J. Pharm. Pharmacol. 1991, 43: 162–166
Received May 11, 1990

Saturable, Non-Michaelis-Menten Uptake of Liposomes by the Reticuloendothelial System

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Abstract—Multilamellar vesicles (300–350 nm) were infused into the rat femoral vein at the rate of 4, 40 and 400 nmol phosphatidycholine min^{-1} for 6 h using [^{3}H]inulin as an aqueous marker. The time courses of blood concentration of vesicles, normalized for infusion rate, were not superimposable, showing the non-linearity of liposome disposition in the blood circulation. These time courses of blood concentration were well fitted by a single Michaelis-Menten equation. On the other hand, the time courses of tissue content could not be so accommodated. Additionally, the observed relationship between the uptake of liposomes by the liver and their clearance from it and other organs differed essentially from a simulation based on Michaelis-Menten type saturable kinetics. Therefore, it is suggested that there is a time-dependent non-Michaelis-Menten type process in the phagocytosis of macrophages in the reticuloendothelial system.

Many studies have been made of the factors which determine the disposition of liposomes used as drug delivery systems, emphasizing the importance of diameter (Juliano & Stamp 1975), surface charge (Gregoriadis & Neerunjun 1974; Juliano & Stamp 1975), and dose (Abra & Hunt 1981; Kao & Juliano 1981). Liposomes >200 nm in diameter are considered to be cleared from the blood circulation by phagocytosis. The kinetic characterization of this process has been carried out by Abra & Hunt (1981) who showed that the uptake of large liposomes decreased in liver and increased in spleen as the dose increased (dose range: 4·3–513 μmol lipid kg^{-1}), and by Chow et al (1989) who showed that the extent of distribution of small unilamellar vesicles decreased both in Kupffer cells and endothelial cells with increase in dose, and increase in hepatocytes in proportion to dose. The result of Chow et al (1989) was explained by saturable transport of the vesicles into Kupffer cells and endothelial cells and their linear transport into hepatocytes.

The saturable process is mediated by macrophages, such as Kupffer cells in liver, and the Michaelis-Menten equation has been used for quantitative analysis (Beaumier et al 1983; Hwang 1987; Sculier et al 1989). Phagocytosis is a different phenomenon, involving the active participation of actin-containing microfilaments (Darnell et al 1990). The objective of the present study was to re-evaluate the kinetics of the phagocytotic process of multilamellar vesicle uptake by the liver by examining the effect of a wide range of infusion rates on the clearance of multilamellar vesicles.

Materials and Methods

Hydrogenated phosphatidylcholine (PC) was kindly donated by Nippon Fine Chem. Co. (Osaka, Japan). Dicetyl phosphate (DCP) was purchased from Nacalai Tesque (Kyoto, Japan). Cholesterol (CH) was analytical grade (Wako Pure Chem., Osaka, Japan), and recrystallized from ethanol. All other reagents were commercial analytical grades.

Liposome preparation was performed by modification of conventional lipid film hydration methods (Bangham et al 1965; Kiwada et al 1985) to give lipid ratios of PC/DCP/CH 5/1/4 with PC concentrations of 50, 5 and 0·5 mM. [^{3}H]Inulin (0·1 MBq mL^{-1}, 48·1–62·9 GBq mol^{-1}, Amersham Japan, Tokyo, Japan) in pH 7·4 phosphate buffered saline (PBS) was used as an aqueous marker. Liposomes were sized by extrusion through a 400 nm polycarbonate filter (Nucleopore membrane, CA, USA). Non-encapsulated inulin was removed by dialysis (Spectra/Por 7, mol. wt 50 000, Spectrum, CA, USA) against frequent changes of PBS for at least 3 days. The trapping efficiency of inulin was 20–25% at 50 mM PC. The mean diameter of liposomes measured by a dynamic laser scattering method (LPA-3100, Otsuka Electronics, Osaka, Japan) was 300–350 nm.

Non-fasted male Wistar rats, 230–300 g, under light ether anaesthesia, were cannulated via the left femoral vein (PE-20, Natsume, Tokyo, Japan), artery (PE-50) and bladder (PE-20 and PE-50 tied together in parallel). Each rat was fixed in a Bollman's cage and left for at least 1 h to recover from anaesthesia. Liposomes were infused (8 μL min^{-1}) (Model 22, Harvard Apparatus, MA, USA or KN-micro infusion pump H-type, Natsume, Tokyo, Japan) at the rate of 400, 40, 4 nmol PC min^{-1} through the femoral vein cannula (high, medium, low infusion rate, respectively). At 0, 3, 5, 15, 30, 60, 120, 240, 360 min after the initiation of the infusion, 0·2 mL of blood was sampled through the femoral artery cannula, and urine was collected from the bladder by washing with 2–3 mL of saline. The urine was diluted up to 10 mL. At 360 min, rats were killed by bolus injection of sodium pentobarbitone (50 mg). Liver and spleen were removed immediately. Rats were also killed at 15, 30, 60, 120, 240, 360 min after sampling of blood and urine, and liver and spleen removed. Whole liver was homogenized with about 30 mL of distilled water, and diluted to 50 mL. KOH (2 M in isopropanol, 0·2 mL) and H_2O_2 (200 μL) were added to liver homogenate (0·5 mL), blood (0·1 mL) or diluted urine (1 mL), and each sample was left overnight. Samples were

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Table 1. The recovery of liposomes at 360 min after the initiation of the infusion.

Infusion rate	Recovery (%)[a] Blood[b]	Urine	Liver	Spleen	Total
High	70·6±4·3	4·3±0·4	15·5±2·0	13·5±1·4	103·9
Medium	25·5±3·0	4·5±0·4	18·9±1·0	20·1±1·8	69·0
Low	9·5±0·2	4·6±0·2	32·5±1·2	25·2±2·4	71·8

[a] Each value represents the mean±s.e.m. (n=3–5). [b] The volume of blood compartment is assumed to be 20 mL.

neutralized with 0·4 mL 10% acetic acid and 10 mL scintillation fluid added (Scintisol EX-H, Dojin Lab., Kumamoto, Japan). Whole spleen was incubated with 2 mL 2 M KOH and 2 mL H_2O_2 at 37°C overnight for dissolving the tissue. The solution was neutralized by 2 mL 10% acetic acid and distilled water to make a volume of 10 mL. A 1 mL portion of the sample was mixed with 10 mL scintillation fluid and ^3H was measured by liquid scintillation spectrometry (LSC-700, Aloka C., Tokyo, Japan).

Data analysis and simulation were performed on a microcomputer using the Runge-Kutta-Gill method and a non-linear least square program MULTI(RUNGE) (Yamaoka & Nakagawa 1983), to fit the data to:

$$V_d \cdot \frac{dC}{dT} = k_o - \frac{V_{max} \cdot C}{K_m + C} \quad (1)$$

where C is the blood concentration of liposomes (nmol mL^{-1}), k_o is the infusion rate (nmol min^{-1}), V_{max} (nmol min^{-1}) is the maximum uptake rate, K_m is the Michaelis constant (nmol mL^{-1}) and V_d is the volume of distribution (mL).

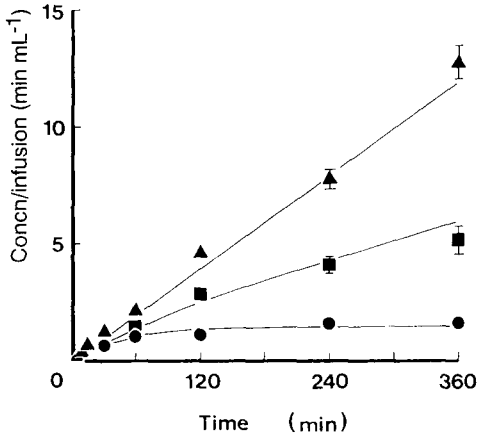

FIG. 1. Liposome concentration in blood, normalized for infusion rate. Symbols represent infusion rate: ●; 4 nmol min^{-1}, ▲; 40 nmol min^{-1}, ■; 400 nmol min^{-1}. Each point and vertical bar represents the mean±s.e.m. (n=4–5). The solid lines are fitted curves (see Methods).

Results

The concentration of intact liposomes retaining inulin (as PC equivalents) was calculated from the [^3H]inulin. Liposome concentration in blood, normalized for infusion rate were plotted against time (Fig. 1). For the low infusion rate, the blood concentration reached a plateau after 60 min. The curves were not superimposable showing that a non-linear process is involved in the disposition of the liposomes. The data for the three infusion rates was fitted simultaneously to the Michaelis-Menten equation (eqn 1) assuming a tissue uptake process. The calculated line is shown in Fig. 1 and fitted values were $K_m = 38{\cdot}8$ nmol mL^{-1}, $V_{max} = 29{\cdot}7$ nmol min^{-1} and $V_d = 28{\cdot}3$ mL. The simulation lines agreed well with the observed data.

The recovery of injected liposomes in blood, urine, liver and spleen, 360 min after initiation of the infusion is shown in Table 1. The recovery was incomplete at lower infusion rates suggesting a contribution by other organs for liposome uptake in a saturable manner.

Fig. 2 represents the time course of liposome uptake, normalized for infusion rate, in liver and spleen. The amount of liposomes in liver increased linearly with time, while in spleen, the liposome content increased in parabolic fashion. If the uptake process of multilamellar vesicles into the liver and spleen followed Michaelis-Menten kinetics, a unique parameter set could be estimated by fitting the observed values of three infusion rates simultaneously using the following equations;

$$\text{Blood; } V_d \cdot \frac{dC_{blood}}{dT} = k_o - \frac{V_{max}^{blood} \cdot C_{blood}}{K_m^{blood} + C_{blood}} \quad (2)$$

$$\text{Organ; } \frac{dX_{organ}}{dT} = \frac{V_{max}^{organ} \cdot C_{blood}}{K_m^{organ} + C_{blood}} \quad (3)$$

V_{max}^{blood}, K_m^{blood}, V_d in equation were fixed at the values estimated from the time courses of blood concentration, described above. Although fitting was tried using various initial parameter sets, tissue uptake could not be described by equation 3.

Organ clearance was calculated by dividing the amount of PC in the organ by the area under the blood concentration-time curve (AUC) from time zero until the end of infusion. If the uptake process can be described by Michaelis-Menten kinetics, the relationship between the blood concentration of liposomes and the uptake clearance should be independent of the infusion rate. In this study, the plot of the hepatic clearance against the blood concentration was not independent of the infusion rate (data not shown). Fig. 3A shows the relationship between the amount of liposomes taken up by the organs and organ clearance. The uptake clearance decreased as the amount of liposomes taken up by the organs

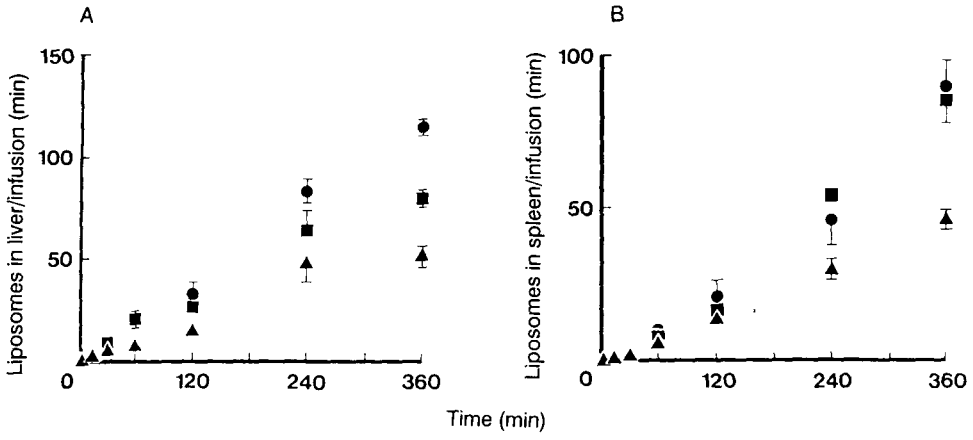

FIG. 2. Liposome amounts in liver (A) and spleen (B) normalized for infusion rate. Symbols are the same as those in Fig. 1. Each point and vertical bar represents the mean ± s.e.m. (n = 3–5).

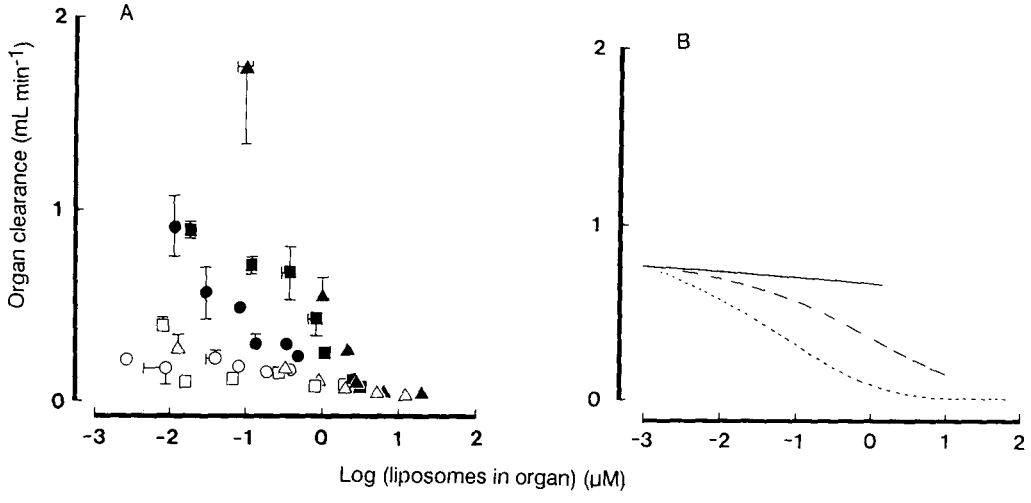

FIG. 3. Relationship between the amount of liposome taken up by liver or spleen and the organ clearance. (A) Observed. Closed and open symbols represent liver and spleen, respectively. Symbols are the same as those in Fig. 1. Each point and vertical bar represents the mean ± s.e.m. (n = 3–5). (B) Simulated. The smooth line represents low infusion rate, the broken line medium infusion rate, and the dotted line high infusion rate. The simulation was performed using the parameter set estimated for the values in Fig. 1 (see Results).

increased, especially in liver, clearly showing that the uptake process of liposomes by organs is non-linear. For spleen, the effect of infusion rate was not remarkable compared with that in liver, and there was a decreasing tendency to non-linearity in spleen. Fig. 3B is the simulated relationship between the uptake and the clearance based on the Michaelis-Menten equation using equation 1 with the fitted parameter set obtained previously. At the low infusion rate, clearance decreased slightly as the blood concentration is similar to the liver concentration. At the higher infusion rate, clearance decreased as the amount taken up in tissue increased because of the high blood concentration. The importance of this pattern is that the higher the infusion rate, the lower the clearance at the same amount in the tissue. On the other hand, the observed value in Fig. 3A shows the opposite pattern, i.e. lower clearance at the lower infusion rate. This suggests that the uptake process of liposomes by the reticuloendothelial system is different from a conventional Michaelis-Menten type saturable process.

Fig. 4 shows the change of organ clearance with time. In both spleen and liver, clearance decreased with time and the higher the infusion rate, the faster the decrease. In spleen, little decrease occurred at the low infusion rate.

Discussion

According to our analysis of liposome size, the diameter ranged from 150 to 600 nm and we could not detect the smaller sizes. The contribution of liposomes < 150 nm in diameter (which are not detected by our equipment) would be much smaller than that by normal liposomes, because the amount of the entrapped marker is proportional to the cube of the diameter.

We used inulin as an aqueous marker and measured inulin excretion via the urine to evaluate the degradation of the liposomes in the blood. As shown in Table 1, the recovery of inulin in the urine was 4–5% of the injected dose at each infusion rate, indicating that more than 95% of the lipo-

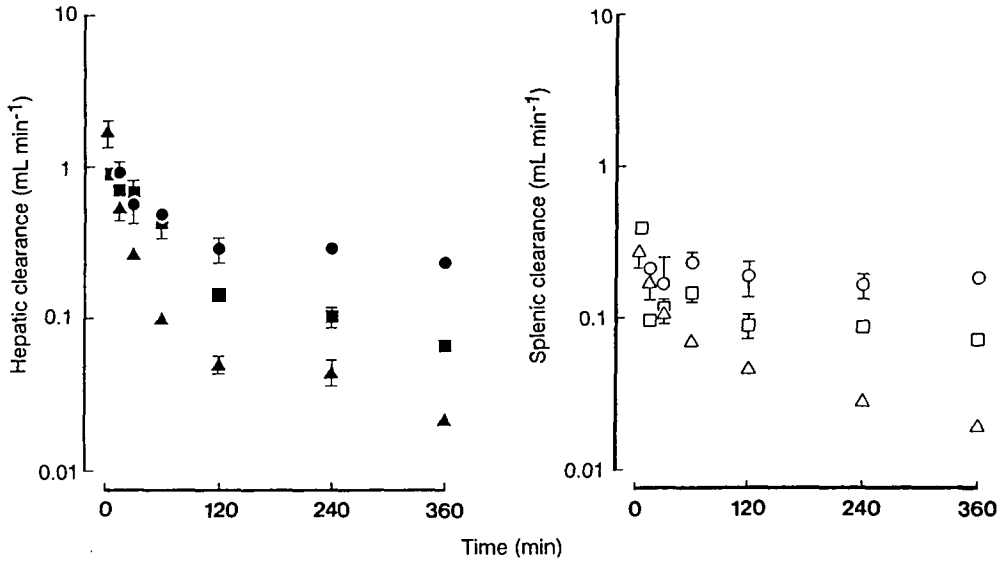

FIG. 4. Uptake clearance into liver (A) and spleen (B). Symbols are the same as those in Fig. 3. Each symbol and vertical bar represents the mean ± s.e.m. (n = 3–5).

somes were intact (in terms of inulin leakage) during the study.

As shown in Fig. 1, the elimination process of large size liposomes can be described by conventional Michaelis-Menten kinetics. Beaumier et al (1983) reported that elimination of liposomes (mean diameters 18·7 nm) from the circulation can be explained by the sum of Michaelis-Menten type and first order processes. In our study, the elimination phenomenon could be described solely by the Michaelis-

containing sphingomyelin, which is hardly taken up by the reticuloendothelial system (Scherphof et al 1987) and their smaller liposomes will also be subject to pinocytosis.

Although the blood concentration results were successfully fitted to Michaelis-Menten kinetics, the uptake of liposomes in the liver and spleen was not. If the blood volume is assumed to be 20 mL for a 250 g rat, the recovery of administered liposomes from blood, urine, liver and spleen at the high infusion rate was approximately 100%, while at the low infusion rate, it was approximately 70%. The contribution of the non-linear uptake of liposomes by other tissues, such as lung, bone marrow, endothelial cells of the capillaries, adipose and brain may be the cause of this different recovery.

The efflux from liver or spleen to blood is negligible for at least 8 h (unpublished data) and therefore the uptake clearance into the organ can be calculated as $X(t)/AUC_{0\to t}$ (Fig. 3). The amount of intact liposomes taken up by the organ can be calculated from [^{3}H]inulin as an aqueous marker (contained within the liposomes). Fig. 3A, comparing the hepatic clearances at the same amount in the tissue shows that the higher the infusion rate, the higher the clearance. This pattern is different from the Michaelis-Menten simulation (Fig. 3B).

Sato et al (1986) showed that the uptake of liposomes depends on binding to the reticuloendothelial system assuming a limited number of binding sites. Further studies are required to examine whether this binding model can be applied to the present data or not. In addition, it is considered that other particles and macromolecules (such as beads, acetylalbumin, lipid emulsion, foreign erythrocytes) are taken up by Michaelis-Menten type saturable processes (Normann 1974).

Recently, many peptides have been developed as drugs as a result of progress in molecular biology. However, most of them are labile in the body, and to use their potential as drugs, it is essential to prevent their degradation in the blood and to control the blood concentration. Liposomes are useful drug carriers which can incorporate both lipophilic and hydrophilic compounds. Therefore we need knowledge of liposome disposition, and their clearance by the reticuloendothelial system. To use liposomes as drug carriers in the rational drug delivery system, quantitative analysis based on the clearance concept as described here is essential. Our study gives the basic information on the kinetic characterization of liposome clearance by the reticuloendothelial system.

References

Abra R. M., Hunt, C. A. (1981) Liposome disposition in vivo. III. Dose and vesicle size effects. Biochim. Biophys. Acta 666: 493–503

Bangham, A. D., Standish, M. M., Watkins, J. C. (1965) Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13: 238–252

Beaumier, P. L., Hwang, K. J., Slattery, J. T. (1983) Effects of liposome dose on the elimination of small unilamellar sphingomyelin/cholesterol vesicles from the circulation. Res. Comm. Chem. Pathol. Pharmacol. 39: 277–289

Chow, D. D., Essien, H. E., Padki, M. M., Hwang, K. J. (1989) Targeting small unilamellar liposomes to hepatic parenchymal cells by dose effect. J. Pharmacol. Exp. Ther. 248: 506–513

Darnell, J., Lodish, H., Baltimore, D. (1990) The internalization of macromolecules and particles. In: Molecular Cell Biology 2nd Ed., Scientific American Books, New York, pp 555–582

Gregoriadis, G., Neerunjun, D. E. (1974) Control of the rate of hepatic uptake and catabolism of liposome-entrapped proteins injected into rats. Possible therapeutic applications. Eur. J. Biochem. 47: 179–185

Hwang, K. J. (1987) Liposome pharmacokinetics. In: Ostro, M. J. (ed.) Liposomes. From Biophysics to Therapeutics. Marcel Dekker, New York, pp 109–156

Juliano, R. L., Stamp, D. (1975) The effect of particle size and charge on the clearance rates of liposomes and liposomes encapsulated drugs. Biochem. Biophys. Res. Comm. 63: 651–658

Juliano, R. L. (1988) Factors affecting the clearance kinetics and tissue distribution of liposomes; microspheres and emulsions. Adv. Drug Delivery Rev. 2: 31–54

Kao, Y. J., Juliano, R. L. (1981) Interactions of liposomes with the reticuloendothelial system. Effects of reticuloendothelial blockade on the clearance of large unilamellar vesicles. Biochim. Biophys. Acta 677: 453–461

Kiwada, H., Niimura, H., Fujisawa, Y., Yamada, S., Kato, Y. (1985) Application of synthetic alkyl glycoside vesicles as drug carriers. I. Preparation and physical properties. Chem. Pharm. Bull. 33: 753–759

Normann, S. J. (1974) Kinetics of phagocytosis II. Analysis of in vivo clearance with demonstration of competitive inhibition between similar and dissimilar foreign particles. Lab. Invest. 31: 161–169

Sato, Y., Kiwada, H., Kato, Y. (1986) Effects of dose and vesicle size on the pharmacokinetics of liposomes. Chem. Pharm. Bull. 34: 4244–4252

Scherphof, G. L., Spanjer, H. H., Derksen, J. T. P., Kuiper, F., Vonk, R. J., Daeman, T., Roerdink, F. H. (1987) Delivery of liposome-associated drug to liver cells. Biochem. Soc. Trans. 15: 345–348

Sculier, J. P., Delcroix, C., Brassinne, C., Laduron, C., Hollaert, C., Coune, A. (1989) Pharmacokinetics of amphotericin B in patients receiving repeated intravenous high doses of amphotericin B entrapped into sonicated liposomes. J. Liposome Res. 1: 151–166

Yamaoka, K., Nakagawa, T. (1983) A nonlinear least squares program based on differential equations, MULTI(RUNGE), for microcomputers. J. Pharm. Dyn. 6: 595–606