Distinction Between the Depletion of Opsonins and the Saturation of Uptake in the Dose-Dependent Hepatic Uptake of Liposomes

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Opsonins play a role in the hepatic uptake of particles such as bacteria, lipid emulsion, and liposomes. The objective of this study was to distinguish between opsonin depletion and uptake saturation in the dose-dependent hepatic uptake of liposomes. The uptake of opsonized and unopsonized liposomes was determined in the isolated perfused liver. Serum (2.9 mL) was required to opsonize 1 μmol liposomes fully, indicating that a rat (250 g with 10 mL of serum) can opsonize 3.5 μmol liposomes. Next the dose effect on hepatic uptake of opsonized and unopsonized liposomes was examined. Saturation of uptake was found only for the opsonized liposomes. On the other hand, the hepatic uptake clearance decreased dose dependently from 4.31 to 0.79 (mL/min), with increasing doses from 0.075 to 17 μmol/250 g, respectively, after i.v. administration. Thus, the decrease in the hepatic uptake clearance at the medium dose was due to the saturation of uptake alone, and at the high dose it was due to opsonin depletion as well. These results show that the saturation of liposomal uptake in the liver and the depletion of opsonins occurred at different liposome dosage levels.

KEY WORDS: opsonins; hepatic uptake; liposomes; saturation.

INTRODUCTION

Liposome disposition in the body is regulated by lipid composition (1,2), size (3–5), and dose (6–8). Large liposomes are extensively taken up by the reticuloendothelial system (RES), while small liposomes have longer half-lives (9–11). This kinetic difference is accounted for by the finding that large liposomes cannot pass the fenestration of the sinusoid and are taken up mainly by the Kupffer cells lining on the sinusoid, while small liposomes are principally taken up by endocytosis into parenchymal cells as well as by Kupffer cells (12,13). Saturation of uptake is characteristic of phagocytosis of large liposomes by RES (14).

Having focused on this nonlinear uptake process of liposomes by RES, we postulated a non-Michaelis–Menten-type kinetics *in vivo,* where hepatic uptake clearance was measured by changing the infusion rate (15). The saturation of hepatic uptake clearance was not explained by the blood concentration but, uniquely, by the area under the curve of the blood concentration (AUC) (16). This saturation in hepatic uptake may be due to the nature of the activity of phagocytic cells such as Kupffer cells in the liver. However, opsonins are known to play a role in the uptake of large particles such as bacteria (17), colloidal particles (18), lipid emulsion (19), and liposomes (20) by RES. Therefore, we assessed the depletion of serum opsonin(s) and the saturation of hepatic uptake clearance in the dose-dependent uptake of liposomes, to clarify the underlying mechanisms of large liposome saturation kinetics.

IgG and complement are known to be the major serum opsonins, but the contribution of these, especially that to the hepatic uptake of liposomes, is unknown (10,21). To what extent is the opsonized form of the liposomes taken up by the liver after i.v. administration? Which will occur first at higher doses, the depletion of opsonins or the saturation of hepatic uptake? To answer these questions, it is essential to distinguish between the "opsonized" and the "unopsonized" forms of liposome uptake. Most studies intending to elucidate the liposome uptake mechanism utilized cell systems; however, it is difficult to quantitatively discuss *in vivo* hepatic uptake based on the results obtained with isolated cells. The perfused liver is a better system to quantitate the hepatic uptake of opsonized and unopsonized liposomes, because it maintains the anatomical architecture and the cell damage that occurs during isolation is excluded.

The objective of this study was to clarify the effect of dose on the depletion of serum opsonins and saturation of hepatic uptake using both isolated perfused liver and *in vivo* systems.

MATERIALS AND METHODS

An isolated perfused liver system was prepared to measure the opsonic capacity of the serum and the dose dependency of opsonized and unopsonized liposomes. Then, the dose-dependent disposition of liposomes was studied *in vivo* by changing the dose from 0.075 to 17 μmol/250 g. The serum opsonic activity at each dose was examined using perfused liver also.

Materials. The preparation of liposomes, surgery, and assay were basically the same as described previously (15). In brief, liposomes were prepared to give a lipid ratio of PC/DCP/CH = 5/1/4 with the mean diameter of 780 nm, where PC, DCP, and CH represent hydrogenated phosphatidylcholine, dicetyl phosphate, and cholesterol. PC was kindly donated by Nippon Fine Chem. 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 of Miyauchi *et al.* (22). Under light ether anesthesia, the bile duct was cannulated with PE-10 and the portal vein was rapidly catheterized with PE-240, which was attached to the perfusion system containing perfusate at 37°C, and infusion of the perfusate was started immediately. The inferior vena cava was catheterized through the right atrium with PE-240, and the inferior vena cava was then ligated. The liver was perfused with Krebs–Ringer bicarbonate buffer with 100 mg/dL glucose and 20 μ*M* taurocholate, oxygenated with 95% O_2–5% CO_2 to give pH 7.4 at 37°C. The perfusate was circulated using a constant-rate infusion pump. The flow rate

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was maintained at 2.5–3.0 mL/min/g liver and all studies were performed under single-pass conditions. 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 amount of liposomes was measured according to the method reported previously (15). The uptake of liposomes was expressed by the extraction (total amount of liposomes taken up by the liver in 10 min divided by 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 μL/min/g liver, and (ii) serum glutamic oxaloacetic transaminase (S-GOT) in the effluent was less than 10 Karmen units.

Effect of Opsonization. The importance of the opsonization of the liposomes was examined using the perfused liver described above. Liposomes were incubated with freshly isolated serum by changing the serum-to-liposome ratio from 0.714 to 14.3 (mL serum/μmol PC) for 10 min just before injection into the liver. Then opsonized liposomes with serum 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. This condition was used throughout the study except for the high dose in Fig. 3. Then the extraction of the opsonized liposomes was measured as described above. In our preliminary study, the effect of size on the hepatic uptake was examined. The larger the size, the larger the opsonization. Thus, we used the large liposomes (780 nm) in this study.

Saturation of Hepatic Uptake of Liposomes. Dose dependency in liposome uptake was examined in a perfusion study using opsonized and unopsonized liposomes. Liposomes were opsonized as described above, and a range of liposome doses, from 0.07 to 0.7 (μmol/10 min), was chosen.

In Vivo *Study.* Male Wistar rats weighing 250–300 g were used. The operations for the introduction of catheters for drug administration and blood sampling were performed as described previously (15). Liposomes labeled with ^{3}H-cholesterylhexadecylether were injected intravenously. The doses of liposomes were 0.075, 0.90, and 17 μmol/250 g. Approximately 300 μL of blood was sampled at the indicated time. At the end of each study, the rat was killed by an injection of 50 mg of sodium pentobarbitone, and the liver was excised.

Pharmacokinetic Analysis. The time courses of liposome blood concentration were analyzed based on a one-compartment model, using MULTI (23). The Damping Gauss Newton method was chosen as the algorithm for the nonlinear least-squares method and the inverse of the square of blood concentration was used as the weight. Pharmacokinetic parameters were calculated as follows:

$$V_d = \text{Dose}/C(0) \quad (1)$$

$$CL_{tot} = \text{Dose}/\text{AUC} \quad (2)$$

$$\text{MRT} = 1/ke \quad (3)$$

$$CL^h = X(30)/\text{AUC}(30) \quad (4)$$

$$E^h = CL^h/Q^h \quad (5)$$

where V_d, $C(0)$, CL_{tot}, AUC, MRT, ke, CL_h, $X(30)$, AUC(30), E^h, and Q^h represent the volume of distribution, initial blood concentration of liposomes, total-body clearance, area under the blood concentration–time curve, mean residence time, elimination rate constant, hepatic uptake clearance, liposomal uptake at 30 min, AUC from 0 to 30 min, hepatic extraction, and hepatic blood flow rate, respectively. The hepatic uptake clearance was calculated according to Eq. (4) by neglecting the efflux of liposomes from the liver. This assumption was examined in our previous paper (16). The value for Q^h was cited from the literature as 14.7 mL/min/250 g rat (24).

Dose Effect on the Depletion of Opsonins. The opsonic activity of the serum after the administration of liposomes *in vivo* was examined at each dose. Whole blood was withdrawn after the end of the *in vivo* study and centrifuged at 3000 rpm for 10 min to separate liposomes, serum, and blood cells. The serum was carefully sampled and used for opsonization. There was a possibility of radioactivity remaining in serum, so it was measured and found negligible compared to the newly added radioactivity of ^{3}H-liposomes. Therefore we used this serum for opsonization directly. ^{3}H-Labeled liposomes were opsonized with this serum at 37°C for 10 min and the hepatic extraction of these liposomes was evaluated by isolated perfused liver as described above. The ratio of serum/liposomes was fixed at 2.9 (mL/μmol lipid) so that all liposomes could be well opsonized in the control serum. The amount of liposomes in the liver was measured as described above.

RESULTS AND DISCUSSION

Serum components called opsonins play an important role in that they enhance the phagocytotic uptake of large particles, the alteration of serum opsonic activity having been shown to govern phagocytotic activity (25). Therefore, it is essential to evaluate the saturation of opsonic activity and phagocytosis separately in dose-dependent liposomal uptake by the liver. Isolated perfused liver was chosen to measure the uptake of opsonized and unopsonized liposomes separately considering the advantages described in the Introduction.

The time course of tissue-to-effluent concentration ratio (K_p) of unopsonized liposomes is shown in Fig. 1. The K_p continued to increase linearly for 30 min without lag time. Ten minutes was chosen as the perfusion time in the following study. The extraction remained constant around 10–12% after 5 min of infusion. This K_p time course and low extraction suggest that the amounts taken up by the liver may represent uptake, not binding, because the binding process of the liposomes to cells are considered to occur in or below the order of a second.

The capacity of the opsonic activity of serum was examined by changing the ratio of serum to liposomes (Fig. 2). The result shows that 2.9 mL of serum is enough to opsonize 1 μmol of liposomes, which indicates that a rat (250 g in body weight, 10 mL serum) can opsonize 3.5 μmol of liposomes completely. This result predicts that serum opsonic activity will be maintained for the medium dose (0.9 μmol/250 g) and depleted at the high dose (16.6 μmol/250 g).

IgG and complements are known as major serum op-

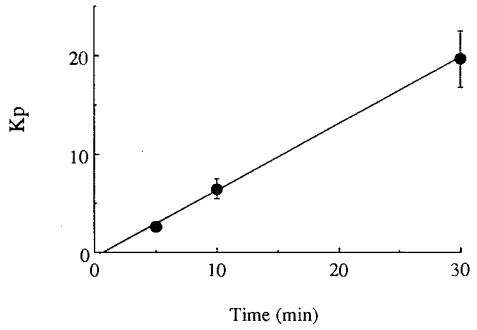

Fig. 1. Time course of K_p in the perfused liver. Kp was calculated as the ratio of liposome concentration in liver to that in effluent. The vertical bar represents the SE of three experiments.

sonins. The latter is heat labile but the former is not. We suspected that complements are acting as an opsonin in this case based on the following two observations. First, our previous study showed that the serum opsonins were heat labile in the isolated perfused study (29). Second, these liposomes activated the complement system through an alternative pathway, where C3b was degraded to iC3i (30), which is known as a ligand for CR3.

It was reported using isolated hepatic nonparenchymal cells that the serum opsonic effect on hepatic uptake is dependent on the cholesterol content and lipid composition. The uptake was inhibited by serum for the cholesterol-rich liposomes (egg PC:CH:DCP = 7:7:1) but enhanced by serum for the cholesterol poor liposomes (egg PC:CH:DCP = 7:2:1) (26). It was also shown using isolated perfused liver that the uptake of liposomes made of dipalmitoylphosphatidylcholine was inhibited by the addition of blood (27). On the other hand, the enhancing effect of serum on the liposomal uptake by the perfused liver was reported for negatively charged liposomes (egg PC:CH:DCP = 6:2:1) or neutral liposomes (egg PC:CH = 9:2) (28). Therefore, these discrepancies in the effect of serum on the uptake of liposomes may stem from differences in the composition of phospholipid, cholesterol, and other components.

Next the effect of liposome dose on extraction was examined for both opsonized and unopsonized liposomes. Opsonization was performed under the condition of 2.9 mL/μmol liposomes to exclude the possibility of opsonin depletion. As shown in Fig. 3, the extraction of opsonized liposomes decreased by 37% ($P < 0.01$) and that of unopsonized liposomes did not. The input concentration of liposomes in the perfusate was calculated by dividing the infusion rate of the liposomes by the perfusion rate. The comparison between the opsonized and the unopsonized liposomes in the perfused liver is also shown in Fig. 3. The opsonized liposomes are taken up by the liver four times more efficiently. Therefore, opsonized liposomes could be taken up by the liver in opsonized form below the 3.5 μmol/250 dose.

The *in vivo* study clearly shows the dose-dependent disposition of liposomes represented by total-body clearance and mean residence time (Table I). This saturation was the result mainly of the saturation of hepatic uptake clearance. Hepatic uptake clearance decreased by 65% even at the medium dose (0.90 μmol/250) and by 82% at the high dose (17

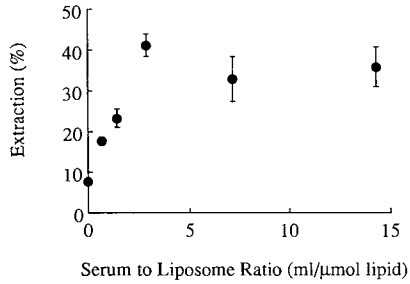

Fig. 2. Effect of opsonization on the uptake of liposomes by the liver. Liposomes were opsonized with fresh rat serum for 10 min before the perfusion study. The ratio of serum to liposomes was changed from 0 to 14.3 and extraction of the liposomes by the perfused liver was measured. The symbol and vertical bar represent the mean and SE of three experiments.

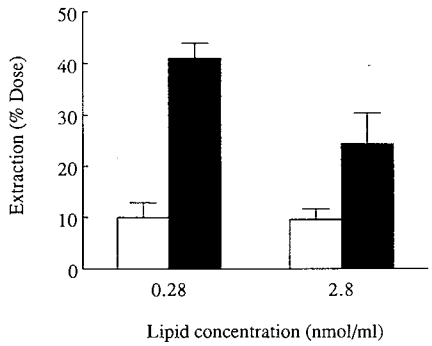

Fig. 3. Effect of dose on the uptake of liposomes by the liver. The extraction for both opsonized (filled bars) and unopsonized (open bars) liposomes was measured at doses of 0.07 and 0.7 μmol/10 min. The input concentrations of liposomes corresponding to these doses were calculated as 0.28 and 2.8 nmol/mL (see Materials and Methods). The vertical bar represents the mean and SE of three experiments.

Table I. Summary of Pharmacokinetic Parameters of Liposomes in Each Dose

	Low	Medium	High
Dose[a]	0.30	3.6	67
V_d[b]	119 ± 24[h]	87.9 ± 30.9	88.3 ± 12.1
CL_{tot}[c]	17.3 ± 8.82	8.63 ± 0.77	5.71 ± 3.38
ke[d]	0.14 ± 0.053	0.11 ± 0.029	0.062 ± 0.028
MRT[e]	7.94 ± 2.83	10.1 ± 2.82	18.7 ± 8.57
CL_h[f]	0.473 ± 0.183	0.148 ± 0.049	0.078 ± 0.05
E_h[g]	25.4 ± 12.7	8.47 ± 1.80	4.52 ± 2.62

[a] The dose of liposomes is expressed as μmol/kg.
[b] V_d was calculated based on Eq. (1) and is expressed as mL/kg.
[c] CL_{tot} was calculated based on Eq. (2) and is expressed as mL/min/kg.
[d] ke was directly obtained in the curve fitting of the time course of blood concentration and is expressed as min^{-1}.
[e] MRT was calculated based on Eq. (3) and is expressed as min.
[f] CL^h was calculated based on Eq. (4) and is expressed mL/min/g liver.
[g] E^h was calculated based on Eq. (5) and is expressed as %.
[h] Every result is expressed as the mean and SE of three experiments.

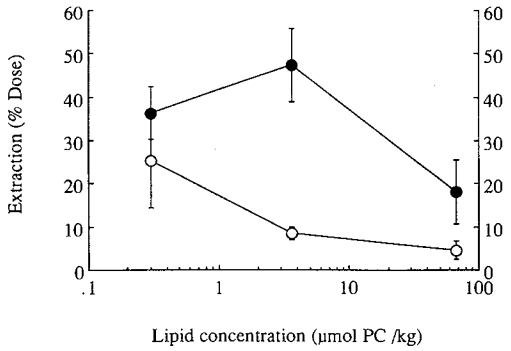

Fig. 4. Dose dependency of hepatic extraction and opsonic activity. The hepatic extraction measured in the *in vivo* study (open circles) and opsonic activity measured by perfused liver (filled circles) are shown. *In vivo* hepatic extraction: Liposomes were administered intravenously and the time course of blood concentration and the uptake amount by the liver were measured. Hepatic uptake clearance was calculated by Eq. (4), then *in vivo* extraction was calculated by Eq. (5). Opsonic activity: The opsonic activity of serum after the administration of liposomes to rats was measured. Liposomes were opsonized by the serum from rats which were preinjected with liposomes at different doses and opsonic activities were assessed by measuring the extraction of liposomes by the perfused liver (see Materials and Methods). The vertical bars represent the mean and SE of three experiments.

μmol/250 g), which is the principal cause of the dose-dependent disposition. Further studies were performed to distinguish the depletion of opsonins and the saturation of hepatic uptake using isolated perfused liver for the evaluation of opsonic activity.

As shown in Fig. 4, the extraction of liposomes opsonized by the serum from the *in vivo* study at the low and medium doses did not decrease, while it decreased significantly at the high dose. This indicates that serum opsonic activity was kept at the baseline in the low and medium dose, while it decreased in the high dose, a result consistent with Fig. 2. Two and nine-tenths milliliters of serum can opsonize 1 μmol liposomes, which means that a rat weighing 250 g with 10 mL of serum can opsonize 3.5 μmol liposomes. Therefore there should be no depletion of serum opsonin at the medium dose (0.9 μmol/250 g). On the other hand, a decrease in extraction was observed at the medium dose in the *in vivo* study, which is attributed to the saturation of hepatic uptake itself and is consistent with the result of saturation of the opsonized liposomes in the perfused liver as shown in Fig. 3. The considerable decrease in hepatic uptake clearance at the high dose resulted from both the saturation of hepatic uptake and the depletion of serum opsonin.

In conclusion, we have distinguished the depletion of opsonins and saturation of hepatic uptake in the dose-dependent disposition of liposomes. The hepatic uptake saturated at a lower dose than that for the depletion of opsonins. At a lower dose, the opsonized liposomes are efficiently taken up by the liver, while the contribution of unopsonized liposomes seems to be larger at a higher dose.

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