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A novel plasma factor initiating complement activation on cetylmannoside-modified liposomes in human plasma

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Abstract

The possibility that the human complement (C) system plays a critical role in both destabilization and opsonization of the liposomes modified with cetylmannoside (Man-MLVs) was demonstrated in our previous in vitro study. In this study, our attention was focused on the underlying mechanism of activation of the C system by the Man-MLVs. It was found that the Man-MLVs had significant ability to activate the C system and the activation proceeded through the C1q-dependent classical pathway. Pretreatment of human plasma with Man-MLVs at low temperature (4°C) diminished the ability of the C system to destabilize the homologous liposomes without affecting the ability to lyse rabbit erythrocytes. However, the C-dependent destabilization did not disappear by the pretreatment with PC-MLVs. The results suggest that a plasma factor, which specifically adsorbs to Man-MLVs but not PC-MLVs, is essential for initiating the C activation. The plasma factor was obviously distinguished from any known classical C pathway (CCP) activators, since the adsorption of the plasma factor to Man-MLVs was not inhibited by treatment with EGTA/Mg^{2+}, soluble sugars (GlcNAc, ManNAc or D-mannose) or Con-A Sepharose. In addition, isolation of the plasma factor was attempted by the following procedure: PEG precipitation, DEAE Sepharose chromatography and gel filtration chromatography. In a series of these isolations, the plasma factor was found to be a protein with higher molecular weight at least than 669 000 Da. It was concluded that the activation of the human C system by the Man-MLVs proceeded through the CCP in the presence of a novel plasma factor.

Keywords: Liposome; Cetylmannoside; Classical complement pathway; Plasma protein; Complement activating factor (CAF)

Abbreviations: ACP = alternative complement pathway; C = complement; CCP = classical C pathway; CF = 5(6)-carboxyfluorescein; Chol = cholesterol; CRP = C-reactive protein; DCP = dicetyl phosphate; GlcNAc = N-acetylglucosamine; PC = hydrogenated egg phosphatidylcholine; PEG = polyethylene glycol; Man = cetylmannoside; ManNAc = N-acetylmannosamine; MBP = mannose-binding protein; MLV = multilamellar vesicle; PBS = phosphate buffered saline; SAP = serum amyloid P component.

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1. Introduction

Clearance rate of liposomes from the circulation is widely believed to be affected by interactions with plasma proteins (Juliano and Lin, 1980; Bonte and Juliano, 1986; Patel, 1992). A variety of plasma proteins are known to bind to liposomes; some of which can cause membrane destabilization. Moreover, high levels of plasma protein binding are associated with rapid clearance of liposomes from the circulation (Chonn et al., 1991a, 1992). In vivo and in vitro studies have shown that the interactions with lipoproteins (Scherphof et al., 1978; Guo et al., 1980; Kirby et al., 1980), complement (C) system (Chonn et al., 1991b; Funato et al., 1992) and serum albumin (Bonte and Juliano, 1986) have been suggested to result in destabilization of liposomes leading to release of their contents. Furthermore, it has been shown that the interactions with the C system (Juliano and Lin, 1980; Chonn et al., 1992; Harashima et al., 1994), fibronectin (Hsu and Juliano, 1982), β2-glycoprotein I (Chonn et al., 1995) and immunoglobulin (IgG) (Senior et al., 1986; Chonn et al., 1991a) mediate the uptake of liposomes by the macrophages in the mononuclear phagocyte system (MPS). However, the major molecular species that determine the fate of liposomes in blood circulation are still not identified.

We have been studying the mechanism of liposomal destabilization in rat plasma or serum as well as the hepatic uptake of liposomes using isolated perfused rat liver. It has become apparent that cetylmannoside-modified multilamellar vesicles (Man-MLVs) were destabilized as a consequence of C activation through the alternative pathway (Funato et al., 1994; Matsuo et al., 1994), and also that the hepatic uptake of the liposomes was enhanced via C-receptor mediated phagocytosis by Kupffer cells (Matsuo et al., 1994). These results show that the C system plays a critical role through the destabilization and opsonization in the clearance of Man-MLVs from blood circulation. Chonn and coworkers also pointed out the importance of the C system in both the opsonization and destabilization of liposomes, and demonstrated that liposomes can activate the C system of several species including human, guinea pig and rat depending on their physicochemical properties and type of used lipid (Chonn et al., 1991b; Devine et al., 1994). Furthermore, Scieszka and coworkers showed that the deposited human C component 3 (C3bi) on liposomes is the opsonin responsible for recognition by the receptor for C3bi (CR3) on the human polymorphonuclear neutrophils (PMNs) (Scieszka et al., 1991). The extent of interaction with the C system may determine the fate of liposomes in blood circulation. Therefore, an understanding of the underlying mechanism of C activation by liposomes allows development of the liposomal system which can be used clinically as a drug carrier for human.

We investigated the underlying mechanism of the liposome-human C system interactions in in vitro assay in this study. Furthermore, we attempted to characterize and isolate a plasma factor responsible for initiating the C activation.

2. Materials and methods

2.1. Materials

Cetylmannoside (Man) and hydrogenated egg phosphatidylcholine (PC) were kindly donated by Nippon Fine Chem. (Osaka, Japan). Dicetylphosphate (DCP) was purchased from Nacalai Tesque (Kyoto, Japan). Cholesterol (Chol) was of analytical grade (Wako Pure Chem., Osaka, Japan). Goat anti-human C1q antiserum was purchased from Organon Teknica Co. (PA, USA). Protein-G Sepharose, Con-A Sepharose, DEAE Sepharose and Sephacryl S-300 were purchased from Pharmacia (Uppsala, Sweden). D(+)-mannose was purchased from Wako Pure Chem. (Osaka, Japan). N-acetylglucosamine (GlcNAc) and N-acetylmannosamine (ManNAc) were purchased from Pfranstiehl Lab. (IL, USA). 5(6)-carboxyfluorescein (CF) was purchased from Eastman Kodak Co. (NY, USA). Polyethylene glycol 4000 (PEG) was purchased from Wako Pure Chem. (Osaka, Japan). Normal human plasma was obtained from the Blood Center of the Japan Red Cross, Tokushima (Tokushima, Japan) and

stored at −80°C. All other reagents were of the highest grade commercially available.

2.2. Preparation of liposomes

Liposomes (mean diameters; 750–870 nm) were composed of Man\PC\Chol\DCP = 3:2:4:1 (Man-MLVs) and = 0:5:4:1 (PC-MLVs) in a molar ratio. Multilamellar liposomes (MLVs) were extruded through polycarbonate filter with the pore size of 0.8 μm. Carboxyfluorescein as aqueous marker was encapsulated to examine liposome destabilization in plasma. Liposomes used for pretreatment of plasma were prepared by entrapping phosphate buffered saline (Na_2PO_4 1.15 g, NaH_2PO_4 0.2 g, NaCl 8 g, KCl 0.2 g/l; pH 7.4, PBS(−)) in place of aqueous marker solution. The diameters of liposomes were checked with a NICOMP-370 HPL submicron particle analyzer (Particle Sizing Systems, CA, USA).

2.3. Destabilization of liposomes in plasma

Destabilization of liposomes after incubation in normal plasma or various treated plasma was assessed by determining the fluorescent intensity of CF released from the liposomes according to the method described previously (Funato et al., 1992). In brief, to 450 μl of normal plasma or various treated plasma, 50 μl of CF entrapped liposomes (containing 10 μmol/ml of total lipid concentration) was added, and the mixture was incubated for 30 min at 37°C. After incubation, the fluorescent intensity (excitation = 490 nm, emission = 520 nm) of the reaction mixture was determined with a fluorospectrophotometer (Hitachi 650, Hitachi Koki, Ibaragi, Japan). One hundred percent release was determined by lysing the liposomes with Triton X-100.

2.4. Plasma pretreatment with heating, chelating and anti-C1q antiserum

Various treated plasma were prepared according to the method given below. Normal human plasma was heated for 30 min at 56°C. Divalent cations (Ca^{2+} and Mg^{2+})-depleted plasma was prepared by incubating 9 vol of normal plasma with 1 vol of 0.1 M EDTA solution for 5 min at 37°C. Likewise, Ca^{2+}-depleted plasma was prepared by adding 0.1 M EGTA/$MgCl_2$ solution instead of EDTA solution. The C1q-depleted plasma was prepared by incubating 9 vol of normal plasma with 1 vol of anti-C1q antiserum for overnight at 4°C. The depletion of C1q was confirmed by Western blot analysis under non-reducing conditions; a 460 kDa band reactive with anti-C1q antiserum was no longer present in the C1q-depleted plasma.

2.5. Complement hemolytic assay

C hemolytic activity was determined with rabbit erythrocytes according to established method (Funato et al., 1992). In brief, 200 μl of rabbit erythrocytes solution (5×10^8 cells/ml) was incubated at 37°C for 60 min with 800 μl of normal or liposome-pretreated plasma . After incubation, 2 ml of cold PBS(−) was added to stop the reaction and the reaction mixture was centrifuged at 3000 rpm for 11 min. Hemolysis (%) was evaluated by measuring the absorbance of the supernatant at 542 nm.

2.6. Reconstitution assay

2.6.1. Plasma factor-adsorbing liposomes

One vol of CF-entrapped Man-MLVs (containing 20 μmol/ml of total lipid concentration) was incubated with 9 vol of normal plasma for 30 min at 4°C. The incubation mixture was centrifuged for 30 min at 15 000 rpm after the addition of excess cold PBS(−). The precipitate of plasma factor-adsorbing Man-MLVs was resuspended with PBS(−) and its lipid concentration was adjusted to 10 μmol/ml. The liposome suspension was immediately subjected to reconstitutional assay.

2.6.2. Plasma factor-depleted plasma

One vol of Man-MLVs (containing 20 μmol/ml of total lipid concentration) was incubated with 9 vol of normal plasma for 30 min at 4°C. After centrifugation (3000 rpm, 30 min), the top float-

ing liposome layer was removed by aspirating. Depletion of the plasma factor was confirmed by assessing the remaining ability to destabilize the Man-MLVs. The plasma factor-depleted plasma was stored at −80°C until used.

2.6.3. Reconstitution

One vol of the plasma factor-adsorbing liposomes (containing 10 μmol/ml of total lipid concentration) was incubated together with 9 vol of the plasma factor-depleted plasma for 30 min at 37°C. By this preparation, the plasma factor-dependent C activation was investigated.

2.7. Plasma fractionation

Pooled human plasma was subjected to PEG fractionation. Initially, the plasma was brought to 5% (v/v) saturation by slow addition, while gently stirring, of a PEG solution at 4°C. After the complete addition of the PEG solution, the suspension was incubated for at least 2 h at 4°C. The precipitated protein and supernatant were separated by centrifugation (15 000 rpm, 30 min, 4°C). After centrifugation, the precipitate was washed in 5% (v/v) PEG solution and redissolved in PBS(+) (PBS(−) containing $CaCl_2$ 0.1 g, $MgCl_2$ $6H_2O$ 0.1 g/l). To the supernatant from the previous step, a PEG solution was added slowly over at least 30 min to bring the final concentration to 7.5% (v/v) at 4°C. After incubation for at least 2 h at 4°C, the precipitated protein (5–7.5% PEG fraction) and supernatant were separated by centrifugation (15 000 rpm, 30 min, 4°C). The 7.5–10%, 10–15% and >15% PEG fractions were subsequently obtained in the same manner as mentioned above. The isolated fractions were dialyzed against PBS(+) overnight at 4°C to remove residual PEG and concentrated to the initial volume with Centriprep (Amicon, MA, USA). After concentration, the fractions were tested for the ability for activating the C system on the plate assay as described below.

The sample, which exhibited the highest ability for activating the C system, was pooled and dialyzed against a DEAE-Sepharose loading buffer (20 mM Tris–HCl, pH 8.0) and applied to a DEAE-Sepharose anion exchange column (50 × 25 mm). The column was washed with a DEAE-Sepharose loading buffer and eluted stepwise with the same buffer containing 100, 135, 170, 200, 300 and 500 mM NaCl. The protein in each fraction was determined with a BCA protein assay kit (Pierce, IL, USA). Each eluate was pooled and dialyzed against PBS(−) for exchanging the buffer. After concentration, the ability for activating the C system in each pooled fraction was tested on the plate assay.

The eluted fraction, which exhibited the highest ability for activating the C system, was pooled and applied to a Sephacryl S-300 column (1.5 × 65 cm) equilibrated with PBS(+) at 4°C. The protein in the collected fraction was determined with a BCA protein assay kit. The protein-containing fractions were tested for ability for activating the C system on the plate assay.

2.8. Plate assay

In order to test the activity for activating the C system immediately after the fractionation, a simple assay system was used. In brief, 50 μl of CF-entrapped Man-MLVs (containing 0.4 μmol/ml of total lipid concentration), 100 μl of the plasma factor-depleted plasma and 100 μl of the fraction were mixed in a well on a 96-well microtiterplate and incubated for 30 min at 37°C. The destabilization of Man-MLVs was assessed according to the method described above.

3. Results

3.1. Destabilization of liposomes in human plasma

Destabilization in human plasma was observed in Man-MLVs but not PC-MLVs and it was increased with increasing plasma concentration (Fig. 1A). When incubated in the plasma preheated at 56°C for 30 min, the destabilization of Man-MLVs disappeared in any plasma concentration. Under this condition, both C activation pathways: classical C pathway (CCP) and alternative C pathway (ACP), were known to be inactivated. Hence, the destabilization of Man-MLVs seems to be mediated as a consequence of C

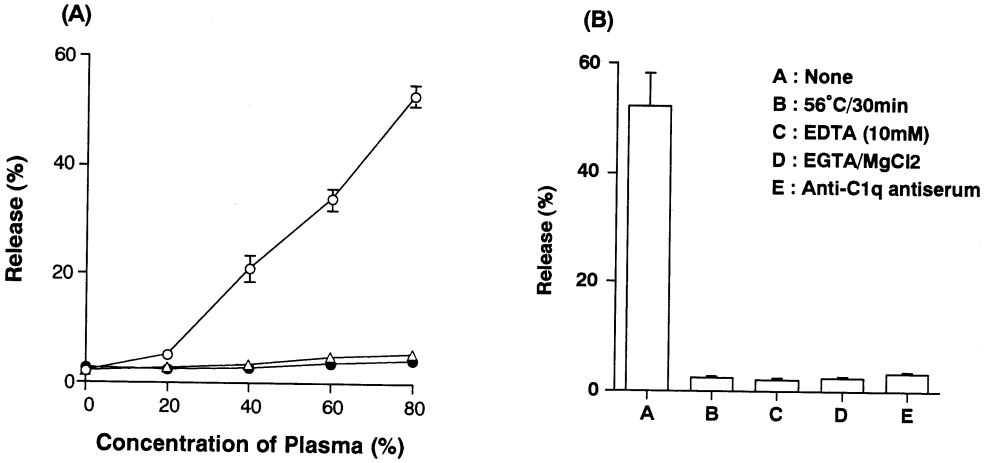

Fig. 1. (A) Plasma concentration-dependent destabilization of Man-MLVs. The effect of plasma concentration was examined on the extent of CF release from either Man-MLVs (○) or PC-MLVs (△). The liposomes were incubated with normal (open symbol) or preheated (closed symbol) human plasma at the indicated plasma concentration and the release of entrapped CF was measured after incubation for 30 min at 37°C. The concentration of liposomes was fixed at 1 μmol/ml. The release was expressed as the percentage of total entrapped CF. The vertical bars represent the standard deviations of three separate experiments. (B) Destabilization of Man-MLVs is mediated by activation of classical complement pathway. CF entrapped Man-MLVs (1 μmol/ml, total lipid concentration) were incubated with normal or various treated plasma (81%, v/v) for 30 min at 37°C. CF release was determined as described in Section 2 (Materials and methods). Each value represents the mean ± S.D. of three separate experiments.

activation by the Man-MLVs. To identify the C pathway activated by Man-MLVs, the effect of treatment with EDTA, EGTA/Mg^{2+} or anti-C1q antiserum on the destabilization of the Man-MLVs in plasma was examined (Fig. 1B). In all experiments, the destabilization of Man-MLVs was completely diminished when compared to that in normal plasma. It is widely accepted that the requirement of divalent cations (Ca^{2+} or Mg^{2+}) for C activation differs for the C activation pathways; the CCP requires both Ca^{2+} and Mg^{2+}, while the ACP requires Mg^{2+} alone. Furthermore, it is also known that the CCP essentially requires C1q to be activated. Therefore, the result that the activation of the C system by Man-MLVs required both Ca^{2+} and C1q shows that the C system is activated through the CCP, but not the ACP.

3.2. Effect of pretreatment with liposomes on the complement activation

It is known that the activation of the C system through the CCP was initiated by the binding of the triggering plasma factors, such as immunoglobulins (IgG or IgM), mannose-binding protein (MBP), C-reactive protein (CRP) and serum amyloid P component (SAP), to the surface of invading foreign particles (Baltz et al., 1982; Volanakis, 1982; Sim and Malhotra, 1994; Turner, 1994). Therefore, we presumed the existence of a plasma factor, which adsorbs to the surface of Man-MLVs and subsequently initiates the activation of the C system through the CCP. To clarify this hypothesis, the remaining C activity in the plasma pretreated with Man-MLVs or PC-MLVs was determined by measuring either CF-release from Man-MLVs or immune damage to rabbit erythrocytes (Fig. 2), a condition in which naturally occurring antibodies against lipids composed of the liposomes or other plasma proteins having specificity for the liposomes were removed (Mold and Gewurz, 1980; Scenkein and Ruddy, 1981; Ihara et al., 1982; Schweinle et al., 1989; Wilson and Kozel, 1992). The pretreatment with the Man-MLVs markedly influenced the destabilization of homologous liposomes, whereas that with the PC-MLVs had no influence on the

destabilization of Man-MLVs. Also, adequate hemolytic activity was retained after pretreatment with the Man-MLVs. These results indicate that the disappearance of the destabilization of Man-MLVs by pretreatment with homologous liposomes is advanced as a consequence of the consumption of a plasma factor responsible for initiating the C activation rather than the consumption of C components responsible for deriving the Man-MLVs destabilization. Consequently, the activation of the C system through the classical pathway by Man-MLVs is likely to be initiated and augmented by the presence of a certain plasma factor, which specifically adsorbs to the Man-MLVs at low temperature.

3.3. Isolation of the plasma factor

Isolation of a plasma factor was attempted by the following procedure: PEG precipitation, ion exchange chromatography on a DEAE-Sepharose column and gel filtration chromatography on a Sephacryl S-300 column. Normal human plasma was subjected to PEG fractionation to obtain a

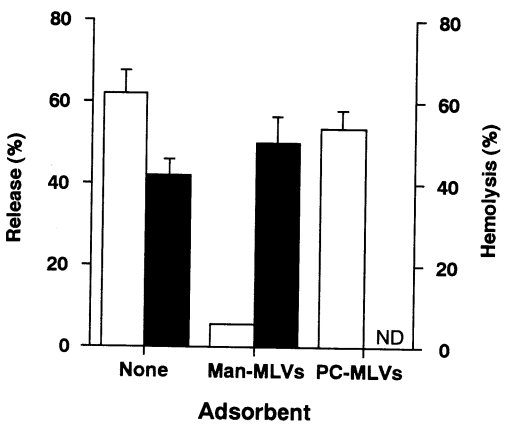

Fig. 2. Effect of pretreatment with liposomes on complement activation by Man-MLVs. Liposomes containing 30 mol% Man (Man-MLVs) or lacking Man (PC-MLVs) adsorbed plasma was incubated with either CF-entrapped Man-MLVs or rabbit erythrocytes for 30 min at 37°C. Both CF release from Man-MLVs (open column) and residual hemolytic activity (closed column) were determined according to the methods described in Section 2 (Materials and methods). Each value represents the mean ± S.D. of three of separate experiments. ND = Not determined.

Table 1
The activity of the plasma components fractionated by PEG-4000 for initating the complement activation

Source of plasma factor		Release (%)	
		Non treated	Protein-G treated
Whole plasma		64.4 ± 1.56	—
Fraction	0–5%	5.84 ± 1.21	—
	5–7.5%	64.4 ± 3.40	64.8 ± 1.61
	7.5–10%	4.40 ± 0.11	—
	10–15%	3.98 ± 0.24	—
	>15%	41.1 ± 7.22	0.04 ± 0.01

Pooled human plasma was fractionated according to the method described in Section 2 (Materials and methods). In each fraction with or without Protein-G preparation, the activity for initiating the C activation was also determined according to the method described in Section 2 (Materials and methods). Each value represents the mean ± S.D. of three separate experiments.

plasma factor capable of activating the C system. In a series of PEG fractionation studies, we found that two fractions, 5–7.5% and >15% PEG, could significantly enhance the C activation (Table 1). The fraction, >15% PEG fraction, has been shown to contain IgG (Freid and Chun, 1971), which indicates that the activity observed in >15% PEG fraction is probably due to IgG, whereas that in another fraction is uncertain. Two fractions, thus, were subsequently treated with Protein-G Sepharose. Although the activity in 5–7.5% PEG fraction remained, that in >15% PEG fraction was diminished following the preparation as expected (Table 1). This finding shows that the activity in >15% PEG fraction results from IgG-dependent C activation, leading to the possibility that a naturally-occurring antibody, particularly IgG, is partly responsible for enhancing the destabilization of Man-MLVs. To further isolate the plasma factor, the 5–7.5% PEG precipitated fraction was applied on a DEAE-Sepharose column. The highest activity was observed in the fraction eluted with DEAE-Sepharose loading buffer containing 135–170 mM NaCl (Fig. 3A). After concentrating the fraction, it was subsequently passed through a Sephacryl S-300 column. The protein was eluted as three minor

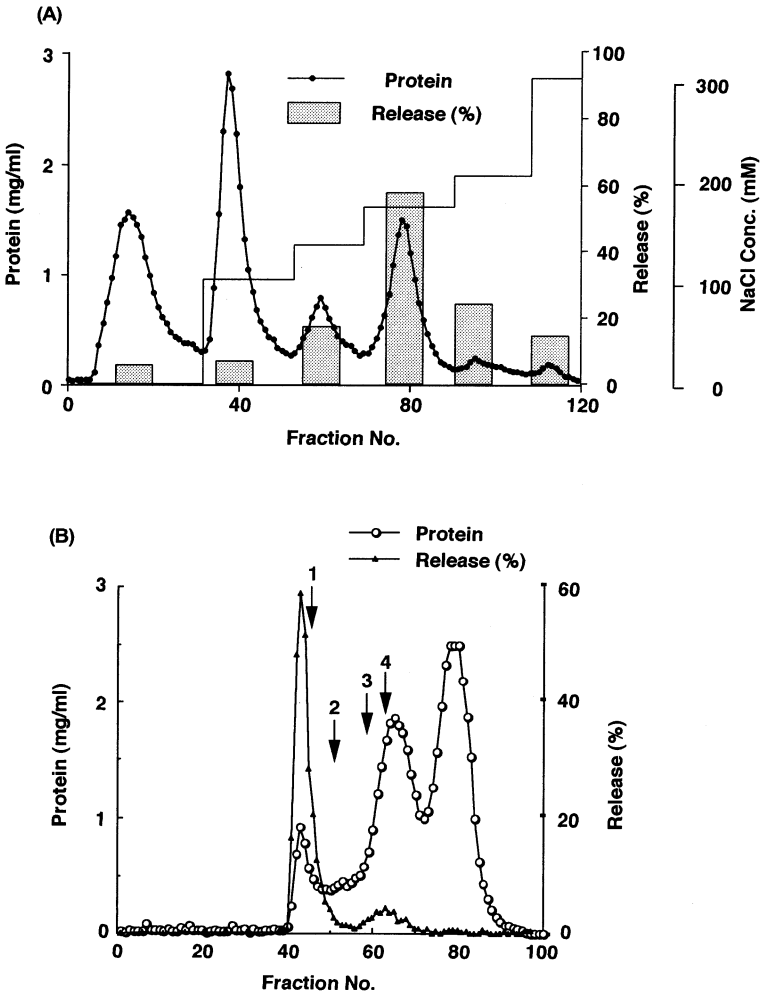

Fig. 3. (A) DEAE-Sepharose chromatography of the plasma components fractionated by PEG precipitation. Concentrated fraction, precipitable by 5–7.5% PEG, was applied to a DEAE-Sepharose column (50 × 25 mm). The column was washed with DEAE-Sepharose loading buffer (20 mM Tris–HCl, pH 8.0) and eluted stepwise with same buffer containing 100, 135, 170, 200 or 300 mM NaCl. Chromatography was carried out at a flow rate of 60 ml/h and fractions of 1 ml were collected. Closed circle (●) and shadow column represent protein and the activity for initiating the C activation, respectively. (B) Gel filtration of isolated plasma factor by an anion exchange chromatography on Sephacryl S-300 column (1.5 × 65 cm) which had been equilibrated with PBS(−). The fractions, which were eluted by the DEAE-Sepharose loading buffer containing 135–170 mM NaCl, were pooled and concentrated. The concentrated fraction was applied to a Sephacryl S-300 column. The flow late was 0.4 ml/min and fractions of 1 ml were collected. Open circle (○) and closed triangle (▲) represent the protein and the activity for initiating the C activation, respectively. The positions of molecular weight markers indicated were established by HMW gel filtration calibration kit (Pharmacia, Uppsala, Sweden): 1 = thyroglobulin 669 kDa; 2 = ferritin 440 kDa; 3 = catalase, 232 kDa; 4 = aldolase, 158 kDa.

peaks and the activity was confined to the first peak around the void volume of the column (Fig. 3B). This observation demonstrates that the plasma factor is a protein with higher molecular weight at least than 669 000 Da of which thyroglobulin used as marker. The results of a typical isolation of the plasma factor are summarized in Table 2.

Table 2
Progress of the plasma factor isolation

Purification step	Protein (mg)	Activity[a] (units)	Yield (%)	Specific activity	(Fold)
Human plasma	1580	100.32	100.0	0.064	(1)
PEG preparation	358.7	59.35	59.2	0.165	(2.57)
DEAE sepharose CL-6B	3.258	10.45	10.4	3.205	(50.08)
Sephacryl S-300	0.828	8.72	8.69	10.53	(164.60)

[a]The activity stands for the ability of the plasma proteins for initiating the C activation. The unit of activity was defined as the dilution ratio in the case of 10% of Man-MLVs was destabilized in a serial diluted sample.

3.4. Characterization of the plasma factor

In order to characterize the plasma factor, we focused on its adsorption mechanism to Man-MLVs for initiating the C activation. The known plasma factors inducing the C activation through the classical pathway have unique mechanisms for binding or adsorbing to their ligands; for example, MBP activates the C system following recognition of mannose or N-acetylglucosamine (GlcNAc) in the presence of Ca^{2+} (Turner, 1994). Hence, we examined the inhibitory effect on adsorption of the plasma factor to Man-MLVs by depletion of Ca^{2+} or addition of soluble sugars (GlcNAc, ManNAc or D-mannose). A 5–7.5% PEG precipitated fraction was subjected to this examination to exclude the IgG contamination. Significant inhibition was not observed in each experiment and the destabilization of Man-MLVs was comparable with that of the case in which the plasma factor-adsorbing Man-MLVs were prepared with a non-treated fraction (Table 3). It has been reported that the depletion of Ca^{2+} influences the binding of MBP, CRP and SAP to their ligands (Pepys et al., 1978; Turner, 1994) and results in the suppression of C activation by them; furthermore, it has been shown that the binding of MBP to its ligand was also inhibited in the presence of soluble sugars, such as ManNAc, GlcNAc or D-mannose (Turner, 1994). Therefore, the findings that significant inhibition was not observed in the absence of Ca^{2+} or presence of soluble sugar suggest that the plasma factor is not analogous to either MBP, CRP or SAP. To further define the plasma factor, the 5–7.5% fraction was treated with Con-A Sepharose. Under this condition, IgM is completely depleted since its 11.8% carbohydrate content is known to form the precipitates with Con-A (Weinstein et al., 1972). The destabilization of Man-MLVs did not decrease after the Con-A Sepharose treatment (Table 3). These results suggest that the plasma factor is a novel human plasma factor initiating the activation of CCP.

Table 3
The effect of either pretreatment (depletion of Ca^{2+}, addition of soluble sugar of removal of IgM) on the reconstitutional experiment

Treatment	Release (%)
None	58.0 ± 3.9
$EGTA/MgCl_2$ (10 mM)	62.4 ± 4.7
GlcNAc (100 mM)	52.6 ± 4.2
ManNAc (100 mM)	54.3 ± 3.3
D-Mannose (100 mM)	56.0 ± 5.2
Con-A Sephrose	50.0 ± 7.7

The 5–7.5% PEG fraction was subjected as a sample to this experiment. One vol of CF-entrapped Man-MLVs (containing 20 μmol/ml of total lipid concentration) was incubated for 30 min at 4°C with 9 vol of the sample with or without various treatment. After the incubation, plasma factor-adsorbd Man-MLVs was recovered from the incubation mixture by cntrifugation (15 000 rpm, 30 min). After the lipid concentration was adjusted to 10 μmol/m, the plasma factor-adsorbed Man-MLVs was incubated for 30 min at 37°C with liposome-adsorbed plasma. The liposome-adsorbed plasma was prepared as describe in Section 2 (Materials and methods). CF release was determined according to the method described in Section 2 (Materials and methods). Each value represents the mean $\pm$ S.D. of three separate experiments.

4. Discussion

The data presented here demonstrate that the human C system is activated by Man-MLVs but not PC-MLVs, and the activation is proceeded through the classical rather than alternative pathway and it is enhanced via adsorption of a plasma factor, C activating factor (mCAF), which could be removed by pretreatment with the Man-MLVs. mCAF was not identical with the known CCP activators, such as immunoglobulins (IgG or IgM), MBP, CRP or SAP, in that the C activation initiated by adsorption of this factor was not inhibited by either depletion of Ca^{2+}, addition of solble sugar or treatment with Con-A Sepharose. Therefore, mCAF is a novel human plasma factor responsible for initiating the C activation.

We have recently reported that the Man-MLVs were destabilized as a consequence of C activation through the alternative pathway in rat plasma and a specific rat plasma factor (mCAF) adsorbed with Man-MLVs was responsible for the C-dependent destabilization of Man-MLVs (Funato et al., 1994). Human mCAF presented here seems to differ from rat mCAF in the mechanisms on C activation; rat mCAF via ACP and human mCAF via CCP. Further, we have indicated another rat factor (CAF) which tended to bind to cholesterol-rich liposomes and has an ability to initiate C activation via ACP (Funato et al., 1994; Ishida et al., 1997; Liu et al., 1997). The results shown in Fig. 1A and Fig. 2 indicate that there is no such CAF in human plasma. Recently, it was reported that the liposome destabilization in plasma or serum and liposome uptake by liver are animal species dependent (Liu et al., 1995, 1997; Harashima et al., 1996; Harashima and Kiwada, 1996; Liu, 1996). Although species difference is a serious problem on predicting the clinical behavior of liposomes in human, their underlying mechanisms are still uncertain. The results presented here and in our earlier reports suggest that species difference is possibly due to differences of the presence, specificity and titer of these plasma factors such as mCAF or CAF which have an ability to initiate C activation among different species, whereas further studies are needed to clarify them.

MBP is a C-type animal lectin which recognizes mannose or GlcNAc via a Ca^{2+}-dependent manner and belongs to the collectin family (Turner, 1994). It has recently been reported that MBP was identical to a component of a Ra-reactive factor (Matsushita et al., 1992) and also could be associated with a novel C1r/C1s-like serine protease designated MASP (MBP-associated serine protease) which has the ability to activate not only C4 and C2 but also C3 directly (Matsushita and Fujita, 1992). Thus, MBP activates the C system in a C1q-independent manner following Ca^{2+}-dependent binding to its ligand. In this study, human mCAF also may be a protein with lectin-like activity, since it specifically adsorbed with the Man-MLVs bearing a carbohydrate moiety (mannose) (Fig. 2). However, the C activating mechanism of mCAF is distinct from that of MBP or MBP-MASP, since the adsorption of the plasma factor to Man-MLVs was observed in the absence of Ca^{2+} or presence of soluble sugars (GlcNAc, ManNAc or mannose) (Table 3) and the C activation initiated by mCAF proceeded through a C1q-dependent classical pathway (Fig. 1B). Moreover, CRP and SAP are known to belong to the pentraxin family and to be able to activate the C system at the C1q level (Baltz et al., 1982; Volanakis, 1982). CRP exhibits Ca^{2+}-dependent binding to pneumococcal C-polysaccharide and phosphorylcholine and SAP binds to zymosan in the presence of Ca^{2+}, and both proteins subsequently promote the C activation through the classical pathway. Although the C activating mechanism of mCAF is analogous with that of CRP and SAP in that they activate the C system at the C1q level, the plasma factor is neither CRP nor SAP, since mCAF significantly adsorbed to the Man-MLVs in the absence of Ca^{2+} (Table 3), and also it had larger molecular weight than both CRP (120–140 kDa) and SAP (150 kDa) (Fig. 3B). mCAF, therefore, belongs to neither the C-type animal lectin nor pentraxin family and seems be a unique factor responsible for initiating the C activation through the classical pathway, although further identification is necessary.

Although mCAF specifically adsorbed to the Man-MLVs (Fig. 2), it has not been determined

that the carbohydrate moiety (Man) in the membrane is the major determinant of reactivity of mCAF, because the competitive inhibition on the adsorption of mCAF did not occur in the presence of soluble sugars (Table 3). This finding suggests that mCAF requirement is unique to the liposome surface. Previously, Hamphries and McConnell (1975) showed that the introducing of Chol into the liposome membrane enhanced hapten exposure on the liposome surface and consequently increased antibody-dependent C fixation. Hence, unique domains are formed by Chol and Man in the membrane and these may play an important role in the liposome-plasma factor interactions. The domains probably construct a dense area of hydroxyl groups, since Chol and Man contain at least a hydroxyl group; in particular, the carbohydrate moiety (Man) has rich-hydroxyl groups. The cluster of hydroxyl groups is assumed to involve the adsorption of mCAF to the liposome surface. Alving et al. (1977) have previously shown an uncharacterized factor; so far as we know, this factor is the only undefined plasma factor responsible for the CCP activation. Interestingly, the uncharacterized factor could be removed by the Chol-rich liposomes (71 mol% Chol), but not by the Chol-poor liposomes (43 mol% Chol). In our experiment, mCAF also could not be depleted by the PC-MLVs containing low levels of Chol (40 mol%) (Fig. 2). It is known that clusters of unobstructed Chol exist in the membrane, when the molar ratio of Chol over other phospholipids amounts to at least 1 (>50 mol% Chol) (Collins and Phillips, 1982). The Chol-rich liposomes is supposed to exhibit the dense area of hydroxyl groups and these areas have the potential ability to interface with the uncharacterized factor. This strongly supports our assumption on the adsorption mechanism of mCAF, whereas it is uncertain whether mCAF is identical with the uncharacterized factor in the present study.

Upon binding to an appropriate recognition site, mCAF is supposed to facilitate the destabilization and opsonization of foreign particles such as liposomes through the activation of the C system, resulting in enhancing the clearance of foreign particles from human circulation. The CRP and SAP are known to promote the activation of the CCP at the C1q level as well as enhance the phagocytosis of invading foreign particles through binding to Fc-receptor on cells of the monocyte/macrophage series, since they have sequence homologies to IgG in the domain responsible for binding of C1q (CH2 domain) and binding to the Fc-receptor on phagocytosis (CH3 domain) (Claus et al., 1977; Levo et al., 1977; Mortensen and Duszkiewicz, 1977). As shown in Fig. 1B, the presence of C1q molecules was essential to the activation of the human C system initiated by mCAF, which indicates that the underlying mechanism of the C activation is similar to that initiated by immunoglobulins (IgG and IgM), CRP or SAP. This leads to the possibility that mCAF has significant sequence homologies to the immunoglobulins, CRP and SAP, although the physiological significance of mCAF is uncertain at present. mCAF, therefore, activates the human C system through the CCP as well as possibly acts as an opsonin through binding to the Fc-receptor on phagocytes, and consequently plays a crucial role in the first-line host defense against certain microorganisms bearing an appropriate recognition site.

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