Plasma Factor Triggering Alternative Complement Pathway Activation by Liposomes

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Several plasma components, such as complement (C) components, play a role in the clearance of liposomes from the circulation. The interactions between liposomes and the C system were investigated in this study. Multilamellar vesicle (MLV) liposomes, which were damaged by activation of the complement, became susceptible depending on the density of cetylmannoside (Man) on the liposome membrane, and activation proceeded through the alternative C pathway as observed for liposomes without Man (PC-MLV) (K. Funato *et al., Biochim. Biophys. Acta* 1103:198–204, 1992). In addition, the capacity of Man-modified liposomes (Man-MLV) to activate the alternative C pathway was abolished by preadsorption of plasma with Man-MLV but not with PC-MLV. The results suggest that a specific plasma factor adsorbed with Man-MLV was responsible for the augmentation of the C activation and, further, that the rapid clearance of Man-MLV from the circulation is caused by both enhanced C-mediated liposome permeability and enhanced C-mediated phagocytosis of liposomes.

KEY WORDS: cetyl α-D-mannoside; liposome; drug delivery; alternative complement pathway; complement activating factor (CAF).

INTRODUCTION

The interaction of blood plasma proteins with liposomes is important for their use as drug carriers. Protein binding depends on the surface properties of the liposomes, resulting in different patterns of liposome clearance from the circulation (1–3). An inverse relation exists between the amount of total blood protein binding to the liposomes and their circulation half-life (4). However, it remains unclear which plasma components are responsible for liposome clearance. Both liposome permeability in blood and liposome uptake by phagocytic cells may be factors determining liposome clearance. We demonstrated that liposomes without surface modification (PC-MLV) activated the alternative pathway of the complement (C) system, thereby causing liposome permeability. Furthermore, the C activation resulted in the deposition of C3 fragments on the surface of liposomes (5). C3 fragments were related to the opsonic effect on liposome uptake by the liver, and the uptake of liposomes depended on the extent of opsonization through C activation (6). These results suggest a common role of the C system as an underlying mechanism in both liposome permeability and liposome uptake. Hence, liposome clearance may depend on their ability to activate the C system.

Liposome clearance *in vivo* was markedly enhanced by grafting mannoside on the liposomal surface (7,8). *In vitro* studies indicated that enhanced liposome clearance was caused by cellular uptake mediated through the mannose receptor present at the cell surface of mononuclear phagocytes. However, it was not clarified whether the clearance of mannosylated liposomes was influenced by the opsonic effect of plasma proteins. We have previously reported that surface modification of liposomes (Man-MLV) by cetylmannoside (Man) results in higher clearance by enhancing both liposome permeability in the blood space and accumulation of liposomes in the liver in comparison with PC-MLV (11). This observation suggests that enhanced clearance of Man-MLV results from facilitating C-mediated liposome permeability and liposome uptake.

In this report, we examine the effect of Man on the activation of C by liposomes. Modification of the liposome surface with Man markedly augmented the activation of C via an alternative pathway, and a plasma factor adsorbed with Man-MLV was found to be required for enhanced C activation.

MATERIALS AND METHODS

Materials. An alkyl glycoside, cetyl α-D-mannoside (Man), was synthesized as described in a previous paper (12). Hydrogenated egg phosphatidylcholine (PC) was a gift from Nippon Fine Chemicals Co. (Osaka, Japan). Cholesterol (CH) and dicetyl phosphate (DCP) were purchased from Wako Pure Chemicals (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. K-76COOH was kindly provided by Otsuka Pharm. Co., Ltd. (Tokushima, Japan).

Preparation of Liposomes and Multilamellar Vesicles (MLV). 5(6)-Carboxyfluorescein (CF)-encapsulated liposomes were prepared by a method described previously (5). PC-MLV were composed of PC/CH/DCP at a molar ratio of 5:4:1. When Man was included in the liposomes, Man-MLV were composed of Man/PC/CH/DCP at a molar ratio of X:5–X:4:1 ($0 < X \leqq 5$) in a manner to be replaced by PC. Liposomes were sized through polycarbonate membranes (Nuclepore Co., CA) with pore sizes of 0.8 μm. Liposomes to be used for adsorption of plasma were prepared by trapping phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(−)] in place of the aqueous marker solution.

Lipsomal Leakage Experiments. Normal rat plasma was obtained from Wistar male rats through polyethylene tubing in the carotid artery into a heparinized test tube. Liposome-adsorbed plasma (90%, v/v) was prepared by incubating 9 vol of normal plasma with 1 vol of liposome solution (containing 20 μmol/mL of total lipid) for 30 min at 0°C. Liposomes were carefully removed by aspirating the top floating layer after centrifuging the incubation mixture for 60 min at 3000g. Liposomal leakage as a result of C reaction was assessed by determining the fluorescent intensity from the CF according to methods described previously (5). To 450 μL of normal plasma or variously treated plasma, 50 μL of liposome solution (containing 10 μmol/mL of total lipid) was added to give a final plasma concentration of 81% (v/v),

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and the mixture was incubated for 60 min at 37°C. The fluorescent intensity of the reaction mixture was determined by excitation and emission wavelengths of 490 and 520 nm, respectively. One hundred percent leakage was measured by lysing the liposomes with Triton X-100.

Complement Hemolytic Activity. Complement hemolytic activity was determined with rabbit erythrocytes (RaE) according to established methods (13). Two hundred microliters of RaE (5×10^8 cells/mL) was incubated at 37°C for 60 min with 800 μL of a dilution of normal, heated (56°C/30 min) or liposome-adsorbed plasma in PBS with Ca^{2+} and Mg^{2+} [PBS(+)]. After incubation, 2 mL of cold PBS(−) was added and the mixture centrifuged at 3000 rpm for 11 min. Hemolysis (%) was evaluated by measuring the absorbance of the supernatant at 542 nm.

RESULTS

Effect of Man on Plasma-Mediated Leakage of Liposomal Contents. To determine if Man had an enhancing effect on the plasma-mediated leakage of liposomal contents, liposomes incorporating various amounts of Man into their membranes were prepared and their reactivity with plasma was examined. In the presence of dilute plasma, the release of CF from liposomes was enhanced in proportion to the increase in the density of Man on the liposome membrane (Fig. 1A). Only liposomes containing 50 mol% Man were potentially leaky in plasma of a high concentration (81%, v/v) (Fig. 1A). We have previously shown, however, that the leakage of an aqueous marker from liposomes containing 30 mol% Man was 3.5-fold higher than that of liposomes without Man after intravenous administration (11). In support of these observations, the lipid concentration affected the leakage of liposomes in plasma under conditions similar to the *in vivo* state (Fig. 1B). The leakage of Man-MLV was extensive in comparison with PC-MLV when the lipid concentration corresponded to that in the *in vivo* experiment (3.25 μmol/mL).

Enhancement of Alternative C Pathway Activation by Man-MLV. To determine whether the damage of liposomes containing Man was mediated by the activation of the C system, the damage of liposomes in plasma preheated at 56°C for 30 min or pretreated with various decomplement reagents was examined as described previously (5) (Table I). The plasma-mediated marker release of liposomes with increasing amounts of Man (0 to 50 mol%) was completely blocked by heating, by C depletion with zymosan, and by blocking of the C5 intermediate step with K-76COOH (14). We next examined the roles of classical and alternative C pathways in the damage of Man-MLV. Compared with the level in normal plasma, the leakage of the marker from Man-MLV was abolished in plasma containing EDTA, a condition in which both pathways are inhibited. On the contrary, it was not abolished in plasma containing EGTA and $MgCl_2$, a condition in which the classical pathway but not the alternative pathway is inhibited (15). These results indicated that the surface modification of liposomes by Man caused potent activation of the C system and that the C activation by Man-MLV proceeded through the alternative pathway in a manner similar to that observed previously with PC-MLV (5).

Effect of Liposome-Adsorbed Plasma on Alternative C Activation. Normal plasma was adsorbed with liposomes lacking PC (50% Man-MLV), containing 30 mol% Man (30% Man-MLV), or lacking Man (PC-MLV) to remove naturally occurring antibodies or other plasma protein such as the mannose-binding protein (MBP) reactive with the surface of each liposome without consumption of the C components (16–20). It was then determined whether plasma adsorbed with each liposome displayed C activation by Man-MLV and PC-MLV (Fig. 2A). Adsorption with homologous liposomes

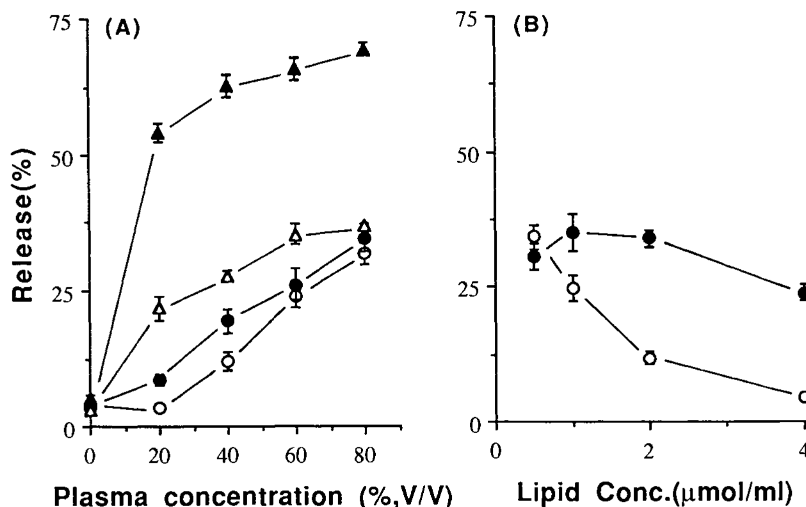

Fig. 1. (A) Effect of Man content on plasma concentration dependence of CF release from liposomes (1 μmol/mL, total lipid conc.). (B) Effect of liposomal concentration on leakage of liposomes in normal plasma (81%, v/v). ○, 5PC 4CH 1DCP (0%); ●, 3Man 2PC 4CH 1DCP (30%); △, 4Man 1PC 4CH 1DCP (40%); ▲, 5Man 4CH 1DCP (50%) (the numbers in parentheses denote the mol% of Man vs total lipid). CF leakage was determined as described under Materials and Methods. Each value represents the mean ± SE of three separate experiments.

Table I. Leakage of Liposomes Is Mediated by Activation of the Alternative Complement Pathway[a]

Treatment	Release (%)		
	50% Man-MLV	30% Man-MLV	PC-MLV
None	69.2 ± 1.2	41.7 ± 1.4	38.4 ± 1.3
56°C/30 min	4.3 ± 0.5	1.8 ± 0.0	2.7 ± 0.0
Zymosan (5 mg/mL)	—	1.7 ± 0.1	2.5 ± 0.0
K-76COOH (2.5 m*M*)	—	1.7 ± 0.1	2.6 ± 0.1
EDTA (10 m*M*)	—	1.6 ± 0.0	2.5 ± 0.0
EGTA/$MgCl_2$ (10 m*M*)	—	47.3 ± 1.2	34.7 ± 1.1

[a] Liposomes (1 μmol/mL, total lipid conc.) composed of Man/PC/CH/DCP at molar ratios of 5/0/4/1 (50% Man-MLV), 3/2/4/1 (30% Man-MLV), or 0/5/4/1 (PC-MLV) were incubated with normal or treated plasma (81%, v/v). CF leakage was determined as described under Materials and Methods. Each value represents the mean ± SE of three separate experiments.

introduced complete disappearance of the activity by each liposome, indicating that a certain liposome-binding plasma factor may be involved in individual C activation. In addition, adsorption with 50% Man-MLV showed results similar to those obtained from the adsorption with homologous liposomes. Adsorption with heterologous liposomes, however, had little effect on C activation by Man-MLV, in contrast to that by PC-MLV. These results suggest that the adsorptive plasma factor responsible for initiation of C activation by Man-MLV and PC-MLV is specific for epitopes unique to each liposome. Further, the inhibition of C activation by adsorption with liposomes was not the result of depletion of the C components needed for activation of the alternative pathway because adsorption with PC-MLV (different activator) did not affect C activation by Man-MLV. Indeed, we observed that adsorption by liposomes did not affect the C hemolytic capacity of plasma (Fig. 2B). Thus, the augmentation of C activation can be accounted for by the adsorptive plasma factor which selectively attaches to Man-MLV.

DISCUSSION

The studies presented here indicate that modification of the liposome surface with Man significantly increases the activation of C via the alternative pathway and that an adsorptive plasma factor is required to initiate individual C activation by Man-MLV and PC-MLV. Further, although Man-MLV captures the adsorptive plasma factor responsible for initiation of C activation by PC-MLV, adsorption with PC-MLV dose not affect C activation by Man-MLV (Fig. 2A). Therefore, the enhanced C activation by Man-MLV is caused by an adsorptive plasma factor, the C-activating factor (CAF), which selectively attaches to Man-MLV (mCAF), regardless of the capture of the adsorptive plasma factor for C activation by PC-MLV (pCAF).

Some lectins have been shown to activate C through the classical pathway (21–23). Ikeda *et al.* have demonstrated that MBP, a lectin specific for mannose or *N*-actylglucosamine, triggers C activation through the antibody-independent classical pathway instead of C1q (23). Calcium is required for specific binding of MBP (17). Recently, MBP was reported to be identical to a component of the Ra-reactive factor (24). In this study, mCAF may also be a protein with lectin-like activity. However, the C activating mechanism of mCAF is distinct from that of MBP, since activation by Man-MLV was found in the absence of calcium and proceeded through the alternative pathway (Table I). Further, zymosan composed primarily of α-D-mannans and β-D-glucans activates the alternative C pathway (25) and IgG antibodies in normal serum facilitate C activation by zymosan (19,20). In addition, MBP markedly enhances C activation via an alternative pathway, which remains poorly understood (18). Both factors can be effectively adsorbed from serum by zymosan (18–20). However, adsorption with zymosan (5 mg/mL) had no apparent inhibitory effect on C activation in plasma preadsorbed with PC-MLV (release of CF from Man-MLV containing 30 mol% Man = 34.6 ± 1.3%; mean ± SE; n = 3) under the same experimental condition (Fig. 2A), indicating that mCAF is neither a natural antibody nor MBP. Therefore, mCAF may represent a novel plasma factor which triggers the activation via an alternative C pathway. The lack of an effect by zymosan adsorption suggests that the mCAF requirement is unique to the liposome surface. Although the reactivity with the liposome containing other carbohydrate residues of mCAF is not yet clarified, the reaction site for mCAF may be mannose residues which are regularly arranged with the CH of the main membrane organization but not the random mannose residues. Further studies of mCAF are in progress.

The binding sites for pCAF are not phosphocholine residues because pCAF can be effectively adsorbed from plasma by liposomes without PC (Fig. 2A), indicating that pCAF is neither CRP (26) nor an unidentified serum factor (16), and therefore, pCAF has specificity for the CH of the liposomal membrane. The antibody-independent classical pathway is activated in the presence of a high concentration of membrane CH, and this activation is triggered by the partly heat-labile serum factor (27). In addition, crystalline CH activates the alternative C pathway (28), and C3 deposited by the alternative C activation was found in the walls of coronary arteries of hypercholesterolemic animals (29). It is possible that pCAF is an analogue of the heat-labile serum factor, and these may affect the pathogenesis of CH-induced atherosclerotic lesions. However, natural antibodies cannot be neglected as another possibility since natural antibodies against CH have been reported to be present in normal serum (28,30).

Liposome clearance is markedly enhanced by grafting mannoside on the liposome surface *in vivo*. The enhanced clearance of those liposomes is thought to be due to uptake mediated through mannose receptors (7,8). However, their interpretations may not account for the behavior of injected liposomes *in vivo*, because the *in vitro* studies were conducted under serum-free conditions or low C activity (9,10). The interaction between liposomes and plasma protein(s) may affect clearance of mannose-modified liposomes *in vivo*. The liver uptake of PC-MLV was shown to be mediated by heat-labile opsonins such as the C component (31). Further, iC3b and C3 fragments are deposited on the surface of PC-MLV (5), and C-mediated phagocytosis of liposomes may play a significant role in liposome clearance from the circu-

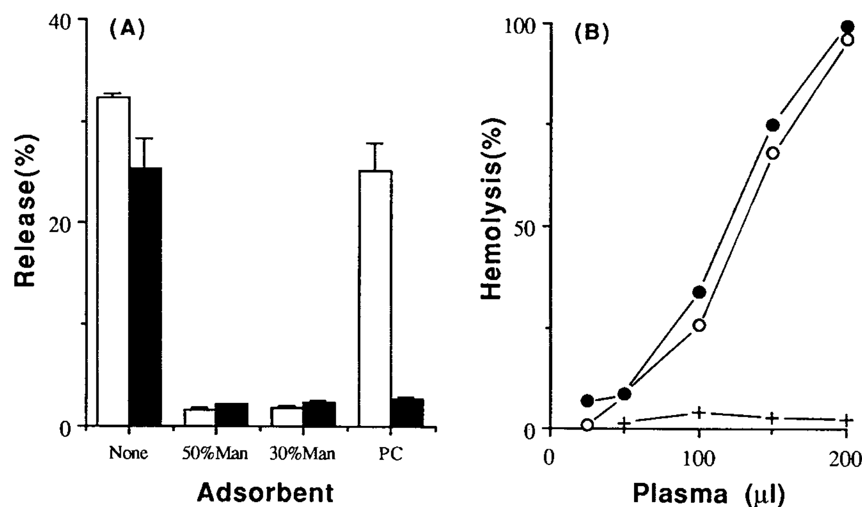

Fig. 2. (A) Effect of cross-adsorption on complement activation by liposomes. Liposomes lacking PC (50% Man)-, containing 30 mol% Man (30% Man)-, or lacking Man (PC)-adsorbed plasma were incubated with liposomes composed of Man/PC/CH/DCP at molar ratios of 3/2/4/1 (Man-MLV; open bars) or PC/CH/DCP at molar ratios of 5/4/1 (PC-MLV; filled bars). CF leakage was determined as described in Table I, footnote *a*. Each value represents the mean ± SE of three separate experiments. (B) Residual hemolytic activity of plasma preadsorbed with liposomes. Normal plasma was adsorbed with liposomes composed of Man/PC/CH/DCP at molar ratios of 3/2/4/1. Two hundred microliters of RaE (5×10^8 cells/mL) was mixed with 800 μL of PBS(+) containing the indicated volumes (90%, v/v) of normal (○), heated (+), or liposome-adsorbed plasma (●). Hemolysis (%) was then determined as described under Materials and Methods.

lation, analogous to C-mediated liposome permeability (6). In addition, we also demonstrated an enhanced clearance rate of Man-MLV from the circulation as a result of both enhanced liposome permeability in blood and enhanced accumulation of liposomes in the liver. The results presented here show that modification of the liposome surface with Man markedly augments activation of the C system, resulting in augmented deposition of C3 fragments as opsonin on the surface of Man-MLV. Thus, the enhanced uptake of Man-MLV by the liver may result from facilitating C-mediated phagocytosis but not mannose receptor-mediated phagocytosis *in vivo*. In fact, the accumulation of Man-MLV in perfused liver was observed to increase in proportion to the serum concentration (data not shown). These observations indicate a correlation between the activation of the C system and the clearance rate of liposomes, and accordingly the clearance rate of liposomes may be strongly dependent on C-mediated liposome permeability and C-mediated phagocytosis of liposomes.

The ability of mCAF to augment C activation may play an important role in the clearance of Man-MLV from the circulation, which is of interest for the use of liposomes as a drug delivery system. Also, we assume that mCAF is a novel plasma factor that triggers the activation of the alternative C pathway and mCAF may play a role in the first-line defense of the nonimmune host, although the role of mCAF in activation is not known at present.

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