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Contribution of complement system on destabilization of liposomes composed of hydrogenated egg phosphatidylcholine in rat fresh plasma

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Large multilamellar vesicles (MLV) composed of hydrogenated egg phosphatidylcholine (HEPC), cholesterol (CH), and dicetyl phosphate (DCP) rapidly release part of an entrapped aqueous marker when incubated with fresh rat plasma and thus have severely limited usefulness as drug carriers. The mechanisms causing the instability of liposomes in plasma were investigated in this study. The leakage of liposomal constituents was completely inhibited by pre-heating at 56°C for 30 min with plasma or by treating with EDTA, K-76COOH, or anti-C3 antiserum but was not inhibited with EGTA/$MgCl_2$. These results indicated that the destabilization of liposomes in fresh rat plasma was induced by activation of the alternative complement pathway (ACP). Furthermore, the complement third component (C3) was detected from the liposomes incubated with fresh plasma by SDS-PAGE followed by Western blotting and immune detection. The C3b deposited on the liposomal surface via ACP was rapidly cleaved to iC3b. The results obtained in the present study suggest a possibility that the liposomes composed of HEPC (without any surface modification) may be effective carriers for macrophages because C3b and its degradative products, iC3b are related to the opsonic function on phagocytosis of foreign particles by macrophages.

Introduction

There has been great interest in the interactions of liposomes with serum or plasma components for the purpose of imaging in the application of liposomes as carriers of therapeutic agents to tissues *in vivo*. Numerous investigators demonstrated that various plasma proteins released entrapped constituents from liposomes or became associated with the liposomal surface. For example, it was found that the destabilization of liposomes was thought to be due to transfer of phospholipid between liposomes and high-density lipoprotein (HDL) [1–3]. In addition, the lipoprotein, other than HDL [4] or the β-globulin fraction of serum protein [5], was also anticipated in liposomal instability. On the other hand, immunoglobulins and fibronectin associate with liposomes [6] and perhaps serve as opsonins which promote the uptake of liposomes by phagocytic cells (neutrophils, monocytes, and macrophages) [7,8]. Furthermore, it is also considered that liposomes composed mainly of phosphatidylcholine (PC) are damaged by activation of the complement pathway followed by deposition of a complement component such as opsonin, since serum-induced leakage from liposomes is reduced when the liposomes are incubated in pre-heated serum (at 56°C for 30 min) [9]. On the contrary, it was reported that the complement-mediated lysis of sheep red cells or levels of C3 in serum were not affected by pre-treatment with liposomes [6]. The heat-labile proteins which facilitated the PC transfer activity of HDL have been considered in addition to complement components [10] in this regard. Recent study has demonstrated, however, that the negatively charged liposomes activate the classical pathway of human or guinea pig complement system [35].

In the previous paper, we examined the stability of liposomes in plasma under condition maintaining *in*

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vivo state as well as possible, and demonstrated that the liposomes consisting of hydrogenated egg phosphatidylcholine was destabilized when incubated with plasma and its destabilization was completely inhibited by pre-heating at 56°C for 30 min [11].

In the present study, we examined the contributions of a complement system on the destabilization of liposomes in plasma with various decomplement reagents and also investigated the deposition of complement-derived proteins on the liposomal surface.

Materials and Methods

Materials. Hydrogenated egg phosphatidylcholine (HEPC) was kindly supplied by Nippon Fine Chem. Co. Ltd., (Osaka, Japan). Dicetyl phosphate (DCP) was purchased from Nacalai Tesque (Kyoto, Japan). Cholesterol (CH) was obtained from Wako Pure Chem. Ind. Ltd. (Osaka, Japan), and recrystallized from ethanol. 5(6)-Carboxyfluorescein (CF) was obtained from Eastman Kodak Co. (New York, NY, USA) and used without further purification. K-76COOH was kindly presented by Otsuka Pharm. Co., Ltd. (Tokushima, Japan). Goat Anti-Rat C3 antiserum and Goat Anti-Rat C3 antibody conjugated with peroxidase were purchased from Organon Teknika Co. (PA, USA). All other reagents were commercial analytical grade.

Preparation of liposome. Liposomes were prepared by the method described previously [12]. A chloroform solution of HEPC, CH, and DCP in a molar ratio of 4:4:1 was evaporated to dryness in a round-bottomed flask on a rotary evaporator. The remaining solvent was completely removed by reducing the pressure with a vacuum pump. Liposomes were prepared as follows; the dried lipid film in the flask was completely hydrated with solution containing an aqueous marker by shaking with a mechanical shaker. The marker solution was prepared by dissolving 25 mmol of CF in 1.4 ml of 5 M NaOH solution and adjusting the pH and osmotic pressure physiologically by dilution with distilled water. The liposomes were extruded through polycarbonate membranes (Nuclepore Co., CA, USA) having pore sizes of 0.8 μm. The total lipid concentration of liposomes in the preparation was 18 μmol/ml. The prepared liposomes were dialyzed in cellulose dialyzing tubing against 2 l of phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS(−), pH 7.4, 285 mosmol/kg, Nissui Pharmaceutical Co., Ltd., Kyoto, Japan) for four days at room temperature to remove unencapsulated aqueous marker. The PBS(−) was changed three times a day. The empty liposomes were prepared by using PBS(−) in place of aqueous marker solution.

Plasma preparation and treatment. Whole blood of a Wistar male rat was drained through polyethylene tubing in the carotid artery into a heparinized test tube. Fresh rat plasma was obtained by centrifugation at 3000 rpm for 10 min at 4°C, and used within 30 min after collection. To partly or entirely inhibit the activity of complement pathway, fresh plasma (0.9 ml) was heated at 56°C for 30 min or at 50°C for 15 min. EDTA, EGTA/$MgCl_2$ (equimolar), K-76COOH, and Anti-Rat C3 antiserum solution (0.1 ml) was added to fresh plasma (0.8 ml), and the plasma treated with them was also used. The treatment with $CaCl_2$ and $MgCl_2$ was carried out in the same manner. The osmotic pressure and pH of various solutions was adjusted physiologically by PBS(−) (25 times concentration) and NaOH.

Stability study in plasma. Fresh plasma or variously treated plasma was pre-incubated at 37°C for 10 min. After the pre-incubation, an aliquot of liposomal suspension (0.1 ml, 1.8 μmol as total lipids) was added to the fresh plasma or treated plasma (0.9 ml, 90–80% plasma concentration) and incubation was continued. At 0, 5, 15, 30, 60 min after the addition of the liposomal suspension, a 10-μl aliquot was transferred into 5 ml of PBS(−). A 1-ml aliquot of the diluted mixture was well mixed with 1 ml of 5% Triton X-100 solution and then 2 ml of distilled water was added.

Release (%) of CF from liposomes was calculated from the fluorescence intensities (excitation = 490 nm, emission = 520 nm) with and without Triton X-100 treatment according to the following equation:

$$\text{Release (\%)} = \frac{F_0}{4F_t} \times 100 \qquad (1)$$

where F_t and F_0 are the fluorescence intensities with or without treatment with 5% Triton X-100, respectively.

Inhibition (%) by various treatment vs. release (%) of liposomes in fresh plasma was calculated according to the following equation:

$$\text{Inhibition (\%)} = \frac{R_a - R_x}{R_a - R_b} \times 100 \qquad (2)$$

where R_a, R_b and R_x are release (%) values in fresh plasma, PBS(−) and treated plasma, respectively.

Acceleration (%) is reflected as an absolute majority of inhibition (%) when inhibition (%) exhibits a minus value.

Detection of complement components on the liposomal surface. Liposomes (0.5 ml) were incubated with fresh plasma or treated plasma (0.5 ml) at 37°C for 30 min, and the mixture was then mixed with Ficoll solution (in phosphate buffered saline with Ca^{2+} and Mg^{2+}, PBS(+), pH 7.4, 285 mosmol/kg) to obtain a Ficoll concentration of 20%. Then 5 ml of 15% Ficoll solution (in PBS(+)) was layered over 3 ml of the mixture, followed by 2 ml of PBS(+). The density gradient

solution was centrifuged for 60 min at 10000 rpm at 4°C, and the liposomes which floated to the PBS/15% Ficoll interface were collected [13]. The liposomes were resuspended with PBS(+) and again centrifuged for 60 min at 10000 rpm at 4°C. The precipitated liposomes were then added to 100 μl of carbonate buffer (pH 11) containing 1% sodium dodecyl sulfate (SDS) and 25 mM methylamine, and incubated for 60 min at 37°C as described for the extraction of C3 from bacteria [14]. The liposomes were removed by centrifugation, and the supernatant (containing extracted C3 fragment) was adjusted to pH 4 with 50 μl of acetate buffer (0.4 M, pH 4). For the purpose of concentration of proteins, the precipitation of proteins with 2 volumes of acetone was carried out, and the supernatant was removed. The precipitated proteins were redissolved with SDS-PAGE sample buffer. In plasma treated with EDTA and EGTA/$MgCl_2$, PBS(−) was used in place of PBS(+) in the processes mentioned above. β-mercaptoethanol (final concentration of 2%) was added to the solubilized samples, and the samples were subjected to SDS-PAGE in a 7% acrylamide gel under reducing condition [15]. The separated proteins were transferred to nitrocellulose paper by western blotting [16]. The nitrocellulose was incubated in PBS(−) containing 5% skim milk to block nonspecific protein binding sites and was then incubated with peroxidase conjugated primary rat antibody against C3 diluted in PBS(−) for immune detection [17]. The nitrocellulose was washed five times in PBS(−), followed by washing three times in 100 mM Tris (pH 7.0), and developed with 100 mM Tris containing 0.05% *o*-dianisidine and 0.025% H_2O_2 (pH 7.0).

Results and Discussion

Instability of liposome in plasma

The time courses of release (%) of CF encapsulated in liposomes during incubation with fresh rat plasma or PBS at 37°C are shown in Fig. 1A. The release of the marker from liposomes in PBS(−) was not observed for at least 60 min, but about 25% of the marker was released at 15 min when exposed to fresh rat plasma. The degree of the leakage of liposomal constituents was proportional to the plasma concentration (Fig. 1B). These observations indicated that liposomes were destabilized by components in the plasma. Moreover, the destabilization of liposomes in plasma proceeded only slightly after 15 min. To pursue the reason, the destabilization of liposomes in plasma which was pretreated at 37°C for 30 min with empty liposomes was examined (Fig. 1A). The release in plasma treated with empty liposomes apparently decreased, compared with that in fresh plasma. This result suggested that the activation of destabilizing factor(s) was reduced by interaction with the liposomes. Because, the destabilizing activity of plasma which was preincubated for extra 30 min was maintained in magnitude as well as that in fresh plasma (data not shown). Therefore, it was considered that the destabilizing factor(s) were not reversible in activity and consumed by interaction with liposomes.

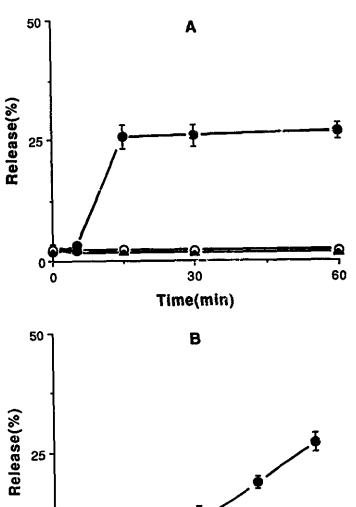


Fig. 1. (A) Time courses of release of CF liposomes incubated at 37°C and effect of treating with empty liposomes. Liposomes containing CF were incubated with fresh plasma (●), PBS (○), and plasma treated with empty liposomes (1.8 μmol) for 30 min (▲). Each value represents the mean ± S.E. of three experiments. (B) Plasma concentration dependence of CF release from liposomes. Liposomes containing CF were incubated at 37°C for 60 min in plasma diluted with PBS (●). Each value represents the mean ± S.E. of three experiments.

Effect of pre-heating at 56°C for 30 min and EDTA on plasma-mediated marker release

In our previous investigation, we reported that the release from liposomes decreased significantly in the

presence of heat-inactivated plasma [11]. The other investigators have also reported a reduction in plasma-mediated marker release has been partially achieved by pre-heating at 56°C for 30 min [9]. In general, it has been known that the complement system which is a self defense mechanism is inactivated by heating at 56°C for 30 min, and that Ca^{2+} or Mg^{2+} is necessary for its activation [18]. The stability of liposomes in plasma pre-heated or pre-treated with EDTA solution was examined. The inhibition (%) of the release after 60 min in fresh plasma by various pre-treatments was calculated by Eqn. 2 and is summarized in Table I. The release from liposomes was completely inhibited by pre-heating at 56°C for 30 min at the same level as in PBS. This result suggested that the liposomes were destabilized by plasma component(s) which were inactivated by heating at 56°C for 30 min. Moreover, it was completely inhibited by 5 mM EDTA at the same total molar concentration as in the incubation mixture. The EDTA is kown as a chelating agent for divalent cations such as Mg^{2+} or Ca^{2+}, and the concentration of 5 mM was generally considered to be sufficient for chelating both Ca^{2+} and Mg^{2+} present in plasma [19]. These results indicated the possibility that the destabilizing factor(s) are complement components. However, the apolipoprotein has been reported to be heat-labile [20], and it has also been reported that the phosphatidylcholine transfer activity of HDL is facilitated by a heat-labile factor in plasma [10]. Therefore, participation of these proteins cannot be neglected as another possibility at present.

Lysis of liposomes by the complement pathway

The complement-mediated damage to membranes arises via either the classical or the alternative pathway, and both pathways can be activated at the level of C3, followed by formation of C5 convertase. These processes can be initiated the assembly of the cytolytic membrane attack complex. Thus, the damage of the membrane is preceded by activation of the complement system and can be inhibited by treatment with Anti-C3 antiserum or K-76COOH which specifically blocks the C5 step [21]. Fig. 2 shows the inhibitory effect of Anti-C3 anti serum or K-76COOH on plasma-mediated marker release in the same manner as described in Table I. The plasma-mediated marker release was completely inhibited by Anti-C3 antiserum in a dose-dependent manner (Fig. 2A). Moreover, about 50% inhibition of release was obtained when the concentration of K-76COOH was about 0.5 mM, and the release was completely inhibited above approximately 1.5 mM of K-76COOH (Fig. 2B). These results indicated that the destabilization of liposomes when exposed to fresh rat plasma was caused by the activation of the complement system in either the classical or alternative pathway. Therefore, the exhaustion of the destabilizing factor(s) by liposomes mentioned in Fig. 1 may be due to the comsuption of the lytic activity of the complement [22].

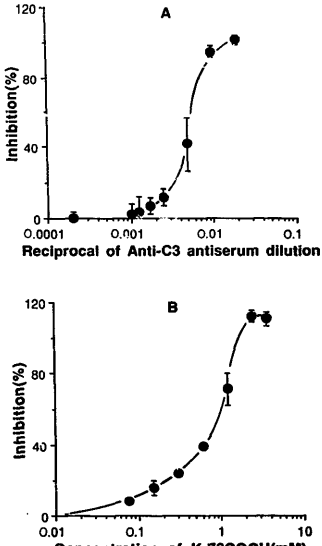


Fig. 2. Effect of Anti-C3 antiserum(A) and K-76COOH(B) on plasma-mediated marker release. Inhibition values were obtained in the same manner as in Table I. Each value represents the mean ± S.E. of three experiments.

TABLE I

Effect of pre-heating and EDTA on plasma-mediated marker release

Liposomes were incubated with fresh plasma, PBS and treated plasma at 37°C for 60 min, inhibition values were calculated by Eqn. 2. Each value represents the mean ± S.E. of three experiments.

Treatment	Inhibition (%)
56°C/30 min	100.6 ± 0.2
EDTA (mM)	
0.0	0.0
2.5	23.5 ± 1.2
5.0	114.4 ± 1.5
10.0	112.3 ± 2.3

Numerous investigators have studied the effect of lipoproteins such as HDL on liposomal instability, and it has been known that the release of marker from liposomes arose from them [2,3]. In addition, the effect of lipoprotein on the liposomal instability has been reported to occur strongly when the fluidity and the curvature of the liposomes were high, such as the small unilamellar liposomes composed of egg phosphatidylcholine [2,3]. We also observed that the release of CF from egg phosphatidylcholine liposomes without cholesterol was not inhibited by treatment with Anti-C3 antiserum and K-76COOH (data not shown). On the other hand, the results presented indicated that the destabilization of liposomes consisting of HEPC was related to the complement system rather than lipoprotein (Fig. 2). Consequently, the reason why the complement system participates in the destabilization of even the plain liposomes used in this study is not clear; however, it seems to be related to liposomal fluidity and curvature. Further studies on the mechanism of these phenomenon are in progress in our laboratory.

Lysis of liposomes via the alternative complement pathway

In general, it has been known that the complement activity of the classical pathway is necessary to both Ca^{2+} and Mg^{2+} and that of the alternative pathway is required only for Mg^{2+}. Since the classical complement pathway (CCP) does not work in EGTA/$MgCl_2$, the lysis by plasma in EGTA/$MgCl_2$ was thought to be mediated by the activation of ACP [18]. The factor B participating in ACP can be inactivated by pre-heating at 50°C for 15 min [18]. Thus, the complement-mediated marker release of the liposomes in plasma which treated with EGTA/$MgCl_2$ or heated at 50°C for 15 min was examined (Fig. 3). The lysis of liposomes in plasma was thoroughly reduced by heating at 50°C for 15 min, and the lytic potency of complement-mediated marker release in EGTA/$MgCl_2$-treated plasma (10 mM) was fully retained. These findings indicated that the lysis of liposomes in fresh plasma was induced by the activation of ACP. The lytic activity of plasma in EGTA/$MgCl_2$ accelerated approx. 75% compared with that in fresh plasma. The lytic activity in $CaCl_2$-treated plasma (10 mM) did not appreciably accelerate, but the degree of acceleration in $MgCl_2$-treated plasma (10 mM) was the same as that in the EGTA/$MgCl_2$-treated plasma (10 mM). It was reported that the lytic potency of CCP was enhanced by addition of either Ca^{2+} or Mg^{2+} [23]. That result raises the possibility that the lytic potency of ACP is induced by Mg^{2+} added to fresh plasma.

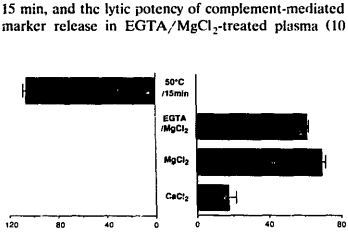


Fig. 3. Involvement of alternative pathway in complement activation by liposomes in fresh plasma. Liposomes were incubated at 37°C for 60 min in plasma with 10 mM of EGTA/$MgCl_2$ (equimolar), $MgCl_2$ or $CaCl_2$. Inhibition values were obtained in the same manner as in Table I. Each value represents the mean ± S.E. of three experiments.

Recent study has demonstrated that the negatively charged liposomes activate the classical pathway whereas the positively charged liposomes activate the alternative pathway of human or guinea pig complement system [35]. Our observations that the negatively charged liposomes containing 10 mol% DCP activated the alternative pathway rather than the classical pathway were in conflict with their conclusions. As one of the reasons for this discrepancy, the imperfection of function of CCP is considered because rat C4 is particularly unstable [37]. However, the all plasma samples were used within 30 min after blood was collected, and the function of rat CCP would be completely maintained [37]. The fundamental properties of liposomes on complement activation is very diverse among some different experimental conditions, and the certain inconsistencies are observed between the individual investigations [22,35,38–40]. Our experimental condition is different from theirs [35] in many points, i.e. surface charge density, size, fluidity, lipid composition, animal species and ratio of plasma protein to liposomal lipid. In fact, we have obtained different results with other lipid composition or size of liposomes, and have also observed that the liposomes used in this experiment does not activate human complement in preliminary experiments (data not shown). The discrepancy, therefore, may arise from some difference as discribed above. The mechanism of destabilization of the liposomes and its extent presented in this study may be considered to reflecte most faithfully those *in vivo* when the liposomes administer for drug delivery because we used fresh plasma of high concentration (80–90%) as *in vivo* state.

Binding of C3 on the liposomal surface by the activation of ACP

The third component (C3) is the most abundant of the complement proteins and consists of two disulfide-linked polypeptide chains, α (125 000 daltons) and β (75 000 daltons) [24]. The enzymatic cleavage of C3 by C3 convertase removes C3a (9000 daltons) from the α-chain and forms C3b. On activation, C3b obtains the ability to form a covalent bond (ester or amide bond)

with a variety of particle surfaces [25]. The combination of detergents and nucleophilic reagents such as SDS-methylamine (pH 11) the most effectively disrupts the ester linkage of C3 fragments on the particle surfaces but not the amide bond [14,25]. The SDS-methylamine (pH 11) extracts were analyzed by the immunoblot technique, since liposomes appeared in absence of nitrogen-containing species in this study. The binding of C3 on the liposomal surface by incubation with fresh, EDTA-treated and EGTA/$MgCl_2$-treated plasma was investigated. Two bands with an apparent molecular mass of 66000 daltons and 42000 daltons were detected from extracts by incubating for 30 min with either fresh plasma or EGTA/$MgCl_2$-treated plasma but little detected with EDTA-treated plasma (Fig. 4, tracks 1–3). These results suggested that the peptide delivered from C3 was deposited on the liposomal surface via ACP. It is known that C3b is dissociated to an α'-chain (113000 daltons) and a β-chain (75000 daltons) under reducing conditions [26]. However, the band of the α'-chain of C3b was little detected from the extracts with either fresh or EGTA-treated plasma, and an additional band with an apparent molecular mass of 42000 daltons is shown in Fig. 4 (tracks 1 and 3). The activation of ACP was regulated by factors I (C3b inactivator) and H(β1H globulin). The α'-chain of bound C3b on the particle surfaces is cleaved by the factors I and H, and the resulting products are iC3b consisting of two peptides of 68000 daltons and 43000 daltons which remain covalently bound to the β-chain [26] (see Scheme I). Actually, it

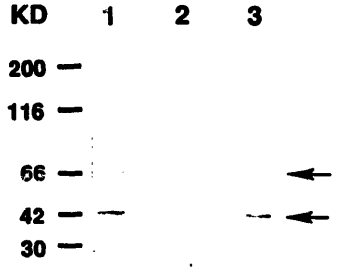
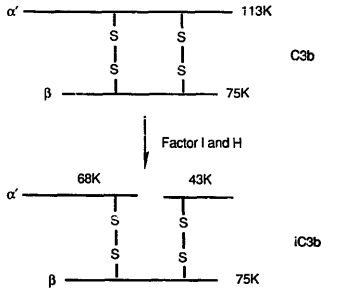


Fig. 4. The deposition of iC3b on the liposomal surface via ACP. Liposomes were incubated with fresh plasma (track 1), EDTA-treated plasma (track 2) and EGTA/$MgCl_2$-treated plasma (track 3). The extracts were separated by SDS-PAGE and stained specifically using peroxidase conjugated antibody against C3 by Western immunoblotting.



Scheme I. The cleavage of C3b to iC3b by factor I and H [26].

was reported that C3b deposited on yeast or rabbit erythrocytes via ACP was rapidly cleaved to iC3b within 30 min, and further cleavage of iC3b to the final C3 fragment (C3dg) did not take place [27]. Thus, it was indicated that the peptide of 42000 daltons which was detected from the extracts was the smaller fragment consisting of the α'-chain of iC3b and that of 66000 daltons was another larger fragment of iC3b. Therefore, it is suggested that C3b deposited on the liposomal surface via ACP is rapidly cleaved to iC3b by the factors I and H. Although the band of the β-chain (75000 daltons) of iC3b was not found as shown in Fig. 4 (tracks 1 and 3), it was considered that either the antibody used in this experiment was specific for the α-chain or the band of the β-chain was existed as a double band comprising larger fragment of α'-chain of iC3b [28].

The complement system also has a role as opsonin in the host defense system against some microorganisms [29]. C3b and iC3b are specifically recognized by the type 1 (CR1) and type 3 (CR3) of complement receptors [29–30] on phagocytic cells, respectively. The C3b and iC3b deposited on the foreign particle surface most likely mediates the clearance of the particles by binding to the complement receptors [29]. In a recent paper, it was indicated that CR3 was primarily responsible for the uptake of liposomes by polymorphonuclear leukocytes [31]. We previously observed that the uptake of liposomes consisting of the same lipid composition by prefused rat liver was enhanced by addition of serum; furthermore, the activity was remarkably decreased with pre-heating of the serum [32]. These results suggest that the uptake of liposomes by liver is enhanced by heat-labile opsonins as complement components. Moreover, it was suggested that iC3b was rapidly deposited on the liposomal surface by the acti-

vation of ACP (Fig. 4). Kupffer cells as phagocytic cells in liver have been reported to express CR1 or CR3 which are receptors for surface-bound fragments of C3 [33]. Consequently, although the mechanism of uptake of liposomes by liver is not yet clarified, the liposomes may possibly be taken up via CR3 on Kupffer cells which recognize iC3b. The involvement of opsonins or complement receptors on the uptake of liposomes by liver is under investigation. We speculate that the facts recognized here are available for the elucidation of the uptake mechanism of liposomes by liver, and the complement-mediated phagocytosis by Kupffer cells plays an important role in the tissue distribution of liposomes *in vivo* as described in a recent study [34]. In this study, therefore, the pronounced interaction between the complement system and the liposomes is very interesting for application as drug carriers, and it suggests a possibility that the liposomes composed of HEPC may be effective carriers for macrophages such as kupffer cells. It has been well known, however, that the complement activation is different among animal species [36] as we have also observed. The biological fate of liposomes is considered to be affected by the difference, and accordingly we need to take account of it for the application of liposomes as drug carriers.

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