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The complement system enhances the clearance of phosphatidylserine (PS)-liposomes in rat and guinea pig

Tran Minh Huong [1], Tatsuhito Ishida, Hideyoshi Harashima [2], Hiroshi Kiwada *

Department of Pharmacokinetics and Pharmaceutics, Graduate School of Pharmaceutical Sciences, The University of Tokushima, 1-78-1, Shomachi, Tokushima 770-8505, Japan

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Abstract

In this study, we investigated the contribution of the complement system to the biodistribution of phosphatidylserine (PS)-containing liposomes in rat and guinea pig. It appeared that the inclusion of PS in the liposome formulation accelerates the rate of liposome uptake by liver, resulting in rapid elimination of the liposomes from blood circulation. Pretreatment with K76COOH (K76), an anti-complement agent, decreased the rapid uptake of PS-containing liposomes by guinea pig liver, resulting in increasing blood concentration of the liposomes. Significant complement-dependent liposome destabilization was observed in vitro in both animals, whereas the complement-dependent destabilization in vivo was likely only a part of the process of the clearance of the PS-containing liposomes. This discrepancy suggests that the rate of complement-dependent liposome uptake by liver is much faster than the rate of complement-dependent liposome destabilization in vivo. Pretreatment of K76 dramatically inhibited the binding of C3 fragments, one of dominant opsonins, to PS-containing liposomes in guinea pig under both in vivo and in vitro conditions. This finding suggests that the C3 fragments in the system are responsible for the clearance of the PS-containing liposomes in guinea pig. In rat, in contrast to guinea pig, in vivo binding of C3 fragments was not inhibited by K76-pretreatment, while in vitro binding was inhibited. This discrepancy may be due to different experimental conditions between in vitro and in vivo assay. Nevertheless, based on the observations in this study, the complement components are most likely involved in the clearance of the PS-containing liposomes in rat. Taken together, the activity of PS in enhancing the liposome clearance appears to be mediated by the complement

Abbreviations: AUC, area under the blood concentration-time curve; C3, third component of the complement; CF, 5(6)-calboxyfluorescein; CHOL, cholesterol; CL, clearance; CLh, hepatic clearance; CLrel, renal clearance; CLs, splenic clearance; CLtot, total clearance; DCP, dicetylphosphate; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; K76, K76COOH; MAC, membrane attack complex; MPS, mononuclear phagocyte system; PA, phosphatidic acid; PBS, phosphate-buffered saline; PC, hydrogenated egg phosphatidylcholine; PG, phosphatidylglycerol; PL, phospholipid; PS, hydrogenated egg phosphatidylserine.

* Corresponding author. Tel./fax: + 81-886-33-9506.

E-mail address: hkiwada@fc.ph.tokushima-u.ac.jp (H. Kiwada).

[1] Present address: Hanoi College of Pharmacy, 13 Le Thanh Tong Str., Hanoi, Vietnam.

[2] Present address: Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12, Nishi 6, Sapporo 060-0812, Japan.

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components, presumably C3 fragments, in both guinea pig and rat. This is a first report showing the mechanism on the hepatic uptake of the PS-containing liposomes in guinea pig. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Liposomes; Biodistribution; Complement system; Rat; Guinea pig

1. Introduction

Liposomes have been extensively evaluated as a potential drug carrier system for therapeutic applications because of their ability to alter the pharmacokinetics and reduce the toxicity of their associated drugs (Hwang, 1987; Allen and Hansen, 1991). Another important factor to further improve the therapeutic efficacy is that one needs to control the blood kinetics and tissue distribution of the liposomes, and the release rate of the contents from the liposomes. For example, liposomes which can stay in circulation for prolonged time to extravasate the leaky vessels of solid tumor are very useful in delivering antitumor drugs for cancer chemotherapy (Huang et al., 1992; Gabizon et al., 1994; Huang et al., 1994). To achieve this goal, however, we will have to understand the physical, chemical and biological factors that regulate the blood clearance and tissue distribution of liposomes.

The clearance of the liposomes from the blood is mainly dependent on liposome composition and diameter (Juliano and Stamp, 1975; Gabizon and Papahadjopoulos, 1988; Liu et al., 1992). Generally, liposomes containing negatively charged lipids such as phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA) or cardiolipin are cleared much faster than those containing neutral lipids. With respect to the effect of liposome size on the rate of liposome clearance, the large sized liposomes are cleared faster than the small sized liposomes of the same composition. However, the basic principles behind these well-known phenomena remain unknown.

The exposure of negatively charged lipid, PS, is thought to serve as a signal for the removal of the liposomes (Liu et al., 1995a) and various abnormal cells such as aged erythrocytes and apoptotic cells (Sambrano and Steinberg, 1995). The mechanisms, which control this process, are not fully understood. The complement system has been suspected to be a possible candidate for major dominant opsonin in the liposome clearance process (Devine and Marjan, 1997; Devine and Bradley, 1998; Harashima et al., 1998; Szebeni, 1998). In fact, our laboratory have already showed the potential role of the complement system on the destabilization of the liposomes (Funato et al., 1992; Harashima et al., 1995; Ishida et al., 1997) and the uptake of liposomes by Kupffer cells in an in situ perfused liver system (Kiwada et al., 1987; Harashima et al., 1994; Matsuo et al., 1994; Harashima and Kiwada, 1996a,b; Harashima et al., 1998). In this study, therefore, we designed experiments to clarify the role of the complement system in the clearance of PS-containing liposomes. The liposomes sized at 800 nm were chosen, since the larger liposomes were observed to be much effective complement activators than the smaller liposomes (Devine et al., 1994; Harashima et al., 1994).

We quantitatively determined the biodistribution of PS-containing liposomes after intravenously administration to either guinea pig or rat. Complement-dependent liposome destabilization in vitro and in vivo was examined by measuring release of the encapsulated contents from the liposomes. In addition, the amount of C3 fragments associated with the liposomes in vitro and in vivo was determined by using an assay system that we have recently developed (Ishida et al., 2000). Our findings demonstrated that the complement system is most likely involved in the process of the clearance of the PS-containing liposomes in both guinea pig and rat. It appeared that complement components, presumably C3 fragments, serve as an opsonin that enhances phagocytosis of the PS-containing liposomes by liver macrophages.

2. Materials and methods

2.1. Materials

Hydrogenated egg phosphatidylserine (PS) and hydrogenated egg phosphatidylcholine (PC) were kindly donated by Nippon Fine Chemical (Osaka, Japan). Dicetylphosphate (DCP) was purchased from Nacalai Tesque (Kyoto, Japan). Cholesterol (CHOL) was of analytical grade (Wako Pure Chemical, Osaka, Japan). All the lipids were used without further purification. K76COOH (K76) was a gift from Otsuka Pharmaceutical Co. (Tokushima, Japan). 5(6)-Carboxyfluorescein (CF) was purchased from Eastman Kodak Co. (NY, USA). ^{3}H-Cholesterylhexadecyl ether (^{3}H-CHE) and ^{3}H-inulin were purchased from NEN Research Products (MA, USA). All other reagents were of analytical grade.

2.2. Preparation of liposomes

Preparation of multilamellar vesicles (MLV) were performed by modification of a conventional lipid film hydration method described previously (Funato et al., 1992). Liposomes were composed of PS/PC/CHOL/DCP at a molar ratio of 3:2:4:1 (PS (30%)-liposomes) and 1:4:4:1 (PS (10%)-liposomes), and sized by extrusion method through polycarbonate membrane filters (Nuclepore Co., CA, USA) with pore size of 800 nm. Mean diameters of liposomes (760–830 nm) were determined using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). Either ^{3}H-inulin or CF was encapsulated as an internal space marker to follow biodistribution of liposomes in vivo or to measure liposome destabilization in vitro, respectively. For determining C3 fragments associated with liposomes, the liposomes were labeled with a trace amount of ^{3}H-CHE (1 μCi/40 μmol of total lipids) as a nonexchangeable lipid phase marker (Pool et al., 1982; Derksen et al., 1987).

2.3. Biodistribution and pharmacokinetics of liposomes

Male Hartley guinea pigs (300–400 g) (Inoue Experimental Animal, Kumamoto, Japan) were anesthetized by an intraperitoneal injection of sodium phenobarbitone (50 mg/kg body wt.) and male Wistar rats (180–230 g, Inoue Experimental Animal, Kumamoto, Japan) were anesthetized with ether. Under anesthesia, the animals were cannulated via the left femoral vein (PE-20, Natsume, Tokyo, Japan), artery (PE-50) and bladder (PE-20 and PE-50 tied together in parallel). To evaluate the biodistribution of the liposomes, the liposomes containing encapsulated ^{3}H-inulin were administered intravenously through the femoral vein cannula into rats. The injected volume was 2.5 ml/kg body wt. with a dose corresponding to 12 μmol as total lipid/kg body wt. for guinea pigs or 15 μmol as total lipid/kg body wt. for rats. In order to examine contribution of the complement system on the liposome biodistribution, several animals were intravenously administered with K76 at a dose of 100 mg/kg body wt. 1 h prior to the liposome injection. K76, a sesquiterpene was initially found to inhibit the C5 step in the cascade of complement activation (Miyazaki et al., 1980). In addition, C3 depression was observed in K76 treated rat (Tanaka et al., 1996). At indicated time points after injection, blood was sampled through the femoral artery cannula, and urine was collected from the bladder by washing with 2–3 ml of saline. Liver and spleen were collected from animals after sampling of blood and urine. ^{3}H-Inulin in blood, urine and tissues were assayed as described earlier (Kume et al., 1991). We have previously demonstrated that ^{3}H-inulin in blood and organs reflect the amount of intact liposomes, and the level of ^{3}H-inulin in urine represents the free inulin released from the liposomes (Harashima et al., 1992). Therefore, the radioactivity of ^{3}H-inulin detected in blood, urine, liver and spleen represents the blood concentration, destabilization in the circulation, the uptake of liposomes by liver and spleen, respectively. The pharmacokinetic parameters of liposomes were determined as described previously (Ishida et al., 1997).

2.4. In vitro liposome destabilization assay

Liposome destabilization in serum was assessed by determining the fluorescence intensity of re-

leased CF according to an earlier described method (Funato et al., 1992).

2.5. Determination of amount of C3 fragments associated with isolated liposomes

For in vitro interaction, to 110 μl of liposome solution labeled with ^{3}H-CHE (20 μmol/ml of total lipid), 990 μl of serum was added and the liposomes-serum mixture was incubated at 37°C for 30 min. For some experiments, the serum pretreated with K76 (1.5 mg/ml) was used to examine effect of inactivation of the complement system. The incubation mixture was then subjected to the spin-column to isolate the liposomes. The incubation mixture (1100 μl) was then loaded onto a 10 ml BioGel A-15m, 200–400 mesh size (Bio-Rad) spin column to isolate liposomes from serum components, including very low density and low density lipoproteins, as described earlier (Chonn et al., 1991). For in vivo interaction, the liposomes labeled with ^{3}H-CHE were administered intravenously through the femoral vein cannula into animals. The injected volume was 2.5 ml/kg body wt. with a dose corresponding to 12 μmol as total lipid/kg body wt. for guinea pigs or 15 μmol as total lipid/kg body wt. for rats. To examine the effect of deficient of the complement system on the C3 binding, several animals were treated with K76 (100 mg/kg) 1 h before the liposome administration. Two minutes after liposome injection, the blood was collected via carotid artery. The blood was immediately cooled at 4°C to avoid further activation of the complement system. Serum was separated from the cooled blood by centrifugation (1000 rpm, 30 min). The serum containing the liposomes was then loaded onto a 10 ml BioGel A-15m, 200–400 mesh size (Bio-Rad) spin column to isolate liposomes from serum components. The amount of C3 fragments extracted from collected liposomes was determined according to the method described earlier (Ishida et al., 2000). Total C3 fragments associated with the liposomes were expressed as ng per μmol of liposomal lipid.

2.6. Statistics

Statistic analyses were performed using StatView software (Abacus Concepts, Inc., CA, USA).

3. Results

3.1. Biodistribution of liposomes and pharmacokinetic analysis

Fig. 1A illustrates the blood concentration-time profile of PS-containing liposomes in guinea pig, in the presence or absence of an anti-complement agent, K76. Higher amount of PS (30%) in the formulation caused the liposomes to clear more rapidly than those containing a lower amount of PS (10%). The pharmacokinetics of PS (30%)-containing liposomes was characterized by an area under the blood concentration-time curve (AUC) (14.7 ± 7.0% dose.min/ml) and a total clearance (CLtot) of 7.86 ± 3.43 ml/min (Table 1). The pharmacokinetics of PS (10%)-containing liposomes was characterized by an AUC (72.8 ± 37.6% dose.min/ml) and a CLtot of 1.64 ± 0.81 ml/min (Table 1). The decrease of PS concentration in the formulation caused a 5-fold increase in AUC ($P < 0.05$) as well as a 5-fold decrease in CLtot ($P < 0.05$). In the presence of K76, the blood concentration of PS (30%)-containing liposomes was higher than that of same liposomes observed in the absence of K76 (Fig. 1A). An AUC (166.1 ± 57.7% dose.min/ml) and a CLtot (0.66 ± 0.27 ml/min) were obtained (Table 1). Compared with the parameters in the absence of K76, this represented a significant ($P < 0.05$) 11-fold increase in AUC, confirming slower liposome clearance from the blood circulation in the presence of K76.

Fig. 1B illustrates the blood concentration-time profile of PS-containing liposomes in rat. The blood concentration-time data were fitted with a two-compartment model, characterized by a rapid phase concentration decrease, and a slower terminal elimination phase. Lower amount of PS (10%) in the formulation caused the liposomes to clear slightly slower than the liposomes containing a higher amount of PS (30%). The pharmacokinetic parameters were characterized by an AUC (315.0 ± 124.7% dose.min/ml) and a CLtot (0.35 ± 0.14 ml/min) for PS (10%)-liposomes, and an AUC (165.6 ± 88.5% dose.min/ml) and a CLtot (0.76 ±

0.46 ml/min) for PS (30%)-liposomes (Table 2). There were no significant differences in AUC and CLtot.

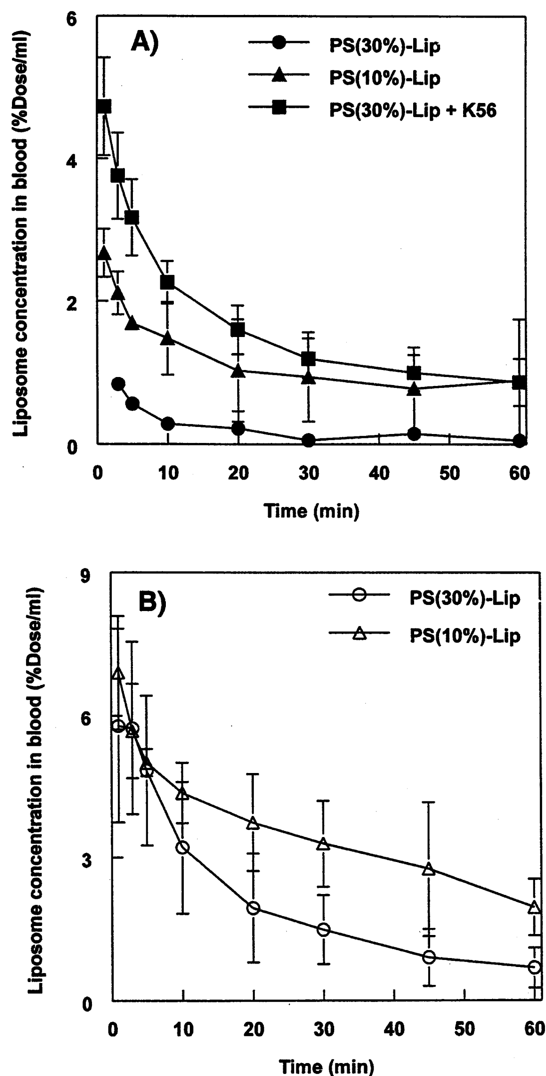

Fig. 1. Biodistribution of liposomes. ^{3}H-Inulin was encapsulated into liposomes which were composed of PS/PC/CHOL/DCP = 3:2:4:1, mol/mol (●, ○, ■) or 1:4:4:1, mol/mol (▲, △). Liposomes were administered by intravenous injection into either (A) male guinea pigs (12 μmol total lipid/kg) or (B) male Wistar rats (15 μmol total lipid per kg). For some experiments, guinea pigs were treated with K76 at a dose of 100 mg/kg body wt. 1 h prior to the liposome injection. At each time point, blood was collected. Radioactivity of ^{3}H-inulin in blood was determined by scintillation counting. Each value represents the mean ± S.D. of three separate experiments.

Hepatic and splenic clearances of liposomes (CLh and CLs) reflect accumulation of liposomes in liver and spleen, respectively. Renal clearance of liposomes (CLrel) reflects liposome destabilization in blood circulation. In guinea pig, values of CLh, CLs and CLrel for PS (30%)-containing liposomes were much higher than those for the PS (10%)-containing liposomes ($P < 0.05$) (Table 1). K76 significantly altered the pharmacokinetic parameters of PS (30%)-containing liposomes when coadministered in the liposomes, as indicated by comparable values for CLh, CLs and CLrel (Table 1). Significant ($P < 0.05$) decreases in CLh, CLs and CLrel were observed in the presence of K76. In rat, values of CLh, CLs and CLrel for PS (30%)-containing liposomes were also much higher than those for the PS (10%)-containing liposomes ($P < 0.05$) (Table 2). This is consistent with the observation in guinea pig as shown in Table 1.

3.2. Release of CF from liposomes in serum

Destabilization of liposomes in vitro was examined by measuring release of the contents (CF) from liposomes. To examine contribution of the complement system to liposome destabilization, sera from guinea pig and rat were pretreated with K76 (1.5 mg/ml) or by heating at 56°C for 30 min. The release of CF from PS (30%)-containing liposomes following incubation in untreated serum, pretreated sera or PBS is shown in Fig. 2. The liposomes significantly released their contents (CF) in untreated serum from either guinea pig or rat. In K76-treated or heated serum (complement-inactivated sera), the release was reduced up to that in PBS (control) ($P < 0.001$). It appeared that the PS (30%)-containing liposomes were destabilized through activation of the complement system in vitro. In addition, the CF release in untreated rat serum was much higher than that in untreated guinea pig serum ($P < 0.001$).

3.3. Amount of C3 fragments associated with PS-containing liposomes

Liposomes were recovered from a liposome-serum mixture either 30 min after incubation at

Table 1
Pharmacokinetic parameters of PS-containing liposomes in guinea pig[a]

Liposome composition (PS/PC/CHOL/DCP, mol/mol)	AUC	CLtot	CLh	CLs	CLrel
1/4/4/1 (PS(10%))	72.8 ± 37.6	1.64 ± 0.81	0.31 ± 0.24	0.12 ± 0.02	0.08 ± 0.03
3/2/4/1 (PS(30%))	14.7 ± 7.0^b	7.86 ± 3.43^b	2.95 ± 1.45^b	0.55 ± 0.16^b	0.46 ± 0.08^b
3/2/4/1 (PS(30%))+K76	166.1 ± 57.7	0.66 ± 0.27	0.50 ± 0.18	0.06 ± 0.02	0.06 ± 0.02

[a] Liposomes containing ^{3}H-inulin (12 μmol total lipid/kg) were administered by intravenous injection into male guinea pigs. Blood and urine at each time point were collected. At 1 h after injection, liver and spleen were collected. ^{3}H-inulin in blood, urine and each tissue were assayed. Pharmacokinetic parameters were calculated as described in Section 2. Each value represents mean ± S.D. of three separate experiments.

[b] Statistically significant at $P < 0.05$ (one-way ANOVA).

37°C in vitro or 2 min after intravenously administration in vivo. C3 fragments associated with the liposomes were extracted and determined by ELISA. As shown in Fig. 3, bound C3 fragments were detectable both in vivo and in vitro, regardless of species difference (guinea pig and rat). In guinea pig, the amount of bound C3 fragments significantly decreased when the animal was pretreated with K76 ($P < 0.05$). In vitro assay demonstrated significant decrease ($P < 0.05$) on bound C3 fragments in the presence of K76. In rat, the treatment with K76 inhibited binding of C3 fragments in vitro ($P < 0.05$), whereas the treatment failed to inhibit the binding of C3 fragments in vivo.

4. Discussion

Liposome accumulation in the MPS and liposome destabilization in blood are important factors which affect liposome circulation lifetimes. In both guinea pig and rat, the liposomes containing higher amount of PS (30%) significantly accumulated into the liver and destabilize in blood circulation, resulting in rapid clearance from circulation compared with liposomes containing less PS (10%) (Fig. 1, Tables 1 and 2). As shown in Figs. 2 and 3, the destabilization of the liposomes in heated serum (56°C, 30 min) and K76-treated serum, as well as the binding of C3 fragments to the liposomes in K76-treated serum in vitro were decreased. The treatments are well-known to inactivate the complement system (Miyazaki et al., 1980; Okada et al., 1982; Tanaka et al., 1996). Therefore, the higher accumulation of the liposomes into liver and destabilization of the liposomes in vivo would be due to PS-dependent complement activation.

Interestingly, although in vitro higher complement-dependent destabilization in the PS (30%)-containing liposomes was observed (Fig. 2), in vivo destabilization which reflects the CLrel shared only a part of the clearance process of the liposomes (Tables 1 and 2). It appeared that the complement-dependent hepatic uptake of the liposomes was much faster than the in vivo complement-dependent liposome destabilization. The complement system is referred to as a cascade reaction through several pathways, because the individual proteins circulate in an inactive form as zymogens and are sequentially activated by other members of the complement components. Once the complement system was activated, activated C3, C3b, covalently bound to the target surface, subsequently the bound C3b formed active serine proteases with other complement components and then assembly of the MAC (C5b-9) started. It is well-known that the bound C3 fragments enhances hepatic uptake of liposomes and the assembled MAC enhances the release of the contents by forming a pore through the bilayer. Therefore, the observation may be explained by assuming that the rate of binding of C3 fragments to the liposomes was much faster than the rate of assembling of MAC on the surface of the liposomes, resulting in rapid hepatic uptake of the liposomes. Alternatively, the observation may be explained by the report by Richard et al. that the channels by the MAC are only transiently open in

Table 2
Pharmacokinetic parameters of PS-containing liposomes in rat[a]

Liposome composition (PS/PC/CHOL/DCP, mol/mol)	AUC	CLtot	CLh	CLs	CLrel
1/4/4/1 (PS(10%))	315.0 ± 124.7	0.35 ± 0.14	0.21 ± 0.01	0.02 ± 0.01	0.013 ± 0.002
3/2/4/1 (PS(30%))	165.6 ± 88.5	0.76 ± 0.46	0.66 ± 0.19[b]	0.06 ± 0.01[b]	0.047 ± 0.012[b]

[a] Liposomes containing ^{3}H-inulin (15 μmol total lipid/kg) were administered by intravenous injection into male Wistar rats. Blood and urine at each time point were collected. At 1 h after injection, liver and spleen were collected. ^{3}H-inulin in blood, urine and each tissue were assayed. Pharmacokinetic parameters were calculated as described in Section 2. Each value represents mean ± S.D. of three separate experiments.

[b] Statistically significant at $P<0.05$ (Student's t-test).

liposomes; they either close, or are readily lost (Richards et al., 1986). The MAC assembled on the liposomal membrane may be lost or closed rapidly, resulting in limiting the in vivo liposome destabilization.

The C3 fragment is well-known as a major opsonin which enhances the uptake of foreign particles, such as pathogens, by Kupffer cells via complement receptor type 1 and 3 (CR1 and 3) (Brown 1991). We have identified the role of the complement system, especially C3 fragments, in enhancing the hepatic uptake of the liposomes composed of PC, CHOL and DCP via complement receptor-mediated phagocytosis by using an isolated perfused rat liver system (Harashima et al., 1994; Matsuo et al., 1994; Harashima and Kiwada, 1996b; Harashima et al., 1998). Liu and co-workers have also reported that liver uptake of PS-containing liposomes are mediated by complement components in rat (Liu et al., 1995b). The CLh which reflects hepatic uptake of the liposomes (Table 1), as well as the binding of the C3 fragments to the liposomes (Fig. 3) decreased significantly when guinea pig was pretreated by K76. It appeared that the liposome opsonization by the C3 fragments leads to enhanced uptake of the liposomes by phagocytic cells in liver in guinea pig. In addition, in rat, complement-dependent destabilization and in vitro C3 binding were inhibited by K76-pretreatment (Figs. 2 and 3), while in vivo C3 binding was not inhibited (Fig. 3). This discrepancy may be due to different experimental conditions between in vitro and in vivo assay as discussed below. Nevertheless, taken together with our and Liu and co-workers' earlier results, the uptake of PS-containing liposomes in rat is mediated by the complement system, presumably bound C3 fragments.

As shown in Fig. 3, the treatment of rat serum by K76 significantly decreased the binding of C3

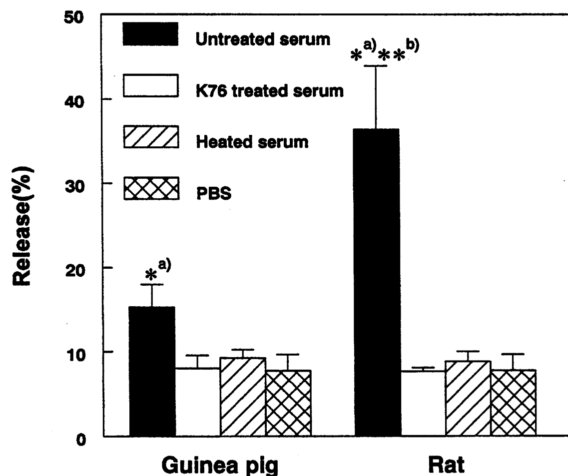

Fig. 2. Release of CF from liposomes in either guinea pig or rat serum. Liposomes (PS/PC/CHOL/DCP = 3:2:4:1, mol/mol) containing encapsulated CF were incubated with untreated serum (closed column), K76 treated serum (opened column), heated serum (56°C, 30 min) (hatched column) or PBS (checkered column) at 37°C for 30 min. The fluorescence intensity of released CF was determined. Total releasable CF from the liposomes was measured by lysing the liposomes with Triton-X 100 solution (5% v/v). Percentage of released CF from liposomes was calculated by dividing the fluorescence intensity of the reaction mixture by that of total encapsulated CF in the liposomes. Each value represents the mean ± S.D. of three to five separate experiments. (a) Statistically significant at * $P<0.001$ (one-way ANOVA). (b) Statistically significant at ** $P<0.0001$ (Student's t-test) versus the value of CF release in untreated guinea pig serum.

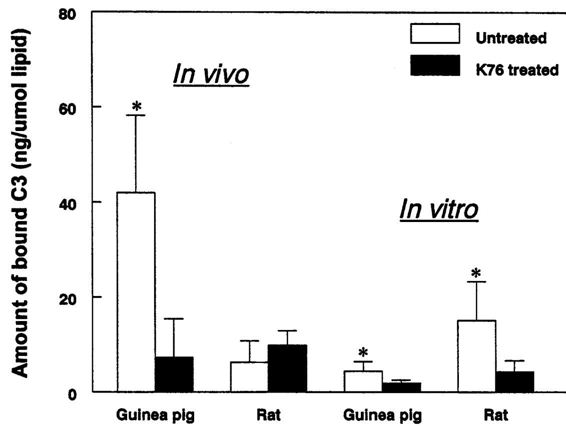

Fig. 3. Amount of bound C3 fragments on liposomes. In vivo interaction, the liposomes (PS/PC/CHOL/DCP = 3:2:4:1, mol/mol) labeled with ^{3}H-CHE were administered intravenously into animals. To examine the effect of deficient of the complement system, several animals were treated with K76 (100 mg/kg) 1 h before the liposome administration. Two minutes after liposome injection, the blood was collected via carotid artery. Serum was separated from the collected blood by centrifugation (1000 rpm, 30 min). The serum containing the liposomes was then loaded onto a spin column to isolate liposomes from serum components. C3 fragments were extracted from the liposomes and quantitated using a sandwich ELISA. In vitro assay, liposomes (PS/PC/CHOL/DCP = 3:2:4:1, mol/mol) were incubated with guinea pig or rat serum at 37°C for 30 min. After the incubation, the liposomes were separated from unbound serum proteins using a spin column. C3 fragments were extracted from the liposomes and quantitated using a sandwich ELISA. Amount of C3 fragments is expressed as nanograms per micromole liposomal lipid. Each value represents the mean $\pm$ S.D. of three to five separate experiments. Statistically significant at * $P < 0.05$ (Student's t-test)*.

fragments. However, the in vivo treatment of rat with K76 failed to inhibit the binding of C3 fragments (Fig. 3). This discrepancy would be due to that the in vivo assay reflects the more complex nature of the in vivo system. Binding of proteins that associated with liposomes in vivo and not in isolated serum may compete with the binding of the fragments resulting from proteolytic activation of the complement system. We have shown a possibility that the in vitro assays for the bound C3 fragments to liposomes and release of the contents from liposomes should be predictive of their clearance behavior in vivo (Ishida et al., 1997, 2000). However, the finding in this report indicates the importance of carefully characterizing the blood protein associated with the liposomes in vivo.

In this study, we showed that the inclusion of PS in the liposome formulation accelerates the rate of complement-dependent liposome uptake by liver, resulting in rapid elimination of the liposomes. In addition, the C3 fragments in the complement system appeared to be responsible for the clearance of the PS-containing liposomes in both guinea pig and rat. As far as we know, there is little report indicating the mechanism for the hepatic uptake of PS-containing liposomes in guinea pig. Hence, we suppose that this is a first report showing that the hepatic uptake of PS-containing liposomes in guinea pig is mediated by the complement components, though further experimental evidence would be needed.

References

Allen, T.M., Hansen, C.B., 1991. Pharmacokinetics of Stealth versus conventional liposomes: effect of dose. Biochim. Biophys. Acta 1068, 133–141.

Brown, E.J., 1991. Complement receptors and phagocytosis. Curr. Opin. Immunol. 3, 76–82.

Chonn, A., Semple, S.C., Cullis, P.R., 1991. Separation of large unilamellar liposomes from blood components by a spin column procedure: towards identifying plasma proteins which mediate liposome clearance in vivo. Biochim. Biophys. Acta 1070, 215–222.

Derksen, J.T., Morselt, H.W., Scherphof, G.L., 1987. Processing of different liposomal markers after in vitro uptake of immunoglobulin-coated liposomes by rat liver macrophages. Biochim. Biophys. Acta 931, 33–40.

Devine, D.V., Marjan, J.M., 1997. The role of immunoproteins in the survival of liposomes in the circulation. Crit. Rev. Ther. Drug Carrier Syst. 14, 105–131.

Devine, D.V., Bradley, A.J., 1998. The complement system in liposome clearance: can complement deposition be inhibited? Adv. Drug Del. Rev. 32, 19–29.

Devine, D.V., Wong, K., Serrano, K., Chonn, A., Cullis, P.R., 1994. Liposome-complement interactions for liposome survival studies. Biochim. Biophys. Acta 1191, 43–51.

Funato, K., Yoda, R., Kiwada, H., 1992. Contribution of complement system on destabilization of liposomes composed of hydrogenated egg phosphatidylcholine in rat fresh plasma. Biochim. Biophys. Acta 1103, 198–204.

Gabizon, A., Papahadjopoulos, D., 1988. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc. Natl. Acad. Sci. USA 85, 6949–6953.

Gabizon, A., Catane, R., Uziely, B., Kaufman, B., Safra, T., Cohen, R., Martin, F., Huang, A., Barenholz, Y., 1994. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. Cancer Res. 54, 987–992.

Harashima, H., Kiwada, H., 1996a. Liposomal targeting and drug delivery: kinetic consideration. Adv. Drug Del. Rev. 19, 425–444.

Harashima, H., Kiwada, H., 1996b. Studies on the mechanism of uptake of liposomes using an isolated perfused liver system. J. Liposome Res. 6, 61–75.

Harashima, H., Kume, Y., Yamane, C., Kiwada, H., 1992. Non-Michaelis-Menten type uptake of liposomes in the rat. J. Pharm. Pharmacol. 44, 707–712.

Harashima, H., Sakata, K., Funato, K., Kiwada, H., 1994. Enhanced hepatic uptake of liposomes through complement activation depending on the size of liposomes. Pharm. Res. 11, 402–406.

Harashima, H., Hiraiwa, T., Ochi, Y., Kiwada, H., 1995. Size dependent liposome degradation in blood: in vivo/in vitro correlation by kinetic modeling. J. Drug Targeting 3, 253–261.

Harashima, H., Matsuo, H., Kiwada, H., 1998. Identification of proteins mediating clearance of liposomes using a liver perfusion system. Adv. Drug Delivery Rev. 32, 61–79.

Huang, S.K., Mayhew, E., Hong, K., Friend, D.S., Papahadjopoulos, D., 1992. Pharmacokinetics and therapeutics of sterically stabilized liposomes in mice bearing C-26 colon carcinoma. Cancer Res. 52, 6774–6781.

Huang, S.K., Stauffer, P.R., Hong, K., Guo, J.W., Phillips, T.I., Huang, A., Papahadjopoulos, D., 1994. Liposomes and hyperthermia in mice: increased tumor uptake and therapeutic efficacy of doxorubicin in sterically stabilized liposomes. Cancer Res. 54, 2186–2191.

Hwang, K.J., 1987. Liposome pharmacokinetics. In: Ostro, M.J. (Ed.), Liposomes: from Biophysics to Therapeutics. Marcel Dekker, New York, pp. 109–156.

Ishida, T., Funato, K., Kojima, S., Yoda, R., Kiwada, H., 1997. Enhancing effect of cholesterol on the elimination of liposomes from circulation is mediated by complement activation. Int. J. Pharm. 156, 27–37.

Ishida, T., Kojima, H., Harashima, H., Kiwada, H., 2000. Biodistribution of liposomes and C3 fragments associated with liposomes: evaluation of their relationship. Int. J. Pharm., in press.

Juliano, R.L., Stamp, D., 1975. Effects of particle size and charge on the clearance of liposomes and liposome-encapsulated drugs. Biochim. Biophys. Acta 1113, 651–658.

Kiwada, H., Miyajima, Y., Kato, Y., 1987. Studies on the uptake of mechanism of liposomes by perfused rat liver. II. An indispensable factor for liver uptake in serum. Chem. Pharm. Bull. 35, 1189–1195.

Kume, Y., Maeda, H., Harashima, H., Kiwada, H., 1991. Saturable, non-Michaelis-Menten uptake of liposomes by reticuloendothelial system. J. Pharm. Pharmacol. 43, 162–166.

Liu, D., Mori, A., Huang, L., 1992. Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of G_{M1}-containing liposomes. Biochim. Biophys. Acta 1104, 95–101.

Liu, D., Liu, F., Song, Y.K., 1995a. Recognition and clearance of liposomes containing phosphatidylserine are mediated by serum opsonin. Biochim. Biophys. Acta 1235, 140–146.

Liu, D., Hu, Q.G., Song, Y.K., 1995b. Liposome clearance from blood: different animal species have different mechanisms. Biochim. Biophys. Acta 124, 277–284.

Matsuo, H., Funato, K., Harashima, H., Kiwada, H., 1994. The complement-but not mannose receptor-mediated phagocytosis is involved in hepatic uptake of cetylmannoside-modified liposomes in situ. J. Drug Target 2, 141–146.

Miyazaki, K., Tamaoka, H., Shinohara, M., Kaise, H., Izawa, T., Nakano, T., Kinoshita, K., Hong, K., Inoue, K., 1980. A complement inhibitor produced by Stachybotrys complementi, nov. sp. K76, a new species of fungi imperfecti. Microbiol. Immunol. 24, 1091–1108.

Okada, N., Yasuda, T., Tsumita, T., Okada, H., 1982. Activation of the alternative complement pathway of guinea-gip by liposomes incorporated with trinitrophenylated phosphatidylethanolamine. Immunology 45, 115–124.

Pool, G.L., French, M.E., Edwards, R.A., Huang, L., Lumb, R.H., 1982. Use of radiolabelled hexadecyl cholesterol ether as a liposome marker. Lipids 17, 445–452.

Richards, R.I., Habbersett, R.C., Scher, I., Janoff, A.S., Schieren, H.P., Mayer, L.D., Cullis, P.R., Alving, C.R., 1986. Influence of vesicle size on complement-dependent immune damage to liposomes. Biochim. Biophys. Acta 855, 223–230.

Sambrano, G.R., Steinberg, D., 1995. Recognition of oxidatively damaged and apoptotic cells by an oxidized low density lipoprotein receptor on mouse peritoneal macrophages: role of membrane phosphatidylserine. Proc. Natl. Acad. Sci. USA 92, 1396–1400.

Szebeni, J., 1998. The interaction of liposomes with the complement system. Crit. Rev. Ther. Drug Carrier Syst. 15, 57–88.

Tanaka, M., Murase, N., Qing, Y., Miyazaki, W., Nomoto, M., Miyazawa, H., Manez, R., Toyama, Y., Demetris, A., Todo, S., Starz, T., 1996. Effect of anticomplement agent K76COOH on hamster-to-rat and guinea pig-to-rat heart xenotransplantation. Transplant 62, 681–688.