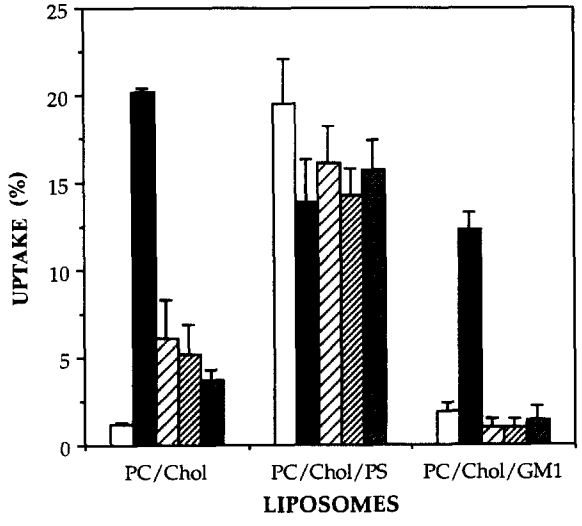

Fig. 2. Effect of inhibition of the complement system in human serum on the liposome uptake by the perfused mouse liver. For serum treatments, 200 μl freshly collected human serum were preincubated with either 40 μl of EDTA (60 mM), EGTA/Mg^{2+} (60 mM/15 mM) or Cobra venom factor (160 units/ml) for 10 min prior to incubation with liposomes. Other conditions were the same as those described in the method section. (□) without serum, (■) with serum, (light-shaded bar) with serum treated with EDTA, (shaded bar) with serum treated with EGTA/Mg^{2+} and (dark-shaded bar) with serum treated with cobra venom factor.

effect of these treatments on GM_1 liposomes. As it is shown in Fig. 2, serum activity in enhancing the liver uptake of GM_1 liposomes was completely blocked by all three treatments, proving that the complement system in human serum plays an important role in mediating the liver uptake. However, blocking complement activation did not have much of an effect on the activity of human serum in reducing the uptake of PS liposomes. This would suggest that the activity of serum in reducing the total liver uptake of PS containing liposomes by the mouse liver may not involve complement activation.

4. Discussion

It has been well documented that intravenously injected liposomes are cleared from blood by the RES, predominantly by the Kupffer cells in the liver [22–24]. Liposome size, surface characteristics and membrane fluidity play important roles in affecting the clearance rate [3–5]. The emphasis of the present work, therefore, was not to simply confirm some of these conclusions, but instead to use selected liposome compositions to demonstrate that the blood circulation time (or blood clearance kinetics) of liposomes could be very different in different animal species. This point has been clearly demonstrated in Fig. 1. Liposomes composed of PC and Chol displayed a moderate blood half-life in mice. Inclusion of GM_1 or PEG5000-PE into such liposomes dramatically increases their blood half-life, while inclusion of PS greatly decreases it. Interestingly, the clearance kinetics for these liposome compositions were different in rats. In contrast to the moderate circulation time in mice, liposomes composed of PC and Chol showed a rather long blood circulation time in rats (Fig. 1B). Furthermore, instead of further prolonging the blood half-life, inclusion of GM_1 into liposomes dramatically reduced the liposome circulation time. Finally, the function of PEG5000-PE in prolonging blood resident time of liposomes observed in mice is not obvious in rats. Liposomes composed of PC and Chol with or without PEG-PE showed similar blood half-lives (Fig. 1B).

Liposomes with a short half-life in blood accumulate mainly in the liver with the Kupffer cells being responsible for their uptake [22–24]. It is apparent from the results presented in Table 1 that liver Kupffer cells in mice and rats employ different mechanisms to remove liposomes from the blood. In mice, Kupffer cells appear to be capable of recognizing the surface characteristics of liposomes without the help of serum components. In fact, serum appears to interfere this recognition (Tables 2 and 3). The function of the liver Kupffer cells of mice in recognizing the surface properties of liposomes appears to be shared with other mouse-derived macrophages. Using isolated mouse bone marrow cells, an elevated uptake of PS containing liposomes in the absence of serum has been demonstrated [25]. It has also been reported that the established murine macrophage-like cell lines take up more

liposomes containing PS than those containing either GM_1 or PEG-PE [26]. On the contrary, recognition of liposomes by rat Kupffer cells appears to depend on the serum components. The function of rat Kupffer cells in differentiating surface properties of liposomes does not seem to be important in the clearance of liposomes. This conclusion, although requiring testing with additional experiments, seems to be true based on the limited number of experiments presented in this report. Experiments designed to extend our understanding of the functional difference between mouse and rat Kupffer cells are yet to be performed.

While it is evident that mouse Kupffer cells are capable of recognizing different liposomes, these cells are also able to recognize opsonized liposomes (Table 3). For example, the total liver uptake of PC/Chol and PC/Chol/GM_1 liposomes is greatly enhanced when these liposomes are preincubated with human or bovine serum (Table 3). The total liposome uptake by perfused mouse liver increased approx. 10-fold when human serum was included compared to that of mouse liver when either no serum or mouse serum was present. These results would suggest that there are major difference in opsonization activity of the serum among different animal species. Mouse serum may be unique in lacking opsonization activity for liposomes composed of PC/Chol, PC/Chol/PS and PC/Chol/GM_1. It is also important to note that the opsonization activity of serum varied significantly among different animal species. Human serum showed highest opsonization activities for all liposomes tested so far except for those containing PEG-PE. This activity appeared to be higher than that of bovine serum (Table 3). Rat serum did not appear to possess opsonization activity for PC/Chol liposomes (Table 2). Additionally, variability in the opsonization activity of serum for GM_1 liposomes has been observed among the human subjects [27]. Such variability, however, has not yet been found among individuals of mice and rat population. It is not clear for bovine since the bovine serum used in our experiments was from one single mixed batch collected from a local slaughter house.

The physiological function of phosphatidylserine has been extensively studied in the past. PS is exclusively located at the inner leaflet of the cell membrane [28]. Exposure of PS on the outer surface of cells is believed to activate the coagulation process and cause a local blood clotting [28–30]. Furthermore, it has also been reported that exposure of PS on the outer surface of red blood cells results in the rapid removal of these cells from the blood circulation by elements of the RES [29,30]. We also found in rats that the clearance of PS containing liposomes was mediated by the activation of complement system via the alternative pathway. Large liposomes appear to activate the complement system more efficiently than small ones [17]. As shown in Table 4, the situation in mice was just the opposite, and mouse serum appeared to decrease the liposome uptake by mouse liver, suggesting that the clearance mechanisms between mice and rats are different. It is interesting to note that the negative effect of serum on the uptake of PS-containing liposomes by mouse liver is also size dependent. As shown in Table 4, small liposomes seem to be more sensitive to the serum effect than large ones. Although the detailed mechanism for this phenomenon requires further experimentation, we believe that the size dependent phenomenon is related to the interaction between liposomes and serum components. It is possible that serum proteins may insert into the bilayer of small liposomes more easily than those with larger diameters due to their high curvature on the outer surface. A clear correlation between the amount of serum proteins bound and liposome diameter has previously been reported [31]. It is likely that more proteins may bind to the surface of small liposomes than large ones. The bound proteins could interfere with recognition or binding of the PS containing liposomes to the mouse Kupffer cells through a PS specific ligand on the surface of the Kupffer cells. The activity of serum in reducing the liver uptake of PS containing liposomes is likely due to a nonspecific interaction since mouse, bovine or human serum show almost identical activity (Tables 2 and 3). Lack of sensitivity to the treatments used to block complement activation (Fig. 2) would suggest that serum components that are responsible for reducing PS liposome uptake by mouse liver are different from those that enhance liposome uptake.

The opsonin activity of serum for different liposomes has been reported by a number of groups (for review, see [32]). The involvement of the complement system in opsonization of liposomes containing PS [17] or dicetylphosphate [18] has recently been demonstrated. We and others have hypothesized that complement component 3 (C3) is the possible opsonin and the results summarized in Fig. 2 seem to support this hypothesis. For both PC/Chol and PC/Chol/GM_1 liposomes, treatment of human serum with cobra venom factor, which is known to specifically remove C3 from serum, removes about 90% (for PC/Chol liposomes) and 100% (for GM_1 containing liposomes) of the activity from human serum. Blockade of serum activity in reducing liposome uptake by the liver by both EDTA and EGTA/Mg^{2+} would suggest that the activation of the complement system is through the classical pathway, indicating the involvement of anti-liposome antibodies. In the case of liposomes composed of PC and Chol, this would imply that antibodies against either phospholipids, cholesterol, or both are present in human serum. For GM_1 containing liposomes, we have recently demonstrated that the antibodies in human serum that are responsible for the liver uptake of GM_1 liposomes are IgM [27]. Even though the physiological significance of anti-lipid antibodies in human serum is still unknown, antibodies against phospholipids, cholesterol and different glycolipids have been found in humans and many other animal species (for review, see [33]).

While it is evident from this report that different animal species have different mechanisms to remove liposomes

from the blood circulation, inclusion of PEG-PE molecules into liposomes seems to be a very efficient way to block these clearance mechanisms (Fig. 1, Tables 2 and 3). The function of PEG on the surface of liposomes in prolonging liposome circulation appeared to be two-fold. In mice, PEG provides steric hindrance to the binding of liposomes composed of PC and Chol to the Kupffer cells possibly through surface receptors. Secondly, in those instances where complement activation is involved, PEG may either prevent the antibodies from binding to the surface of liposomes, block the activation of complement system or prevent the activated complement components from binding to liposome surface. Therefore, PEG-PE containing liposomes, even in the presence of opsonins to the matrix lipids, can still avoid recognition and blood clearance by the RES, suggesting that PEG-PE is a good reagent for making long circulating liposomes.

In summary, four major conclusions can be made from the results presented in this report. First, liposomes with same lipid composition may have different blood half-lives in different animals (Fig. 1). Second, the mechanisms of liposome uptake by the RES, predominantly by Kupffer cells in the liver, may vary among different animal species (Tables 2 and 3). In mice, the liver uptake of liposomes is directly determined by the surface characteristics of liposomes and no specific serum opsonins are involved. Rapid removal of PS-containing liposomes from blood by the liver in mice is likely due to the recognition of PS by the Kupffer cells. In contrast to such a direct recognition mechanism, hepatic uptake of liposomes in rats appears to depend on the serum opsonins. It is the serum opsonins that determine the rate of blood clearance of liposomes in rats. Third, opsonin activity for a given liposome composition may vary among animal species (Tables 2 and 3). Lastly, complement component C3 in human serum is likely to be the opsonin for liposomes composed of PC/Chol and PC/Chol/GM_1 (Fig. 2). Given the fact that animals are commonly used as a model to predict the clinical behavior of liposomes in humans, these conclusions are important not only for our current understanding of the mechanism of clearance but also for choosing the appropriate animal model for the development of liposome-based drug delivery systems. Avoiding activation of the complement system or preventing the binding of C3 to liposomes may be critical in the future design of long circulating liposomes for clinical use.

Acknowledgements

We thank Dr. Leaf Huang for his assistance in providing DTPA-SA and for the use of particle analyzer. Critical review of the manuscript by Dr. Joseph Knapp is acknowledged. We would also like to thank Feng Liu for his technical assistance in some of the experiments. This work was supported by a seeding grant to Dexi Liu from the University of Pittsburgh.

References

[1] Senior, J.H. (1988) Crit. Rev. Ther. Drug Carrier System 3, 123–193.
[2] Juliano, R.L. (1988) Adv. Drug Delivery Rev. 2, 31–54.
[3] Gabizon, A. and Papahadjopoulos, D. (1988) Proc. Natl. Acad. Sci. USA 85, 6949–6953.
[4] Juliano, R.L. and Stamp, D. (1975) Biochem. Biophys. Res. Commun. 63, 651–658.
[5] Liu, D., Mori, A. and Huang. L. (1992) Biochim. Biophys. Acta 1104, 95–101.
[6] Allen, T.M. and Chonn, A. (1987) FEBS Lett. 223, 42–46.
[7] Klibanov, A.L., Maruyama, K., Torchilin, V.P. and Huang, L. (1990) FEBS Lett. 268, 235–237.
[8] Allen, T.M., Hansen, C., Martin, F., Redemann, C. and Yau-Young, A. (1991) Biochim. Biophys. Acta 1066, 29–36.
[9] Senior, J., Delgardo, C., Fisher, D., Tilcock, C. and Gregoriadis, G. (1991) Biochim. Biophys. Acta 1062, 77–82.
[10] Allen, T.M. (1992) J. Liposome Res. 2, 289–305.
[11] Papahadjopoulos, D., Allen, T.M., Gabizon, A., Mayhew, E., Mathay, K., Huang, S.K., Lee, S.K.D., Woodle, M.C., Lasic, D.D., Redemane, C. and Matrtin, F.J. (1991) Proc. Natl. Acad. Sci. USA 88, 11460–11464.
[12] Huang, L. (1992) J. Liposome Res. 2, 451–454.
[13] Allen, T.M. (1994) Trends Pharmacol. Sci. 15, 215–220.
[14] Kabalka, G., Buonocore, E., Hubner, K., Moss, T., Norley, N. and Huang, L. (1987) Radiology 163, 255–258.
[15] Wu, M.S., Robbins, J.C., Bugianesi, R.L., Ponpipom. M.M. and Shen, T.Y. (1981) Biochim. Biophys. Acta 674, 19–26.
[16] Sjostrand, T. (1962) In Handbook of Physiology (Hamilton, W.F., ed.), Vol. 1, pp. 51–62, American Physiological Society, Washington.
[17] Liu, D., Liu, F. and Song, Y.K. (1995) Biochim. Biophys. Acta 1235, 140–146.
[18] Harashima, H., Sakata, K., Funato, K. and Kiwada, H. (1994) Pharm. Res. 11, 402–406.
[19] Wassef, N.M. and Alving, C.R. (1987) Methods Enzymol. 149, 124–134.
[20] Mayer, M.M. (1961) In Experimental Immunology (Kabat, E.A. and Mayer, M.M., eds.), pp. 133–240, Charles C. Thomas, Springfield.
[21] Cochrane, C.G., Muller-Eberhard, H.J. and Aikin, B.S. (1970) J. Immunol. 105, 55–69.
[22] Segal, A.W., Willis, E.J., Richmond, J.E., Slavin, G., Black. C.D.V. and Gregoriadis, G. (1974) Br. J. Exp. Pathol. 55, 320–327.
[23] Scherphof, G., Roerdink, F., Dukstra, J., Ellens, H., De Zanger, R. and Wisse, E. (1983) Biol. Cell 47, 47–58.
[24] Huang, S.K., Lee, K.D., Hong, K., Friend, D.S. and Papahadjopoulos, D. (1992) Cancer Res. 52, 5135–5143.
[25] Allen, T.M., Austin, G.A., Chonn, A., Lin, L. and Lee, K.C. (1991) Biochim. Biophys. Acta 1061, 56–64.
[26] Lee, K.D., Hong, K., and Papahadjopoulos, D. (1992) Biochim. Biophys. Acta 1103, 185–197.
[27] Liu, D., Song, Y.K. and Liu, F. (1995) Pharm. Res., in press.
[28] Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71.
[29] Schwartz, R.S., Tanaka, Y., Fidler, I.J., Chiu, D., Lubin, B. and Schroit, A.J. (1985) J. Clin. Inves. 75, 1965–1972.
[30] Lubin, B., Chiu, D., Bastacky, J., Roelofsen, B. and Van Deenen, L.L.M. (1981) J. Clin. Invest. 67, 1643–1649.
[31] Litzinger, D., Buiting, A.M.J., Van Rooijen, N. and Huang, L. (1994) Biochim. Biophys. Acta 1190, 99–107.
[32] Patel, H.M. (1992) Crit. Rev. Ther. Drug Carrier System 9, 39–90.
[33] Alving, C.R. and Swartz, Jr. G.M. (1991) CRC Crit. Rev. Immunol. 10, 441–453.