Complement Dependent and Independent Liposome Uptake by Peritoneal Macrophages: Cholesterol Content Dependency

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The uptake mechanisms of liposomes by rat peritoneal macrophages (PMs) were investigated. Incubation of liposomes with fresh rat serum enhanced the uptake of liposomes depending on the liposome size and cholesterol (CH) content. The binding of liposomes was also enhanced by serum, and this increase depended on the size and CH content as in the case of liposome uptake, which suggested that the binding of opsonized liposomes with PMs govern the extent in liposome uptake. The rate constant for the internalization (kint) was calculated by measuring both uptake and binding. The kint cannot explain the variation of liposome uptake for different sizes and CH contents. The kint values for liposomes with high (44%) and medium (33%) CH contents were constant (2.5 h−1), while those for liposomes with low (22%) CH content were significantly elevated (5–9 h−1). These results indicate the presence of at least two kinds of uptake mechanisms of liposomes. Treatment of serum with anti-C3 antibody completely inhibited the enhanced uptake of CH-high, large liposomes, which suggested that complement receptor-mediated phagocytosis may be an uptake mechanism for CH-high and -medium liposomes. In addition, complement-independent enhanced uptake was suggested for CH-low liposomes, since no inhibition was observed for CH-low liposomes by anti-C3 antibody and these liposomes were disintegrated in serum via complement-independent pathway. These results provided evidence that PMs take up liposomes via complement-dependent and independent mechanisms depending on the CH content of the liposomes.

Key words phagocytosis; binding; internalization; opsonin; C3; clearance

Macrophages play an important role in eliminating foreign particles from blood circulation and extracellular space, and extensive studies have been performed to clarify the mechanisms of liposomes by macrophages.1–4 The role of complements as opsonins has been shown using an isolated rat perfused liver system,5–7 and size-dependent enhancement of liposome uptake was observed.8,9 It was opsonins which distinguished the size of liposomes, and enhanced hepatic uptake resulted from the extent of opsonization.8,9 This size-dependent opsonization explained well the variation in hepatic uptake clearance of different sized liposomes.9 In our previous report, the effect of liposome size and cholesterol (CH) content in the hepatic uptake of liposomes were examined systematically, and a synergistic effect between liposome size and CH content was found in enhancing the hepatic uptake of liposomes.10 This interactive effect was explained to result from the size- and CH-dependent complement activation by these liposomes in rat serum.10

In the present study, rat peritoneal macrophages (PMs) were chosen as a model system for the uptake of liposomes, and the effect of liposome size and CH content on the binding, as well as uptake of these liposomes, were examined with or without the presence of serum. The uptake process was divided into binding and internalization to evaluate their contributions in the uptake of these liposomes. In addition, the involvement of a complement system as opsonins was assessed using anti-C3 antibody. Two kinds of uptake mechanisms were suggested based on the kinetic and mechanistic experiments in the serum-enhanced uptake of liposomes by PMs.

MATERIALS AND METHODS

Materials and Animals Hydrogenated egg phosphatidylcholine (HEPC) was a gift from Nippon Fine Chemicals Co., Ltd. (Osaka, Japan). Dicetyl phosphate (DCP) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). CH was obtained from Wako Pure Chemicals Co., Ltd., (Osaka, Japan). [3H]Cholesterylhexadecylether ([3H]CHE) was purchased from NEN (Boston, MA, U.S.A.). Thiglycollate medium, Hanks’ solution without sodium bicarbonate, and RPMI 1640 medium Nissui 1 without sodium bicarbonate were all purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Gentamicin and fetal bovine serum (FBS) were from Gibco BRL. FBS was inactivated by treatment at 56 °C for 30 min. Goat IgG fraction (antibody) to rat complement C3 was from Organon Teknika Corporation (Durham, NC 27704). All other reagents were of commercially analytical grade.

Male Wistar rats (body weight about 180–230 g) were purchased from Inoue Experimental Animals (Kumamoto, Japan).

Preparation and Characterization of [3H]CHE-Labeled Liposomes The preparation of liposomes was basically the same as described previously.10 Briefly, HEPC, CH and DCP in different molar ratios of 4 : 4 : 1 (44%, CH-high), 5 : 3 : 1 (33%, CH-medium), and 6 : 2 : 1 (22%, CH-low) were dissolved with the [3H]CHE (10 μCi/μmol lipid) in chloroform, followed by evaporation to make a thin lipid film. The lipid film was completely hydrated by PBS to obtain a liposome suspension (2 μmol lipid/ml). Liposomes were sized by extrusion using polycarbonate membrane filters with pore sizes of 800, 400, and 200 nm in diameter. The diameter of liposomes was measured by Nicomp 370 (Particle Sizing Systems, Santa Barbara, CA, U.S.A.).

Opsonization of Liposomes Blood was obtained from rat carotid artery under ether anesthesia by introducing a polyethylene tube (PE-50), and was left at room temperature for one hour. Serum was obtained by centrifugation at 3000 rpm for 30 min. The serum was frozen at −20 °C until

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opsonization of the liposomes. Liposomes were incubated with rat serum at the lipid concentration of 333 nmol/ml at 37°C for 20 min as reported previously. The opsonized liposomes with serum were used immediately for the binding or uptake experiments.

**Treatment of Serum by Anti-C3 Antibody** Rat serum was mixed well with goat IgG fraction (antibody) to the third component of the complement system (C3) of rat (6 mg/ml). The optimum concentration of Anti-C3 antibody was determined in a preliminary experiment at 100-fold dilution. Rat serum was incubated with Anti-C3 antibody for 6 h on ice. The complex of rat C3 and Anti-C3 antibody was removed by centrifugation at 3000 rpm for 20 min at 4°C. The supernatant was used as Anti-C3 treated serum.

**Preparation and Culture of Peritoneal Macrophages** Resident peritoneal phagocytes were elicited by the intraperitoneal injection of warmed thioglycolate medium (10 ml medium/100 g body weight). Three days later, rats were killed by decapitation and peritoneal macrophages were collected from the abdominal cavity by adding Hanks’ solution (finally 50 ml). The cells were centrifuged at 800 rpm at 4°C for 10 min. The supernatant was removed and the cells were washed two times. The number of cells was counted and viability was checked with Trypan blue dye exclusion. The cells were plated at 2×10⁶ cells/ml in RPMI 1640 medium Nissui 1 plus Gentamicin (60 μg/ml) and sodium bicarbonate (3 mg/ml) per 35 mm dish (Becton Dickinson, Japan) and incubated for 60 min at 37°C to allow cell adherence. Non-adherent cells were removed by washing with RPMI 1640 medium, and adherent cells were cultured with RPMI 1640 medium supplemented by 5% inactivated FBS for 20 h.

**Binding and Uptake of Liposomes by PMs** After 20 h incubation, the culture medium was replaced by 2 ml of RPMI 1640 medium without FBS. The liposome suspension with or without serum was added (100 nmol lipid/dish) and incubated at 37°C with 5% CO₂ for the uptake experiments and incubated on ice for the binding experiments. At the end of 6 h-incubation, the medium was removed and the cells were washed with cold Hanks’ solution three times. The cells were solubilized with 0.5 ml of NaOH (1 n) solution for overnight at room temperature and neutralized with 0.5 ml of acetic acid (1 n). The radioactivity of [³H]CHE was measured after adding scintillation liquid (10 ml) with a scintillation counter (LSC-700, Aloka, U.S.A.). Protein content was also measured by a DC-protein assay kit (Bio-Rad Laboratories, CA, U.S.A.).

**Calculation of Internalization Rate Constant** The uptake process can be considered to consist of both binding and internalization, as follows:

\[ [L-PM]_{in} = [L-PM]_{b} = [L-PM]_{h} \]

where [L] and [PM] represent the amounts of liposomes and their binding sites on PMs, respectively. The [L-PM]ₙ and [L-PM]ₜ represent the liposomes bound and internalized to PMs, respectively. Equilibrium of binding between liposomes and PMs was assumed, and the following differential equation for the rate of liposome uptake can be described:

\[ \frac{d[L-PM]_{in}}{dt} = k_{int}[L-PM]_{in} \]

where \( k_{int} \) represents the rate constant for the internalization process. This equation was integrated from time 0 to \( t \) to obtain \( k_{int} \) as follows:

\[ k_{int} = \frac{[L-PM]_{in}(t)}{\int_0^t [L-PM]_{in}(t)\,dt} \]

The amount of internalized liposomes \([L-PM]_{in}(t)\) can be substituted by the difference between the amount of liposomes taken up and bound by PMs.

Thus,

\[ k_{int} = \frac{[L-PM]_{in}(t) - [L-PM]_{b}(t)}{\int_0^t [L-PM]_{in}(t)\,dt} \]

where \([L-PM]_{in}(t)\) and \([L-PM]_{b}(t)\) represent the bound and taken up liposomes by PMs until time \( t \), respectively. In our preliminary experiments, the binding of liposomes to PMs increased linearly with time, making the area under the binding-time curve triangular in shape, thus, Eq. 3 can be approximated as follows:

\[ k_{int} = \frac{([L-PM]_{in}(t) - [L-PM]_{b}(t)) \times \sqrt{2}}{([L-PM]_{in}(t))} \]

RESULTS

**Binding of Liposomes to PMs** The effects of opsonization as well as liposome size and CH content on the binding of liposomes to PMs are shown in Fig. 1. Incubation of lipo-

![Fig. 1. Effects of CH Content and Size of Liposomes on the Binding to PMs](A) Binding of non-opsonized liposomes. (B) Binding of opsonized liposomes. Symbols: O, 800 nm; □, 400 nm; △, 200 nm.)
Cultured PMs (2×10⁶ cells/dish) were incubated with non-opsonized or opsonized [³¹]CHE labelled liposomes (100 nmol lipid/dish) for 6 h at 37°C. Data was the mean±S.D. of 6—8 experiments. (A) Uptake of non-opsonized liposomes. (B) Uptake of opsonized liposomes. (C) Opsonin-induced uptake by PMs, which was obtained by subtracting the uptake of non-opsonized liposomes from that of opsonized ones. Symbols: ○, 800 nm; □, 400 nm; △, 200 nm.

Fig. 3. Relationship between Uptake and Binding or Internalization Rate Constant of Liposomes by PMs
(A). Correlation between uptake and binding of CH-poor liposomes (dotted line with r²=0.797). Correlation between uptake and binding of CH-medium or high liposomes (dashed line with r²=0.986). (B) Uptake and internalization rate constant. There was no statistically significant correlation between the uptake and internalization rate constant. Symbols: circles, CH-high; squares, CH-medium; triangles, CH-low liposomes. Closed, 800 nm; darkened, 400 nm; opened, 200 nm diameter.

Liposomes with rat serum significantly enhanced the binding of liposomes with PMs for every composition of liposomes examined in this study (p<0.001). The binding of liposomes increased with the increase of liposome size or CH content for both non-opsonized and opsonized liposomes.

Uptake of Liposomes by PMs Figure 2 represents the effects of opsonization, as well as liposome size and CH content, on the uptake of liposomes by PMs. In the absence of serum, the size and CH content slightly influenced the uptake of liposomes. On the other hand, the incubation of liposomes with serum enhanced the macrophage uptake of every liposome preparation. This opsonization depended on liposome size as well as CH content, and the size-dependent opsonization was prominent in CH-high and low liposomes. In turn, CH content-dependent opsonization was prominent in large liposomes.

Relationship between Uptake and Binding or k_int There is a global correlation between liposome uptake and binding, while no correlation was observed between liposome uptake and k_int (Fig. 3). There is a good correlation between uptake and binding among CH-high and -medium liposomes and there is also a good correlation among CH-low liposomes with a different slope (Fig. 3A). The k_int values for CH-high and -medium liposomes were around 2 (h⁻¹), while those for CH-low liposomes showed larger values (5—9 h⁻¹). These results indicate a distinct uptake mechanism between CH-high and -medium liposomes and CH-low liposomes.

Fig. 4. Role of Complement Component on the Uptake of Liposomes by PMs
Liposomes were incubated with cultured PMs under the conditions described in Fig. 1. Dotted bar represents the uptake of liposomes opsonized by fresh serum. Closed bar represents the uptake of liposomes opsonized by serum pre-treated with anti-C3 antibody. Hatched bar represents the uptake of non-opsonized liposomes. Data represent the mean with S.D. of 6—8 experiments.
Complement-Dependent and Independent Uptake Mechanisms

The enhanced uptake for CH-high (800 nm) liposomes was completely inhibited by the pre-treatment of serum with anti-C3 antibody to the level of non-opsonized liposomes (Fig. 4). On the other hand, the serum-induced enhancement was not remarkably inhibited for CH-low (800 nm) liposomes. There is a slight increase in the uptake of CH-high (200 nm) liposomes in the presence of serum, and this enhancement was inhibited by anti-C3 antibody; however, there was no statistically significant difference among these uptakes.

DISCUSSION

The role of the complement system in the enhanced hepatic uptake of liposomes was extensively studied in our laboratory. The effects of serum opsonins have been studied with a rat perfused liver system, and the role of the complement system was clarified in the enhanced hepatic uptake of liposomes via complement receptor-mediated phagocytosis. Based on the fact that the CH-high, large sized liposomes released the entrapped carboxyfluorescein (CF) via an alternative pathway in rat plasma, the hydroxyl-group of CH was considered to play an important role in the activation of the complement system; therefore, systematic study was performed to examine the effect of CH-content as well as liposome size under the in vitro disposition as well as an in vitro release study. These studies revealed the synergistic effect between liposome size and CH content in the activation of the complement system, and the hepatic uptake clearance increased depending on both liposome size and CH content.

In this study, we have separated liposome uptake into two steps, binding and internalization, as described in Eq. 1. As shown in Figs. 1 and 2, the opsonization of liposomes enhanced both the binding and uptake of liposomes for all kinds of liposomes examined in this study. There is a similar pattern in the enhanced binding of opsonized liposomes (Fig. 1) as found in the in vivo experiments. However, quite a different pattern was observed in the enhanced uptake of opsonized liposomes (Fig. 2). This difference seemed to result from the size-dependent enhancement for CH-low liposomes in the uptake by PMs, which was not seen in the in vivo disposition study. These results suggested another uptake pathway distinct from complement receptor-mediated phagocytosis for PMs. In a further analysis, we examined the relationship between binding and internalization to clarify the uptake mechanism of liposomes by PMs.

A good correlation was observed between the uptake and binding of opsonized liposomes composed of CH-high and -medium content (Fig. 3A), while the \( k_{in} \) remained unchanged (Fig. 3B). These results suggested that the uptake of CH-high and -medium liposomes was determined by the binding step, not the internalization. This finding is in agreement with the results obtained from the perfused rat liver, where the binding of opsonins to liposomes depends on the size of liposomes, and the enhanced uptake of liposomes by the liver is governed by the extent of opsonization. Moreover, the pre-treatment of serum by anti-C3 antibody decreased the uptake of CH-high, large liposomes to the level of non-opsonized ones (Fig. 4), which suggests that the complement system may contain the principal opsonins responsible for the uptake of these liposomes by PMs, as in the case of the liver.

On the other hand, the uptake of CH-low liposomes showed a different slope in the relationship between the uptake and the binding (Fig. 3A), which is reflected in the larger \( k_{in} \) for these liposomes (Fig. 3B). In addition, the enhanced uptake of CH-low, large liposomes was not remarkably inhibited by the pre-treatment of serum with anti-C3 antibody (Fig. 4). These results indicated that CH-low liposomes were taken up by a mechanism with low binding and efficient internalization that differed from the complement-dependent mechanism. The complement-independent mechanism is also supported by the in vitro release study in which CH-low, large liposomes released CF in rat serum via a complement-independent manner.

The bilayer-packing defects are believed to expose hydrophobic domains on the surface of the bilayer which enhance protein binding, leading to rapid clearance. The HEPc-based liposomes with high and medium CH contents were relatively solid, and may not be ready for the binding of general serum proteins to hydrophobic domains. In fact, the effect of serum components such as HDL was not observed for these liposomes in release experiments. Moreover, the ability of plasma proteins to bind to distearoylphosphatidylcholine (DSPC)-liposomes was shown recently to be reduced dramatically by the inclusion of CH in the bilayer. However, in our laboratory, the plasma proteins were suggested to be C3 fragments (mainly C3b and iC3b) which are bound to the liposome surface directly or mediated by a certain factor through the CH molecule. This interaction between liposomes and the complement system is different from the non-specific binding of plasma proteins to liposomes through hydrophobic interaction. In addition, the complement activation was not observed by liposomes composed of unsaturated egg phosphatidylcholine (data not shown), similar to the observation by Devine et al. that liposomes containing unsaturated lipids are much less effective complement activators than those containing saturated lipid with the same acyl chain length.

There was a correlation between hepatic uptake clearance and liposome uptake by PMs as shown in Fig. 5A. However, the synergistic effect between liposome size and CH contents was not observed in the uptake by PMs. Better in vivo/in vitro correlation can be obtained in CH-high and -medium liposomes and in CH-low liposomes, as shown in Figures 5B and C.

Chomn et al. have shown the profile of proteins associated with various anionic large unilamellar vesicles (LUVs) by sodium dodecyl sulfate-polyarylamide gel electrophoresis (SDS-PAGE), and they found a difference in the protein profiles between in vivo and in vitro. Thus, the difference in the experimental conditions between in vitro and in vivo or in situ cannot be excluded as a cause of different uptake mechanisms between liver and PMs. Cell-derived proteins and proteolytic fragments such as a coagulation system could be candidates for the opsonin of the complement-independent uptake mechanism. Moghimi and Patel have shown that the uptake of liposomes with low CH contents by Kupffer cells was promoted by heat-stable serum components, whereas the uptake by peritoneal macrophages was enhanced by heat-
Labile components. They suggested the existence of specific opsonins for different macrophages such as Kupffer cells, splenic macrophages, peritoneal macrophages and bone marrow macrophages, besides differences in membrane receptors.

In conclusion, we have found that opsonins enhance the uptake of liposomes by PMs via increasing the liposome binding to PMs. Not the internalization process, but the binding process, governed the extent of uptake of opsonized liposomes depending on the CH contents as well as the size of the liposomes. Two kinds of uptake mechanisms were suggested based on the kinetic and mechanistic examination: one is a complement-dependent uptake and the other is a complement-independent uptake. The CH-high and -medium liposomes were taken up by PMs via a complement-dependent pathway, while CH-low liposomes were taken up via a complement-independent pathway.

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