ELSEVIER

International Journal of Pharmaceutics 224 (2001) 69–79

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Effect of cholesterol content in activation of the classical versus the alternative pathway of rat complement system induced by hydrogenated egg phosphatidylcholine-based liposomes

Tatsuhiro Ishida, Kazumi Yasukawa, Hiroko Kojima, Hideyoshi Harashima [1], Hiroshi Kiwada *

Department of Pharmacokinetics and Biopharmaceutics, Graduate School of Pharmaceutical Sciences, The University of Tokushima, 1-78-1 Sho-machi, Tokushima 770-8505, Japan

Received 21 February 2001; received in revised form 16 May 2001; accepted 20 May 2001

Abstract

Liposomes composed of hydrogenated egg phosphatidylcholine (HEPC) and cholesterol (CHOL) were found to activate the rat complement (C) system in a CHOL content-dependent manner. Liposomes containing 22 or 33 mol% CHOL activated the C system in a Ca^{2+}-dependent manner, suggesting that C activation occurred via the classical pathway. Liposomes containing 44 mol% CHOL activated the C system in a Ca^{2+} independent manner, suggesting that C activation occurred via the alternative pathway. The CHOL content appeared to dictate the pathway by which the C system was activated. This C activation was inhibited by removal of serum component(s), which adsorb to the liposomes. Activation of the alternative pathway, induced by the liposomes, was reduced by the depletion of IgG and IgM, whereas the classical pathway activation was reduced by the depletion of IgG, but not IgM. In addition, the removal of adsorbed serum component(s) by treatment with 44 mol% CHOL-containing liposomes decreased serum IgG and IgM levels that adsorb to the same liposomes, whereas the removal of adsorbed serum component(s) by treatment with 22 mol% CHOL-containing liposomes only slightly decreased serum IgG levels, which adsorbs to the same liposomes. Collectively, both IgG and IgM, which are specifically adsorbed to the liposomes in a CHOL-content dependent manner, were responsible for C activation via the alternative pathway induced by the 44 mol% CHOL containing liposomes. IgG alone would be partially responsible for C activation via the classical pathway induced by 22 or 33 mol% CHOL-containing liposomes. The discovery of this unique C-activating property of liposomes will be

Abbreviations: Ab, antibody; C, complement; CF, 5(6)-carboxyfluorescein; CHOL, cholesterol; CRP, C-reactive protein; DCP, dicetylphosphate; E, erythrocytes; [^{3}H]-CHE, [^{3}H]-cholesterylhexadecyl ether; HEPC, hydrogenated egg phosphatidylcholine; MAC, membrane attack complex; PL, phospholipid.

* Corresponding author. Tel.: +81-886-33-7260; fax: +81-886-33-5190.

E-mail address: hkiwada@fc.ph.tokushima-u.ac.jp (H. Kiwada).

[1] Present address: Department of Molecular Design and Pharmaceutics, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12, Nishi 6, Sapporo 060-0812, Japan.

PII: S0378-5173(01)00737-2

of value in attempts to decipher the underlying mechanism of C activation by providing a useful model membrane system.

Keywords: Liposomes; Drug delivery system; Complement activation; Antibodies

1. Introduction

The complement (C) system plays a crucial role in humoral defense against infectious agents such as microbial pathogens (Müller-Eberhard, 1975, 1988; Porter and Reid, 1979; Reid, 1983). The deposition of activated C3 fragments (C3b or iC3b) onto microbial surface leads to two major effects, (I) the components serve as opsonins for C3 receptor-bearing phagocytes; and (II) they act as a stable C5 convertase, resulting in the formation of a membrane attack complex (MAC), which is capable of lysing susceptible bacteria. The C system can be activated by any of three pathways, designated the alternative, classical and lectin pathways (Szebeni, 1998).

Liposomes have been utilized as potent drug carriers, which improve the therapeutic index of the drug associated with them. Liposomal formulations of some anti-cancer drugs, most importantly sterically stabilized liposomal doxorubicin (Doxil®), have been reported to cause immediate hypersensitivity reactions that cannot be explained by the conventional paradigm of IgE-mediated (type-I) allergy (Uziely et al., 1995; Alberts and Garcia, 1997; Skubitz and Skubitz, 1998). Recently, Szebeni et al. (2000) presented a rationale and experimental evidence for the concept that these novel-induced hypersensitivity reactions can be referred to as a C activation-related pseudoallergy. Therefore, it is important to understand the interaction of the C system with liposomes when considering liposomes for use as drug carrier systems, since C activation will reduce the effectiveness of liposomes by assisting the body in removal of the drug carrier from circulation (Szebeni, 1998) as well as causing the hypersensitivity reactions related to C activation (Szebeni et al., 2000).

To date, activation of C system by liposomes that do not contain any specific ligands has been reported for the human (Alving et al., 1977; Chonn et al., 1991; Marjan et al., 1994), the rat (Devine et al., 1994; Funato et al., 1992; Harashima et al., 1996; Ishida et al., 1997) and the guinea pig (Chonn et al., 1991; Huong et al., 2001). Chonn et al. (1991) reported that for both human and guinea pig serum the surface charge is a key determinant of C-activating liposomes; negatively charged liposomes activate the C system via the classical pathway, while positively charged liposomes activate the C system via the alternative pathway. In rat serum, however, both negatively and positively charged liposomes activated the C system via the classical pathway (Devine et al., 1994; Szebeni et al., 1994). In our earlier investigations, activation of the C system via the alternative pathway was observed in rat plasma or serum by negatively charged liposomes composed of hydrogenated egg phosphatidylcholine (HEPC), cholesterol (CHOL) and dicetylphosphate (DCP) (Funato et al., 1992). The apparent inconsistencies in the C activation by liposomes might be due to differences in experimental conditions (e.g. liposome composition, liposome size or animal species studied). This suggests the importance of the specific conditions employed in the study of liposome-induced C activation. In this study, therefore, controlled experimental conditions were used to investigate the induction of C activation, based on the chemical and physical properties of liposomes. Three compositionally different liposomes were prepared. Each contained saturated phospholipid (PL) and 11 mol% DCP to maintain identical surface net charges. The CHOL content was varied (22, 33 or 44 mol%) and the size was maintained at a mean diameter of 800 nm. We chose to concentrate on the CHOL content, because of its widespread distribution in mammalian membranes and its ability to affect the fluidity of the biomembrane. Our findings show, using rat serum, that the CHOL content in the liposome composition appears to dictate the pathway by which the C system is activated; the 22 and 33

mol% CHOL-containing liposomes activate the C system via the classical pathway whereas 44 mol% CHOL-containing liposomes activate the C system via the alternative pathway. We also show that natural Abs are responsible for C activation induced by the liposomes.

2. Materials and methods

2.1. Preparation of liposomes

Multilamellar vesicles (MLVs) composed of HEPC/CHOL/DCP (44:44:11 mol/mol), HEPC/CHOL/DCP (55:33:11 mol/mol) and HEPC/CHOL/DCP (66:22:11 mol/mol) were prepared by an extrusion procedure through a Nuclepore polycarbonate filter having a defined pore size (800 nm) (Costar, MA, USA) as described in detail elsewhere (Funato et al., 1992). HEPC was kindly donated by Nippon Fine Chem. (Osaka, Japan). DCP was purchased from Nacalai Tesque (Kyoto, Japan). CHOL was of analytical grade from Wako Pure Chem. (Osaka, Japan). These lipids were used without further purification. 5(6)-Carboxyfluorescein (CF) (Eastman Kodak Co., NY, USA) was encapsulated into the liposomes in order to investigate their destabilization. Once prepared, the liposomes were dialyzed in cellulose dialyzing tubing against PBS(−) (Na_2HPO_4 8.10 mM, NaH_2PO_4 1.47 mM, NaCl 137 mM, KCl 2.68 mM: pH 7.4, PBS(−)) at 4 °C to remove unencapsulated CF. For preparing the liposome-treated rat serum, liposomes were hydrated by using PBS(−) in place of the CF solution. These liposomes were suspended at a concentration of 20 μmol/ml in PBS(−). The average size of the liposomes was 775 ± 65 nm ($n = 6$) as determined with a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). A nonexchangeable, radiolabeled lipid marker, [^{3}H]-cholesterylhexadecyl ether (CHE) (NEN, CA, USA) (0.02 μCi/μmol of total lipid), was incorporated to quantify the concentration of liposomes (Pool et al., 1982). The specific radioactivity of liposomes was determined by measuring their radioactive content using standard liquid scintillation counting methods.

2.2. Serum and antibodies

Blood was collected from the carotid arteries of Wistar male rats (Inoue Experimental Animal, Kumamoto, Japan) through polyethylene tubing into a test tube and allowed to clot at room temperature for 30 min. Serum was prepared by centrifugation (3000 rpm, 30 min, 4 °C), which removed blood cells and other insoluble debris, and stored at 4 °C until used. Peroxidase-conjugated anti-rat C3 goat IgG, anti-rat IgG (whole molecule) rabbit IgG and anti-rat IgM (μ-chain) goat IgG were purchased from Organon Teknika (PA, USA).

2.3. Pretreatment of rat serum

Liposome-treated serum was prepared according to previously described methods (Funato et al., 1994). In brief, 300 μl of liposomes (20 μmol/ml) was incubated with 2700 μl of rat serum at 4 °C for 30 min. The liposome/serum mixture was centrifuged (60 min, 3000 rpm, 4 °C) (Hitachi Himac CR5B2, Tokyo, Japan) and the upper layer, which comprised the liposome/serum proteins complex was removed by aspiration. The clear serum was pooled as liposome-treated serum and stored at 4 °C. The hemolytic activity of the liposome-treated serum remained unchanged after the incubations and centrifugation (data not shown)

Serum, depleted of Ca^{2+} and Mg^{2+}, was obtained by treatment of rat serum with EDTA (10 mM, pH 7.4) for 5 min. Ca^{2+}-depleted serum was obtained by treatment with 10 mM EGTA containing 100 mM $MgCl_2$ for 5 min (pH 7.4). C-inactivated serum was obtained by heating at 56 °C for 30 min.

Natural Abs (IgG or IgM)-depleted sera were prepared by incubation of rat serum with either anti-rat IgG Ab or anti-rat IgM Ab. In brief, 300 μl of Ab solution (10 mg/ml) was incubated with 2700 μl of rat serum at 4 °C for 4 h. The incubation mixtures were centrifuged (60 min, 13000 rpm, 4 °C) (Hitachi Himac CR15D, Tokyo, Japan) and the clear serum was separated from the sediment, pooled and stored at 4 °C. The absence of IgG was confirmed by sodium dodecyl

sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). All bands detected in normal rat serum were also detected in the treated serum with the exception of those bands corresponding to IgG. A sample of pure IgG was run on the same gel to confirm that the molecular weight of the depleted protein corresponded to that of IgG. The depletion of IgG was further confirmed by Westernblot analysis under non-reducing conditions; a 150 kDa band, reactive with anti-rat IgG Ab in untreated serum, was not detected in the treated serum. Similarly, the depletion of IgM was confirmed by Westernblot; under reducing conditions, the band which had been reactive with anti-rat IgM Ab in untreated serum was no longer detectable in the treated serum.

2.4. Liposome-destabilization assay in rat serum

The liposome-destabilization assay is described in detail elsewhere (Funato et al., 1992). An amount of 50 μl of CF encapsulated liposome (6 μmol/ml) was added to 450 μl of either untreated serum (90%, v/v), liposome-treated serum or natural Abs-depleted serum. The resulting mixture was then incubated for 30 min at 37 °C. The fluorescence intensity of 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 fluorescence intensity of the total releasable CF was measured by lysing the liposomes with 5% (v/v) Triton X-100 solution. The extent of release of CF from liposomes (release (%)) was calculated by dividing the fluorescence intensity of the reaction mixture by the fluorescence intensity of total releasable CF.

2.5. Complement hemolytic assays

Total or residual C hemolytic activity in either untreated or liposome-treated serum was measured using established methodology (Tanaka et al., 1987; Funato et al., 1994). The C induced hemolysis of rabbit erythrocytes (E), involve activation via the entire alternative pathway and the level of hemolysis is proportional to the level of C activity present in the serum (Tanaka et al., 1987; Funato et al., 1994). A reduction in C hemolysis activity in the serum, after the removal of the serum component(s) which adsorb to the liposomes implies the depletion of a C system initiator. In brief, 200 μl of rabbit E solution (5×10^8 cells per ml) was incubated at 37 °C for 60 min with 800 μl of either untreated or liposome-treated rat serum. After the incubation, 2 ml of cold PBS(−) containing 40 mM EDTA (pH 7.4) was added to terminate the reaction. The reaction mixture was centrifuged at 3000 rpm for 11 min to remove unlysed E. Hemolysis (%) was evaluated by measuring the absorbance of the supernatant at 542 nm.

2.6. Westernblot analysis of C3 fragments associated with liposomes after incubation with serum

To 1 ml of a liposome suspension (20 μmol/ml), 9 ml of untreated serum was added and the resulting liposome/serum mixture was incubated at 37 °C for 15 min, in order to detect C3 binding to the liposomes. The liposomes were isolated by centrifugation (12 000 rpm, 30 min, 4 °C) after the addition of 40 ml of ice-cold PBS(−) to the incubation mixture, followed by three washings with 50 ml portions of cold PBS(−). The final liposome pellet was dissolved in 700 μl PBS(−) and the liposome concentration was adjusted to 10 μmol/ml with PBS(−) by measuring the radioactivity of the [^{3}H]-CHE incorporated into the liposomes. Proteins were extracted from the liposomes (5 μmol) according to a published method (Wessel and Flugge, 1984). In brief, 4 ml of methanol, 1 ml of chloroform, and 1.5 ml of distilled water was added to 1 ml of the resuspended liposomes with vortexing after each addition. The resulting two phase-system was separated by centrifugation (20 min, 3000 rpm). The majority of the upper phase was removed by aspiration but a slight amount was left, to cover the protein at the interface. Then, 1.5 ml of methanol was added, resulting in the protein precipitated. The mixture was subjected to centrifugation (30 min, 3000 rpm) and the supernatant removed by aspiration leaving the pellet, which was subsequently dried under nitrogen. The dried

pellet was resuspended in 1 ml of PBS(−) containing 0.5% SDS (w/v). Protein separation was performed by SDS-PAGE on 7.5% polyacrylamide gels. Prestained SDS-PAGE standards (Bio-Rad, CA, USA) were used to estimate the molecular weight of the proteins. For immunodetection of C3 fragments, the SDS-PAGE separated proteins were electrophoretically transferred onto a nitrocellulose membrane. The blotted nitrocellulose membrane was incubated in blocking buffer, which consisted of PBS(−) containing 1% (w/v) dried skim milk, for 30 min at room temperature. The membrane was then incubated with 5000-fold diluted peroxidase-conjugated anti-rat C3 goat IgG for 4 hr at room temperature. The dilution buffer consisted of PBS(−), which contained 0.1% (w/v) BSA. Finally, the labeled C3 fragments (C3b/iC3b) were developed via the use of ECL Western blotting detection reagents (Amersham, England). The Westernblot analyses were repeated twice.

2.7. Solid-phase ELISA for the detection of natural Abs (IgG or IgM) reactive with the liposomes

Solid-phase ELISA, to ascertain the identity of the natural Abs that have reactivity to the liposomes, was carried out according to methods described earlier (Szebeni et al., 1994) with minor modifications. In brief, the wells of a 96-well plate (Nunc-immuno plate, Nunc, Denmark) were coated with a liposome suspension (100 nmol/ml) by incubation overnight at 50 °C. After cooling to room temperature, the plates were blocked with 100 μl of 0.3% (w/v) gelatin in PBS(−) for 3 h at room temperature, then washed with PBS(−), and subsequently incubated with 100 μl of untreated or liposome-treated serum at a specific dilution ratio overnight at 4 °C. The plates were then washed three times with PBS(−), and 100 μl of 3000-fold diluted peroxidase-conjugated anti-rat IgG (whole molecule) goat IgG or peroxidase-conjugated anti-rat IgM (μ-chain) rabbit IgG. After incubation for 2 h at room temperature, the plates were washed three times with PBS(−). Color was developed by adding *o*-phenylene diamine (OPD) (1 mg/ml) in substrate buffer (citric acid 36.4 mM, Na_2HPO_4 66.7 mM) with 0.018% H_2O_2. After incubation for 5 min at room temperature, the reaction was terminated by adding 100 μl of 1 M H_2SO_4 and the absorbance at 490 nm was measured using a Microplate Reader (Model 450, Bio-Rad).

2.8. Statistics

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

3. Results and discussion

3.1. CHOL content-dependent C activation induced by liposomes

The release of an entrapped aqueous and membrane impermeable dye, CF (Haugland, 1992), from liposomes, which is directly related to the extent of liposome destabilization was determined following incubation at 37 °C for 30 min in either untreated rat serum, treated serum or buffer (PBS(−)) (Table 1). In untreated serum, the liposomes released their contents (CF), and the extent of CF release increased with increasing CHOL content of the liposomes. The release of CF from liposomes of all compositions was higher in the presence of untreated serum, compared with those in the absence of serum (in PBS(−)). In addition, the release of CF from the liposomes was diminished when either heated serum or divalent cation (Ca^{2+} and Mg^{2+})-depleted serum was used. These findings indicate that heat labile serum component(s) and divalent cations (Ca^{2+} and Mg^{2+}) are involved in the destabilization of the liposomes in rat serum. It is well-known that C activity is completely inactivated by heating serum at 56 °C for 30 min (Okada et al., 1982) as well as by excluding the divalent cations (Ca^{2+} and Mg^{2+}) from serum (Okada et al., 1982). Collectively, it appears that the release of CF from the liposomes was caused by CHOL-content dependent C activation induced by the liposomes.

To further verify that the destabilization of liposomes occurs as a result of C activation, resulting in the fixation of the activated components

Table 1
Effect of serum treatment on release of entrapped CF from the liposomes containing various cholesterol contents

Serum treatment	Release (%)[a]		
	HEPC/CHOL/DCP (66:22:11)	HEPC/CHOL/DCP (55:33:11)	HEPC/CHOL/DCP (44:44:11)
Untreated	18.5 ± 3.4	30.3 ± 1.5	40.1 ± 2.0
PBS(−)	3.4 ± 0.2***	2.9 ± 0.3***	5.9 ± 0.3***
EDTA (10 mm)	3.0 ± 0.6***	3.3 ± 0.5***	4.8 ± 0.4***
56 °C/30 min	3.0 ± 0.1***	4.1 ± 0.1***	6.9 ± 0.1***

Liposomes were incubated at 37 °C for 30 min with untreated serum, treated serum (90% v/v) or PBS(−). The fluorescence intensity of released CF was determined as described in Section 2 and expressed as a percentage of the total releasable CF by addition of 5%(v/v) Triton-X solution.

[a] Values represent mean ± S.D. from three independent experiments. ***, $P<0.005$ vs. untreated serum.

onto the liposomal surface, C3 fragments, one of major components of the C system, which were associated with the liposomes after incubation in untreated serum at 37 °C were isolated and analyzed by Westernblot analysis. Four bands (43, 68, 75 and 116 kDa) corresponding to C3 (Wessel and Flugge, 1984) were detected under reducing conditions (data not shown).

3.2. Activation pathway of the C system induced by the liposomes

The effect of Ca^{2+}-depletion on the destabilization of the liposomes was examined. The destabilization of both liposomes containing both 22 and 33 mol% CHOL was nearly completely abolished in Ca^{2+}-depleted serum, compared with that in untreated serum, whereas the destabilization of the liposomes containing 44 mol% CHOL was not affected (Fig. 1). The liposome-destabilization pattern changed as a function of the CHOL content. By excluding Ca^{2+} from the serum, the alternative pathway activation can be distinguished from the classical pathway activation since the initiation complex of the classical pathway (C1qr2s2) requires Ca^{2+} (Müller-Eberhard, 1975). Therefore, we concluded that the liposomes, which contain 22 or 33 mol% CHOL activated the C system via the classical pathway and the liposomes containing 44 mol% CHOL activated the C system via the alternative pathway. It appeared that the CHOL content dictates the pathway by which the C system was activated.

3.3. Serum component(s) involved in the C activation which are induced by the liposomes

Rat serum was incubated with each of the three liposome formulations at 4 °C for 30 min. Subsequently, the liposomes, along with any surface-adsorbed proteins, were removed by centrifugation (liposome-treated serum). Finally, the C-depen-

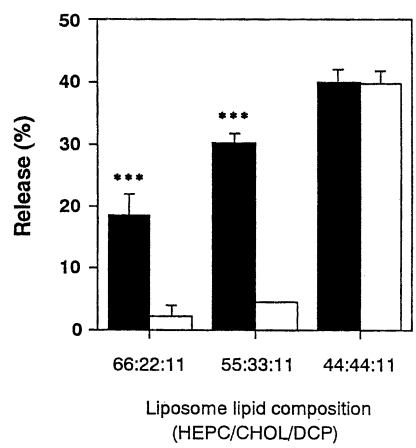

Fig. 1. Complement-mediated destabilization of liposomes in Ca^{2+}-depleted serum. Carboxyfluorescein (CF) entrapped liposomes composed of either HEPC/CHOL/DCP (66:22:11), HEPC/CHOL/DCP (55:33:11) or HEPC/CHOL/DCP (44:44:11) were incubated at 37 °C for 30 min with either 90% untreated serum (filled bars) or Ca^{2+}-depleted serum (open bars). The fluorescence activity due to the released CF from liposomes was determined as described in Section 2, and is expressed as a percentage of the total releasable CF by the addition of 5% (v/v) Triton X-100 solution. Values represent mean ± S.D. ($n=3$). ***, $P<0.005$ vs. Ca^{2+}-depleted serum.

dent destabilization of each liposome formulation was determined in each of the liposome-treated sera at 37 °C (Fig. 2A). The removal of serum component(s), which are adsorbed to the 22 mol% CHOL-containing liposomes significantly reduced the destabilization of, not only the same liposomes, but the 33 mol% CHOL-containing liposomes as well, whereas it had very little effect on the destabilization of the 44 mol% CHOL-containing liposomes (Fig. 2A, column 2). The removal of serum component(s), which adsorbed to the 33 mol% CHOL-containing liposomes significantly reduced the destabilization of, not only the same liposomes but 22 mol% CHOL-containing liposomes as well (Fig. 2A, column 3). This treatment also exhibited an inhibitory effect on the destabilization of 44 mol% CHOL-containing liposomes. The removal of serum component(s), which adsorbed to the 44 mol% CHOL-containing liposomes significantly reduced the destabilization of the liposomes (Fig. 2A, column 4). In all cases, the removal of serum component(s), which adsorbed to the liposomes significantly reduced the C activation induced by the same liposomes. In addition, C activation via the alternative pathway, which was induced by the 44 mol% CHOL-containing liposomes, was also reduced by this treatment in a CHOL content-dependent manner. These results indicate that serum component(s), which normally adsorb to the liposomal surface are involved in the initiation of individual C activation induced by the liposomes. It should be noted that the removal of serum component(s), which adsorbed to the 22 mol% CHOL-containing liposomes significantly reduced the classical pathway activation without reducing the alternative pathway activation (Fig. 2A,

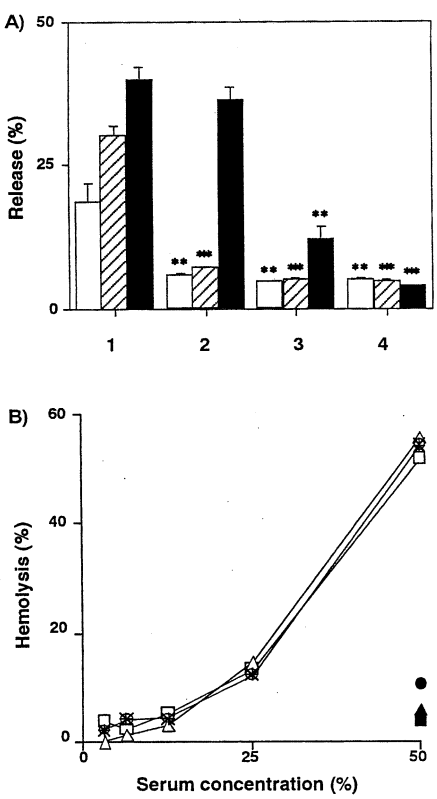

Fig. 2.

Fig. 2. Effect of removal of serum component(s) adsorbed to the liposomes from serum on either complement-mediated destabilization of the liposomes or hemolysis of rabbit erythrocyte. (A) Carboxyfluorescein (CF) entrapped liposomes composed of either HEPC/CHOL/DCP (66:22:11) (open bars), HEPC/CHOL/DCP (55:33:11) (hashed bars) or HEPC/CHOL/DCP (44:44:11) (filled bars) were incubated at 37 °C for 30 min in untreated serum (column-1), the liposome-treated serum (90% v/v) in which the serum component(s) which are adsorbed to the liposomes composed of HEPC/CHOL/DCP (66:22:11) were removed (column-2), the liposome-treated serum (90% v/v) in which the serum component(s) which are adsorbed to the liposomes composed of HEPC/CHOL/DCP (55:33:11) were removed (column-3), and the liposome-treated serum (90% v/v) in which the serum component(s) adsorbed to the liposomes composed of HEPC/CHOL/DCP (44:44:11) were removed (column-4). The liposome-treated serum was prepared as described in Section 2. The fluorescence activity due to the released CF was determined as described in Section 2, and is expressed as a percentage of the total releasable CF by the addition of 5% (v/v) Triton X-100 solution. Values represent mean ± S.D. ($n = 3$). **, $P < 0.01$; ***, $P < 0.005$ vs. untreated serum. (B) Rabbit E (5×10^8 cells per ml) were incubated at 37 °C for 30 min with untreated serum (★) or each liposome (HEPC/CHOL/DCP (66:22:11))-treated serum (○), liposome (HEPC/CHOL/DCP (55:33:11))-treated serum (△) and liposome (HEPC/CHOL/DCP (44:44:11))-treated serum (□) in the absence (open symbol) or presence of 10 mM EDTA treatment (filled symbol), diluted with PBS(+) (PBS(−) with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$) as indicated. The liposome-treated serum was prepared as described in Section 2. The remaining hemolytic activities were expressed as a percentage of hemolysis in each sera.

column 2). This indicates that rat serum contains at least two separate serum components, which are responsible for activation of the classical and alternative pathway.

There is a possibility that the non-specific binding of C components to the liposomes due to electrostatic and/or hydrophobic interactions and their subsequent removal by centrifugation decreased the C activity in the serum, thereby reducing C-dependent liposome destabilization. To test this, the hemolysis of rabbit E with the adsorbable protein-removed sera (liposome-treated sera) was examined. A reduction in the remaining hemolytic activity signifies the depletion or consumption of C component(s) by such treatment. Diluting untreated serum with PBS(+) decreased the levels of hemolytic activity (Fig. 2B). The dilution curve for hemolysis in each liposome-treated sera was not reduced when compared with that in untreated serum. Indeed, the hemolytic activity of serum with or without the removal of liposome-adsorbed serum component(s) was reduced by excluding the divalent cations (Ca^{2+} and Mg^{2+}). Therefore, the reduction of C activation by the removal of serum component(s), which adsorbed to the liposomes (Fig. 2A) was not the result of the depletion or consumption of C component(s) required for liposome destabilization.

3.4. Contribution of natural Ab to the liposome-induced C activation

A likely candidate for the serum component responsible for the initiation of liposome-induced C activation is natural Ab, particularly in view of reports that sera from some animal species contain Abs of the IgG and IgM classes which are reactive with liposomes (Wassef et al., 1990; Alving and Swartz, 1991). Therefore, the destabilization of the 22 mol% CHOL-containing liposomes (classical pathway activator) and the 44 mol% CHOL-containing liposomes (alternative pathway activator) was examined in IgG- or IgM-depleted serum. The extent of destabilization of these liposomes was significantly reduced by IgG-depletion (75.9% inhibition for 44 mol% CHOL-containing liposomes, 44.9% inhibition for 22 mol% CHOL-containing liposomes) (Table 2). In the use of IgM-depleted serum, a reduction was observed in 44 mol% CHOL-containing liposomes (87.4% inhibition) and little reduction was observed in the 22 mol% CHOL-containing liposomes (5.7% inhibition) (Table 2). These results suggest that IgG and IgM class natural Abs contribute to the alternative pathway activation, which is induced by the 44 mol% CHOL-containing liposomes and the IgG class of natural Abs contributes, in part, to the activation of the classical pathway. Furthermore, IgG and IgM class natural Abs, which are both associated with each liposome formulation (22 or 44 mol% CHOL-containing liposomes) were determined with solid phase ELISA. This assay showed the presence of natural Abs (IgG and IgM), which adsorb to the liposomes in rat serum (Fig. 3). The amount of natural Abs associated with 44 mol% CHOL-containing liposomes was larger than those associated with 22 mol% CHOL-containing liposomes. These findings, along with earlier results in this study, suggest that natural Abs affect the C activation induced by the liposomes and play an important role in liposome-induced C activation. The removal of

Table 2
Effect of depletion of natural antibodies from rat serum on C-mediated release of entrapped CF from the liposomes containing of various cholesterol contents

Serum treatment	Release (%)[a]	
	HEPC/CHOL/DCP (66:22:11)	HEPC/CHOL/DCP (44:44:11)
Untreated	45.5 ± 2.7	62.0 ± 5.3
IgM-depleted	42.9 ± 1.3	7.8 ± 0.2***
IgG-depleted	25.7 ± 0.6**	14.9 ± 0.5***

Either anti-rat IgG Ab or anti-rat IgM Ab was incubated at 4 °C for 4 h with rat serum and then the mixture was centrifuged to remove insoluble conjugate. Carboxyfluorescein (CF) containing liposomes composed of HEPC/CHOL/DCP (66:22:11) or HEPC/CHOL/DCP (44:44:11) were incubated at 37 °C for 30 min with the IgG-depleted or IgM-depleted serum. The fluorescence intensity due to the released CF was determined as described in Section 2, and expressed as a percentage of the total releaseable CF by the addition of 5%(v/v) Trition X-100 solution.

[a] Values represent mean ± S.D. from three independent experiments. **, $P<0.01$; ***, $P<0.005$, vs. untreated serum.

serum component(s) which adsorbed to the 44 mol% CHOL-containing liposomes reduced the amounts of natural Abs (both IgG and IgM classes) associated with the same liposomes, while the removal of serum component(s) which adsorbed to the 22 mol% CHOL-containing liposomes reduced the amount of IgG, not IgM, associated with the same liposomes (Fig. 3). The reduction patterns were similar to the reductions found for the destabilization of liposomes in either IgG- or IgM-depleted serum (Table 2). Therefore, it appears that the IgG and IgM class natural Abs both contribute to C activation via the alternative pathway, which is induced by 44

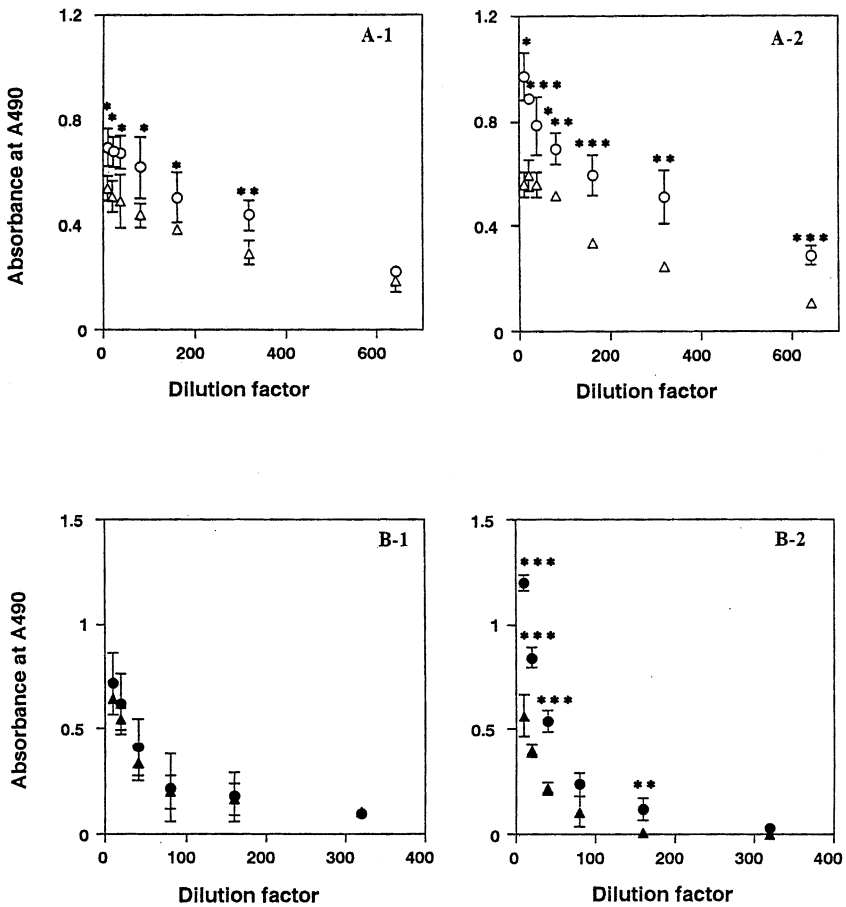

Fig. 3. Natural antibodies in rat serum, which have a reactivity with the liposomes in untreated or the liposome-treated serum. The plate coated with liposomes composed of either HEPC/CHOL/DCP (66:22:11) or HEPC/CHOL/DCP (44:44:11) was incubated with untreated serum or liposome-treated serum overnight. The liposome-treated serum was prepared as described in Section 2. The detectable Abs (IgG or IgM) reactive with the liposomes were determined with the peroxidase-conjugated anti-rat IgG goat IgG or peroxidase-conjugated anti-rat IgM rabit IgG. (A-1) Detectable IgG class natural Abs reactive with the liposomes (HEPC/CHOL/DCP (66:22:11)) in untreated serum (○) or in the liposome (HEPC/CHOL/DCP (66:22:11))-treated serum (△). (A-2) detectable IgG class natural Abs reactive with the liposomes (HEPC/CHOL/DCP (44:44:11)) in untreated serum (○) or in the liposome (HEPC/CHOL/DCP (44:44:11))-treated serum (△). (B-1) detectable IgM class natural Abs reactive with the liposomes (HEPC/CHOL/DCP (66:22:11)) in untreated serum (●) or in the liposome (HEPC/CHOL/DCP (66:22:11))-treated serum (▲). (B-2) detectable IgM class natural Abs reactive with the liposomes (HEPC/CHOL/DCP (44:44:11)) in untreated serum (●) or in the liposome (HEPC/CHOL/DCP (44:44:11))-treated serum (▲). Values represent mean ± S.D. for triplicate wells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

mol% CHOL-containing liposomes, and IgG class natural Ab plus another adsorbable serum component(s) contributes to C activation via the classical pathway, which is induced by 22 and 33 mol% CHOL-containing liposomes.

A possible candidate for the other, presently unknown, serum component responsible for the C activation via classical pathway induced by the 22 or 33 mol% CHOL-containing liposomes is C-reactive protein (CRP). CRP exhibits Ca^{2+}-dependent binding to pneumococcal C-polysaccharide and phosphorylcholine, and subsequently promotes classical pathway activation at the C1q level (Volanakis, 1982). Liposomes, which contain large amounts of phosphatidylcholine exhibit dense areas of phosphorylcholine groups in the membrane (Collins and Phillips, 1982). Such dense areas could mediate the binding of CRP onto liposomal membrane and thereby cause Ab-independent classical pathway activation. The involvement of CRP in the liposome-induced classical pathway activation is presently under investigation in our laboratory.

The findings presented here show that the amount of CHOL in the liposomes is a determinant of the activation pathway of the C system (classical vs. alternative pathway). This suggests that optimal membrane properties might exist, such as fluidity, which serve as requirements for the binding of serum component(s) to initiate C activation via the alternative pathway or classical pathway. The fluidity of biological and liposomal membranes is largely determined both by the PL fatty acyl unsaturation and by the CHOL content of the membrane (Oldfield and Chapman, 1971). The fluidity of the liposomes used here would be expected to be increased by the addition of CHOL into a membrane composed of saturated PL (HEPC); however, we found previously that the fluidity remained virtually unchanged by the addition of CHOL (Nagayasu et al., 1996), indicating that the change in the extent of C activation and the activation pathway, which is dependent on the CHOL content, are not solely due to the fluidity of the liposomal membrane. It has been shown that changes in membrane fluidity affect both the initiation of C activation and the assembly of membrane attack complex of C on liposome membranes (Humphries and McConnell, 1975). This complication has prevented our understanding of the underlying mechanism of C activation by liposome systems. The liposome system described here represents a potentially useful model with which to study the underlying mechanism of the activation of the C system because the fluidity remains virtually unchanged (Nagayasu et al., 1996).

CHOL has widely been studied as a fundamental and ubiquitous constituent because of its widespread distribution and important biological roles. Recently, CHOL-dependent C activation has been implicated as a possible mechanism for the pathogenesis of atherosclerosis (Alving, 1998; Alving and Swartz, 1991). Furthermore, natural Abs to CHOL would serve a useful normal function through C activation for removing debris derived from damaged or dead cells due to either necrosis or apoptosis. Therefore, the liposomal system described here represents a potentially useful tool for investigating how C activation occurs in vivo and how the C system plays a role in the pathogenesis of atherosclerosis and for removing debris derived from self-cells in vivo.

Acknowledgements

The authors are grateful to Dr Milton S. Feather for helpful comments and English corrections on the manuscript for this paper.

References

Alberts, D.S., Garcia, D.J., 1997. Safety aspects of pegylated liposomal doxorubicin in patients with cancer. Drugs 4 (Suppl.), 30–45.

Alving, C.R., 1998. Antibodies to cholesterol: principles for practical application. In: Janoff, A.D. (Ed.), Liposomes: Rational Design. Marcel Dekker, New York, pp. 159–180.

Alving, C.R., Swartz, G.M. Jr, 1991. Antibodies to cholesterol, cholesterol conjugates, and liposomes: implications for atherosclerosis and autoimmunity. Crit. Rev. Immunol. 10, 441–453.

Alving, C.R., Richards, R.L., Guirguis, A.A., 1977. Cholesterol-dependent human complement activation resulting in damage to liposomal model membranes. J. Immunol. 118, 342–347.

Chonn, A., Cullis, P.R., Devine, D.V., 1991. The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. J. Immunol. 146, 4234–4241.

Collins, J.J., Phillips, M.C., 1982. The stability and structure of cholesterol-rich codispersions of cholesterol and phosphatidylcholine. J. Lipid Res. 23, 291–298.

Devine, D.V., Wong, K., Serrano, K., Chonn, A., Cullis, P.R., 1994. Liposome-complement interactions in rat serum: implications for liposome survival studies. Biochim. Biophys. Acta 1191, 43–51.

Funato, K., Yoda, R., Kiwada, H., 1992. Contribution of complement system on destabilization of liposomes composed of hydrogenated egg phosphatidylcholine in rat fresh plasma. Biochim. Biophys. Acta 1103, 198–204.

Funato, K., Yamashita, C., Kamada, J., Tominaga, S., Kiwada, H., 1994. Plasma factor triggering alternative complement pathway activation by liposomes. Pharm. Res. 11, 372–376.

Harashima, H., Huong, T.M., Ishida, T., Manabe, Y., Matsuo, H., Kiwada, H., 1996. Synergistic effect between size and cholesterol content in the enhanced hepatic uptake clearance of liposomes through complement activation in rats. Pharm. Res. 13, 1702–1707.

Haugland, R.P., 1992. Handbook of Fluorescent Probes and Research Chemicals. Molecular Probes, Oregon.

Humphries, G.M., McConnell, H.M., 1975. Antigen mobility in membranes and complement-mediated immune attack. Proc. Natl. Acad. Sci. USA 72, 2483–2487.

Huong, T.M., Ishida, T., Harashima, H., Kiwada, H., 2001. The complement system enhances the clearance of phosphatidylserine (PS)-liposomes in rat and guinea pig. Int. J. Pharm., 215, 197–205.

Ishida, T., Funato, K., Kojima, S., Yoda, R., Kiwada, H., 1997. Enhancing effect of cholesterol on the elimination of liposomes from circulation if mediated by complement activation. Int. J. Pharm. 156, 27–37.

Marjan, J., Xie, Z., Devine, D.V., 1994. Liposome-induced activation of the classical complement pathway does not require immunoglobulin. Biochim. Biophys. Acta 1192, 35–44.

Müller-Eberhard, H.J., 1975. Complement. Ann. Rev. Biochem. 44, 697–724.

Müller-Eberhard, H.J., 1988. Molecular organization and function of the complement system. Ann. Rev. Biochem. 57, 321–347.

Nagayasu, A., Uchiyama, K., Nishida, T., Yamagiwa, Y., Kawai, Y., Kiwada, H., 1996. Is control of distribution of liposomes between tumors and bone marrow possible? Biochim. Biophys. Acta 1278, 29–34.

Okada, N., Yasuda, T., Tsumita, T., Okada, H., 1982. Activation of the alternative complement pathway of guinea-pig by liposomes incorporated with trinitrophenylated phosphatidylethanolamine. Immunology 45, 115–124.

Oldfield, E., Chapman, D., 1971. Effects of cholesterol and cholesterol derivatives on hydrocarbon chain mobility in lipids. Biochem. Biophys. Res. Commun. 43, 610–616.

Pool, G.L., French, M.E., Edwards, R.A., Huang, L., Lumb, R.H., 1982. Use of radiolabelled hexadecyl cholesterol ether as a liposome marker. Lipids 17, 445–452.

Porter, P.R., Reid, K.B.M., 1979. Activation of the complement system by antibody-antigen complexes: the classical pathway. Adv. Prot. Chem. 33, 1–71.

Reid, K.B.M., 1983. Proteins involved in the activation of the classical pathways of human complement. Biochem. Soc. Tran. 11, 1–12.

Skubitz, K.M., Skubitz, A.P., 1998. Mechanism of transient dyspnea induced by pegylated-liposomal doxorubicin (Doxil). Anticancer Drugs 9, 45–50.

Szebeni, J., 1998. The interaction of liposomes with the complement system. Crit. Rev. Therap. Drug Car. Syst. 15, 57–88.

Szebeni, J., Wassef, N.M., Spielberg, C., Rudolph, A.S., Alving, C.R., 1994. Complement activation in rats by liposomes and liposome-encapsulated hemoglobin: evidence for anti-lipid antibodies and alternative pathway activation. Biochem. Biophys. Res. Commun. 205, 255–263.

Szebeni, J., Baranyi, L., Savay, S., Lutz, H.U., Jelezarova, E., Bunger, R., Alving, C.R., 2000. The role of complement activation in hypersensitivity to pegylated liposomal doxorubicin (Doxil®). J. Liposome Res. 10, 467–481.

Tanaka, S., Kitamura, F., Suzuki, T., 1987. Studies on the hemolytic activity of the classical and alternative pathway of complement in various animal species. Complement 4, 33–41.

Uziely, B., Jeffers, S., Isacson, R., Kutsch, K., Wei-Tsao, D., Yehoshua, Z., Libson, E., Muggia, F.M., Gabison, A., 1995. Liposomal doxorubicin: antitumor activity and unique toxicities during two complementary phase I studies. J. Clin. Oncol. 13, 1777–1785.

Volanakis, J.E., 1982. Complement activation by C-reactive protein complexes. Ann. New York Acad. Sci. 389, 235–250.

Wassef, N.M., Swartz, G.M. Jr, Alving, C.R., 1990. Antibodies to liposomal phosphatidylcholine and phosphatidylsulfocholine. Biochem. Cell Biol. 68, 54–58.

Wessel, D., Flugge, U.J., 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. 138, 141–143.