

## Enhanced Hepatic Uptake of Liposomes Through Complement Activation Depending on the Size of Liposomes

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The objective of this study was to differentiate the roles of opsonins and phagocytic cells in the size-dependent hepatic uptake of liposomes in the submicron region. The extent of opsonization decreased with the decrease in size of liposomes (from 800 to 200 nm in diameter) and no enhancement of uptake was observed at 200 nm. There was no effect of liposome size on the uptake of unopsonized liposomes. Serum was pretreated with empty liposomes of each size and its opsonic activity was measured in the perfused liver. The small liposomes could not consume the opsonic activity, while the larger ones did so substantially. These results suggest that opsonins bind to liposomes depending on the size of liposomes and phagocytic cells take up liposomes in proportion to the extent of opsonization. Size-dependent liposome degradation in serum was also found, which was consistent with the size-dependent complement activation, because liposomes with this composition have been shown to be degraded by complement. The mechanism of opsonization was examined by treating serum at 56°C for 30 min or with anti-C3 antiserum. Since both treatments inhibited the opsonic activity, the hepatic uptake of liposomes is considered to occur via complement receptor. In conclusion, the size of liposomes affected complement recognition, and the liposomes were taken up by the liver depending on the extent of opsonization.

**KEY WORDS:** liposomes; opsonin; size; phagocytosis; endocytosis; complement; receptor.

### INTRODUCTION

Hepatic nonparenchymal cells form a protective barrier for the systemic circulation by distinguishing between foreign and endogenous substances and removing potentially harmful materials such as bacterial endotoxins (1), immune complexes (2,3), microorganisms (4,5), or tumor cells (6). Recognition is mediated through endocytosis with specific cell surface receptors. Endocytosis is classified into two mechanistic categories: receptor-mediated endocytosis (RME; and/or pinocytosis) and phagocytosis. RME (or pinocytosis) is assigned to the internalization of small compounds, while phagocytosis is for engulfment of large particles (7,8). The mean diameter of pinosomes (or endosomes) lies in the range of approximately 100–200 nm (9), while larger particles are taken up through phagocytosis. Immunoglobulin G and complements are known as major opsonins for the uptake of large particles such as aged red blood cells,

bacteria, and tumor cells, and specific receptors are present on the phagocytic cell surface to enhance phagocytosis (10,11).

Liposomes were chosen as model submicron particles which can be easily sized and modified. Multilamellar vesicles (MLV) with a diameter of 200–1000 nm had been shown to be rapidly taken up by the reticuloendothelial system (RES) depending on size rather than lipid composition and surface charge (12–15). We have shown that the uptake of MLV with hydrogenated egg phosphatidylcholine (HEPC), cholesterol (CH), and dicylphosphate (DCP) is enhanced by opsonization with serum (16). This effect was dependent on the size of liposomes, and small liposomes were not opsonized (17). However, the mechanism underlying the relationship between opsonization and liposome size has not been studied in these studies. As a possible candidate for opsonin, complement was suspected because liposomes were shown to activate the alternative complement pathway (18). Complement component 3 (C3) was deposited on the surface of liposomes and degraded to C3b and iC3b, which are known to be the ligands for complement receptors 1 and 3 (CR1 and CR3) (10). Size dependence was also reported in the activation of the complement system by liposomes (19). Based on this information, the present study was designed to examine the roles of opsonins, especially on the complement, to distinguish the size of liposomes in the hepatic uptake of liposomes, using isolated perfused rat liver.

### MATERIALS AND METHODS

**Materials.** The preparation of liposomes, surgery, and assay were basically the same as described previously (16). In brief, liposomes were prepared to give a lipid ratio of HEPC/DCP/CH = 5/1/4. For measuring the extraction of liposomes by isolated perfused liver, the lipid phase of liposomes was labeled with <sup>3</sup>H-cholesteryl hexadecyl ether (NEN, Boston, MA), which is known as an inert membrane marker (20). Liposomes were extruded eight times through polycarbonate membrane filters (Nucleopore Co., CA) with diameters of 800, 400, and 200 nm as indicated. For measuring the degradation of liposomes, 5(6)-carboxyfluorescein (CF) was introduced as an aqueous phase marker. After extrusion, the CF-encapsulated liposomes were dialyzed in a cellulose dialyzing tube against phosphate-buffered saline (PBS) without Ca<sup>2+</sup> and Mg<sup>2+</sup> for at least 4 days at room temperature with frequent changes of PBS to remove unencapsulated CF (18). The HEPC was kindly donated by Nippon Fine Chemical Co. (Osaka, Japan). DCP was purchased from Nacalai Tesque (Kyoto, Japan). CH was analytical grade (Wako Pure Chem., Osaka, Japan) and recrystallized from ethanol. All other reagents were of commercially analytical grades.

**Perfusion Study.** Male Wistar rats weighing 180–230 g were used (Inoue Experimental Animal, Kumamoto, Japan). Perfused liver was prepared according to the method reported previously (16). After a stabilization period of 10 min, liposomes were infused from the portal vein at a constant rate for 10 min. After a 1-min wash with liposome-free perfusate, liver was sampled and weighed, and the radioactivity in whole liver was measured according to the method re-

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ported previously (16). The uptake of liposomes was expressed as extraction (the percentage uptake of liposomes by the liver divided by the total amount of liposomes infused). The viability of the liver was tested routinely by the following two criteria: (i) the bile flow rate was greater than 1  $\mu\text{L}/\text{min}/\text{g}$  liver, and (ii) serum glutamic oxaloacetic transaminase (S-GOT) in the effluent was less than 10 Karmen units.

**Size-Dependent Opsonization.** Fresh serum was obtained from rats and stored at  $-130^\circ\text{C}$  until use. Various sized liposomes were incubated with rat serum at a ratio of 3.5 mL serum/ $\mu\text{mol}$  HEPC for 10 min. Then serum-opsonized liposomes were infused into the perfusate just before the liver. The input concentration of liposomes was fixed at 0.28 (nmol lipid/mL) and the total dose of lipid was about 150 nmol/liver. Unopsonized liposomes were incubated with PBS instead of serum. Then the extraction of the opsonized liposomes was measured as described above.

**Size-Dependent Inhibition of Serum Opsonic Activity.** Serum was preincubated with empty liposomes of differing sizes (6 mmol lipid/mL serum) at  $37^\circ\text{C}$  for 10 min and was then separated from liposomes by ultracentrifugation at 100,000g for 120 min at  $4^\circ\text{C}$ . After removing liposomes, the serum was used for the opsonization of  $^3\text{H}$ -labeled liposomes (800 nm), and the opsonizing activity of pretreated serum evaluated in the isolated perfused liver as described above.

**Size-Dependent Degradation of Liposomes in Serum.** The composition of liposomes described above was shown to be degraded through complement activation (18). In this study, the effect of liposome size on the degradation in serum was investigated in relation to the opsonic activity. Serum was preincubated at  $37^\circ\text{C}$  for 10 min, after which an aliquot of liposomal suspension (0.1 mL, 1.8  $\mu\text{mol}$  as total lipids) was added to the serum and incubated until 60 min. A control study was done with PBS instead of serum. A 1-mL aliquot of the diluted mixture was mixed well with 1 mL of 5% Triton X-100 solution, to which 2 mL of distilled water was then added. Percentage release of CF from liposomes was calculated from the fluorescence intensities with and without Triton X-100 according to the following equation:

$$\text{Release (\%)} = F_o/4F_t \times 100$$

where  $F_t$  and  $F_o$  are the fluorescein intensities with and without treatment with 5% Triton X-100, respectively. The result was expressed after subtracting the percentage release with PBS as the baseline.

**Mechanism of Opsonization.** The following inhibition experiments were performed to examine the mechanism of opsonization. Serum was preincubated at  $56^\circ\text{C}$  for 30 min or treated with goat anti-rat C3 (complement component 3) antiserum ( $\times 50$ ) and the opsonizing activity was measured in perfused liver as described above. To examine the effect of C5a (complement component 5a) on uptake, liposomes were opsonized in the presence of K-76COOH, an inhibitor of C5a (21).  $^3\text{H}$ -Labeled liposomes were opsonized with these treated sera and extraction was measured as described above. The size of all liposomes was 800 nm in this study.

## RESULTS

**Size-Dependent Opsonization.** As shown in Fig. 1, the opsonic activity depended on the size of the liposomes.

Small liposomes, with a mean diameter of 200 nm, were not opsonized, while larger liposomes were opsonized depending on the increase in diameter. On the other hand, there was no size effect in the uptake of unopsonized liposomes.

Based on these observations, we posed the following hypothesis that opsonins bind to liposomes independent of size, but phagocytic cells take up liposomes preferentially, depending on the size. According to this hypothesis, pretreatment of serum with liposomes will consume opsonins regardless of liposome size. To test this hypothesis, we examined the effect of liposome size on the consumption of opsonic activity. As shown in Table I, serum opsonic activity was inhibited by pretreatment with liposomes of larger size. No inhibition was observed by preincubation of serum with liposomes of 200-nm diameter. This result is inconsistent with the hypothesis described above. Therefore, we altered the hypothesis as follows: Opsonins bind to liposomes depending on the size of liposomes and the uptake of liposomes by the liver depends on the extent of opsonization.

**Size-Dependent Degradation.** The new hypothesis was examined by measuring the degradation of liposomes in serum. As shown in Fig. 2, the degradation increased with the increase in size of liposomes. Since the mechanism of degradation has been shown to be via the alternative complement pathway (18), the activation of complement by these liposomes may be size dependent.

**Mechanism of Opsonization.** As shown in Table II, both heat treatment and anti-C3 antiserum remarkably inhibited the opsonic activity, pushing it down to the unopsonized level. These results are consistent with the hypothesis that complements are the opsonins in the hepatic uptake of liposomes. The effect of K-76COOH on the hepatic uptake of liposomes was also examined. Uptake was inhibited by K-76COOH in a dose-dependent fashion (Fig. 3), which suggested the activation of phagocytes by C5a.

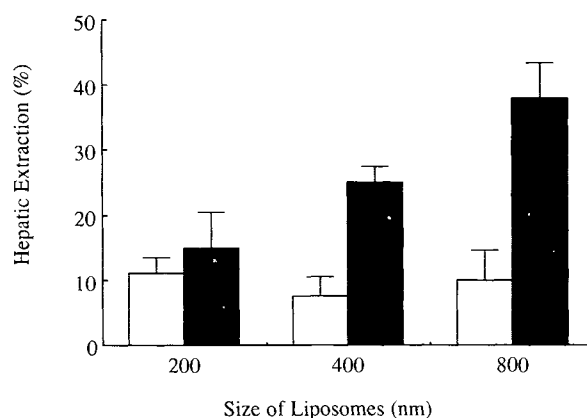


Fig. 1. Size-dependent opsonization of liposomes. The effect of opsonization on hepatic extraction was examined with various sizes of liposomes in the isolated perfused liver. The liposomes were opsonized by incubation in serum at  $37^\circ\text{C}$  for 10 min, then infused into the liver for 10 min at an input concentration of 0.28 nmol lipid/mL. The uptake of liposomes is expressed as extraction calculated by dividing the amount taken up by the liver by the total amount of liposomes infused times 100. The open bar represents the extraction of unopsonized liposomes, while the filled bar represents that of opsonized liposomes. The vertical bars represent the standard deviation of three to six experiments.

**Table I.** Size-Dependent Consumption of Complement Components<sup>a</sup>

Treatment	Extraction (%)	Inhibition <sup>b</sup>
Unopsonized	9.9 ± 3.0	—
Opsonized	41.1 ± 2.8	—
Pretreated (200) <sup>c</sup>	41.6 ± 3.5	$P > 0.05$
Pretreated (400) <sup>d</sup>	31.0 ± 1.0	$P < 0.05$
Pretreated (800) <sup>e</sup>	26.9 ± 5.9	$P < 0.01$

<sup>a</sup> The opsonizing activity of serum was evaluated using <sup>3</sup>H-liposomes with a diameter of 800 nm after preincubation of serum with liposomes of different sizes. The hepatic uptake of liposomes is expressed as extraction. The results are the mean of three or four experiments, with standard deviation.

<sup>b</sup> Inhibition of opsonizing activity by pretreatment of liposomes was statistically examined against the opsonized value.

<sup>c</sup> Serum was preincubated with liposomes with a diameter of 200 nm, then used for opsonization of <sup>3</sup>H-liposomes.

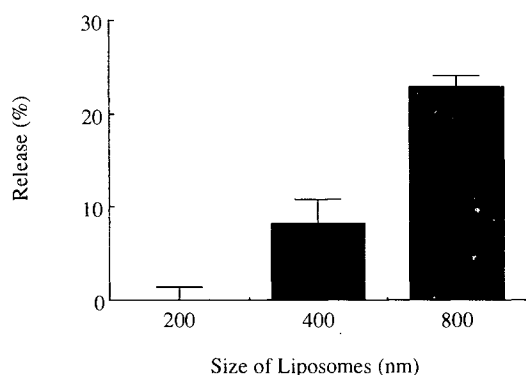
<sup>d</sup> Serum was preincubated with liposomes with a diameter of 400 nm.

<sup>e</sup> Serum was preincubated with liposomes with a diameter of 800 nm.

## DISCUSSION

Size-dependent opsonization was reported in our previous study using the Oldendorf method (17), where there was no size dependence in the liver uptake index of unopsonized liposomes and opsonization was observed only in large liposomes. However, these results did not allow us to distinguish between the size recognition capabilities of opsonins and those of phagocytic cells. In the present paper, we have shown that the extent of opsonization of liposomes was dependent on their size, while there was no size-dependent difference in the extraction of unopsonized liposomes. The pretreatment of serum with empty liposomes showed that small liposomes did not consume the opsonins (Table I). Based on these observations, we hypothesized that opsonins bind to liposomes depending on their size, and the uptake by the liver depends on the extent of opsonization.

Size-dependent liposome degradation was also shown in this study (Fig. 2). Since liposomes with a mean diameter of 800 nm have been shown to be degraded through the activa-



**Fig. 2.** Size-dependent degradation of liposomes in serum. The effect of size on the degradation of liposomes in serum was measured. The entrapped aqueous phase marker 5(6)-carboxyfluorescein was released by complement activation and the fluorescein intensity measured after 60 min of incubation of liposomes with serum at 37°C. The vertical bars represent the standard deviation of three experiments.

**Table II.** Inhibition of the Uptake of Liposomes by Complement Inhibition<sup>a</sup>

Treatment	Extraction (%)	Inhibition (%) <sup>b</sup>
Unopsonized	9.9 ± 3.0	—
Opsonized <sup>c</sup>	41.1 ± 2.8	—
56°C, 30 min <sup>d</sup>	12.7 ± 1.8	91
Anti-C3 antiserum <sup>e</sup>	14.6 ± 0.6	85

<sup>a</sup> Liposomes with a mean diameter of 800 nm were used in this study.

<sup>b</sup> The effect of inhibition of the complement system was also expressed as a percentage of the enhanced extraction by subtracting the extraction of unopsonized liposomes from each extraction.

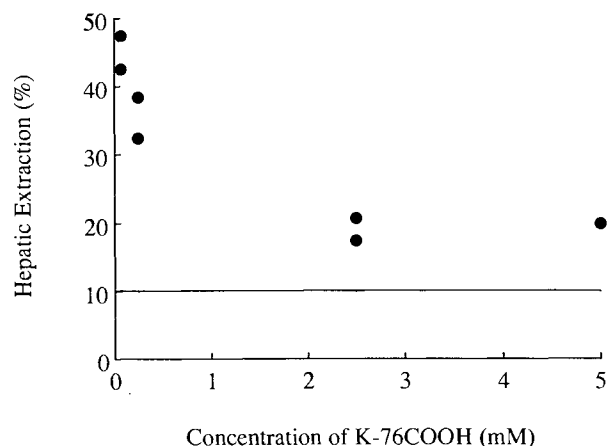
<sup>c</sup> Liposomes were opsonized with serum at 37°C for 10 min before infusion into the perfused liver.

<sup>d</sup> Liposomes were opsonized with serum which was pretreated at 56°C for 30 min to inactivate the heat-labile complements.

<sup>e</sup> Liposomes were opsonized with serum which was pretreated with anti-C3 antiserum.

tion of the alternative complement pathway (antibody-independent pathway) (18), the size-dependent degradation suggests size-dependent complement activation. Z. Liu *et al.* also reported that the complements preferentially attack medium- to larger-sized liposomes (larger than 200 nm) composed of dimyristoyl-phosphatidylcholine:cholesterol:dicetylphosphate, 4:3:1 (19). These results suggest complements as a common underlying mechanism in both opsonization and degradation of liposomes. As expected, a good correlation was seen between opsonization and degradation (correlation coefficient, 0.993), which suggests that complement—possibly C3 components—may play an important role for both opsonization and degradation of these liposomes.

Complements and IgG are major serum opsonins, and they opsonize a wide variety of particles, including many strains of bacteria and inert polymers (22). Complements are active at 37°C and lose activity when they are heated at 56°C



**Fig. 3.** Effect of K-76COOH, an inhibitor of C5a, on hepatic extraction. Liposomes were opsonized in the presence of K-76COOH for 10 min at 37°C, then infused into an isolated perfused liver at a constant rate for 10 min. After 1 min of washing with liposome-free buffer, radioactivity in the liver was measured. The extraction is expressed as the percentage of the total infused liposomes. The line represents the extraction of unopsonized liposomes. The concentration range of K-76COOH was from 0.075 to 5 mM.

for 30 min (23), while IgG is heat resistant. The loss of opsonizing activity after heat inactivation and anti-C3 antiserum administration indicates complements as opsonins (Table II). The complement receptor (CR) on the surface of monocytes and polymorphonuclear leukocytes (PMN) only binds complement-coated particles and does not ingest them so far as PMN are not activated by phorbolmyristate acetate (PMA) or chemotactic substances such as C5a (24,25). The K-76COOH inhibits both the classical and the alternative pathway at 1–5 mM without inhibiting proteases and esterases (21). Indeed, we observed this inhibition in the reported concentration ranges. Therefore, opsonized liposomes might be taken up by the liver after activation through C5a, similar to the finding of the uptake mechanisms of liposomes by neutrophils through CR3 (complement receptor type 3) (26).

Since cholesterol is the only molecule with a hydroxy group on the liposome surface which is recognized by C3b, various "clustering states" of cholesterol molecules in the membrane caused by the size of liposomes could be an underlying mechanism in size-dependent complement activation.

It was shown in this study that the complement system was activated and enhanced hepatic uptake depending on the size of liposomes. Figure 4 represents our hypothetical view on the uptake of foreign particle as a self-defense mechanism. As the uptake mechanism of foreign particles by the liver, endocytosis removes small particles such as viruses (27,28) and low-density lipoproteins (LDL) (29), whereas phagocytosis removes large ones such as red blood cells (RBC) (7,23), bacteria (4,5) and tumor cells (6). In both cases, uptake is enhanced by specific receptor binding. The

submicron region is the boundary between endocytosis and phagocytosis and might be critical for the self-defense system to shift the uptake mechanism from phagocytosis to endocytosis. Complement system may play a *complementary* role for phagocytic cells by enhancing recognition of foreign particles.

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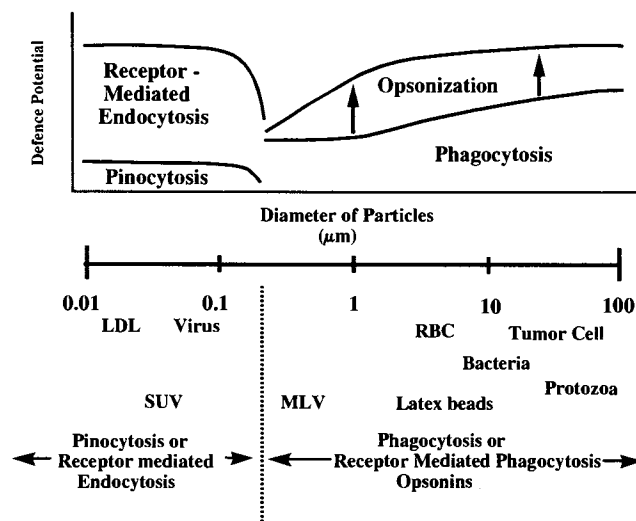


Fig. 4. Conceptual view of the uptake of foreign particles as a self-defense mechanism. The size of foreign particles ranges from nanometers (LDL, virus) to microns (RBC, bacteria, protozoa, and tumor cells). Depending on the particle size, two kinds of uptake mechanisms are assigned as self-defense systems: endocytosis or pinocytosis for small particles and phagocytosis for large particles. Opsonins enhance the uptake of large particles. The submicron region is considered to be a boundary for switching between these two mechanisms. The complement system acts as an opsonins to enhance the hepatic uptake of liposomes through phagocytosis.

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