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Is control of distribution of liposomes between tumors and bone marrow possible?

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Abstract

The objective of this study is to clarify to what extent the accumulation of liposomes from the blood into the tumor and bone marrow can be controlled by liposome size and membrane fluidity. Liposomes with different diameters (50–400 nm) and different membrane fluidity were prepared from hydrogenated egg phosphatidylcholine (HEPC) or egg phosphatidylcholine (EPC), cholesterol (Ch) and dicetylphosphate in various molar ratios. These liposomes were injected intravenously into rats bearing Yoshida sarcoma, and the ratios of the accumulation of liposomes in the tumor to those in the bone marrow, liver and spleen were compared. The tumor-to-bone marrow accumulation ratio increased with the decrease in liposome size from 400 to 50 nm. This ratio was greater than those for the liver and spleen at all sizes. Although tumor-to-liver accumulation ratios of 50- and 100-nm HEPC-containing liposomes were higher than those of EPC-containing liposomes, no obvious difference in tumor-to-bone marrow or tumor-to-spleen accumulation ratios was found between these liposomes. Tumor-to-bone marrow accumulation ratio of HEPC-containing liposomes increased remarkably with the decrease in Ch content from 40 to 30 or 20 mol% compared with ratios for the liver and spleen. Interestingly, the tumor uptake clearance of liposomes of the same size was constant regardless of their membrane fluidity. These findings show that the increases in these accumulation ratios are due to their decreased uptake clearance by the bone marrow. Furthermore, the uptake of 50-nm HEPC-containing liposomes by the bone marrow was specifically inhibited by preinjection of other liposomes, but not when they were exposed in advance to in vivo components. These observations suggest the involvement of in vivo component(s) in the uptake of these liposomes by the bone marrow. We conclude that small HEPC-liposomes with low Ch content show their significantly decreased uptake by the bone marrow due to their decreased recognition by this tissue.

Keywords: Liposome; Distribution; Size; Membrane fluidity; Tumor; Bone marrow

1. Introduction

The change in the biodisposition of an antitumor agent by encapsulation in liposomes has been expected to increase its accumulation in the tumor or reduce its toxicity, thereby enhancing its therapeutic usefulness. The encapsulation of an antitumor drug in liposomes has been reported to effectively reduce systemic toxicity [1] and toxicity in organs such as the heart [2] and kidney [3] due to the slow release of the drug from liposomes in blood circulation or reduced drug accumulation in these organs. However, pharmacokinetic alteration by liposomes includes the possibility of an increase in toxicity in some organs or generation of new toxicity [4]. Liposomes tend to accumulate more in tissues belonging to the reticuloendothelial system (RES) such as liver, spleen and bone marrow, compared with others [5]. Thus, toxicity in the RES may be increased or generated by the administration of liposomes containing an antitumor agent.

In particular, myelosuppression is the most important toxicity in the RES, since it serves to greatly limit the dose of many antitumor agents. Indeed, for doxorubicin [6] and cisplatin analog [3] encapsulated in liposomes, leukopenia is likely to be the dose-limiting toxicity in humans. We have also observed that the considerable decrease in num-

Abbreviations: HEPC, hydrogenated egg phosphatidylcholine; EPC, egg phosphatidylcholine; Ch, cholesterol; DCP, dicetylphosphate; RES, reticuloendothelial system; *P*, steady-state fluorescence polarization; AUC, area under the curve.

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ber of leukocytes in the blood is induced by daunorubicin which is encapsulated in liposomes composed of hydrogenated egg phosphatidylcholine, cholesterol and dicetylphosphate in a molar ratio of 5:4:1, although this liposome preparation showed superior antitumor activity to free daunorubicin [7]. To minimize the myelosuppression, it would be necessary to formulate liposomes so that their accumulation in the tumor tissue may be as high as possible relatively to that in the bone marrow, a major hematopoietic and lymphopoietic tissue. Such liposomes will be very useful drug-carriers for cancer chemotherapy.

The uptake of liposomes by RES is well known to be influenced by various their physicochemical factors such as their size [8], surface charge [9], surface hydrophilicity [10] and membrane fluidity [11]. These factors are thought to affect the interaction of opsonins with the liposomal surface. Several investigators [12–16] have shown that serum contains specific opsonins for macrophages in the liver, spleen and bone marrow. Thus, the mechanisms underlying the uptake of liposomes by these organs may not always be common. Recently, we [17] and others [14,18] reported that the circulation time of liposomes in the blood and their size was important in determining their accumulation in tumor tissue. Furthermore, we demonstrated that the accumulation of liposomes from the blood into the tumor was dependent only on their size, and not on their circulation time in the blood [17]. These findings suggest that different mechanisms are involved in the accumulation of liposomes from the blood into the tumor and bone marrow, and therefore, the accumulation of liposomes into these tissues may be controlled by utilizing these differences.

In this study, we investigated the effects of the physicochemical characteristics of liposomes on their accumulation in the tumor and bone marrow using tumor-bearing rats. We focused here on the size and membrane fluidity of liposomes as their physicochemical factors.

2. Materials and methods

2.1. Materials

Hydrogenated egg phosphatidylcholine (HEPC) and egg phosphatidylcholine (EPC) were kindly supplied by Nippon Fine Chemical (Osaka, Japan) and Nippon Oil & Fats (Tokyo, Japan), respectively. Cholesterol (Ch) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Dicetylphosphate (DCP) was obtained from Nacalai Tesque (Kyoto, Japan). [^{3}H]Cholesteryl hexadecyl ether was purchased from NEN (Boston, MA, USA). Other chemicals used were of reagent grade.

2.2. Preparation of liposomes

All liposome preparations were labeled with a trace of [^{3}H]cholesteryl hexadecyl ether (1 μCi/40 μmol of total lipids) as a lipid phase marker. This lipid label has been demonstrated to be non-metabolizable [19] and non-exchangeable [19,20]. The radiolabeled liposomes composed of HEPC or EPC, Ch and DCP in a molar ratio of 5:4:1, 6:3:1 or 7:2:1 were prepared as reported previously [20] with a minor modification. In brief, these lipids were dissolved in chloroform with a trace of [^{3}H]cholesteryl hexadecyl ether and the solvent was evaporated in vacuo. The obtained lipid films (200 μmol as total lipids) were hydrated in 5 ml of phosphate-buffered saline (Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g, NaCl 8 g, KCl 0.2 g/l; pH 7.4; PBS). Some of these liposome suspensions were extruded through a polycarbonate membrane filter (Nuclepore, Pleasanton, CA, USA) with a pore size of 0.4, 0.2 or 0.1 μm. The others were sonicated into small unilamellar vesicles using a probe-type sonicator (UR-200P, Tomy, Tokyo, Japan) until they became transparent for 90 min on ice, and then centrifuged at $100\,000 \times g$ for 60 min to remove large particles and make their size homogeneous. The final concentration of total lipids was approx. 40 mM. The liposome size was determined by the dynamic light scattering with a submicron particle analyzer (Nicomp model 370, Particle Sizing Systems, Santa Barbara, CA, USA).

2.3. Steady-state fluorescence polarization (P) of liposome membranes

The degree of fluorescence polarization of diphenylhexatriene in liposomes at 37°C was used as a parameter to determine membrane fluidity, and determined according to the method of Shinitzky and Barenholz [22] with slight modifications. 12 ml of 0.5 mM liposome suspensions were incubated with 0.4 μl of 6 mM diphenylhexatriene in *N,N*-dimethylformamide at 37°C for 75 min. The fluorescence intensity of diphenylhexatriene was then measured by excitation and emission wavelengths of 360 and 425 nm, respectively, at 37°C, and the degree of fluorescence polarization (P) was calculated by the following equation.

$$P = (I_{vv} - G \cdot I_{vh})/(I_{vv} + G \cdot I_{vh})$$

$$G = I_{hv}/I_{hh}$$

where I_{vv} and I_{vh} are the vertical and horizontal components of fluorescence intensity of diphenylhexatriene excited by vertically polarized light, respectively. G is the correction factor, and I_{hv} and I_{hh} are the vertical and horizontal components of fluorescence intensity obtained using horizontally polarized light, respectively.

2.4. Inoculation of tumor cells into rats

Yoshida sarcoma cells ($5 \cdot 10^6$ cells) were subcutaneously implanted into the right backs of male Donryu rats (5 weeks old, SLC, Shizuoka, Japan) as described previously [17]. The implantation site was located between the

axilla and thoractic spine. Rats were used for experiments 4 or 5 days after implantation.

2.5. Biodistribution of liposomes

^{3}H-Labeled liposomes were intravenously injected via the tail vein at a dose of 100 μmol lipid/kg. After specified periods, the animals were anesthetized by diethyl ether and blood samples were then taken from the abdominal vein. Immediately, the abdominal vein and artery were cut and the animals were sacrificed by bleeding. Tumor, liver and spleen were then removed and weighed. A femoral bone was also removed and vertically divided into two pieces. Bone marrow was taken from these pieces and weighed. After decolorization with 35% H_2O_2 and dissolution with 2 M KOH in isopropyl alcohol [21], the radioactivity in each sample was measured with an Aloka LSC-700 liquid scintillation counter. The weights of blood and bone marrow were assumed to be 6.5 and 3% of the body weight, respectively [23]. The area under the blood concentration–time curve (AUC) was calculated by the trapezoidal rule [24]. The tumor-to-tissue accumulation ratio and the uptake clearance by each tissue were calculated by dividing the amount (% dose/g) of liposomes accumulated in the tumor by that obtained for each tissue and by the corresponding AUC, respectively.

In another set of experiments, ^{3}H-labeled liposomes (200 μmol lipid/kg) were injected intravenously into rats, and recovered from the blood 15 min after injection. Immediately, these blood samples were centrifuged (3000 rpm, 4°C, 15 min) to obtain the plasma containing ^{3}H-labeled liposomes. The obtained plasma or ^{3}H-labeled liposomes without exposure to in vivo components were injected at a dose of 50 μmol lipid/kg into another group of rats preinjected 1 or 24 h previously with unlabeled liposomes (100 μmol lipid/kg). 1 h after the injection, blood samples, bone marrow and other tissues were obtained as described above. In these experiments, since there was plenty of the radioactivity in the blood 1 h after injection, we corrected the blood content in each tissue. Correction factors for the blood content in tissues were determined using the [^{51}Cr]RBC method [14] (Kiwada, H., unpublished data).

2.6. Statistics

Following the F-test, statistical analysis was performed using Student's t-test, Aspin-Welch test or Cochran test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of liposome size on distribution of HEPC-containing liposomes in tumor and bone marrow

Fig. 1 shows the effects of liposome size on tumor-to-bone marrow, -liver and -spleen accumulation ratios of liposomes composed of HEPC, Ch and DCP in a molar ratio of 5:4:1 (HEPC/Ch/DCP (5:4:1)-liposomes) 24 h after intravenous injection into Yoshida sarcoma-bearing rats. The ratio for the bone marrow was greater than those for the liver and spleen at all liposome sizes and decreased with the increase in the size. Similar size-dependency was observed in the ratio for the spleen. The ratio for the liver was the highest with liposomes of about 100 nm in diameter.

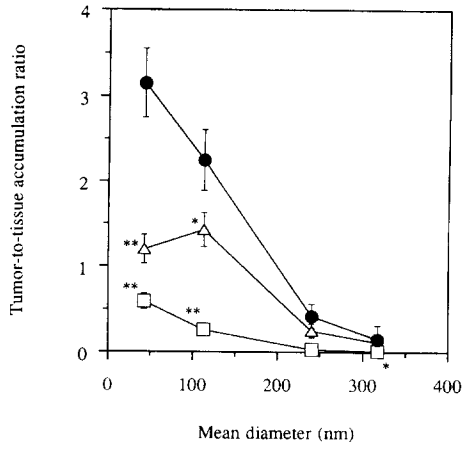

Fig. 1. Effects of liposome size on tumor-to-tissue accumulation ratios of HEPC/Ch/DCP (5:4:1)-liposomes 24 h after intravenous administration in Yoshida sarcoma-bearing rats. ^{3}H-Labeled liposomes were injected intravenously into tumor-bearing rats at a dose of 100 μmol lipid/kg. Tumors, bone marrow (●), livers (△) and spleens (□) were removed and weighed 24 h after intravenous injection. Values are the mean ± S.E. of three or four rats. Significantly different from tumor-to-bone marrow ratio: * $P < 0.05$; ** $P < 0.01$.

3.2. Effects of HEPC and Ch on membrane fluidity of liposomes

The membrane fluidity of liposomes is known to be regulated by the inclusion of a saturated phospholipid with high phase-transition temperature (such as HEPC) [25] and Ch [9,12] in the bilayers. Fig. 2 shows effects of Ch content on the membrane fluidity of HEPC- and EPC-containing liposome. The membrane fluidity was expressed as P-value. This parameter decreases with the increase in membrane fluidity. When the Ch content was decreased from 40 to 30 or 20 mol%, the P-value of HEPC-containing liposomes was virtually unchanged, while that of EPC-containing liposomes decreased.

3.3. Effects of membrane fluidity of liposomes on their distribution in tumor and bone marrow

Fig. 3 shows the effects of membrane fluidity on tumor uptake clearance of liposomes with diameters of 50 and 100 nm. The clearance of liposomes of the same size was constant regardless of membrane fluidity or lipid composition.

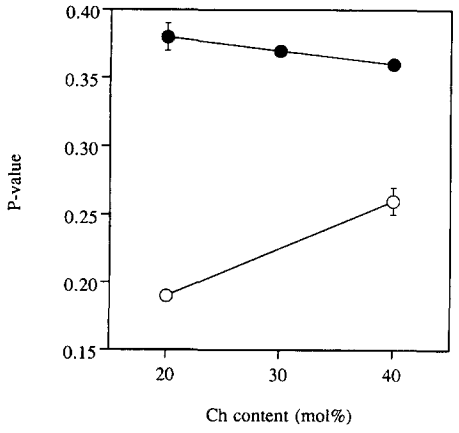

Fig. 2. Effects of HEPC and Ch on membrane fluidity of liposomes. The *P*-value, an order parameter of membrane fluidity, was measured at 37°C as described in Section 2, using 50- or 100-nm liposomes composed of HEPC or EPC, Ch and DCP in a molar ratio of 5:4:1, 6:3:1 or 7:2:1. Only the size of HEPC/Ch/DCP (7:2:1)-liposomes was 100 nm, since 50-nm ones could not be prepared in this molar ratio. The size of liposomes with other lipid compositions was 50 nm. Closed and open symbols represent HEPC- and EPC-containing liposomes, respectively. Values are the means ± S.E. of two or three experiments.

Fig. 4A and B show the tumor-to-bone marrow, -liver and -spleen accumulation ratios of 50- and 100-nm liposomes with various membrane fluidity, respectively. These ratios, especially the tumor-to-bone marrow accumulation ratio, were greater at low Ch content ($\leq$ 30 mol%) for both sizes of HEPC-containing liposomes, whose fluidity

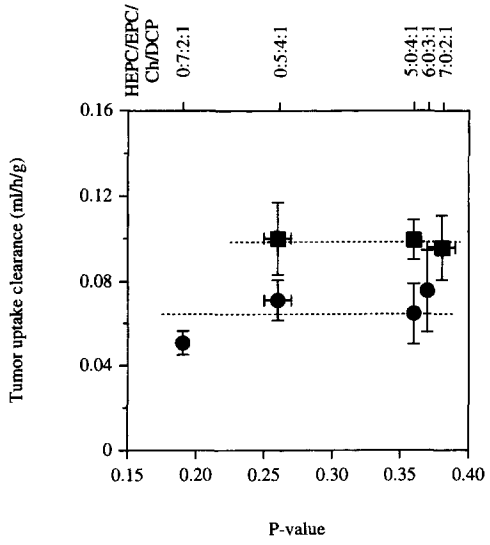

Fig. 3. Tumor uptake clearance of liposomes with various membrane fluidity. ^{3}H-Labeled liposomes with a diameter of about 50 nm (●) or 100 nm (■) were injected intravenously into tumor-bearing rats at a dose of 100 μmol lipid/kg. At appropriate intervals up to 24 h after intravenous injection, blood samples were taken via the abdominal vein and then tumors were removed and weighed. The tumor uptake clearance of liposomes was calculated by dividing the total amount of liposomes accumulated in the tumor until 24 h by the corresponding AUC. Values of tumor uptake clearance are the means ± S.E. of three or four rats. *P*-values are the means ± S.E. of two or three experiments.

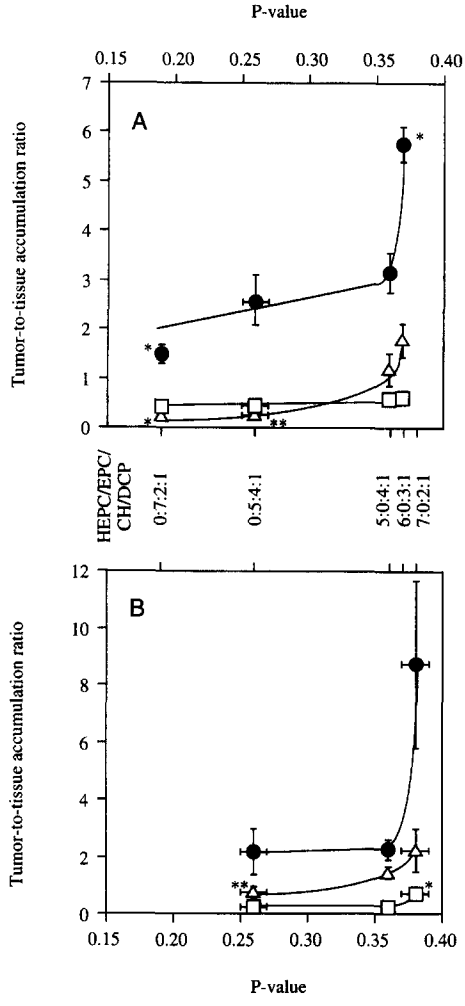

Fig. 4. Effects of membrane fluidity on tumor-to-tissue accumulation ratios of liposomes with diameters of about 50 nm (A) and 100 nm (B) 24 h after intravenous injection into Yoshida sarcoma-bearing rats. The details of this experiment and symbols are the same as in Fig. 1. Values are the means ± S.E. of three or four rats. Significantly different from HEPC/Ch/DCP (5:4:1)-liposomes: * $P < 0.05$; ** $P < 0.01$.

was almost the same in the Ch content range (20–40 mol%). However, these ratios were not significantly different in EPC-containing liposomes with different Ch content, which showed obviously different fluidity. Although HEPC-containing liposomes showed higher tumor-to-liver accumulation ratios than EPC-containing liposomes, there was no obvious difference in tumor-to-bone marrow or tumor-to-spleen accumulation ratios between both types of liposomes.

3.4. Effects of in vivo components on distribution of liposomes in bone marrow

To clarify the involvement of in vivo component(s) in the uptake of liposomes by the bone marrow, we investigated whether the accumulation of ^{3}H-labeled liposomes in the bone marrow was inhibited by the preinjection of unlabeled liposomes and whether that of ^{3}H-labeled lipo-

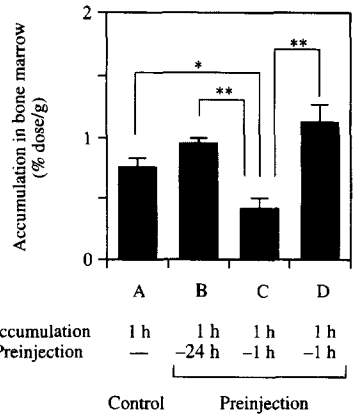

Fig. 5. Effects of in vivo components on accumulation of 50-nm HEPC/Ch/DCP (5:4:1)-liposomes in bone marrow. Column A (control, no preinjection): ^{3}H-labeled liposomes (100 μmol lipid/kg) were injected intravenously into rats. Columns B and C: ^{3}H-labeled liposomes (50 μmol lipid/kg) were injected intravenously into rats preinjected 24 and 1 h previously with unlabeled liposomes (100 μmol lipid/kg), respectively. Column D: ^{3}H-labeled liposomes associated with in vivo components were injected intravenously into rats preinjected with unlabeled liposomes under the same condition as in the column C. Liposomes associated with in vivo components were obtained as follows: ^{3}H-labeled liposomes (200 μmol lipid/kg) were injected intravenously into rats and recovered from the blood 15 min after injection; the blood samples were immediately centrifuged (3000 rpm, 4°C, 15 min) to obtain the plasma containing ^{3}H-labeled liposomes. Bone marrow was removed 1 h after injection of ^{3}H-labeled liposomes. Values are the means ± S.E. of four rats. Significant difference: * $P < 0.05$; ** $P < 0.01$.

somes associated with in vivo components was affected by the preinjection of unlabeled liposomes. In these experiments, 50-nm HEPC/Ch/DCP (5:4:1)-liposomes were used, and liposomes associated with in vivo components were obtained by the exposure to in vivo components for 15 min after intravenous injection into rats. The results of these experiments are shown in Fig. 5. The preinjection of unlabeled liposomes 24 h prior to administration of labeled liposomes did not inhibit their accumulation in the bone marrow 1 h after their intravenous injection (column B). However, the preinjection of unlabeled liposomes 1 h prior to administration of labeled liposomes significantly inhibited their accumulation in the bone marrow 1 h after intravenous injection (column C). When labeled liposomes exposed to in vivo components for 15 min were intravenously injected 1 h after the preinjection of unlabeled liposomes, no inhibitory effect of preinjection of unlabeled liposomes was observed (column D).

4. Discussion

We demonstrated previously that the uptake clearance of liposomes by tumors depended primarily on their size and that of liposomes with a diameter of 100 nm was maximum [17]. The observation that the tumor uptake clearance of liposomes of the same size was constant regardless of their membrane fluidity or lipid composition (Fig. 3) is consistent with this finding. The tumor-to-tissue accumulation ratio is equal to the tumor-to-tissue uptake clearance ratio, since the uptake clearance is calculated by dividing the accumulation amount by AUC. Thus, the increased ratios for the bone marrow or spleen of 50-nm HEPC-containing liposomes (Fig. 1) are due to decreased uptake clearance by these tissues. Also, as the tumor uptake clearance of liposomes of the same size was constant (Fig. 3), the increased tumor-to-tissue accumulation ratios as shown in Fig. 4 are due to decreased uptake clearance of liposomes by the liver, spleen and bone marrow. On the other hand, the decreased tumor-to-liver accumulation ratio of 50-nm HEPC-containing liposomes (Fig. 1) is due to decreased their uptake clearance by the tumor, because the uptake clearance of HEPC-containing liposomes by the liver decreased with the decrease in liposome size (data not shown). Therefore, it is considered that, at smaller size and lower Ch content, the uptake clearance of HEPC-containing liposomes by the bone marrow was dramatically reduced without changes in their tumor uptake clearance. Such decreased uptake clearance of liposomes by the bone marrow could be attributed to the difference between the mechanisms by which they accumulate in the tumor and bone marrow.

The marked change in tumor-to-bone marrow accumulation ratio of Ch-poor HEPC-containing liposomes suggests some in vivo component(s) involved in the uptake of liposomes by the bone marrow. The results shown in Fig. 5 strongly support this possibility. These results also show that the in vivo component(s) is (are) subject to temporary depletion at an early stage after intravenous injection of liposomes.

Although the mechanism of the uptake of liposomes by the bone marrow is unknown, several in vitro studies have shown Ch-dependent uptake of multilamellar liposomes by macrophages in the bone marrow. Moghimi and Patel [16] suggested the involvement of serum components in the uptake of Ch-rich EPC-containing liposomes by reticuloendothelial cells in the bone marrow. The in vivo findings of the present study using small liposomes (Figs. 4 and 5) are consistent with this finding, and thus the uptake of these liposomes by the bone marrow may be mediated by similar serum components. This possibility is supported by a microscopic observation [26] that about 100-nm liposomes containing monosialoganglioside localized exclusively in the resident macrophages in the bone marrow. Allen et al. [27] presented a contrary report that the bone marrow macrophages showed low affinity for Ch-rich EPC-containing liposomes compared with those poor in Ch. However, this may not apply to our in vivo results, since it was obtained in the absence of serum.

Recently, a Ch-specific factor which associates with Ch molecules on the surface of PC-containing multilamellar liposomes to activate the alternative complement pathway was found in rat plasma [28]. Moghimi and Patel [16] suggested the possibility of the involvement of serum

components other than complement in the uptake of Ch-containing liposomes by bone marrow macrophages. Unfortunately, we have no evidence to clarify the nature of the factor(s) adsorbed on the surface of small liposomes. The decreased bone marrow uptake in Ch-poor HEPC-containing liposomes of small size may be due to the decreased interaction of the in vivo component(s) with the liposome surface.

The size of liposomes is a factor determining the magnitude of their interaction with plasma proteins such as opsonins [29]. The small size tends to decrease the interaction of plasma proteins with liposomes. This may explain the size-dependent decrease in the uptake clearance of liposomes by the bone marrow.

The composition of both HEPC and Ch appears to be responsible for the decreased bone marrow uptake in Ch-poor HEPC-containing liposomes, because an incomplete correlation was observed between the membrane fluidity or Ch content of liposomes and the tumor-to-bone marrow accumulation ratio (Fig. 4). The decrease in the fluidity of liposome membranes has been shown to decrease the interaction of plasma proteins with the membranes [9,11], and thus HEPC-containing liposomes with less membrane fluidity (Fig. 3) may show less interaction with proteins. However, Ch-poor HEPC-containing liposomes showed remarkably high tumor-to-bone marrow ratios, although their membrane fluidity was almost comparable to that of Ch-rich liposomes (Figs. 3 and 4). This suggests that the decreased uptake clearance of HEPC-containing liposomes by the bone marrow may be dependent on Ch content rather than membrane fluidity. The decreased Ch content may directly reduce Ch-dependent interaction of in vivo component(s) with the bilayers of HEPC-containing liposomes, and this may be assisted by the decreased membrane fluidity.

There are discrepancies between the dependency of the accumulation ratios for the bone marrow, liver and spleen on liposome size and fluidity (Figs. 1 and 4). This suggests different mechanisms underlying the accumulation of liposomes in these RES tissues. Several in vitro studies also suggest that liposomes are taken up by macrophages in these tissues through different opsonins [12,13,16,27]. Therefore, the accumulation of liposomes in the tumor and liver or spleen, as well as bone marrow, may be controlled by utilizing different mechanisms of the uptake of liposomes by these tissues. The answer to the title of this report is 'yes'.

The control of the distribution of liposomes between the tumor and bone marrow is more significant than the control of their distribution between the tumor and liver or spleen, because their accumulation in the bone marrow will determine the magnitude of myelosuppression induced by liposomal antitumor agents. Liposomes which allow controlled accumulation in the tumor, bone marrow and other organs may be utilized to lower the bone marrow toxicity of the liposome-encapsulated antitumor agent without reduction of antitumor activity.

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