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Biodistribution of liposomes and C3 fragments associated with liposomes: evaluation of their relationship

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Abstract

The biodistribution of liposomes with two different kind phospholipids (hydrogenated egg phosphatidylcholine and egg phosphatidylcholine) plus cholesterol (CHOL) were investigated after intravenous administration to rats. Elimination of liposomes from blood circulation was affected by the lipid composition. It appeared that the inclusion of CHOL in liposomes accelerates the rate of liposome uptake by liver, resulting in rapid elimination of liposomes. The amount of C3 fragments bound to liposomes was quantitatively determined to assess the contribution of the complement system to liposome accumulation into organs and liposome destabilization in vivo and in vitro. The amount of bound C3 fragments was directly proportional to CHOL content, and the amount was also proportional to the CLh, CLs as well as CLrel. This relationship suggests that the complement system is responsible for the elimination of liposomes from blood circulation, presumably as a consequence of opsonization by C3 fragments and assembly of membrane attack complex (MAC) onto liposomes. In addition, substitution of cholesteryl methyl ether into the liposome formulation for CHOL significantly diminished not only the binding of C3 fragments but also the CLh, CLs and CLrel, resulting in increased mean resident time (MRT) of the liposomes. This result suggests that the hydroxyl-group on CHOL is a binding site for C3 fragments on the liposomes and that CHOL in a liposome formulation promotes the accumulation of liposomes into the liver and spleen, probably due to their uptake by phagocytic cells, and impairs the stability of the liposomes in blood circulation, via a mechanism involving the complement system.

Keywords: Liposomes; Complement; C3; Cholesterol; Drug delivery system (DDS)

Abbreviations: AUC, area under the blood concentration–time curve; CF, 5(6)-calboxyfluorescein; CHOL, cholesterol; C3, third component of the complement; CL, clearance; CLh, hepatic clearance; CLs, splenic clearance; CLrel, renal clearance; DCP, dicetylphosphate; ELISA, enzyme-linked immunosorbent assay; EPC, egg phosphatidylcholine; EPC-MLV, MLV composed of EPC, CHOL and DCP; HEPC, hydrogenated egg phosphatidylcholine; HEPC-MLV, MLV composed of HEPC, CHOL and DCP; MAC, membrane attack complex; MLV, multilamellar vesicles; MPS, mononuclear phagocyte system; MRT, mean residence time; PBS, phosphate-buffered saline; PL, phospholipid.

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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). After administration of liposomes in vivo, opsonins are adsorbed onto the surface of liposomes by a process called opsonization, triggering recognition and liposome uptake by the mononuclear phagocyte system (MPS) through receptor-mediated phagocytosis or endocytosis (Senior, 1987; Patel, 1992; Liu, 1997). Furthermore, the functional importance of serum proteins in destabilization of liposomes has been supported by in vitro studies (Funato et al., 1992; Hernandez-Caselles et al., 1993; Harashima et al., 1996; Ishida et al., 1997; Panagi et al., 1998). The complement system has been suspected to be a possible candidate for major dominant opsonin in the clearance process because it plays a critical role in elimination of particulate materials, such as pathogens, through initiating membrane lysis and enhancing the uptake by the MPS (Porter and Reid, 1979; Pangburn, 1983; Tomlinson, 1993).

The third component of the complement system (C3) is one of the principal opsonins, enhancing the uptake and clearance of liposomes by the MPS. When the complement system is activated, activated C3 fragments termed C3b bind to hydroxyl or amino groups on the target, and bound C3 fragments termed C3b or iC3b enhance uptake of the target by the MPS through complement receptor-mediated phagocytosis (Brown, 1991; Wright, 1992). Our laboratory and others have shown the potential role of the complement system in the uptake of liposomes by Kupffer cells in an in situ perfused liver system (Kiwada et al., 1987; Harashima et al., 1994, 1998; Matsuo et al., 1994; Liu et al., 1995a,b; Liu and Liu, 1996; Harashima and Kiwada, 1996a,b) or isolated Kupffer cells (Dijkstra et al., 1984, 1985; Moghimi and Patel, 1988). However, a quantitative method to estimate the contribution of the complement system, especially the C3 fragments, to in vivo biodistribution of liposomes has not been developed. Since the complement system is expected to influence liposome biodistribution by assisting the body in removal of the drug carrier from circulation, understanding of the correlation between the amount of bound C3 fragments and liposome biodistribution can help us to predict the fate of liposomes after administration, thus helping develop a more desirable liposomal drug delivery system.

In this study, we quantitatively determined the biodistribution of liposomes after intravenous administration to rats. We developed an in vitro assay system to evaluate the amount of associated C3 fragments onto liposomes after exposure to isolated rat serum. The relationship between the biodistribution of liposomes and the amount of bound C3 fragments was examined. Our findings demonstrated that the complement system is responsible for elimination of liposomes from blood circulation. Cholesterol present in liposomes accelerates the elimination of liposomes from circulation via a mechanism involving complement activation.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC) and hydrogenated egg phosphatidylcholine (HEPC) 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). Cholesteryl methyl ether was purchased from Sigma (CA, USA). All lipids were used without further purification. 5(6)-Carboxyfluorescein (CF) was purchased from Eastman Kodak (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) was carried out by modification of a conventional

lipid film hydration method described previously (Funato et al., 1992). Liposomes were composed of EPC/CHOL/DCP (EPC-MLV) or HEPC/CHOL/DCP (HEPC-MLV) at a molar ratio of 4:4:1 (high-CHOL liposomes), 5:3:1 (medium-CHOL liposomes), and 6:2:1 (low-CHOL liposomes), and sized by extrusion through polycarbonate membrane filters (Nuclepore, CA) with a pore size of 400 nm. Mean diameters of liposomes (380–420 nm) were determined using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA). [^{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 Wistar rats weighing 180–230 g (Inoue Experimental Animal, Kumamoto, Japan) 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). 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 25 μmol as total lipid/kg body wt. 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 previously (Kume et al., 1991).

The time courses of liposome concentration in the blood was determined using the following Eq. (1) with MULTI (Yamaoka et al., 1981). The Damping Gauss Newton method was chosen as an algorithm for the nonlinear least squares method, and the inverse of blood concentration was used as a weight. Pharmacokinetic parameters were calculated as follows:

$$\mathrm{Cb} = A\exp(-\alpha t) + B\exp(-\beta t) \quad (1)$$

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

$$\mathrm{MRT} = (A/\alpha^2 + B/\beta^2)(A/\alpha + B/\beta) \quad (3)$$

where α and β are the apparent rate constants, A and B are the corresponding zero-time intercepts, and t is time. The AUC and MRT are the area under the blood concentration–time curve and the mean residence time, respectively.

The hepatic clearance (CLh), splenic clearance (CLs) and renal clearance (CLrel) were calculated as follows:

$$\mathrm{CLh} = X(t)\mathrm{h}/\mathrm{AUC}(0\rightarrow\mathrm{t}) \quad (4)$$

$$\mathrm{CLs} = X(t)\mathrm{s}/\mathrm{AUC}(0\rightarrow\mathrm{t}) \quad (5)$$

$$\mathrm{CLrel} = X(t)\mathrm{rel}/\mathrm{AUC}(0\rightarrow\mathrm{t}) \quad (6)$$

where $X(t)$h, $X(t)$s and $X(t)$rel are the hepatic uptake, splenic uptake and cumulative urinary excretion of [^{3}H]inulin at time t. AUC($0\rightarrow$t) is the area under the blood concentration–time curve from time 0 to t.

2.4. In vitro liposome destabilization assay

The complement-inactivated rat serum was prepared by heating at 56°C for 30 min and by addition of EDTA (100 mM) (Okada et al., 1982). Liposome destabilization in rat serum was assessed by determining the fluorescence intensity of released CF according to a previously described method (Funato et al., 1992). In brief, 50 μl of liposomes containing CF (6 μmol/ml of total lipid) was added to 450 μl of serum, and the mixture was incubated for 30 min at 37°C. The fluorescence intensity in the reaction mixture was determined with a Hitachi 650-40 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 490 and 520 nm, respectively. The total amount of CF encapsulated in the liposomes was measured by lysing the liposomes with Triton X-100 solution (5%

(v/v)). Percentage of CF released from liposomes was calculated by dividing the fluorescence intensity of the reaction mixture by that of the total encapsulated CF in the liposomes.

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

To 110 μl of liposome solution labeled with [^{3}H]CHE (20 μmol/ml of total lipid), 990 μl of rat serum was added and the liposomes–serum mixture was incubated at 37°C for 15 min. The incubation mixture was then cooled to 4°C using an ice bath to stop the reaction of the complement system. 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 previously (Chonn et al., 1991a). The fractions containing liposomes were pooled and concentrated by centrifugation (75 000 rev./min, 60 min on an Optima TLX Ultracentrifuge, Beckman, CA) at 4°C. Radioactivity of [^{3}H]CHE in the liposome suspension was assayed to determine the concentration of the liposomes with an Aloka LSC-3500 liquid scintillation counter (Aloka, Tokyo, Japan). To assay for C3 fragments associated with liposomes, the liposome suspension was mixed with equal amounts of Triton X-100 solution (2% v/v) to destabilize the liposomal membrane. By addition of a threefold amount of 1,1,2-trichloro 1,2,2-trifluoroethane (Wako Pure Chemical, Osaka, Japan), the liposome-associated C3 fragments were extracted into aqueous phase and delipidated. Extracted C3 fragments were determined in a Sandwich ELISA using a combination of anti-Rat C3 monoclonal antibody (produced in our laboratory) and HRP-conjugated anti-Rat C3 polyclonal antibody (Cappel, NA, USA). The number of liposomes in the collected liposome suspension was calculated according to the equations described previously (Pidgeon and Hunt, 1981). Total C3 fragments associated with the liposomes were expressed as μg per liposome vesicle.

2.6. Statistics

Statistical analyses were performed using StatView software (Abacus Concepts, CA).

3. Results

3.1. Biodistribution of liposomes and pharmacokinetic analysis

To evaluate the biodistribution of liposomes, liposomes containing [^{3}H]inulin were administered intravenously to rats. The time course of blood concentration of liposomes is shown in Fig. 1. For liposomes (HEPC-MLVs) containing fully saturated phospholipid (PL) (HEPC), higher and medium amounts of CHOL in the formulation caused the liposomes to clear more rapidly than one containing a lower amount of CHOL (Fig. 1A). For liposomes (EPC-MLVs) composed of unsaturated PL (EPC), higher and lower amounts of CHOL caused the liposomes to clear slightly more quickly than the liposomes containing a medium amount of CHOL (Fig. 1B). Pharmacokinetic parameters for liposome biodistribution were calculated according to equations described in Section 2, and are summarized in Table 1. PL degree of saturation and amount of CHOL content altered pharmacokinetic parameters. Hepatic and splenic clearance 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. The values of CLh, CLs and CLrel in the HEPC-MLVs were much higher than those in the EPC-MLVs. CLh increased with an increase of CHOL content regardless of difference of PL. CLrel increased with an increase in CHOL content in the HEPC-MLVs, while CLrel slightly decreased by increase of CHOL content in the EPC-MLVs. AUC decreased as CHOL content was increased in the HEPC-MLVs, whereas there were no CHOL-dependency on the AUC in the EPC-MLVs. The mean residence time (MRT) of liposomes decreased with an increase in CHOL content regardless of degree of PL saturation.

3.2. Amount of C3 fragments associated with liposomes

Liposomes were recovered from a liposome–serum incubation mixture after incubation at 37°C for 15 min. Serum proteins bound to the liposomes were extracted from the separated liposomes. The amount of C3 fragments associated with liposomes is shown in Fig. 2. HEPC-MLVs bound more C3 fragments than did EPC-MLVs, regardless of the CHOL content. The amount of C3 fragments increased with increasing CHOL content, but this effect was less pronounced for EPC-MLVs than for HEPC-MLVs.

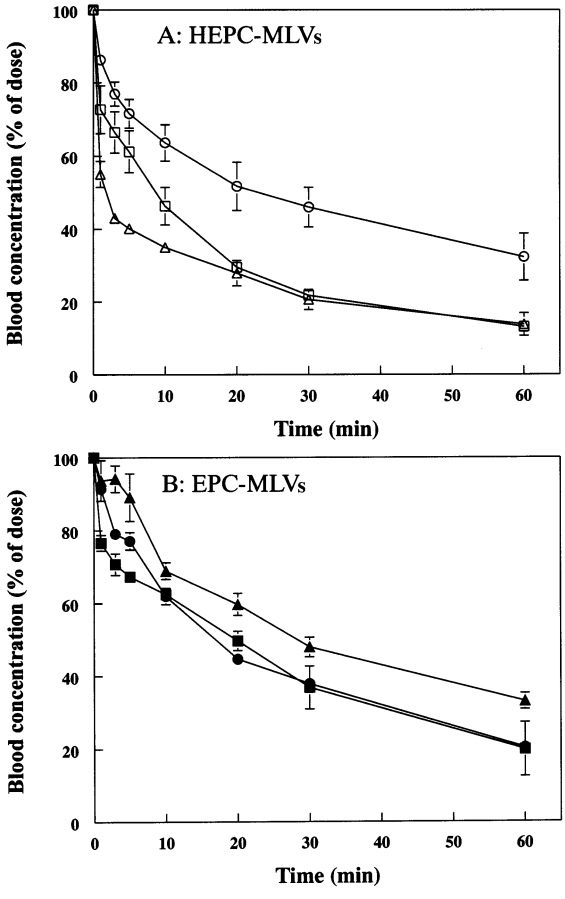

Fig. 1. Biodistribution of liposomes. [^{3}H]Inulin was encapsulated into liposomes which were composed of (A) HEPC/CHOL/DCP = 6:2:1 (○), 5:3:1 (△), 4:4:1 (□) or (B) EPC/CHOL/DCP = 6:2:1 (●), 5:3:1 (▲), 4:4:1 (■). Liposomes (25 μmol total lipid/kg) were administered by intravenous injection into male Wistar rats. At each time point, blood was collected. [^{3}H]Inulin in blood was determined by scintillation counting. Each value represents the mean ± S.E. of three separate experiments.

3.3. Release of carboxyfluorescein (CF) from liposomes

Release of CF from liposomes in untreated rat serum, pretreated sera or PBS is shown in Fig. 3. For HEPC-MLVs, the release of CF from the liposomes in untreated serum increased with an increase in CHOL content. On the other hand, for EPC-MLVs, the release of CF from the liposomes in untreated serum decreased with an increase in CHOL content. In heated or EDTA-treated serum (complement-inactivated sera), the release of CF from all HEPC-MLVs formulations and EPC-MLVs containing 22 mol% CHOL (EPC/CHOL/DCP = 6:2:1) was reduced. It appeared that all HEPC-MLVs formulations and EPC-MLVs containing 22 mol% CHOL were destabilized through activation of complement system.

3.4. Correlation between amount of bound C3 fragments and in vivo pharmacokinetic parameters

Bound C3 fragments (Fig. 2) versus individual organ clearances (CLh, CLs or CLrel) (Table 1) are plotted in Fig. 4. The amount of bound C3 fragments affected all organ clearances. There is a good correlation between the amount of bound C3 fragments and CLh ($r^2 = 0.889$, $P < 0.01$, Fig. 4A), CLs ($r^2 = 0.880$, $P < 0.01$, Fig. 4B) or CLrel ($r^2 = 0.895$, $P < 0.01$, Fig. 4C).

3.5. Influence of incorporation of cholesteryl methyl ether in liposome formulation on the binding of C3 fragments and the biodisposition of the liposomes

Liposomes containing cholesteryl methyl ether (CHOL-O-Me) instead of CHOL (HEPC/CHOL-O-Me/DCP = 4:4:1) were prepared according to the method described in Section 2. The liposome size was 398 ± 21 nm in diameter. We examined the binding of C3 fragments to the liposomes, and the biodistribution of liposomes. As shown in

Table 1
Pharmacokinetic parameters of the liposomes[a]

Lipid composition of liposomes	AUC (% dose min/ml)	CLh (ml/min)	CLs (ml/min)	Clrel (ml/min)	MRT (min)
EPC/CHOL/DCP					
6:2:1	180 ± 15	0.143 ± 0.014	0.063 ± 0.005	0.091 ± 0.020	70.8 ± 17.8
5:3:1	252 ± 2	0.166 ± 0.018	0.054 ± 0.003	0.085 ± 0.006	50.6 ± 3.0
4:4:1	147 ± 15	0.183 ± 0.044	0.051 ± 0.013	0.035 ± 0.011	32.6 ± 0.7
HEPC/CHOL/DCP					
6:2:1	357 ± 79	0.216 ± 0.109	0.074 ± 0.003	0.019 ± 0.016	60.4 ± 11.3
5:3:1	143 ± 32	0.324 ± 0.010	0.091 ± 0.004	0.330 ± 0.058	51.9 ± 11.3
4:4:1	102 ± 8	0.399 ± 0.018	0.104 ± 0.009	0.372 ± 0.041	27.9 ± 1.8

[a] Liposomes containing [^{3}H]inulin (25 μmol total lipid/kg) were administered by i.v. 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 was assayed. Pharmacokinetic parameters were calculated as described in Section 2. Each value represents mean ± S.E. of three separate experiments.

Table 2, the binding of C3 fragments to liposomes with CHOL-O-Me substituted for CHOL was significantly diminished compared to CHOL-containing liposomes. The pharmacokinetic parameters are also summarized in Table 2. AUC of the CHOL-O-Me-containing liposomes increased almost twofold compared to that of the CHOL-containing liposomes. CLh, CLs and CLrel of the CHOL-O-Me-liposomes were dramatically reduced compared to those of the CHOL-containing liposomes. In addition, MRT was increased threefold when CHOL-O-Me was incorporated into the formulation instead of CHOL. These results suggest that the hydroxyl-group on the liposome surface dramatically affects in vivo liposome pharmacokinetics.

4. Discussion

Liposome accumulation in the MPS and liposome destabilization in blood are important factors which affect liposome circulation lifetimes. CHOL-dependent CLh and CLs increases were observed for HEPC-MLVs but not for EPC-MLVs. In vivo and in vitro CHOL-dependent liposome destabilization was also observed in the HEPC-MLVs (Table 1 and Fig. 3). This CHOL-dependency in the HEPC-MLVs is due to CHOL-dependent complement activation, since the destabilization of HEPC-MLVs in rat serum was decreased in both heated serum (56°C, 30 min) and EDTA-treated serum (Fig. 3), treatments which inactivate the complement system (Okada et al., 1982). This conclusion is further supported by our findings for quantitative determination of bound C3 fragments following incubation with untreated serum. The increase of bound C3 fragments was proportional to the increase of CHOL content in the composition (Fig. 2); the amount of bound C3 fragments was also proportional to CLh, CLs and CLrel (Fig. 4). Recently, we have reported, for HEPC-MLVs having 800 nm diameter, that a complement-activating factor (CAF), which adsorbed onto the liposomes in a CHOL-dependent manner, triggers CHOL-dependent complement activation in rat serum (Ishida et al., 1997). Hence, in this study, we assume that the CAF adsorbed to the HEPC-MLVs initiated the complement activation, causing both the binding of C3 fragments and bilayer permeability changes by assembling the membrane attack complex (MAC), resulting in rapid liposome elimination.

Interestingly, for EPC-MLVs, CHOL-dependent in vivo and in vitro liposome destabilization was not observed (Table 1 and Fig. 3), whereas CHOL-dependent C3 binding was observed (Fig. 2). It appeared that incorporation of CHOL into the EPC-MLVs increased liposome stability in vivo and in vitro (Fig. 3 and Table 1). This

finding is consistent with previous results (Senior and Gregoriadis, 1982). In the case of an unsaturated PL such as EPC, having low transition temperature, the high membrane fluidity is conducive to removal of PL and CHOL by serum HDL

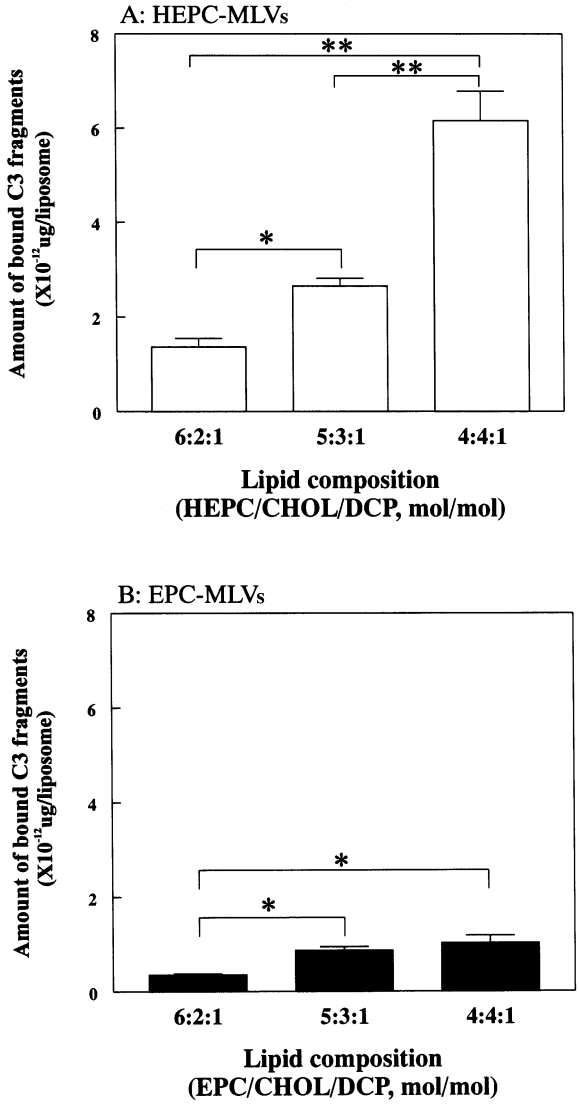

Fig. 2. Amount of bound C3 fragments on liposomes. Liposomes were incubated with rat serum at 37°C for 15 min. After the incubation, the liposomes were separated from unbound serum proteins using a spin column. C3 fragments were extracted from the liposomes and quantitated using a sandwich ELISA. Amount of C3 fragments is expressed as micrograms per liposome vesicle. Each value represents the mean ± S.E. of three separateexperiments. *$P < 0.05$, **$P < 0.01$.

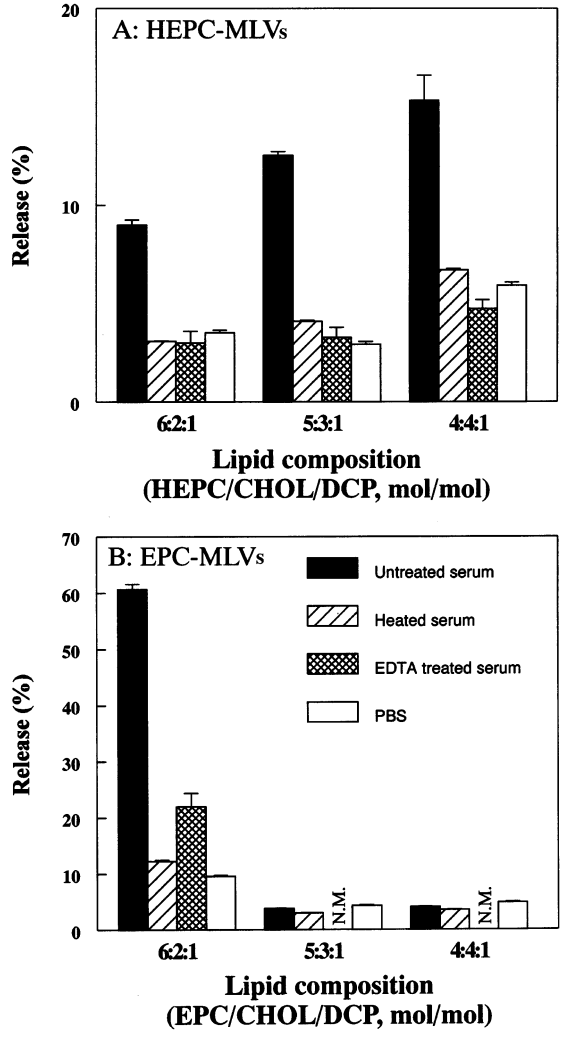

Fig. 3. Release of CF from liposomes in rat serum. Liposomes containing encapsulated CF were incubated with untreated rat serum, treated sera or PBS at 37°C for 30 min. The fluorescence intensity of released CF was determined. Total releasable CF from the liposomes was measured by lysing the liposomes 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. (A) Release of CF from the liposomes composed of HEPC/CHOL/DCP. (B) Release of CF from the liposomes composed of EPC/CHOL/DCP. Each value represents the mean ± S.E. of three separate experiments. N.M., not measured.

and pore formation in the bilayers, resulting in an increased release of liposomal contents (Kirby and Gregoriadis, 1981). It has been reported that

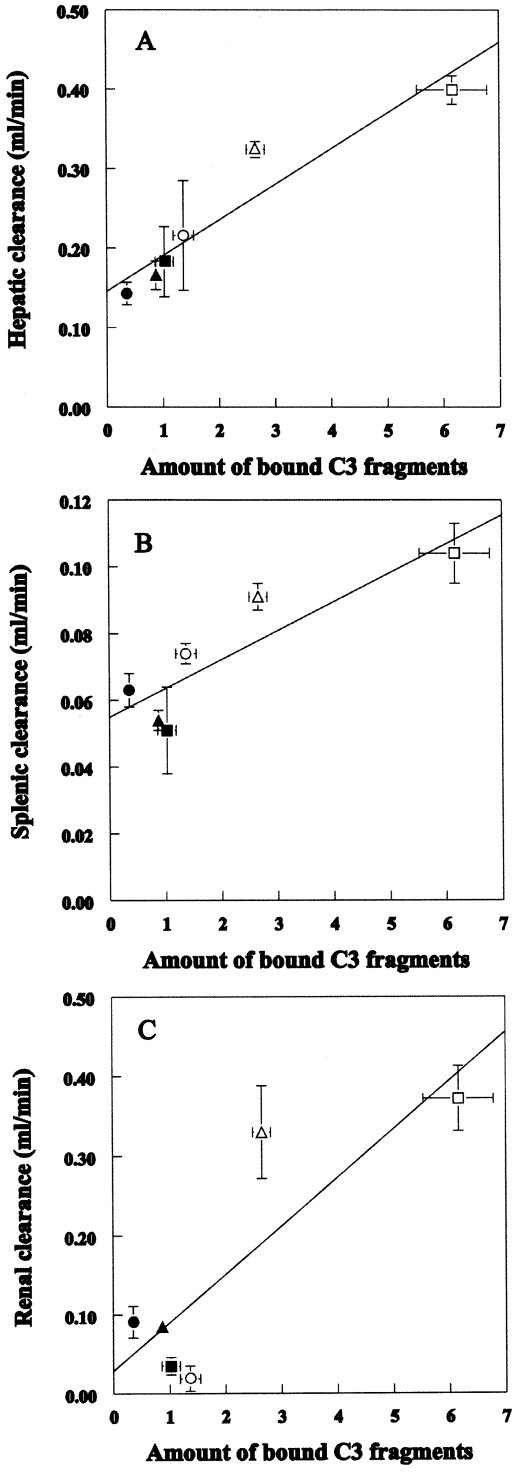

Fig. 4.

incorporation of CHOL into such liposomes reduces the membrane fluidity by increasing the transition temperature of the lipid membrane, resulting in increased stability of the membrane (Senior and Gregoriadis, 1982). Therefore, we assume for EPC-MLVs that the inclusion of CHOL makes the membrane less susceptible to liposome destabilization by serum components, preventing complement-activated pore formation by MAC and exchange of PL and CHOL by serum HDL.

We have previously reported the use of an isolated perfused liver system to identify the role of serum opsonins in the hepatic uptake of liposomes (MLVs), and have identified the role of the complement system in enhancing the hepatic uptake of liposomes via complement receptor-mediated phagocytosis (Matsuo et al., 1994; Harashima et al., 1994, 1998; Harashima and Kiwada, 1996a). In this study, we quantitatively determined C3 fragments associated with the liposomes after incubation in untreated serum as an indication of complement activation. C3 plays a critical role in complement activation through both classical and alternative pathways, and C3 is well known as a specific ligand for complement-receptor on phagocytic cells in liver and spleen (Porter and Reid, 1979; Pangburn, 1983; Brown, 1991; Wright, 1992; Tomlinson, 1993). The amount of bound C3 fragments increased with an increase in CHOL content regardless of the PL in the formulations (Fig. 3), and there is a direct correlation between the amount of associated C3 fragments and CLh, CLs and CLrel (Fig. 4). We have previously shown that the C3b/iC3b, activated C3 fragment, bound to HEPC-MLVs of 800 nm diameter by Western blotting (Funato et al., 1992; Matsuo et al., 1994). These results reported here clearly indicate that liposome opsonization

Fig. 4. Correlations between the organ CL and the amount of bound C3 fragments on the liposomes. (A) Correlation between the amount of bound C3 fragments and CLh. (B) Correlation between the amount of bound C3 fragments and CLs. (C) Correlation between the amount of bound C3 fragments and CLrel. Values for pharmacokinetic parameters are from Table 1. Values for bound C3 fragments are from Fig. 2. The lipid compositions of liposomes are HEPC/CHOL/DCP = 6:2:1 (○), 5:3:1 (△), 4:4:1 (□) or (B) EPC/CHOL/DCP = 6:2:1 (●), 5:3:1 (▲), 4:4:1 (■), respectively.

by the C3 fragments (C3b/iC3b) leads to enhanced uptake of liposomes by phagocytic cells in liver and spleen.

As expected, incorporation of CHOL-O-Me instead of CHOL into the liposome formulation reduced the binding of C3 fragments to the liposomes to below the detection level of our assay system, and decreased clearance rates in vivo (increased AUC and MRT) (Table 2). These results provide definitive evidence for a critical role of CHOL in liposome biodistribution and complement activation triggered by liposomes. Once the complement system is activated by the liposomes, the activated C3 fragment, C3b, binds to the hydroxyl group of CHOL on the liposomes. CHOL is the only molecule on the liposome surface with a hydroxyl group which can be recognized by C3b. A part of the bound C3b is rapidly cleaved to form iC3b, which is a specific ligand for complement receptor on phagocytic cells. Bound iC3b enhances the uptake of liposomes by phagocytic cells. Another part of the bound C3b makes some convertase complexes with other complement components such as factor B and C5 fragments, to further enhance C3b deposition or assembly of MAC on the liposomes. Use of CHOL-O-Me would prevent specific binding of C3b onto the liposomes, resulting in decreased complement activation by the liposomes, and increased liposome circulation time.

Various clustering states of CHOL in liposomal membrane caused by the different lipid composition might be an underlying mechanism in CHOL-dependent complement activation. Fluidity in liposomal membrane might also be one factor which affects the interaction of the complement system with liposomes. Membrane fluidity is dramatically changed by the fatty acyl composition of the PL and by the CHOL content (Oldfield and Chapman, 1971). Recently, Nagayasu et al., showed that membrane fluidity of EPC-MLVs is higher than that of HEPC-MLVs, regardless of the CHOL content (Nagayasu et al., 1996). In the present paper, the amount of C3 fragments associated with liposomes was higher for HEPC-MLVs than for EPC-MLVs (Fig. 2). Therefore, liposomal membrane fluidity is also likely to affect the interaction of activated C3 molecules with the liposome surface; rigid membrane having low fluidity appear to be more susceptible to binding the C3 fragments. However, the precise mechanism of binding of C3 fragments to the liposomes is not clear, thus, this hypothesis should be tested experimentally in further studies.

Table 2
Bound C3 fragments and pharmacokinetic parameters of liposomes containing cholesteryl methyl ether[a]

Lipid composition of liposomes	C3 fragments[b] % dose	AUC (ml/min)	CLh (ml/min)	CLs (ml/min)	CLrel (ml/min)	MRT (min)
HEPC/CHOL-O-Me/DCP = 4:4:1	N.D.	216 ± 11*	0.165 ± 0.029***	0.069 ± 0.015*	0.266 ± 0.023*	82.2 ± 4.8*
HEPC/CHOL/DCP = 4:4:1	6.16 ± 0.63	102 ± 8	0.399 ± 0.018	0.104 ± 0.009	0.372 ± 0.041	27.9 ± 1.8

[a] The amount of bound C3 fragments was determined as described in Section 2. Liposomes (HEPC/CHOL-O-Me/DCP = 4:4:1, 25 μmol total lipid/kg) containing [^{3}H]inulin was administered by i.v. injection into male Wistar rats. At each time point, blood was collected. At 1 h after injection, liver and spleen were collected. Radioactivities of [^{3}H]inulin in blood, urine and various tissues were assayed. The pharmacokinetic parameters were calculated as described in Section 2. Each value represents mean ± S.E. of three experiments. The values for the HEPC-MLV (HEPC/CHOL/DCP = 4:4:1) were taken from Fig. 2 and Table 1. N.D., not detectable.

[b] $\times 10^{-12}$ μg/liposome.

* $P < 0.05$.

*** $P < 0.001$.

As discussed above, addition of CHOL-O-Me dramatically reduced the binding of C3 fragments to the liposomes. However, this reduction did not completely prevent hepatic clearance (Table 2). We have previously reported that in an in situ perfused liver system, significant liposome uptake was still observed under conditions where complement activity in serum was completely abolished (Harashima et al., 1994; Matsuo et al., 1994). Hepatic uptake of liposomes, probably by Kupffer cells in liver, is by two different mechanisms: complement-dependent and complement-independent (direct recognition). The residual CLh may be due to complement-independent hepatic uptake. The results reported by Liu and coworkers strongly support this hypothesis (Liu et al., 1995a,b; Liu and Liu, 1996).

Complement activation induced by liposomes has been examined using complement consumption and release of liposomal contents after incubation in animal or human sera (Chonn et al., 1991b; Funato et al., 1992; Hernandez-Caselles et al., 1993; Devine et al., 1994; Marjan et al., 1994; Szebeni et al., 1994; Panagi et al., 1998). No studies determined the complement component deposited on the liposome surface and evaluated the involvement of the complement system in liposome biodistribution in a quantitative manner. In this study, therefore, we report the quantitative determination of C3 fragments on liposomes and show that there is a close relationship between liposome biodistribution and the amount of bound C3 fragments. Our results strongly suggest that C3 fragments associated with liposomes affect liposome biodistribution. It may be possible to use the amount of deposited human C3 fragments on liposomes to predict the biodistribution of liposomes in human. Quantitative determination of the amount of deposited human C3 fragments would provide useful information in developing a more optimal composition of liposomes for use in human therapeutics.

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