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Repeated injection of pegylated liposomal antitumour drugs induces the disappearance of the rapid distribution phase

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Abstract

Upon repeated administration, empty pegylated liposomes lose their long-circulating characteristics, referred to as the accelerated blood clearance (ABC) phenomenon. To investigate whether cytotoxic drug-containing pegylated liposomes could also elicit a similar phenomenon, two pegylated liposomal antitumour drugs (doxorubicin and mitoxantrone) were prepared, and they were administrated twice in the same animals with a 10-day interval at a dose level of 8 mg kg^{-1} (pegylated liposomal doxorubicin) and 4 mg kg^{-1} (pegylated liposomal mitoxantrone). By comparing the overall pharmacokinetics after a single-dose injection with that in animals treated with two doses, it was surprising to find that repeated administration of pegylated liposomal antitumour drugs caused the disappearance of rapid distribution phase instead of the ABC phenomenon, resulting in the conversion of a two-compartment model to a one-compartment model. Further investigation revealed that repeated injection induced the decreased uptake of liposomal antitumour drugs by the spleen at the early time point of 0.5–8 h after injection. In contrast, the deposition of liposomal antitumour drugs into liver was not affected. Therefore, the disappearance of the rapid distribution phase might be related to the reduced spleen uptake at the early time point.

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Introduction

Liposomes have long been recognized as promising drug carriers, especially for the delivery of antineoplastic drugs (Gabizon 1994; Allen 1998; Moghimi et al 2001; Medina et al 2004). Liposome encapsulation may alter the pharmacokinetics and biodistribution of therapeutic agents, thus resulting in altered toxicity and efficacy (Gabizon et al 2003; Medina et al 2004; Immordino et al 2006). Provided liposomes are rationally designed, they could selectively deliver drugs into malignant zones due to enhanced permeability and retention effects, and reduce the exposure of healthy tissues to toxic drugs, leading to improved therapeutic indexes (Allen 1998; Moghimi et al 2001; Moghimi & Szebeni 2003).

Conventional liposomes are usually made from phosphatidylcholine (saturated or not) and cholesterol. Following intravenous injection, they are easily recognized by the reticuloendothelial system (RES), resulting in rapid clearance and nonlinear pharmacokinetics (Cattel et al 2003). To reduce endocytosis by the RES and realize the targeted delivery of drugs, polyethylene glycol (PEG) modified liposomes were introduced in the late 1980s and registered as 'stealth technology' by ALZA Company (Allen 1994; Oku & Namba 1994; Coukell & Spencer 1997; Reddy 2000; Moghimi et al 2001; Cattel et al 2003; Moghimi & Szebeni 2003). In 1995, Doxil/Caelyx, a pegylated liposomal doxorubicin formulation, was approved in the USA, representing a milestone technical breakthrough in the field of liposomal drug delivery (Gabizon 1994; Coukell & Spencer 1997; Gabizon & Martin 1997; Gabizon et al 2003; Medina et al 2004; Orditura et al 2004; Vail et al 2004).

Recently, it has been reported that repeated administration of PEG-modified liposomes could induce the disappearance of long-circulating characteristics, termed the accelerated blood clearance (ABC) phenomenon (Ishida et al 2002, 2003a, b, 2004, 2005, 2006a, b, c, 2007, 2008; Wang et al 2005, 2007; Ishida & Kiwada 2008). This

phenomenon might be affected by many factors, such as animal species (mouse or rat), time interval between repeated injections, lipid dose and properties of liposomes (Ishida et al 2003a, b, 2004, 2005). In mice, the maximum reduction in circulation half-time of the second dose usually occurred at 10 days after administration of the first dose. The administration of small-sized liposomes (~60 nm) with ~5% PEG grafting density at a dose of >20 μmol kg^{-1} lipid is enough to induce considerable ABC (Ishida et al 2003b, 2004). Subsequent experiments revealed that the generation of immunoglobin with anti-PEG activity might be responsible for the occurrence of the ABC phenomenon (Ishida et al 2006c, 2007; Wang et al 2007). Some researchers suspect that the presence of this phenomenon might affect the clinical application of pegylated liposomes, especially when repeated administration is needed. However, it should be noted that in almost all the previous studies, empty (drug-free) liposomes coated with PEG polymer were used, not drug-containing liposomes as would be used to treat diseases in the clinic.

We aimed to investigate if repeated administration of pegylated liposomal drugs could induce the ABC phenomenon. Since liposomes are good carriers for antineoplastic drugs, two liposome-entrapped antineoplastic drugs (doxorubicin and mitoxantrone) were prepared, and their plasma pharmacokinetics following repeated administration were investigated.

Materials and Methods

Materials

Doxorubicin hydrochloride and mitoxantrone were provided by Zhejiang Hisun Pharmaceuticals (Zhejiang, China) and Chongqing Kailin Pharmaceuticals (Chongqing, China), respectively. Hydrogenated soybean phosphatidylcholine (HSPC) was a kind gift from Degussa (Freising, Germany). *N*-(Carbonyl-methoxypolyethyleneglycol$_{2000}$)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt (MPEG$_{2000}$-DSPE) was purchased from Genzyme Pharmaceuticals (Liestal, Switzerland). Cholesterol and Sephadex G-75 (medium) were obtained from Sigma Chemical Company (St Louis, MO, USA). All other chemicals used in this study were of analytical or high-performance liquid chromatography (HPLC) grade. Female KM mice (8–10 weeks old) were obtained from Hebei Medical University.

Preparation of liposomes

Pegylated liposomal doxorubicin (PLD) and pegylated liposomal mitoxantrone (PLM) were prepared according to the following procedure. Briefly, a mixture of HSPC, cholesterol and MPEG$_{2000}$-DSPE (9.58:3.19:3.19, mass ratio) were solubilized in chloroform and dried to a thin lipid film under a stream of N_2 gas, followed by incubation overnight under vacuum to remove residual solvent. The dried lipid films were subsequently hydrated in 0.5 L of 300 mmol L^{-1} ammonium sulfate to a final lipid concentration of 15.96 mg mL^{-1}. The hydration process was performed at 65°C for 1 h. This dispersion was then homogenized in the M-110EH Laboratory Microfluidizer Processor (Microfluidics, Newton, MA, USA) at 1500 bar. This procedure formed small unilamellar vesicles, which were then filtered through a 0.22-μm filter membrane at 25°C to remove any large particles.

Remote loading of liposomes

To realize remote loading, a transmembrane ammonium sulfate gradient must be created. Thus, the extraliposomal ammonium sulfate solution was replaced by sucrose/glycine (250/50 mM) buffer using a Millipore Labscale TFF system (with 50 000 nominal molecular weight limit polysulfone filters; Millipore, Billerica, MA, USA). During the tangential flow filtration process, constant feed volume was maintained, and at least seven exchange volumes were used, resulting in liposomes suspended in an exterior aqueous phase composed of 250 mM sucrose and 10 mM glycine.

After diafiltration, 0.25 L of empty liposomes was mixed with 50 mL of doxorubicin solution (or 25 mL of mitoxantrone solution). In both cases, 10 mg mL^{-1} drug solutions were used. Accordingly, the drug-to-total lipid mass ratio was 1:7.98 for PLD and 1:15.96 for PLM. The mixtures were rapidly warmed to 65°C and incubated at the same temperature for 40 min, after which the mixture was rapidly cooled in an ice-water bath. After remote loading, a sample of the liposomes was taken to determine percent encapsulation and to measure the mean particle diameter.

The final liposome preparation was first concentrated to a drug concentration of 2 mg mL^{-1} for PLD and 1 mg mL^{-1} for PLM, then filtered using a sterile 0.22-μm cellulose acetate syringe filter, and stored refrigerated and protected from light until use.

Measurement of mean size and zeta potential

All the measurements were performed at 25°C using a Zetasizer Nano ZS (Malvern Instruments, UK). Before measurement, the samples were diluted in 5% glucose solution with a volume ratio of 1:100. DTS 4.0 software (www.malvern.co.uk) was used to collect the data that were analysed using 'multinarrow modes'. Each sample was analysed at least three times to calculate the mean and s.d.

Pharmacokinetic and tissue distribution studies

Plasma pharmacokinetic analysis and tissue distribution studies were performed in normal KM mice. All the experimental protocols were reviewed and approved by the Animal and Ethics Review Committee of Hebei Medical University.

A total of 195 KM mice (39 groups, five mice per group) received injections of 8 mg kg^{-1} of PLD or 4 mg kg^{-1} of PLM in a single intravenous bolus dose via the tail vein. In both cases, the injection volume was 10 mL kg^{-1}; thus, the liposomes were diluted to a concentration of 0.8 mg mL^{-1} for PLD and 0.4 mg mL^{-1} for PLM before injection. Blood samples were first obtained via cardiac puncture under ketamine/xylazine anaesthesia (intraperitoneal injection of

100 μL of 32 mg mL^{-1} ketamine and 4 mg mL^{-1} xylazine in water to achieve final doses of 160 and 20 mg kg^{-1}) and collected in Eppendorf tubes containing sodium heparin as an anticoagulant at specified time points after administration. The mice were then killed and the liver and spleen were rapidly excised, rinsed in ice-cold normal saline and snap frozen. Blood samples were centrifuged at 2500*g* for 10 min to separate the plasma. The plasma and tissue samples were stored at −20°C until additional analysis.

Doxorubicin and mitoxantrone concentrations in plasma and tissue samples were determined using an HPLC method (Ahmad et al 2005; Cui et al 2007). Before analysis, mouse tissues were first homogenized using a Tissue Tearor equipped with a 7-mm probe (Biospec Products, Inc., Bartlesville, OK, USA). A 10% (w/v) homogenate was prepared in cold purified water for PLD or in 20% ascorbic acid solutions for PLM.

Before doxorubicin analysis, the samples were treated as follows. For 25 μL plasma or 50 μL tissue homogenate, 50 μL 3.0% (v/v) Triton X-100 was added. The mixture was then vortexed for 10 s, and 50 μL of 65% (w/v) 5-sulfosalicylic acid dihydrate and 375 μL (for plasma) or 250 μL (for homogenate) methanol were added. The resulting mixture was vortexed again and then centrifuged at 20 000*g* for 10 min. For homogenate, 250 μL supernatant was pipetted into silanized glass autosampler vials and buffered with 25 μL of 3.0 M sodium acetate. For plasma, 100 μL supernatant was mixed with 50 μL of 3.0 M sodium acetate and 350 μL methanol. The injection volumes for plasma and tissue samples were 20 and 50 μL, respectively.

In contrast, mitoxantrone samples were treated using a simple procedure. For 150 μL plasma or tissue homogenate, 150 μL extraction solution (methanol containing 0.5 M HCl/acetonitrile 90:10 v/v) was added. The resulting mixture was vortexed and allowed to precipitate at −20°C for at least 1 h, and then centrifuged at 20 000*g* for 10 min. The supernatant was collected for analysis. The injection volume for samples was 20 μL.

To analyse doxorubicin levels, an Agilent 1100 Series HPLC system controlled by ChemStation A.8.04 software (www.chem.agilent.com) was used. A Waters fluorescent detector was connected through a signal converter. The autosampler was maintained at 4°C and the column compartment at 30°C. The HPLC separations were achieved using a Diamonsil C18, 250 mm × 4 mm i.d., 5 μm particle size column from Dikma (Beijing, China). A guard column (Diamonsil C18, 4 mm × 8 mm) was installed ahead of the analytical column. The isocratic mobile phase was a mixture of 46 vols of acetonitrile and 54 vols of a solution containing 2.88 g L^{-1} of sodium lauryl sulfate and 2.30 g L^{-1} of phosphoric acid, running at a flow rate of 0.8 mL min^{-1}. Detection was accomplished at $\lambda_{ex}480/\lambda_{em}580$ nm.

For mitoxantrone analysis, a Waters HPLC system controlled by Millennium 32 software was used for chromatographic analysis of mitoxantrone levels, which was composed of a 2690 liquid chromatograph and 996 diode array detector. The HPLC separations were also achieved using a Zorbax C18, 150 mm × 4 mm i.d., 5 μm particle size column. The isocratic mobile phase was a mixture of acetonitrile and a solution containing 6.0 g L^{-1} of sodium 1-heptanesulfonate and 9.0 mL L^{-1} of glacial acetic acid (30:70 v/v) at a flow rate of 1 mL min^{-1}. Detection was accomplished at 650 nm. The retention time for mitoxantrone was ~10 min, the recovery of drug was > 95% and the standard curve had an r value of 0.999.

The pharmacokinetic variables, tissue area under the curve (AUC) and maximum concentration (C_{max}) were calculated using DAS 2.0 software (www.drugchina.net). The difference in drug concentrations between different groups at each time point was examined by a series of independent samples *t*-test. Because individual animals were required to generate each data point and subsequent sampling over time was not possible, the difference in overall pharmacokinetics (plasma or tissue) was assessed with two-way analysis of variance (General Linear Model, Univariate, SPSS 11.5 software; SPSS Inc., Chicago, Illinois, USA). In all cases, $P < 0.05$ was considered to be statistically significant.

Results

Many factors, such as vesicle size, zeta potential and drug-loading efficiency, might have a role in the in-vivo behaviour of liposomal drugs (Drummond et al 1999), thus the liposomes should be well characterized. In our studies, both doxorubicin and mitoxantrone were loaded into pegylated liposomes in response to a transmembrane ammonium sulfate gradient. For both formulations, the percentage encapsulation was ~100% and the drug-to-lipid mass ratio was 1:8 for PLD and 1:16 for PLM. The mean vesicle sizes before loading and after loading were similar, with a value of ~64 nm. Moreover, all the formulations had a narrow size distribution, with a polydispersion index ranging from 0.08 to 0.11. Zeta potential measurement showed that drug loading did not affect surface potential, and all the vesicles were negatively charged, with a zeta potential of ~31 mV (Table 1). The negative charges carried by vesicles resulted from PEG lipids because under physiological pH values HSPC is neutral but PEG-DSPE is negatively charged.

Table 1 Characterization of pegylated liposomal doxorubicin (PLD) and pegylated liposomal mitoxantrone (PLM)

Formulation	Size (nm)	PDI	Zeta potential (mV)	Loading efficiency (%)	Drug-to-total lipid mass ratio
Empty liposomes	63.52 ± 0.29	0.113 ± 0.033	−31.3 ± 1.23		
PLD	64.90 ± 0.50	0.082 ± 0.010	−30.3 ± 3.86	99.8 ± 0.5	1:7.98
PLM	63.80 ± 1.05	0.114 ± 0.002	−31.6 ± 1.67	99.2 ± 0.4	1:15.96

PDI, polydispersion index. Data are presented as means from at least three repeated experiments.

Based on previous studies, if the time interval between the first and second dose is 10 days, repeated administration of pegylated liposomes will induce a dramatic reduction in the circulation time (Ishida et al 2003b, 2004). Therefore, in this study, the second dose of pegylated liposomal formulations was injected into mice 10 days after the first injection. Both formulations were injected into the mice at a dose equivalent to the optimum therapeutic dose level in S180 tumour-bearing models (8 mg kg^{-1} for PLD and 4 mg kg^{-1} for PLM; unpublished data). The plasma drug concentrations versus time profiles are presented in Figures 1 and 2.

The plasma pharmacokinetic parameters were calculated using DAS 2.0 software and the results are listed in Table 2. As shown in Table 2, the intravenous injection of PLD or PLM resulted in a prolonged t½ and small V_d values, approximately equal to the plasma volume of a 20-g mouse, which are the characteristics of pegylated liposomes (Coukell & Spencer 1997). Relative to PLD, PLM displayed rapid clearance kinetics, which might be ascribed to differences in formulation composition. Although both formulations had the same size, internal buffer and lipid composition, the entrapped drugs and drug-to-lipid molar ratio were different.

Two-way analysis of variance revealed that repeated injection of PLD induced no alterations in the plasma pharmacokinetics ($P = 0.257 > 0.05$). In this analysis, the plasma doxorubicin level was the dependent variable, and time and treatment group (single or repeated dose) were set as fixed factors (Univariate, SPSS software). An independent *t*-test performed at each time indicated that only at 2 and 8 h after injection were the plasma doxorubicin levels in animals treated with one dose of PLD significantly different to those in animals that received repeated injections (2/10 time points). For PLM, repeated injection also did not affect the overall plasma kinetics (two-way analysis of variance, $P > 0.05$) even though at certain time points (2, 4 and 24 h), mitoxantrone levels between those two groups were different ($P < 0.05$).

Although two-way analysis of variance revealed that the overall pharmacokinetic profiles exhibited no difference, it is surprising to find that indeed the concentration–time curve after the first injection could be well fitted to a two-compartment model instead of a one-compartment model, regardless of the kind of liposomal drugs ($P < 0.05$, DAS 2.0 software). In contrast, the one-compartment model is more suitable for the data from the second injection. The data calculated according to the optimum compartment are listed in Table 1. According to these data, $t½_{\alpha}$ for PLD and PLM following the first injection was 0.603 h and 0.890 h, respectively. The existence of an α phase might mean the rapid distribution of liposomal drugs into blood-rich organs.

When the animals were treated with liposomal drugs repeatedly, the α phase disappeared. For both formulations, the second injection would also induce a decreased distribution volume and increased AUC values ($P < 0.05$),

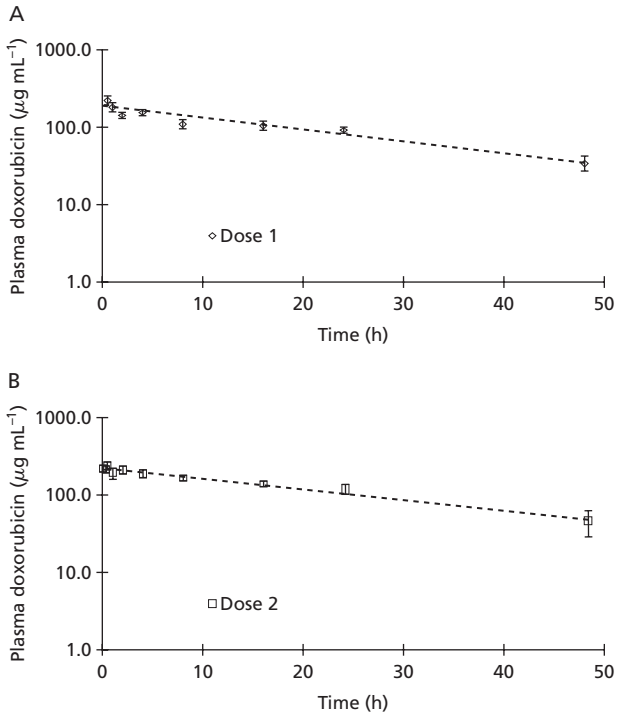

Figure 1 Plasma concentrations of doxorubicin in normal KM mice injected intravenously with a single (A) or repeated (B) dose of pegylated liposomal doxorubicin. The second dose was administered 10 days after the first dose. The doxorubicin dose was 8 mg kg^{-1} and the total doxorubicin levels were assayed using HPLC. Data are shown as mean ± s.d., n = 5.

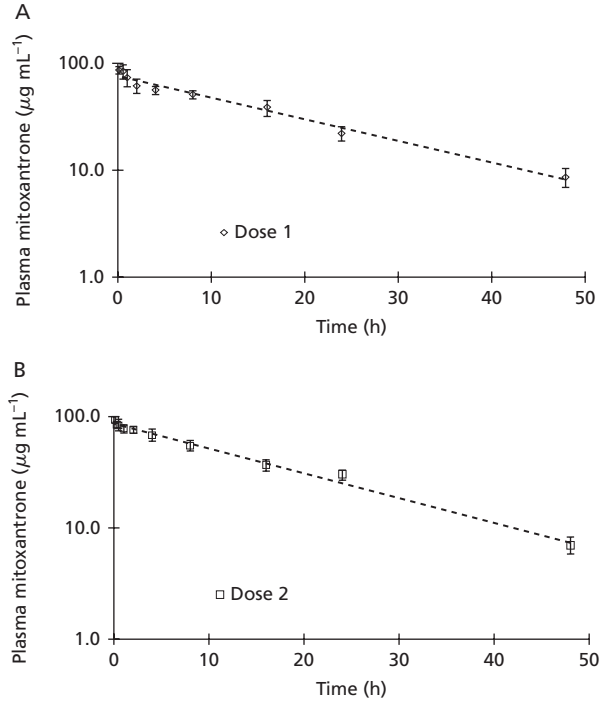

Figure 2 Plasma concentrations of mitoxantrone in normal KM mice injected intravenously with a single (A) or repeated (B) dose of pegylated liposomal mitoxantrone. The second dose was administered 10 days after the first dose. The mitoxantrone dose was 4 mg kg^{-1} and the total mitoxantrone levels were assayed using HPLC. Data are shown as mean ± s.d., n = 5.

Table 2 Pharmacokinetic parameters for pegylated liposomal doxorubicin (PLD) and pegylated liposomal mitoxantrone (PLM)

Parameter	PLD			PLM		
	First dose		Second dose	First dose		Second dose
$t½_{\alpha}$ (h)	20.125	0.890	22.574	15.039	0.603	13.477
$t½_{\beta}$ (h)		22.775			16.046	
V_1 (mL kg^{-1})	42.860	36.739	41.654	51.416	43.887	44.784
CL (mL kg^{-1} h^{-1})	1.476	1.511	1.279	2.370	2.422	2.303
AUC_{0-48} (mg h L^{-1})	4384.710	4195.194	4859.871	1490.745	1445.022	1588.846
AUC_{0-8} (mg h L^{-1})	5419.491	5293.329	6254.704	1687.992	1651.795	1736.658
k_{10} (1/h)	0.034	0.041	0.031	0.046	0.055	0.051
k_{12} (1/h)		0.187			0.266	
k_{21} (1/h)		0.581			0.872	

Pharmacokinetic parameters were calculated for total drugs after the intravenous injection of PLD and PLM (0–48 h). Mice received liposomal doxorubicin via a tail injection at a dose of 8 mg kg^{-1} and liposomal mitoxantrone at 4 mg kg^{-1}. Values represent the mean.

which indicated the decreased blood clearance rate of liposomal drugs. To investigate the phenomenon, we determined the doxorubicin and mitoxantrone levels in liver and spleen during the 48-h period following the first and second injection. It was interesting to find that, compared with the first injection, the second injection led to a marked decrease in spleen mitoxantrone levels, especially at the early time after injection (Figure 3A). However, repeated injection had no influence on liver mitoxantrone levels (data not shown). As shown in Figure 3A, during the 0.5–8-h period after injection, the spleen mitoxantrone levels in animals treated with two doses of liposomal mitoxantrone were significantly lower than those in mice receiving one injection of PLM. In the animals treated with PLD, we observed a similar phenomenon, and the difference between the two groups was also significant ($P < 0.05$, two-way analysis of variance) (Figure 3B). The reduced drug level in spleen might be responsible for the decreased clearance of the second dose, thus leading to the better fitting of the concentration–time data to a one-compartment model.

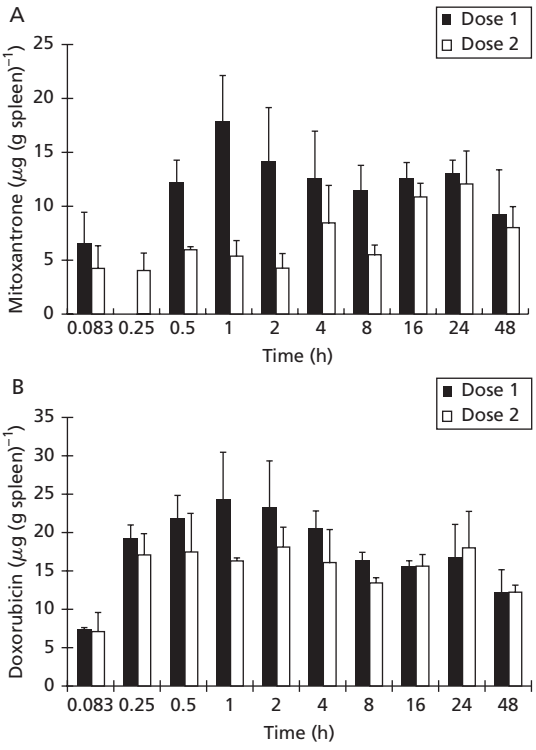

Figure 3 Spleen levels of total drugs were determined in KM mice following administration of 4 mg kg^{-1} mitoxantrone as pegylated liposomal mitoxantrone and 8 mg kg^{-1} doxorubicin as pegylated liposomal doxorubicin. A. Mitoxantrone levels; B. doxorubicin levels. The second dose was administered 10 days after the first dose. Data are shown as mean ± s.d., n = 5.

Discussion

Stealth technology represents a milestone breakthrough in the field of liposomal drug delivery (Allen 1994). Complete coverage of the liposome surface with hydrophilic PEG polymer may help evade recognition of liposomes by the RES. Thus, pegylated liposomes could effectively deliver encapsulated drugs into malignant zones, provided no encapsulated drugs are lost in circulation (Allen 1998; Cattel et al 2003). The first injection of empty pegylated liposomes could induce the ABC of the second dose, which occurs in a time interval dependent manner. The ABC phenomenon might affect the clinical application of pegylated liposomes and so it was interesting to investigate whether the repeated administration of drug-containing liposomes could also cause a similar phenomenon.

PLD and PLM were administered to mice at a dose level equivalent to the optimum therapeutic dose in a tumour-bearing model. The time interval between the two doses was 10 days, which could result in a drastic alteration of the second dose, provided empty liposomes are used (Ishida et al 2003b). However, compared with previous studies, repeated administration of liposomal cytotoxic drugs (PLD and PLM) did not elicit the ABC of the second dose.

Why did pegylated liposomes not induce the enhanced clearance of a second dose after the encapsulation of antineoplastic agents? To answer this question, we must take into account the antitumour mechanism of both drugs. Doxorubicin and mitoxantrone are semi-synthetic

anthracenediones, and they have a similar mode of action by which they exert their cytotoxic effects (Fox 2004; Soloman & Gabizon 2008). The cytocidal effects of anthracenecycles are thought to be related to nucleotide base intercalation, inhibition of topoisomerase and the generation of highly reactive species such as free radical OH. In-vitro studies have also shown that both drugs can inhibit B cell, T cell and macrophage proliferation and impair antigen presentation as well as the secretion of cell factors (Fox 2004; Soloman & Gabizon 2008).

Following intravenous injection, liposomal drugs are deposited into RES-rich organs such as the liver and spleen in spite of modification with PEG polymer. Although the encapsulated drugs are not bioavailable, the released drugs might be harmful to RES cells (Cui et al 2007). Both formulations are slow release formulations, made from solid phosphatidylcholine and loaded via the ammonium sulfate gradient method (Cui et al 2007). Based on previous studies, after accumulation into local tissues, the depletion of the transmembrane gradient might induce the release of trapped drugs (Lasic et al 1995). The drug release kinetics are slow, but taking into account the slow clearance of liposomes from local tissues and delayed occurrence of the ABC phenomenon (Ishida et al 2003b), the leaked drug might be enough to impair the activity of RES systems.

A mechanism underlying the induction of the ABC phenomenon by empty pegylated liposomes has recently been proposed (Ishida & Kiwada 2008). Following the first dose, anti-PEG immunoglobin M will be produced by the B cells in the spleen, which could selectively bind to PEG polymer on the liposomes when the second dose is administered, thus activating the complement system and enhancing the uptake of pegylated liposomes by Kupffer cells in the liver (Ishida & Kiwada 2008). Perhaps, it is just because the cells involved in the ABC phenomenon such as B cells in the spleen and Kupffer cells in the liver are effectively inhibited or killed by the released drugs that the administration of liposomal drugs prevents the occurrence of the ABC phenomenon. Furthermore, since the uptake of liposomes by the spleen was impaired, following intravenous injection, the rapid accumulation of liposomes in this organ might be affected, resulting in the disappearance of the rapid clearance phase.

Based on our observations, it is reasonable to deduce that the uptake of pegylated liposomes by the RES of spleen might occur in a time-dependent manner. Otherwise, it is difficult to explain why after more than 16 h, by repeated injection of PLD (or PLM), the drug levels in the spleen were nearly equal to those of the first injection. Pegylated liposomes could deposit into the spleen via at least two different means, including extravasation through sinusoidal endothelium and uptake by the RES of spleen. Perhaps the RES of the spleen plays a role at the early time following injection. Thus, if these types of cells are impaired by released cytotoxic drugs, the uptake of pegylated liposomes at the early time after injection will be affected, resulting in the decreased spleen drug levels between 0.5 and 8 h after repeated injection. In accordance with this explanation, it is not surprising to find that after more than 16 h, the drug levels in the spleen between the first dose and repeated injection were similar because at that time RES cells might exert no effects.

It should be noted that the reduction of drug levels of doxorubicin in the spleen was different to mitoxantrone (Figure 3). To explain this, the drug release rate and drug sensitivity must be considered. Although both formulations were similar, they had different drug-to-lipid mass ratios and encapsulated different drugs, which may affect the drug release rate and the amount of leaked drugs in the spleen. In addition, the RES cells may exhibit different sensitivity to both drugs. Therefore, following the first injection of PLD or PLM, the impairment of the RES may be different, leading to the reduction of drug levels to different degrees following the second dose.

Our observation that repeated injection of PLD does not induce the ABC phenomenon is consistent with previous studies. Ishida et al (2006b) investigated the influence of the first injected PLD dose on inducing the ABC phenomenon and found that the injection of PLD as a first dose did not affect the pharmacokinetics of empty pegylated liposomes. However, they did not find the disappearance of the α phase and the reduction of the spleen drug level at the early time after the second injection. The reasons that they did not observe the above phenomenon are that: (i) in their study, the first dose was PLD and the second was empty liposomes; and (ii) biodistribution studies were only performed at 24 h after injection. Our study clearly indicate the possible existence of time-dependent clearance of pegylated liposomes by the spleen RES, which may have important implications and needs further investigation.

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