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# Enhancing effect of cholesterol on the elimination of liposomes from circulation is mediated by complement activation

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**Abstract**

The effects of cholesterol (Chol) content on the biodistribution of liposomes as well as the interaction of liposomes with plasma proteins, primarily complement (C) components, were examined in this study. The elimination of liposomes from blood circulation was enhanced by increasing the Chol content in liposomes. Furthermore, included Chol augmented the rate of liposome degradation as measured by the urinary excretion of $^{3}$H-inulin encapsulated in liposomes. We have also examined the effect of liposomal Chol on organ clearance (CL) and renal CL ($CL_{rel}$). The values of organ CL and $CL_{rel}$ reflect the affinity of liposomes for the organ and the degree of liposome degradation in the blood, respectively. Hepatic CL and $CL_{rel}$, but not splenic CL, increased with the rise of Chol content in liposomes. The amount of liposome degradation in vitro, which reflects the extent of C activation, was correlated with degradation observed in vivo ($CL_{rel}$). However, the amount of plasma proteins bound to the liposomes was inversely proportional to the extent of in vitro liposome degradation. We have investigated the role of C activating factor (CAF) (Funato et al., 1994, Plasma factor triggering alternative complement pathway activation by liposomes, Pharm. Res., 11, 372–376) on Chol-dependent-C activation. Our results showed that binding of CAF to the liposomes is directly proportional to the amount of Chol present in the liposome. Thus, C activation by Chol in liposomes may proceed via a mechanism involving CAF. Taken together, these results suggest that increasing the Chol content of liposomes enhances the binding of CAF to the liposomes, which in turn, mediates Chol dependent-C activation, resulting in the augmentation of both degradation in blood and hepatic uptake of the liposomes. © 1997 Elsevier Science B.V.

*Keywords:* Drug delivery system; Liposome; Cholesterol; Complement activating factor; Complement

*Abbreviations:* ACP, alternative complement pathway; AUC, area under the blood concentration-time curve; CCP, classical complement pathway; CF, 5(6)-calboxyfluorescein; Chol, cholesterol; C, complement; CAF, complement activating factor; $CL_{tot}$, total body clearance; $CL_h$, hepatic clearance; $CL_s$, splenic clearance; $CL_{rel}$, renal clearance; DCP, dicetylphosphate; HEPC, hydrogenated egg phosphatidylcholine; MBP, mannose-binding protein; MPS, mononuclear phagocyte system; PBS, phosphate-buffered saline.

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## 1. Introduction

The plasma proteins associated with liposomes have been implicated as one of dominant factors in determining the elimination rate and tissue distribution of liposomes in vivo (Juliano and Lin, 1980; Bonte and Juliano, 1986; Chonn et al., 1992, 1994). The different liposomal surface characteristics determine the composition of plasma proteins bound to the liposomes, and therefore the elimination rate of liposomes depends on surface properties such as charge (Juliano and Stamp, 1975; Chonn et al., 1991), fluidity (Patel, 1992), hydrophobicity (Senior, 1987; Moghimi and Patel, 1989) and size (Juliano and Stamp, 1975; Scherphof and Morselt, 1984). The functional importance of plasma proteins in elimination rate and tissue distribution of liposomes is supported by the studies using a perfused rat liver (Kiwada et al., 1987; Matsuo et al., 1994) and purified Kupffer cells (Dijkstra et al., 1984; Moghimi and Patel, 1988, 1990) demonstrating that the uptake of liposomes is remarkably potentiated by incubating them with serum. However, it is not clear how or which plasma proteins regulate the elimination rate and tissue distribution of liposomes

Recently, Chonn et al. demonstrated that the elimination rates of liposomes are inversely related to the total amount of bound plasma proteins associated with the liposomes and the adsorbed proteins include substantial levels of opsonins such as complement (C) components and IgG, leading to rapid uptake by the phagocytic cells (Chonn et al., 1992). Using perfused rat livers, we have provided evidence that, in the presence of serum, the hepatic uptake of liposomes proceeds via a mechanism requiring C components (Harashima et al., 1994a; Matsuo et al., 1994). Moreover, hepatic uptake of liposomes appears to depend on their size, and this size-dependence is associated with the affinity of C activation (Harashima et al., 1994b). These results suggest that the plasma proteins, in particular C components, function as a key molecule which modifies the biodistribution of liposomes in vivo.

To characterize the mechanism involved in plasma protein-mediated biodistribution of liposomes we prepared three types of liposomes containing different Chol contents without altering the surface charge density, since the inclusion of Chol in the liposomes is known to markedly influence the biodistribution of liposomes (Kirby et al., 1980; Senior and Gregoriadis, 1982; Senior, 1987; Moghimi and Patel, 1989). Using these liposomes, we investigated the possibility that Chol dependent biodistribution of liposomes proceeds via an interaction between liposomes and plasma proteins, in particular C components. In this study, we measured the disposition of liposomes in vivo, namely the organ distribution in the body, degradation in blood, as well as organ clearance of liposomes which is expressed as the affinity of liposomes for a given organ. In addition, to assess which factors affect the Chol-dependent liposome biodistribution, we evaluated the total amount of plasma protein bound to and the extent of C activation by the liposomes.

## 2. Materials and methods

### 2.1. Materials

Hydrogenated egg phosphatidylcholine (HEPC) was kindly donated by Nippon Fine Chem. (Osaka, Japan). Dicetylphosphate (DCP) was purchased from Nacalai Tesque (Kyoto, Japan). Chol was of analytical grade (Wako Pure Chem., Osaka, Japan). 5(6)-carboxyfluorescein (CF) and [$^3$H]inulin were purchased from Eastman Kodak (NY, USA) and NEN Research Products (MA, USA), respectively. All other reagents were of analytical grade.

### 2.2. Preparation of liposomes

Liposomes were prepared by the method described previously (Funato et al., 1992). [$^3$H]inulin and CF as aqueous markers were encapsulated to follow biodistribution of liposomes in vivo (Harashima et al., 1993) and to measure liposome degradation in vitro (Funato et al., 1992). For experiments determining the total amount of plasma proteins associated with liposomes and the pre-adsorption of plasma, the liposomes were pre-

pared in a phosphate buffered saline solution (PBS(-)) as empty liposomes. Multilamellar liposomes (770–850 nm average diameter) were composed of HEPC/Chol/DCP in a molar ratio of 4:4:1 for high-Chol liposomes (44.4 mol% of Chol), 5:3:1 for medium-Chol liposomes (33.3 mol% of Chol) and 6:2:1 for low-Chol liposomes (22.2 mol% of Chol) and sized through polycarbonate membrane filters (Nuclepore Co., CA., USA) with pore size of 0.8 $\mu$m. The diameters of liposomes were determined with a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA).

### 2.3. Distribution of the liposomes

[$^3$H]inulin encapsulated liposomes were administered by intravenous injection into Wistar male rats weighing 180–230 g (Inoue Experimental Animal, Kumamoto, Japan). The injected volume was 2.5 ml/kg body weight with a dose corresponding to 25 $\mu$mol as total lipid/kg body weight. At indicated time points after injection, blood and urine were collected. Subsequently, liver and spleen were collected from the respective animals after sampling of the blood and urine. The radioactivities of [$^3$H]inulin in blood, urine and each tissue were assayed as described previously (Kume et al., 1991).

### 2.4. Pharmacokinetic analysis

Time courses of blood concentration of the liposomes were analyzed based on the following Eq. (1) with MULTI (Yamaoka et al., 1981). The Damping Gauss Newton method was chosen as an algorithm for the nonlinear least square's method and the inverse of blood concentration was used as a weight. Pharmacokinetic parameters were calculated as follows:

$$C_b = A \cdot \exp(-\alpha t) + B \cdot \exp(-\beta t) \quad (1)$$

$$AUC = \frac{A}{\alpha} + \frac{B}{\beta} \quad (2)$$

$$CL_{tot} = \frac{Dose}{AUC} \quad (3)$$

where $\alpha$ and $\beta$ represent the apparent rate constants. $A$ and $B$ represent the corresponding zero-time intercepts and $t$ represents time. The AUC and $CL_{tot}$ represent area under the blood concentration-time curve and the total body clearance (CL), respectively.

The hepatic CL ($CL_h$), splenic CL ($CL_s$) and renal CL ($CL_{rel}$) were calculated as follows (Harashima et al., 1992):

$$CL_h = \frac{X(t)h}{AUC(0 \rightarrow t)} \quad (4)$$

$$CL_s = \frac{X(t)s}{AUC(0 \rightarrow t)} \quad (5)$$

$$CL_{rel} = \frac{X(t)rel}{AUC(0 \rightarrow t)} \quad (6)$$

where $X(t)$h, $X(t)$s and $X(t)$rel represent the amount of hepatic uptake, splenic uptake and cumulative urinary excretion at time $t$. AUC($0 \rightarrow t$) represents area under the blood concentration–time curve from time 0 to $t$.

### 2.5. Degradation of the liposomes in rat plasma

Blood was collected from Male Wistar rats (Inoue Experimental Animal, Kumamoto, Japan) through polyethylene tubing in the carotic artery into a heparinized test tube and plasma was prepared by centrifugation at 3000 r.p.m. for 30 min. The preadsorbed plasma (90%, v/v) was prepared according to a previously described method (Funato et al., 1994). Briefly, 9 volumes of normal plasma were incubated with 1 volume of liposome (containing 36 $\mu$mol/ml of total lipid) containing the various molar ratios of Chol for 30 min at 0°C. The floating liposomes were then carefully removed by aspirating after centrifugation (3000 r.p.m., 60 min). Degradation of the liposomes in rat plasma as a result of C activation was assessed by determining the fluorescent intensity of CF according to a previously described method (Funato et al., 1992). Then 50 $\mu$l of liposome solution (containing 18 $\mu$mol/ml of total lipid) was added to 450 $\mu$l of either fresh or preadsorbed plasma yielding a final plasma concentration of 81% (v/v). The samples were incubated for 60 min at 37°C. The fluorescent intensity of the reaction

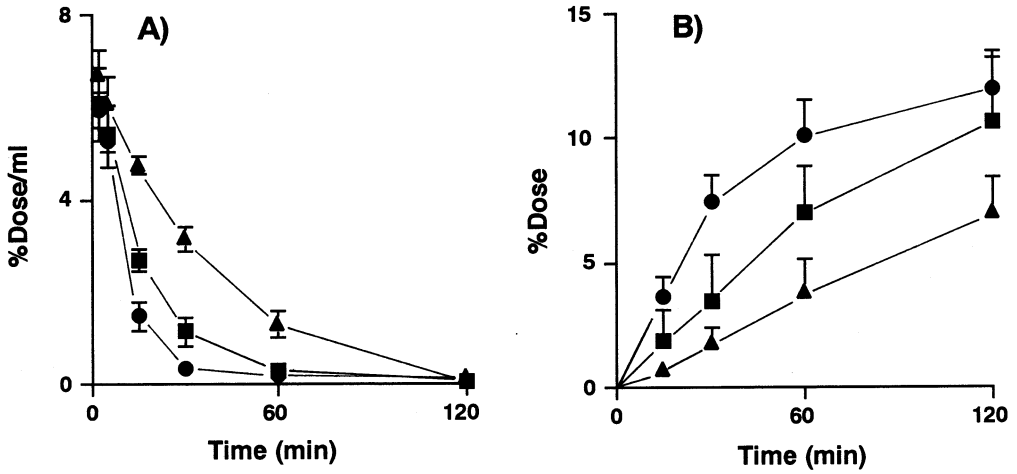


Fig. 1. The effect of Chol content on time courses of (A) blood concentration and (B) urinary excretion after i.v. injection of liposomes to rats. [$^3$H]inulin was encapsulated into liposomes which were composed of HEPC/Chol/DCP = 4/4/1 (●); = 5/3/1 (■); = 6/2/1 (▲). Each value represents the mean ± S.D. of three separate experiments.

mixture was determined at the excitation and emission wave lengths of 490 and 520 nm, respectively. Total amount of CF was measured by lysing the liposomes with Triton X-100 solution (5% v/v).

### *2.6. Quantitation of total amount of plasma proteins associated with the liposomes*

The total amount of plasma proteins associated with the liposomes was determined according to a previously described method (Ferdous et al., 1996). Liposome solution (0.3 ml) containing 18 $\mu$mol/ml of total lipid was incubated with 2.7 ml of normal rat plasma at 37°C for 15 min. The incubation was stopped by adding an excess of cold PBS(-). The liposomes were then separated by centrifugation at 15 000 r.p.m. for 30 min, washed three times with cold PBS(-) and the liposomal pellets were resuspended in 3 ml of PBS(-) for the measurements of Chol and proteins. The content of Chol was measured with a previously reported method (Nagayasu et al., 1994). For the assay of proteins bound to the liposomal surface, the liposome suspension was mixed with equal amounts of Triton X-100 solution (2% v/v) to destabilize the liposome membrane. A 3-fold amount of 1,1,2-trichloro 1,2,2-trifluoroethane (Wako Pure Chem., Osaka, Japan) was then added to extract the lipid. After centrifugation at 3000 r.p.m. for 10 min, protein contained in the supernatant was measured with a DC protein assay kit (Bio-Rad Laboratories, CA, USA). The total amount of plasma proteins bound to liposomes was expressed as grams of protein per total lipid calculated from the Chol content.

## 3. Results

### *3.1. Biodisposition of the liposomes labeled with [$^3$H]inulin*

To evaluate the effect of Chol content on the biodistribution of liposomes, [$^3$H]inulin encapsulated liposomes were administered to rats. Time courses of blood concentration and urinary excretion are shown in Fig. 1. Chol dependency was observed in both the elimination of liposomes and the urinary excretion of [$^3$H]inulin. High-Chol liposomes were cleared from blood circulation faster than the low- and medium-Chol liposomes (Fig. lA). Since the radioactivity in urine represents free [$^3$H]inulin leaked from liposomes, the observed Chol dependent-urinary excretion of [$^3$H]inulin (Fig. lB) reflects liposome degradation

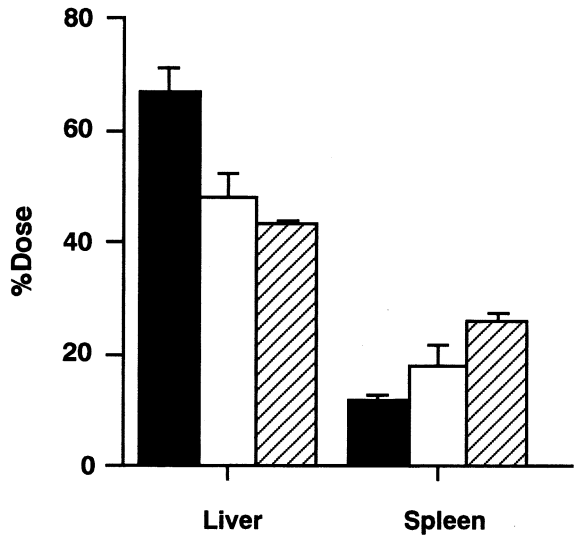


Fig. 2. The effect of Chol content on tissue distributions of liposomes labeled with [$^3$H]inulin at 2 h after i.v. injection of liposomes to rats. [$^3$H]inulin was encapsulated into liposomes which were composed of HEPC/Chol/DCP = 4/4/1 (closed column); = 5/3/1 (open column); = 6/2/1 (hatched column). Each value represents the mean ± S.D. of three separate experiments.

in the blood. This result shows that the inclusion of Chol in liposomes makes them susceptible to degradation in the blood. The included Chol possibly promotes the interaction of the liposomes with plasma proteins which disrupt the liposomes. Furthermore, we examined the effect of Chol content on the uptake of liposomes by the mononuclear phagocyte system (MPS) (liver and spleen) (Fig. 2). Chol dependency was observed in [$^3$H]inulin accumulation for both liver and spleen. Accumulation was directly proportional to the Chol content for case of liver, but inversely proportional for spleen. It is interesting to note that this Chol dependency in hepatic uptake is similar to that of urinary excretion (Fig. 1B). These results show that the Chol content makes the liposomes susceptible to uptake by the liver, as well as to degradation in the blood.

To evaluate liposome affinity for the organs, the total body, renal, and individual organ clearance (CL) were calculated. As shown in Table 1, the total body CL ($CL_{tot}$) increased with the rise in the Chol content, and the high-Chol liposomes showed the highest $CL_{tot}$. The $CL_{rel}$ also increased with rising Chol content. Thus, $CL_h$ has a similar response to that of $CL_{rel}$. Whereas $CL_h$ and accumulation in liver correlate well, this was not the case for $CL_s$ and splenic accumulation. This finding indicates that the effect of Chol on accumulation in the spleen is not due to a change in the affinity of the liposomes for splenic cells. Thus, these results clearly indicate that Chol-dependent elimination of the liposomes from blood circulation (Fig. 1A) is directly related to both degradation of liposomes in the blood and uptake of liposomes by the liver but not spleen. Therefore, it can be assumed that the liposomes exhibiting greater instability in blood have higher affinity for the liver, and that there may be an organ specific mechanism for the uptake of liposomes.

### *3.2. Correlation between the extent of complement activation and the amount of plasma proteins associated with the liposomes*

It has been reported that both the rate of elimination from circulation (Chonn et al., 1992)

Table 1
Effect of Chol content on the pharmacokinetic parameters of the liposomes

| Clearance (CL) | Lipid composition | | |
|---|---|---|---|
| | 4/4/1 (Chol: 44 mol%) | 5/3/1 (Chol: 33 mol%) | 6/2/1 (Chol: 22 mol%) |
| $CL_{tot}$ | 1.401 ± 0.344 | 0.863 ± 0.130 | 0.416 ± 0.060 |
| $CL_h$ | 0.779 ± 0.122 | 0.383 ± 0.051 | 0.171 ± 0.012 |
| $CL_s$ | 0.135 ± 0.021 | 0.138 ± 0.026 | 0.102 ± 0.013 |
| $CL_{rel}$ | 0.137 ± 0.020 | 0.086 ± 0.024 | 0.028 ± 0.008 |

The $CL_{tot}$, $CL_h$, $CL_s$ and $CL_{rel}$ represent total body clearance, hepatic clearance, splenic clearance and renal clearance, respectively. The pharmacokinetic parameters were calculated as described in Section 2. Each value represents mean ± S.D. of three experiments.

Table 2
Effect of Chol content on the release of CF from and the total amount of plasma proteins associated with the liposomes

| | Lipid composition | | |
|---|---|---|---|
| | 4/4/1 (Chol: 44 mol%) | 5/3/1 (Chol: 33 mol%) | 6/2/1 (Chol: 22 mol%) |
| Release (%) | 26.9 ± 1.8 | 12.4 ± 1.1 | 5.6 ± 0.4 |
| $P_B$ (g of protein/mol of total lipid) | 13.6 ± 1.1 | 18.1 ± 1.3 | 25.4 ± 3.6 |

$P_B$ represents the total amount of plasma proteins associated with the liposomes.
The release of entrapped CF was measured after incubation at 37°C for 60 min. The value was expressed as the percentage of total entrapped CF. Each value represents the mean ± S.D. of three experiments. The plasma proteins bound to liposomes were measured by DC protein assay kit as described in the method sections. Each value represents the mean ± S.D. of three experiments.

and extent of degradation in serum (Hernandez-Caselles et al., 1993) might depend directly upon the total amount of plasma proteins associated with the liposomes. However, our recent data have shown that the C system plays a critical role in both degradation and disposition of the liposomes in vivo (Funato et al., 1992; Harashima et al., 1994a). To assess which factors contribute to the Chol-dependent liposome elimination, the total amount of plasma proteins bound to as well as the extent of C activation by the liposomes were evaluated after incubation of liposomes with rat plasma. As shown in Table 2, the total amount of plasma proteins associated with the liposomes decreased with an increase in the Chol content, indicating that the included Chol markedly reduces the interaction of plasma proteins with the surface of the liposomes. On the other hand, the release of CF from liposomes in rat plasma was augmented by an increase in Chol content. Since we previously reported that the degradation of liposomes in rat plasma proceeds through the activation of C (Funato et al., 1992), the release of CF from liposomes reflects the extent of C activation interacted with the liposomes. We also observed that the enhancing effect of Chol content on liposome degradation was completely blocked by preheating at 56°C for 30 min (data not shown). Thus, these findings suggest that the high-Chol liposomes are easier to degrade than the medium- and low-Chol liposomes in rat plasma, and this degradation results from the Chol dependent-C activation. Furthermore, these results are consistent with the in vivo liposome degradation measurements shown in Fig. 1B. Taken together, these observations suggest that the elimination rate of the liposomes from blood closely relates to the extent of C activation on liposomal surfaces rather than to total amount of bound plasma proteins.

### *3.3. Inhibition of complement activation by preadsorption with liposomes*

We recently reported that the C activating factor (CAF), a plasma factor which adsorbs to liposomes with high Chol content, was responsible for the initiation of C activation (Funato et al., 1994). Furthermore, our data provides the possibility that CAF might have a specificity for Chol in the liposomal membrane because CAF can be adsorbed to liposomes without HEPC (Funato et al., 1994). In this study, to elucidate the involvement of CAF in Chol-dependent C activation, we examined the release of CF from the high-Chol liposomes in rat plasma preadsorbed with liposomes containing varying molar ratios of Chol. Preadsorption with the high-Chol liposomes greatly reduced the amount of released CF. The extent of inhibition of release was 76.3%, approximately 4-fold higher when compared with preadsorption with the low-Chol liposomes. The inhibition of C activation depended on the Chol content in the liposomes used for preadsorption (Table 3). We have previously reported that the removal of CAF by preadsorption with high-Chol liposomes does not affect the hemolytic activity of plasma (Funato et al., 1994), indicating that the

Table 3
Effect of pretreatment with the liposomes on the complement-mediated liposome degradation

| | Liposomes for preadsorption | | | |
|---|---|---|---|---|
| | None | 6/2/1 (Chol: 22 mol%) | 5/3/1 (Chol: 33 mol%) | 4/4/1 (Chol: 44 mol%) |
| Release (%) | 19.8 ± 2.8 | 15.9 ± 2.9 | 12.5 ± 0.7 | 4.7 ± 1.2 |
| Inhibition | — | 19.7 | 36.9 | 76.3 |

The plasma preadsorbed with liposomes containing 22 mol%-, 33 mol%-, or 44 mol%-Chol was incubated with CF entrapped liposomes composed of HEPC/Chol/DCP at molar ratio of 4/4/1.
The release of entrapped CF was measured after incubation at 37°C for 60 min. The release was expressed as the percentage of total entrapped CF. Each value represents the mean ± S.D. of five experiments. The inhibition (%) was calculated by dividing the value of release (%) in treated plasma by that in non-treated plasma.

abolishment of C activation by preadsorption is not the result of depletion of C components. Thus, these observations are evidence that CAF has a specificity for Chol in liposomal membranes, and that the binding of CAF to the liposomal surface results in C-activation.

### *3.4. Correlation between in vivo degradation and either affinity for organs or in vitro degradation of the liposomes*

The correlations between $CL_{rel}$ and either individual organ CL or release % (liposome degradation in rat plasma) were plotted in Fig. 3A and B, respectively. The values of these pharmacokinetic parameters and release % are the same as those described in Table 1 and Table 2. The $CL_{rel}$, organ CL, and release % reflect the amount of liposome degradation in vivo, the affinity of the liposomes for each organ, and the extent of C activation in vitro, respectively. Both the $CL_{rel}$ and $CL_h$ increased with increasing amounts of Chol content in liposomes (Fig. 3A). The $CL_{rel}$ was directly proportional to the $CL_h$, but not to the $CL_s$. Furthermore, a significant correlation between the $CL_{rel}$ and the release % was observed as shown in Fig. 3B. These observations indicate that the included Chol mediates both the degradation of liposomes in vivo and in vitro as well as the liposome's high affinity for the liver. Therefore, our findings provide further support for the mechanistic similarity between liposome degradation in blood and liposome uptake by the liver.

## 4. Discussion

Previous studies have suggested that plasma proteins play an important role in determining the biodistribution of liposomes (Juliano and Lin, 1980; Bonte and Juliano, 1986; Gregoriadis, 1988; Patel, 1992; Chonn et al., 1992, 1994). However, direct evidence has been lacking to reveal the specific plasma protein(s) involved in both liposome degradation in blood and uptake by the MPS. In this study, we examined the effect of Chol content on the biodistribution of liposomes with particular regard to the role of plasma protein, in particular C components, interaction with liposomes.

As shown in Fig. 1 and Fig. 2, the biodistribution of liposomes was remarkably influenced by Chol content. Both the degradation of liposomes and its affinity for liver were dramatically increased by the included Chol (Table 1 and Fig. 3A). Therefore, the rapid elimination of the high-Chol liposomes appears to be caused by the additive effects of both degradation in blood and uptake by the liver. As shown in Table 2, the increase in Chol content within the liposomes drastically reduced the interaction of plasma proteins with the liposomal surface. The elimination rate of the liposomes was inversely proportional to the total amount of bound plasma proteins. These findings strongly suggest that the biodistribution is determined by the binding of specific plasma proteins, rather than nonspecific binding proteins such as albumin. Conflicting experimental results have been reported by Chonn

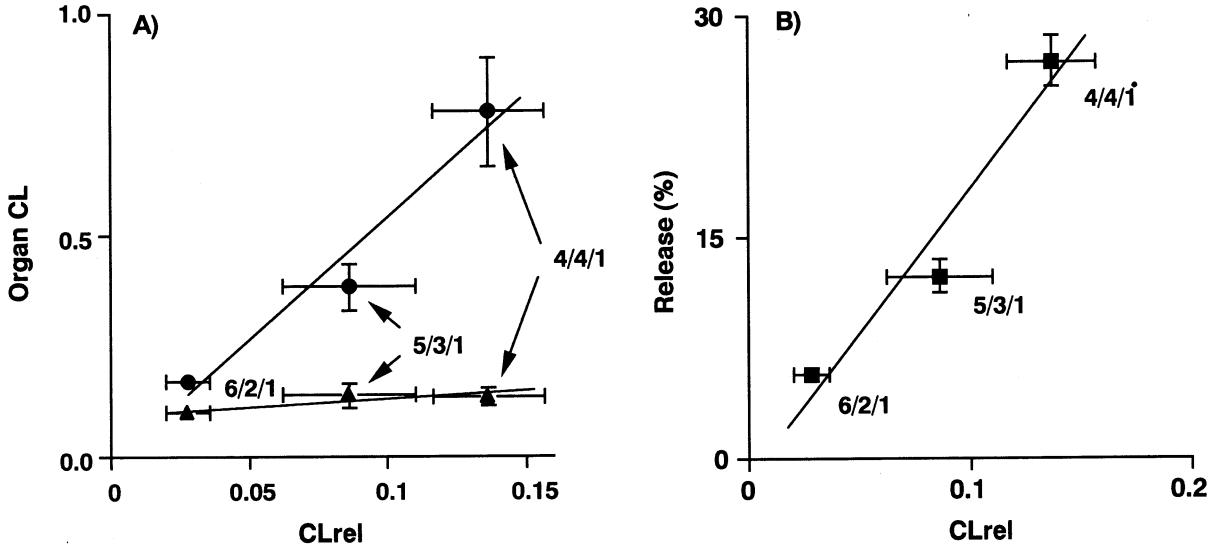


Fig. 3. Correlations between the liposome degradation in vivo and the organ CL or the liposome degradation in vitro. (A) The correlation between $CL_{rel}$ and $CL_h$ (●) or $CL_s$ (▲). The values of the pharmacokinetic parameters were the same as those described in Table 1. (B), The correlation between the $CL_{rel}$ and the percentage of CF release (Release (%))(■) from liposomes in rat plasma. The values of release (%) are the same as those described in Table 2 and reflect the extent of complement activation to the liposomes. The lipid composition of liposomes was presented in respectively figure at molar ratio of HEPC/Chol/DCP.

et al. They have shown that the liposomes which have high reactivity with blood proteins were rapidly cleared from blood circulation (Chonn et al., 1992). However, in their recent study, they showed that the $\beta$2-glycoprotein I is a major protein associated with rapidly cleared liposomes, and plays a primary role in enhancing the elimination of liposomes (Chonn et al., 1995). Thus, it seems reasonable to conclude that specific plasma protein(s) play a primary role in determining the biodistribution of liposomes.

It is known that the C system can opsonize liposomes (Patel, 1992). We have previously reported that liposomes in rat plasma degraded through the activation of the alternative complement pathway (ACP) (Matsuo et al., 1994), and that the uptake of liposomes was mediated by enhanced C-mediated phagocytosis (Harashima et al., 1994a). In this study, we have found that C-mediated liposome degradation is enhanced by increasing the Chol content of liposomes (Table 2), and that there is a direct correlation between the $CL_{rel}$ and $CL_h$ (Fig. 3A). These results suggest that both the rapid degradation and the large amount of hepatic accumulation of the high-Chol liposomes result from the enhanced of affinity for C activation mediated by the inclusion of Chol to liposomes.

In contrast, although the splenic uptake of the liposomes was dependent on the Chol content, the $CL_s$ was unaffected (Fig. 2 and Table 1). These results indicate that the affinity of the liposomes for splenic cells is not affected by the Chol content of the liposomes. It has been known that the introducing of polyethylene glycol, ganglioside (GM1) or hydrogenated phosphatidyl inositol in liposomal membranes to prolong the half-life of liposomes in the blood circulation enhanced the uptake of liposomes by the sinusoidal and nonsinusoidal spleen (Klibanov et al., 1991; Liu et al., 1991; Gabizon and Papahadjopoulos, 1992). The high-splenic accumulation of these liposomes is thought to result from high-blood concentration by avoiding the hepatic accumulation. Alternatively, Frank has performed 17 in vivo studies in which he found that the IgG-coated erythrocytes were rapidly phagocytosed in spleen, while the C-coated ones were disposed by the liver (Frank,

1983). This suggests that there is little involvement of the C system in the uptake mechanism of liposomes by the spleen, a conclusion that is in complete agreement with our results as shown in Fig. 2. In addition, Moghimi and Patel, using in vitro uptake experiments (Moghimi and Patel, 1988), have shown that serum contains opsonins specific for hepatic and splenic cells. This suggests that serum proteins play an important role in enhancing the splenic uptake of liposomes. There are two possible explanations for the uptake mechanism of liposomes by the spleen: one possibility is that the accumulation of liposomes reflects its concentration in the blood, or alternatively, the accumulation is mediated by opsonins specific for spleen. The definitive explanation is unclear (even though our results favor the former), since data supporting were obtained under different experimental conditions (e.g. lipid compositions, sizes and species difference) and thus cannot be compared directly.

We recently reported that CAF, which adsorbs onto liposomes with high Chol content, has the ability to initiate C activation (Funato et al., 1994). As shown in Table 3, the specificity of CAF for the Chol in liposome membranes and the ability of CAF to activate the C system was confirmed. Recently, various activators of both the classical C pathway (CCP) and ACP (Reid and Turner, 1994; Sim and Malhotra, 1994) have been identified. Some of them, such as the mannose-binding protein (MBP), have the ability to activate the C system via $C1_q$-independent CCP, known as the lectin pathway (Holmskov et al., 1994). Specific sites on potential pathogen are recognized by these C activators. MBP, for example, has 18 been known to interact with suitable carbohydrate ligands containing high mannose (Turner, 1994). Based on our current and past results, we speculate that CAF may have the ability to recognize the unique liposomal surface characteristic produced by the formation of Chol clusters and/or the alteration of membrane fluidity as a result of changes in the Chol density. Interestingly, Szebeni et al. showed that hemoglobin-containing liposomes (LEH), which have high Chol content (45 mol% of Chol), cause significant C activation via natural antibody-dependent ACP (Szebeni et al., 1994). Natural antibodies to Chol are widespread in all animal species. However, CAF appears not to be identical to natural antibodies specific for Chol since C activation by the high-Chol liposomes in human plasma was not observed (our unpublished data). Thus, it seems likely that CAF is a novel serum factor involved in the first line of immune defence, although further studies are needed to clarify its physiological roles.

The studies presented here clearly indicate that Chol in liposomes enhances both liposome accumulation in liver and degradation in blood, and the biodistribution closely interacts with C system. We propose the following mechanism. The C activation is initiated by the binding of the CAF to liposomes and the Chol-dependency on C activation is mediated according to different affinities of the CAF for liposomal surface. Subsequently, both liposome accumulation in the liver and liposome degradation in the blood depend upon the CAF-mediated C activation. Thus, CAF may be a dominant factor governing the behavior of liposomes in the body. Although the exact role of CAF in C activation is not clear, clarifying its function should provide important information regarding the use of liposome as a drug carrier.

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