Species Difference in the Disposition of Liposomes Among Mice, Rats, and Rabbits: Allometric Relationship and Species Dependent Hepatic Uptake Mechanism

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Purpose. The species difference in the pharmacokinetics of liposomes was investigated in mice, rats and rabbits.

Methods. Liposomes were intravenously injected at doses of 1, 10 and 100 (nmol/g body weight), and the time courses of liposomes in blood, liver and spleen were measured. Pharmacokinetic parameters were regressed as a function of body weight (BW) and dose of liposomes (D). The uptake mechanism of liposomes was also examined with the isolated perfused liver between rats and mice.

Results. Mean residence time increased with the increase of BW and D of liposomes. This increase of mean residence time resulted from the decreased total body clearance, which was principally explained by the species difference in the hepatic uptake clearance (CLh) of liposomes. The parameter CLh was regressed well by a multiple regression as a function of BW and D. In this analysis, an exponent for BW was around 0.5, which clearly indicates that smaller animals have higher uptake clearance per unit BW. Immunohistochemical analysis revealed that there was no significant difference in the density of Kupffer cells among these species. This suggest that the species difference in CLh resulted not from the density of Kupffer cells but from the uptake ability of Kupffer cells amoung species. In the isolated perfused liver, the hepatic uptake of liposomes was mainly explained by opsonin dependent uptake in rats, while opsonin independent uptake in mice.

Conclusions. These quantitative and qualitative information on the species difference of liposome disposition will provide an useful information for constructing a drug delivery system using liposomes.

KEY WORDS: liposome; species difference; pharmacokinetics; drug delivery system.

INTRODUCTION

Adolph first reported the allometric relationship between body weight (BW) and physiological parameters (1). There is a general tendency in the exponents of the allometric equations. As to organ volumes, the exponents tend to lie around 1.0, which indicates a 1:1 anatomic proportionality. On the other hand, the exponents for metabolic rates such as heat production, inulin clearance, urine output etc. tend to lie around 0.75, which indicates that smaller animals maintain a higher metabolic state than larger animals. This phenomenon is partly explained by a body surface area with the exponent of 0.67.

The allometric relationship in pharmacokinetic parameters was extensively studied in 1980s (2,3). There are good allometric relationships between BW and each pharmacokinetic parameter in small as well as large molecular weight compounds (4). There are many reports on the disposition of liposomes and physico-chemical factors such as lipid composition (5), charge (6), size (7) and dose (8) were shown to be important in determining the pharmacokinetics of liposomes. However, little has been known for the species difference in the disposition of liposomes.

We have examined the effect of dose (D) of liposomes on their hepatic uptake *in vivo* (9) and clarified the uptake mechanism of liposomes composed of hydrogenated phosphatidylcholine (HEPC): cholesterol (CH): dicetylphosphate (DCP) = 5:4:1 (10). This composition of liposomes are taken up by the liver through complement receptor mediated phagocytosis (10). However, it is still unknown whether the finding in rats can be extrapolated to other experimental animals, and then to humans.

In this study, we examined the relationship in the species difference of liposome disposition using mice (30 g), rats (300 g) and rabbits (3,000 g), changing the dose of liposomes from 1 to 100 nmol HEPC/g. Using a multiple regression analysis, each pharmacokinetic parameter was described well as a function of BW and D of liposomes. In addition, the hepatic uptake mechanism was compared between rats and mice using isolated perfused liver.

MATERIALS AND METHODS

Materials

Hydrogenated egg phosphatidylcholine was kindly donated by Nippon Fine Chem. Co. (Osaka, Japan). Dicetyl phosphate was purchased from Nacalai Tesque (Kyoto, Japan). Cholesterol was of analytical grade (Wako Pure Chem., Osaka, Japan). ^{3}H-cholesterylhexadecylether (^{3}H-CHE) was purchased from NEN (Boston, MA, USA). All other reagents were of commercially analytical grades.

Preparation of Liposomes

The method of preparing liposomes was basically the same as described elsewhere (9). Briefly, liposomes (multilamellar vesicles) were prepared to give the lipid molar ratio of HEPC/DCP/CH = 5/1/4. After hydration by phosphate buffered saline (PBS) at pH 7.4, liposomes were extruded through polycarbonate filters with pore sizes of 0.8 and 0.4 μm (Nuclepore, CA, USA) eight times each. The distribution of liposome diameter was determined by a dynamic laser scattering method (LPA-3100, Otsuka Electronics, Osaka, Japan). For measuring the degradation of liposomes, 5(6)-carboxyfluorescein (CF) was introduced as an aqueous phase marker. After extrusion, the CF

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ABBREVIATIONS: AUC: area under the blood concentration time curve; BW: body weight; Cb: blood concentration; CF: 5(6)-carboxyfluorescein; CH: cholesterol; CHE: cholesterylhexadecylether; CLh: hepatic uptake clearance; CLmax: the maximum uptake clearance; CL_{res}: uptake clearance by RES; CLs: splenic uptake clearance; CLtot: total body clearance; D: dose; DCP: dicetylphosphate; HEPC: hydrogenated egg phosphatidylcholine; MRT: mean residence time; P: pharmacokinetic parameter; PBS: phosphate buffered saline; RES: reticuloendothelial system; Vc: volume of distribution for central compartment; Xmax: the maximum uptake amount.

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encapsulated liposomes were dialyzed in a cellulose dialyzing tubing against PBS.

In Vivo Study

Male ddy mice (30 ± 5 g), Wistar rats (300 ± 35 g), and Japanese White rabbits (3,000 ± 300 g) were purchased from Inoue Experimental Animal (Kumamoto, Japan). The catheterization for liposome administration and blood sampling in rats (9) and rabbits (11) was performed as described previously. The doses of liposomes were fixed at 1 (low), 10 (medium) and 100 (high) nmol HEPC/g BW. At the end of the experiment, rats and rabbits were killed with an injection of excess sodium pentobarbitone and tissue was sampled for assay. In mice, liposomes were injected into the tail vein. Blood and tissue were sampled at indicated times after decapitation. The duration of sutdy was as follows: in mice, low (20 min), medium (60 min) and high (360 min); in rats, low (60 min), medium (120 min) and high (360 min); in rabbits, low (30 min), medium (120 min) and high (360 min). Since ^{3}H-CHE is a non-metabolizable lipid phase marker and not released from liposomes in the presence of serum (12), the total radioactivity in blood represents the liposomal ^{3}H-CHE. The radioactivity of each sample was assayed as described previously (9). The mean and standard deviation of liposome diameter were 0.436 ± 0.202 μm. The results were expressed as the mean ± standard deviation of three or four animals.

Pharmacokinetic Analysis

The time courses of blood concentration (Cb) of liposomes were analyzed by the non-linear least squares program, MULTI (13) and the total body clearance (CLtot), the volume of central compartment (Vc), and the mean residence time (MRT) of liposomes were calculated. The uptake clearance of liposomes by liver, spleen (CLh, CLs) were calculated by the uptake amount (X) divided by the area under the blood concentration time courve from time 0 to t (AUCo-t). Since liposomes used in this study were principally taken up by the liver and spleen, the uptake clearance by the reticuloendothelial system (CL_{res}) was defined as the sum of CLh and CLs.

Multiple Regression Analysis

Each pharmacokinetic parameter (P) was regressed by the Eq. (1) as a function of BW and D of liposomes.

$$\log P = a + b \log BW + c \log D \quad (1)$$

Saturation kinetics of CLh

The saturation kinetics of hepatic uptake of liposomes was described by the Eq. (2).

$$CLh = \Sigma Xmax, j/AUC[1 - \exp(-CLmax, j/Xmax, j*AUC)] \quad (2)$$

where CLmax and Xmax represent the maximum hepatic uptake clearance and the maximum hepatic uptake amount, respectively (9). The j represents a high-affinity/low-capacity and a low-affinity/high-capacity clearance. The CLh in mice were analysed according to the Eq. (2) and obtained parameters were compared with those in rats reported previously (9). In case of rabbits, the number of data points were too small to estimate these parameters reliably, because each time course of blood concentration was measured from one animal by sampling the arterial blood from cannula and CLh was measured only at the final sampling point.

Immunohistochemistry

To explain the species dependent CLh, the density of Kupffer cells were measured immunohistochemically. Liver specimens were fixed in periodate-lysine-paraformaldehyde for 4 hr at 4°C. After fixation, the specimens were washed with PBS containing 10, 15, and 20% sucrose for 4 hr, rinsed in PBS containing 20% sucrose and 10% glycerol for 1 hr, and then embedded in OCT compound (Miles, Elkhart, IN, USA). They were frozen in dry ice-acetone, cut by a cryostat into 6 μm thick sections, and dried in air. After inhibition of endogenous peroxidase activity by the method of Isobe et al. (14), the sections were incubated with a primary antibodies followed by a secondary antibody. To visualize peroxidase activity, the sections were incubated with 3,3′-diaminobenzidine (DAB) and H_2O_2 as substrate. Counterstaining was done with hematoxylin. As control, sections were incubated with PBS instead of primary antibody and then processed by the same procedures described above. The positive cells with nuclei per 1 mm^2 were counted with a light microscope. The specimens were prepared from three animals and the results were expressed as the mean and standard deviation.

Liposome Degradation in Fresh Serum

Serum was preincubated at 37°C for 10 min, after which an aliquot of liposomal suspension was added to the serum and incubated until 60 min (final concentration at 100 nmolHEPC/ml). A control study was done with PBS instead of serum. The result was expressed as % release of CF after subtracting the % release in PBS.

Perfusion Study

The hepatic uptake mechanism of liposomes in rats has already been reported (10). We have developed the same perfusion system for mice by scaling down the method for rats. The perfusate was infused at a constant rate with a peristalic pump and the flow rate was maintained at 2.5–3.0 ml/min/g liver. All studies were performed under single pass conditions. After a stabilization period of 10 min, liposomes labeled with ^{3}H-CHE were infused into the portal vein cannula at a constant rate for 10 min. After a one-minute wash with liposome-free perfusate, the liver was sampled and weighed, and the radioactivity was measured according to the method reported previously (10). The uptake of liposomes was expressed as the extraction (the hepatic uptake amount divided by the total input dose times 100). The viability of the liver was tested routinely by checking the value of glutamic oxaloacetic transaminase in the effluent. Fresh blood was obtained from mouse carotid artery, and the serum was separated by centrifugation and stored at −130°C until use. Liposomes were incubated with serum at 100 nmol HEPC/ml for 10 min. The total dose of liposomes in mice and rats were 18 and 150 nmol HEPC/liver, respectively. The calculated input concentration of liposomes were ~0.6

(nmolHEPC/ml) for both mice and rats. Unopsonized liposomes were incubated with PBS instead of serum.

RESULTS

Pharmacokinetic Study

The time courses of blood disappearance curves in each dose of liposomes are shown in mice, rats and rabbits in Figure 1. Effect of liposome dose on the disposition was seen in each species. It is clear from the blood disappearance curves that liposomes are cleared much faster in smaller animals when the same dose of liposomes per unit BW was administered. These curves were analyzed pharmacokinetically and parameters are shown in Figure 2 and Table I. The MRT increased with the increase of BW, which clearly shows the species dependent disposition of liposomes. A remarkable dose dependency was also observed in MRT in each species, which mainly resulted from the dose dependent CLtot, while the Vc increased in proportion to the BW independent of the dose of liposomes. It was also shown in the Figure 2 that the CLh largely contributes to CL_{res} and the CLtot is governed mainly by the CLh, although CLs has a large variation.

Each pharmacokinetic parameter was examined by a multiple regression analysis as a function of BW and D of liposomes. The results were summarized in Table II. The regressions were significant and the coefficients of determination (r^2) are higher than 90% except for CLs. The exponent of log(BW) for CLh is 0.54, which indicates that the smaller animals have much higher CLh (per unit BW) than larger animals.

Saturation Kinetics of CLh

The saturation characteristics of CLh in mice was described well with Eq. (2) and each parameter was compared to the corresponding value in rats reported previously (9). As shown in Table III, both CLmax and Xmax in mice are higher than those in rats in both uptake pathways. The remarkable difference was observed in the Xmax of the low-affinity/high capacity clearance pathway.

Immunohistochemistry

The number of Kupffer cells per mm^2 were measured in mice, rats and rabbits as 203 ± 45, 232 ± 54 and 109 ± 38 (mean ± standard deviation of three measurements), respectively. These values did not correlate with the order of CLh (mice > rats > rabbits). Thus, the density of Kupffer cells are not the principal cause of the species dependent CLh.

Liposome Degradation in Fresh Serum

As reported previously, the leakage study *in vitro* can be used as an index of complement activation (15). In rats, liposomes were degraded through an alternative complement pathway (15) and the activation of complement system also enhanced the hepatic uptake as opsonins (10). As shown in the Table IV, there was no degradation of liposomes in mouse serum. This suggests no activation of complement system by the liposomes in mice serum.

Perfusion Study

The hepatic uptake of liposomes was enhanced in rats by incubating liposomes with serum before infusion. On the other hand, no enhancement was observed in mice as shown in Table IV. These results show a difference in the hepatic uptake mechanism of liposomes between rats and mice. In rats, the hepatic uptake of liposomes was opsonin dependent, while opsonin independent in mice.

DISCUSSION

The allometric relationship in pharmacokinetic parameters has been extensively studied (2–4). In most cases, the smaller animals have higher clearance per unit BW, which is usually described by the exponent value for BW in the log-log relation-

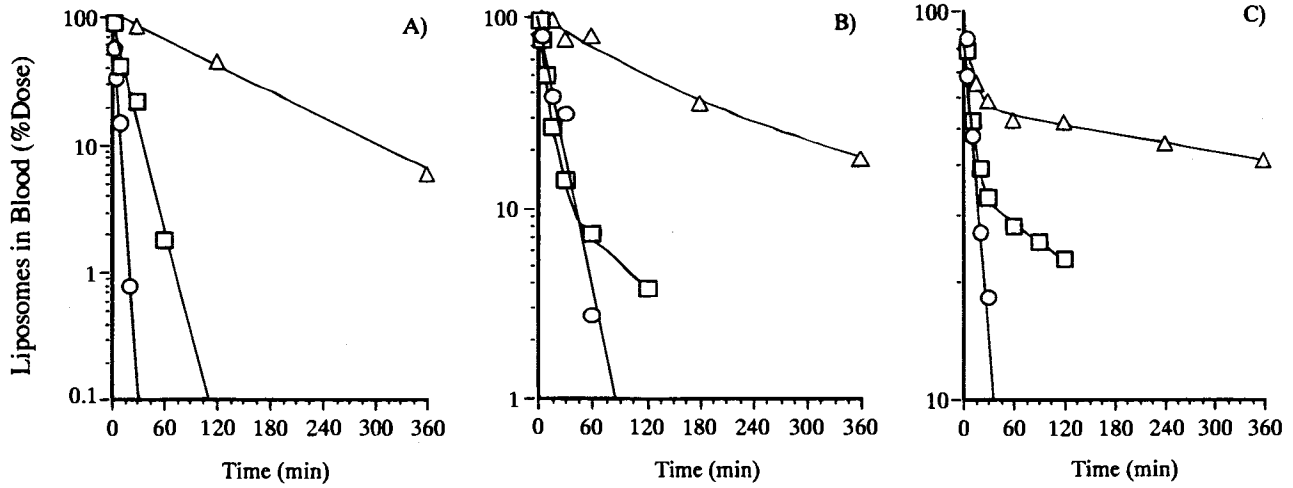

Fig. 1. Blood disappearance curves of liposomes in mice (A), rats (B), and rabbits (C) with changing dose of liposomes. The dose of liposomes was fixed at 1 (○), 10 (□), 100 (△) nmolHEPC/g BW. The percent of liposomes in blood compartment was calculated by multiplying blood concentration (% dose/ml) by Vc. Each symbol represents the mean value of three or four experiments. The solid line represents the fitting curve based on following equation.: $Cb = \Sigma[A_i \exp(-\alpha_i t)]$, where A_i and α_i represent the hybrid pharmacokinetic constants.

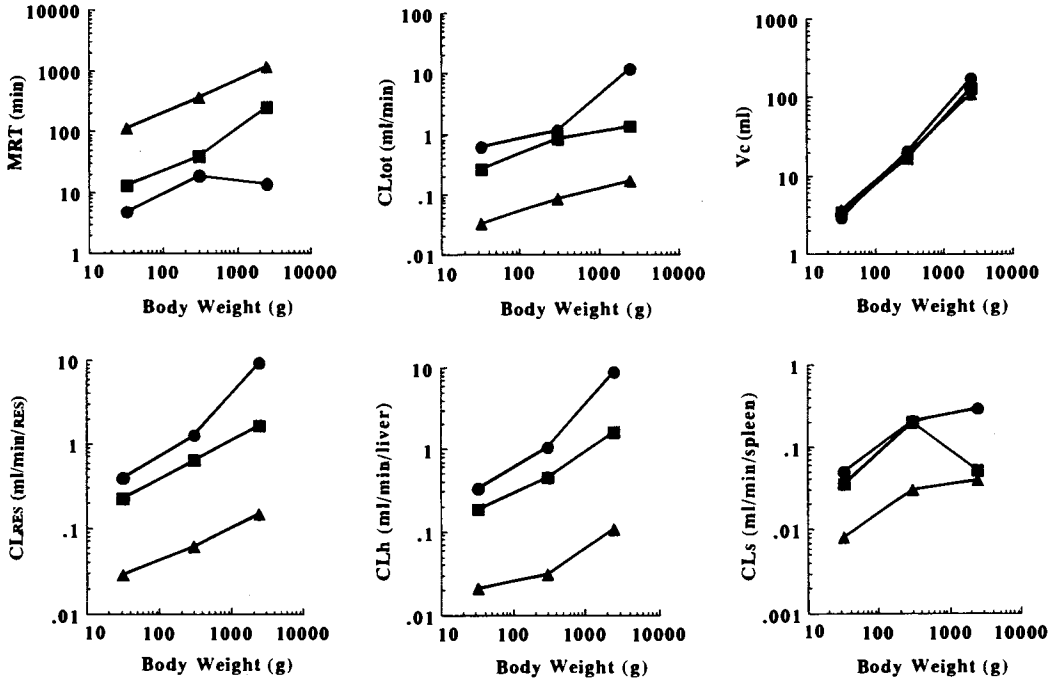

Fig. 2. Allometric relationship of pharmacokinetic parameters in mice, rats and rabbits. Relationship between BW and pharmacokinetic parameters such as MRT, CLtot, Vc, CLres, CLh and CLs are shown for each dose of liposomes. Each value represents the mean value of three or four experiments. The dose of liposomes was fixed at 1 (●), 10 (■), 100 (▲) nmolHEPC/g BW.

ship. As to pharmacokinetics of liposomes, species difference has not been evaluated systematically, mainly because the results in different group of study could not be compared due to the variation of physico-chemical factors such as lipid composition, size, surface charge etc., which significantly influence the pharmacokinetics of liposomes. In this study, we fixed the lipid composition of liposomes by which we have investigated the uptake mechanism (10) as well as dose dependency (9) in rats.

Remarkable species difference was observed in the MRT, which was mainly explained by the difference of CLh. According to the multiple regression analysis, the CLh increased with BW with the exponent value of 0.54 in Eq. (2). This value is smaller than commonly observed exponent values for metabolic clearances of drugs, probably due to the difference of underlying mechanism for CLh between liposome uptake and drug metabolism. In the hepatic uptake of liposomes, the liposomes were mainly taken up via phagocytosis or endocyto-

Table I. Pharmacokinetic Parameters of Liposomes Among Mice, Rats, and Rabbits

	Dose[a]		
	Low	Medium	High
Mice			
CLtot (ml/min)	0.608	0.255	0.0327
MRT (min)	4.80	13.3	113
Vc (ml)	2.91	3.39	3.69
Rats			
CLtot (ml/min)	1.47	0.719	0.052
MRT (min)	11.1	20.5	321
Vc (ml)	16.4	14.8	16.7
Rabbits			
CLtot (ml/min)	12.2	1.31	0.172
MRT (min)	13.9	251	1184
Vc (ml)	170	130	112

Note: Time courses of blood concentration of liposomes were fitted by MULTI (13) and the total body clearance (CLtot), volume of distribution for central compartment (Vc) and mean residence time (MRT) were calculated according to following equations: CLtot = D/AUC; $Vc = D/\Sigma A_i$; $MRT = \Sigma(A_i/\alpha_i^2)/\Sigma(A_i/\alpha_i)$.

[a] Low, Medium and High dose represent the 1, 10 and 100 nmolHEPC/g BW.

Table II. Regression Analysis of Pharmacokinetic Parameters as a Function of BW and D of Liposomes Among Mice, Rats, and Rabbits

Parameters	a	b	c	r^{2a}
Vc (ml)	−0.792*	0.854**	—[b]	99.1**
MRT (min)	−0.188	0.489*	0.766**	94.9**
CLtot (ml/min)	−0.816	0.482*	−0.713**	93.7**
CLh (ml/min)	−1.07	0.542*	−0.778**	93.1**
CLres (ml/min)	−0.983	0.523*	−0.710**	94.2**
CLs (ml/min)	−1.52	0.291	−0.426	78.8

Note: Vc, MRT and CLtot were calculated as described in Table I. CLh, CLs and CLres were calculated as follows: $CLh = Xh/AUC_{0\text{-}t}$, $CLs = Xs/AUC_{0\text{-}t}$ and CLres = CLh + CLs. Each pharmacokinetic parameter except Vc was analyzed by Eq. (1): $\log P = a + b \log BW + c \log D$. The symbols, * and **, represent the significance of the coefficient at levels $p < 0.005$ and $p < 0.001$, respectively.

[a] The r^2 represents the coefficient of determination.

[b] Vc was analysed as a function of BW only.

Table III. Saturation Kinetics of the Hepatic Uptake Clearance of Liposomes in Mice and Rats

Parameter	Mice[a]	Rats[b]	Ratio[c]
CLmax,[1] (ml/min/g liver)	0.29 ± 0.037	0.0867 ± 0.0218	3.3
Xmax,[1] (μmolHEPC/g liver)	0.130 ± 0.048	0.0569 ± 0.0064	2.3
CLmax,[2] (ml/min/g liver)	0.0127 ± 0.0072	0.0033 ± 0.0008	3.8
Xmax,[2] (μmolHEPC/g liver)	107 ± 155	1.71 ± 0.87	63

[a] Each parameter was obtained by the fitting based on Eq. (2) in this study.
[b] Each parameter was cited from our previous study (9).
[c] The value represents the ratio of each parameter of rat to mouse.

sis, and interspecies relationship of these uptake processes are not clarified quantitatively.

The CLh is smaller than hepatic blood flow rate in each case and the CLh of liposomes is intrinsic clearance limited. According to Adolph (1),

$$LW = 0.082\ BW^{0.87}$$

where LB represents the liver weight (g). The exponent value is 0.87 which indicated the smaller animals have larger liver based on the unit BW. However, this effect does not explain the observed exponent value of 0.5 for CLh. Therefore the species difference of CLh may result from the species dependent uptake ability *per se*. Then, the density of Kupffer cells in the liver was examined as a cause of species dependency in CLh. However, histochemical analysis did not explain the species difference of CLh.

The saturation characteristics of the dose dependent CLh was analyzed by Eq. (2) in mice and rats. In this analysis, the uptake clearance was characterized by CLmax and Xmax, which represent the affinity and capacity of liposome uptake, respectively. As shown in Table III, each parameter of mice is higher than that of rats. Remarkably high capacity was found in mouse Xmax for the low-affinity/high-capacity pathway. This high capacity may explain the higher CLtot in mice (1.09 ml/min/kg) compared to rats (0.178 ml/min/kg) and rabbits (0.057 ml/min/kg) at high dose. Further studies are required to estimate these parameters reliably in rabbits.

Table IV. Species Dependent Heptaic Uptake Mechanism of Liposomes in Rats and Mice

Treatment	Rats	Mice
Degradation in Serum[a]	8.1 ± 1.0	0
Hepatic Extraction[b]		
Unopsonized	1.4 ± 0.3	10.4 ± 0.9
Opsonized	12.6[c] ± 1.9	12.3 ± 0.7

[a] The degradation of liposomes in fresh serum was expressed as percent release of CF from liposomes. The concentration of liposomes in the incubation with serum was fixed at 100 nmol HEPC/ml. There was no detectable release of CF from liposomes in mouse serum.
[b] The concentration of liposomes in the incubation with serum was fixed at 100 nmol HEPC/ml. The mean diameter of liposomes in each case was fixed at 0.4 μm. The results are shown as the mean of three to six experiments with standard deviation.
[c] Significantly different from the unopsonized value by the Student's t-test ($p < 0.001$).

The effect of serum on the hepatic uptake of liposomes was examined in the perfused liver. In rats, the hepatic uptake of liposomes was opsonized by the preincubation with serum, which is consistent with our previous results (10). Our recent study found that there is a good correlation between the degradation of liposomes in serum and opsonization in the hepatic uptake of liposomes in rats (16). On the other hand, no enhancement was observed by the mouse serum. This result suggests that there was no activation of complement system in mouse serum. This result suggests that there was no activation of complement system in mouse serum, which is consistent to the *in vitro* study where there was no significant release of CF in the mouse serum. Thus, these results indicate that liposomes are taken up by the liver via an opsonin independent manner in mice. In rats, the high-affinity/low capacity uptake pathway corresponded to opsonin dependent uptake pathway and the low-affinity/high-capacity uptake pathway corresponded to opsonin independent uptake pathway. While in mice, only opsonin independent pathway was observed in this experimental condition and the underlying mechanisms of the two uptake pathways derived based on the Eq. (2) in mice are not clear at this stage.

In conclusion, the species difference in the pharmacokinetics of liposomes was shown among mice, rats and rabbits and pharmacokinetic parameters were quantitatively analyzed with a multiple regression as a function of BW and D of liposomes. The quantitative difference of the disposition of liposomes was explained principally by the species dependent CLh. This difference was not explained by the density of Kupffer cell according to the histochemical investigation. According to the isolated perfused liver system, liposomes were mainly taken up via opsonin dependent pathway in rats, while via opsonin independent pathway in mice. These quantitative and qualitative analysis on the disposition of liposomes will contribute to the development of rational drug delivery system using liposomes as a drug carrier.

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