Factors Affecting the Accelerated Blood Clearance of Polyethylene Glycol-Liposomes upon Repeated Injection

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ABSTRACT

Previously, we showed that long-circulating polyethylene glycol (PEG)-liposomes are cleared rapidly from the circulation when injected repeatedly in the same animal. In this article, we describe the effects of PEG-coating, the circulation time, the lipid dose, and the presence of encapsulated doxorubicin on the pharmacokinetics upon repeated injection in rats. Furthermore, the role of liver and splenic macrophages was investigated. Liposomes without PEG-coating also showed the so-called “enhanced clearance effect”: blood levels at 4 h post injection decreased from 62.8 ± 13.7% of injected dose (%ID) after the first injection to 0.54 ± 0.21%ID after the second injection. This decrease was independent of the circulation time of the first dose. Decreasing the first lipid dose of PEG-liposomes to 0.05 μmol/kg still led to enhanced clearance of a second dose of 5 μmol/kg. No changes in pharmacokinetics were observed when the second dose was 50 μmol/kg. When hepatosplenic macrophages were depleted, no enhanced clearance of repeated liposome injections was observed. A dose of doxorubicin containing PEG-liposomes (Doxil1 or Caelyx2), injected 1 week after injection of empty PEG-liposomes, was cleared rapidly from the circulation in rats. Our results indicate that hepatosplenic macrophages play an essential role in the enhanced clearance effect and that the change in pharmacokinetic behavior upon repeated injection is a general characteristic of liposomes, unrelated to the presence of PEG. Therefore, these findings may have a considerable impact on the clinical application of liposomal formulations that are administered repeatedly.

Long-circulating liposomes, either small sized liposomes with rigid bilayers or polyethylene glycol (PEG)-coated liposomes are considered suitable carrier systems for targeted drug delivery to tumors and inflammatory foci. In addition, long-circulating liposomes can also be used for diagnostic imaging applications, e.g., magnetic resonance imaging, computed tomography, and scintigraphic imaging (Torchilin, 1997). Critical factors affecting the blood clearance of long-circulating liposomes include the lipid composition, the presence of a polymer coating, and the lipid dose (Laverman et al., 1999a). In general, when incorporating phospholipids with long, saturated acyl chains, the blood residence time will be prolonged (Senior, 1987). Incorporation of cholesterol has a stabilizing effect and will result in a more rigid bilayer and consequently a longer circulation time (Senior and Gre goriadis, 1982). A first attempt to further increase the blood circulation time was by coating the liposomes with monosialo-ganglioside, to mimic the outer surface of erythrocytes (Allen and Chonn, 1987). The search for a clinical safe and inexpensive substitute resulted in the use of the hydrophilic polymer PEG as a liposome coating. When using PEG, the circulation time seems to be independent of the lipid composition (Schiffelers et al., 1999). Inclusion of cholesterol or a negatively charged lipid was shown to have hardly any effect on the blood residence time of PEG-liposomes. The circulation time of long-circulating liposomes may also be influenced by the administered lipid dose. For non-PEGylated long-circulating liposomes, circulation time may positively correlate with the lipid dose given; a lipid dose effect was observed over a broad dose range (Abra and Hunt, 1981); however, when using PEG-coated long-circulating liposomes, a dose effect was observed only at low lipid doses, i.e., lower than 0.5 μmol of phospholipid/kg of body weight in humans and 0.05 μmol/kg in rabbits (Laverman et al., 2000). Additionally, studies have been performed on the influence of the injection

ABBREVIATIONS: PEG, polyethylene glycol; PHEPC, partially hydrogenated egg-phosphatidycholine; DPPC, dipalmitoylphosphatidyl-choline; DSPE, distearoylphosphatidyl-ethanolamine; HYNIC, hydrazinonicotinamide; %ID, percentage of injected dose; p.i., post injection; 99mTc, technetium-99 m; PBS, phosphate-buffered saline.
regimen on the pharmacokinetics of long-circulating liposomes (Goins et al., 1998; Oussoren and Storm, 1999; Dams et al., 2000). Studies on the repeated injection of PEG-liposomes showed no changes in pharmacokinetics when the interval between the injections was 24 or 48 h (Oussoren and Storm, 1999) or 6 weeks (Goins et al., 1998). However, we recently showed that repeated injections of PEG-liposomes can be associated with substantial alterations of the pharmacokinetic behavior of PEG-liposomes (Dams et al., 2000). A second dose of PEG-liposomes was cleared very rapidly (within the first 30 min) from the blood of rats when the interval between the first and second injection was between 5 and 21 days (Dams et al., 2000). The observed effect (further referred to as “enhanced clearance effect”) diminished with subsequent injections. Furthermore, it was shown that the accelerated blood clearance of PEG-liposomes after the second dose is mediated by a soluble heat-labile serum factor (or factors) produced in response to the first injection. Transfusion of blood or serum from rats preinjected with PEG-liposomes 1 week before into nontreated rats could indeed elicit enhanced blood clearance of a first dose of PEG-liposomes in these rats. For the ease of presentation, we discriminate in the enhanced clearance effect two phases: the induction phase, following the first injection in which the biological system is “primed” (reflected in the formation of the fusible serum factor(s)), and the effectuation phase, following the second injection in which the PEG-liposomes are rapidly cleared from the bloodstream (Fig. 1).

In the present study, we investigated the dependence of the enhanced clearance effect on 1) the presence of PEG, 2) the long circulation property, and 3) the lipid dose. Furthermore, we studied the involvement of macrophages in the enhanced clearance effect. Finally, we studied the relevance of the enhanced clearance effect for the pharmacokinetics of the commercially available formulation of doxorubicin in PEG-liposomes (Caelyx or Doxili).

**Experimental Procedures**

**Materials.** Partially hydrogenated egg-phosphatidylcholine (PHEPC) with an iodine value of 35 and dipalmityloxyphosphatidylcholine (DPPC) were a gift from Lipoid GmbH (Ludwigshafen, Germany). Distearoylphosphatidyl-ethanolamine (DSPE) and the polyethylene glycol-2000 (PEG) derivative of DSPE were purchased from Avanti Polar Lipids (Alabaster, AL). N-Hydroxyxysuccinimidyldihydrizoniocitamidene (S-HYNIC) was synthesized as described by Abrams and coworkers (1990) with minor modifications (Claessens et al., 1996). The hydrazinonicotinamide-DSPE conjugate (HYNIC-DSPE) was prepared as described previously (Laverman et al., 1999b). Clodronate (chlorohemethylene-bisphosphonate) was a gift from Roche Diagnostics GmbH (Mannheim, Germany).

**Animals.** Randomly bred Wistar rats (HarlanCPB:WU) were obtained from Harlan Nederland (Horst, The Netherlands). For the experiments, 6- to 8-week-old male rats weighing 180 to 220 g were used. The animals had free access to water and were fed standard laboratory chow (Hope Farms, Woerden, The Netherlands). All experiments were carried out in accordance with the guidelines of the local Animal Welfare Committee.

**Preparation of the Liposomes.** The following liposome preparations were used: 1) small PEG-DSPE/PHEPC/cholesterol/HYNIC-DSPE (molar ratio, 0.15:1.85:1.0:0.07) liposomes (small PEG-liposomes, mean size about 90 nm). HYNIC-DSPE was incorporated in the lipid bilayer as a chelator to facilitate radiolabeling with technetium-99 m (99mTc); 2) small DPPC/cholesterol/HYNIC-DSPE (molar ratio, 1.85:1.0:0.07) liposomes (small DPPC-liposomes, mean size about 100 nm); and 3) large DPPC/cholesterol/HYNIC-DSPE (molar ratio, 1.85:1.0:0.07) liposomes (large DPPC-liposomes, mean size about 600 nm). Liposomes were prepared as described previously (Laverman et al., 1999b). Briefly, the lipids were dissolved in ethanol, and after evaporation of the organic solvent, the resulting lipid film was hydrated in phosphate-buffered saline (PBS, pH 7.4). The liposomes were sized by multiple extrusion through pairs of stacked polycarbonate membranes using a medium pressure extruder (Lexip Biomembranes Inc., Vancouver, BC). The phospholipid recovery after the first injection was 85% on average. The particle size distribution was determined by dynamic light scattering with a Malvern 2000 system equipped with a 25-mW Neon laser (Malvern Instruments Ltd., Malvern, UK). The mean size of the small PEG-liposomes and the small DPPC-liposomes was 90 and 100 nm, respectively, with a polydispersity index < 0.1. The mean size of the large DPPC-liposomes was 600 nm with a polydispersity index of 0.6. In all experiments, the liposomes were administered intravenously via the tail vein at a dose of 5 µmol of phospholipid/kg in a volume of 0.2 ml, unless stated otherwise.

PEG-liposomes encapsulating doxorubicin (Caelyx) were obtained from Schering-Plough NV/SWA (Brussels, Belgium) and consisted of fully hydrogenated soy phosphatidylcholine, cholesterol, and PEG-DSPE in a molar ratio of 1.5:1.0:0.15 and contained 2 mg/ml doxorubicin hydrochloride. The phospholipid concentration was 14 µmol/ml. The mean size was 85 nm, with a polydispersity index < 0.03.

**Radiolabeling.** Technetium-99 m labeling of the liposomes (except for Caelyx) was performed as described previously (Laverman et al., 1999b) with minor modifications. Briefly, various volumes of liposomes (85 µmol of phospholipid/ml) were adjusted to a final volume of 100 µl with HEPES-buffer, and a mixture of 10 µg of Tricine, 10 µg of stannous sulfate, and 99mTcO4– (Mallinkrodt, Petten, The Netherlands) in saline was added. The solution was incubated for 20 min at room temperature. Radiochemical purity was determined using instant thin-layer chromatography on silica gel strips (Gelman Sciences, Inc., Ann Arbor, MI) with 0.15 M sodium citrate buffer, pH 5.5, as the mobile phase. When radiochemical purity was less than 95%, unbound radiolabel was removed by gel filtration on a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden) with PBS as the eluent. A phospholipid dose of 5 µmol/kg of body weight 99mTc-labeled liposomes per rat (10 MBq of 99mTc) was injected intravenously, unless stated otherwise.

The commercially available liposome preparation Caelyx was radiolabeled with indium-111 (111In). Briefly, 30 MBq of 111In-oxine (Mallinkrodt) diluted in 0.2 M Tris(hydroxymethyl)aminomethane (pH 8.0) was added to the liposomal formulation and incubated at room temperature for 30 min. Radiolabeled liposomes were purified by gel filtration on a PD-10 column with PBS as the eluent. A dose of 2.2 MBq of 111In-labeled liposomes per rat was injected intravenously.

**Imaging and Tissue Distribution Studies.** In all animal experiments, the in vivo distribution of the radiolabel was monitored by gamma camera imaging up to 4 h post injection (p.i.). The animals were anesthetized with a mixture of enflurane (Ethrane, Abbott BV, Amstelveen, The Netherlands), nitrous oxide, and oxygen, and they were placed prone on a single-head gamma camera equipped with a parallel-hole, low-energy collimator (99mTc-liposomes) or a medium-
energy collimator ($^{111}$In-liposomes). Images (300,000 counts/image) were obtained using either a symmetric 15% window at 140 keV ($^{99m}$Tc-liposomes) or a 15% symmetric window for both the 172- and 246-keV photo peaks ($^{111}$In-liposomes) and stored in a 256 $\times$ 256-pixel matrix. Four hours p.i., after acquisition of the last image, rats were killed by CO$_2$ suffocation. Blood was obtained by cardiac puncture. The blood concentration of the liposomes was calculated assuming that the total amount of blood counted for 6% of the body weight of rats (Baker et al., 1979). After cervical dislocation, liver, spleen, and kidney were dissected and weighed, and their activity was measured in a shielded well type gamma counter (Wizard, Pharmacia-LKB, Uppsala, Sweden). To correct for physical decay and to calculate uptake of the radiolabeled liposomes in each tissue sample as a fraction of the injected dose, aliquots of the injected dose were counted simultaneously. The results are expressed as percentage of the injected dose per organ (%ID).

**Macrophase Depletion Experiments.** To study the involvement of macrophages in the induction and the effectuation of the enhanced clearance effect occurring after the second injection of PEG-liposomes, liver and spleen macrophages were depleted by intravenous injection of unsized multilamellar EPC-cholesterol-liposomes containing dichloromethylene-bisphosphonate (clodronate). Clodronate containing liposomes were prepared as described previously (van Rooijen and Sanders, 1994) and contained approximately 5 mg/ml clodronate.

One milliliter of clodronate liposomes was injected either 48 h before the first injection or 48 h before the second injection of $^{99m}$Tc-PEG-liposomes. Injection of liposomes without clodronate (i.e., containing PBS) served as control. The interval of 48 h was chosen because macrophage depletion in liver and spleen is maximal at that time-point (van Rooijen and Sanders, 1994). Macrophage depletion was confirmed by acid phosphatase staining on cryostat sections of liver and spleen (Zysk et al., 1997). The interval between the first and second PEG-liposome injection was set at 14 days because the macrophage population is recovered 12 days after clodronate-liposome injection (Wacker et al., 1986; van Rooijen and Sanders, 1990). Therefore, the macrophage population of the rats depleted before the first injection (induction phase) was recovered when the second injection was given. After recording the final images, rats were killed, tissues were dissected, and the biodistribution of the radiolabel was determined as described above.

**Statistical Analysis.** All mean values are expressed as mean ± S.D. Statistical analysis was performed using a Welch’s corrected unpaired t test or one-way analysis of variance using GraphPad InStat software (version 4.00, GraphPad Software, San Diego CA). The level of significance was set at $p < 0.05$.

**Results**

**Effect of Presence of PEG-Coating.** To investigate whether a PEG-coating is required to induce the enhanced clearance effect, we injected rats with long-circulating liposomes, either PEG-liposomes or small non-PEG-liposomes (DPPC-liposomes). One week later, rats of both groups were injected with $^{99m}$Tc-PEG-liposomes. The blood levels of both first injections at 4 h p.i. were comparable (76.4 ± 2.8 versus 62.8 ± 13.7%ID, PEG- versus DPPC-liposomes). After the second injection with PEG-liposomes, the blood levels of the PEG-liposomes at 4 h p.i. were dramatically lower (0.58 ± 0.28 and 0.54 ± 0.21%ID after PEG- and DPPC-liposomes, respectively) as compared with the level after the first injection ($p < 0.001$). These results indicate that the PEG-coating is not required for the induction of the effect.

To study whether only PEG-coated liposomes are subject to the enhanced clearance effect (i.e., during the effectuation phase), we injected rats first with PEG-liposomes and 1 week later rats were injected either with $^{99m}$Tc-PEG-liposomes or with small $^{99m}$Tc-labeled non-PEG-liposomes (DPPC-liposomes). Although the blood level of the small DPPC-liposomes was significantly higher as compared with that of the PEG-liposomes (13.2 ± 6.9 versus 0.57 ± 0.23%ID, 4 h p.i.), this level was still markedly lower as compared with the level 4 h after the first injection ($p < 0.04$). This experiment indicates that not only PEG-coated liposomes are subject to the enhanced clearance but that non-PEGylated liposomes are also cleared rapidly after a second injection.

Finally, rats were injected twice with small non-PEGylated DPPC-liposomes with a 1 week interval. Again, enhanced clearance of the liposomes was observed after the second injection (66.03 ± 11.12 versus 15.33 ± 9.28%ID, 4 h p.i., first and second injections).

**Induction of the Enhanced Clearance Effect: Dependence on Long-Circulation Property.** To study whether a long circulation time is required to induce the enhanced clearance effect, we investigated whether large DPPC-liposomes with a short circulation time can induce rapid clearance of a second injection of small long-circulating DPPC-liposomes. Two groups of three rats were injected with either large (short-circulating) or small (long-circulating) DPPC-liposomes. One week later, both groups received small, long-circulating $^{99m}$Tc-DPPC-liposomes. After 4 h, rats were imaged and organs of interest dissected. Blood levels at 4 h p.i. were low, being 6.95 ± 6.27%ID for the group injected with short-circulating large DPPC-liposomes 1 week before and 13.55 ± 9.8%ID for the group injected with long-circulating small DPPC-liposomes 1 week before. This experiment demonstrated that liposomes with a short circulation time are also able to induce enhanced clearance of long-circulating liposomes injected 1 week later.

**Effect of Lipid Dose.** The effect of the administered lipid dose of PEG-liposomes on both the induction phase and the effectuation phase of the enhanced clearance effect was investigated. To study the effect of the lipid dose on the induction phase, three groups of three rats received $^{99m}$Tc-PEG-liposomes at either 0.05, 0.5, or 5.0 µmol of phospholipid/kg of body weight. All groups received a second injection of 5.0 µmol of phospholipid/kg $^{99m}$Tc-PEG-liposomes 1 week later. All lipid doses given in the first injection induced enhanced blood clearance of the second dose of PEG-liposomes (Fig. 2). Blood levels at 4 h after the second injection decreased to 1.85 ± 0.71, 0.71 ± 0.21, and 0.43 ± 0.07%ID (0.05, 0.5, and 5 µmol/kg, respectively).

To study whether the effectuation phase is affected by the lipid dose, three groups of three rats first received a standard dose of $^{99m}$Tc-PEG-liposomes (5 µmol/kg), and 1 week later rats received either 5.0, 15, or 50 µmol/kg $^{99m}$Tc-PEG-liposomes. Remarkably, when the lipid dose given at the second injection exceeded 15 µmol/kg, the enhanced clearance effect was much less intense. In fact, the blood levels at 4 h after the second injection were almost normal (39.0 ± 6.7 and 51.8 ± 16.1%ID, 15 and 50 µmol/kg, respectively). The blood level 4 h after injection of 50 µmol/kg $^{99m}$Tc-PEG-liposomes was not significantly different from the blood level after the first injection of 5 µmol/kg (75.5 ± 4.6%ID).

**Involvement of Macrophages in the Enhanced Clearance Effect.** We investigated whether macrophages in the liver and spleen are involved in the induction and/or the effectuation of the enhanced clearance effect. In these stud-
ies, the hepatosplenic macrophages were depleted by i.v. administration of clodronate containing liposomes.

To study the involvement of the macrophages in the induction of the enhanced clearance effect, PEG-liposomes were injected in macrophage-depleted rats. Two weeks later, when the macrophage population had recovered, a second $^{99m}$Tc-PEG-liposome dose was injected. These radiolabeled PEG-liposomes showed a long circulation time and low hepatosplenic uptake, indicating that the enhanced clearance effect did not occur when the macrophages were depleted during the induction phase.

To investigate whether the macrophages also play a role in the accelerated clearance of a second liposome dose (the effectuation phase), $^{99m}$Tc-PEG-liposomes were injected in rats that had received PEG-liposomes 2 weeks before. Additionally, in one group of animals the macrophage population was depleted at the time of injection of the second dose. Again, a normal biodistribution of the second liposome dose was observed in this group, characterized by a long blood residence time and low liver uptake (Fig. 3). These results strongly suggest involvement of the liver and splenic macrophages in both the induction and the effectuation of the enhanced clearance effect. The biodistribution of the $^{99m}$Tc-PEG-liposomes is presented in Fig. 4. A control experiment showed that neither the clodronate-containing liposomes themselves, nor the empty clodronate liposomes, induced the enhanced clearance effect: no changes in the biodistribution of $^{99m}$Tc-PEG-liposomes given 1 week later were observed (data not shown).

Relevance of the Enhanced Clearance Effect for the Commercial Product Caelyx (Doxil).

The effect of serial injections of PEG-liposomes with encapsulated doxorubicin (Caelyx or Doxil) on their pharmacokinetics and biodistribution was investigated. Three groups of five rats were injected with either Caelyx, PEG-liposomes without doxorubicin (similar lipid formulation), or PBS. One week later, all groups were injected with $^{111}$In-labeled Caelyx. The injected lipid dose was 5 $\mu$mol/kg, corresponding to 0.7 mg/kg doxorubicin. Imaging and dissection was performed at 4 h p.i.

When rats had received unlabeled Caelyx, the pharmacokinetics and biodistribution of a second $^{111}$In-labeled Caelyx injection 1 week later was similar to the pharmacokinetics and biodistribution of the control group (i.e., after a first injection with PBS). However, the pharmacokinetics and biodistribution of the $^{111}$In-Caelyx altered dramatically when empty PEG-liposomes were injected 1 week before (Fig. 5). As expected, the control group (given PBS as first injection)
showed a pharmacokinetic and biodistribution profile with a long blood residence time of the radiolabeled Caelyx (78.9 ± 2.7%ID in blood, 4 h p.i.) as its main characteristic (Fig. 6).

**Discussion**

In the present study, the influence of several factors on the pharmacokinetics of 99mTc-labeled PEG-liposomes after repeated injections was investigated to further characterize the recently observed enhanced clearance effect (Dams et al., 2000). When 99mTc-PEG-liposomes were administered weekly, the pharmacokinetics and biodistribution of 99mTc-PEG-liposomes were dramatically altered. Here, we show that the enhanced clearance of subsequent doses is not solely induced by PEG-liposomes, but can also be caused by conventional, non-PEGylated liposomes. This observation implies that the enhanced clearance effect can be a potential problem associated with various liposomally formulated drugs requiring multiple dosing schemes.

We also investigated whether the induction phase and/or the effectuation phase is affected by the lipid dose. All lipid doses of PEG-liposomes tested in the induction phase (0.05, 0.5, and 5 μmol of phospholipid/kg) led to rapid clearance of a subsequent injection of PEG-liposomes. While the induction of the enhanced clearance effect was independent of the administered dose, the effectuation seemed to be lipid dose-dependent. Increasing the lipid dose of the second injection (up to 50 μmol/kg) led to an attenuation of the effect that the predose imposes on the pharmacokinetics of the second dose. This observation is in line with the earlier suggestion (Dams et al., 2000) that the effect is mediated by the presence of an opsonic factor (or factors) in the blood stream capable of facilitating the clearance of a limited amount of liposomes. Experimental evidence for the induction of circulatory opsonin(s) by the first injection has been presented in a previous study (Dams et al., 2000). It was proposed that a transfusible, heat-labile factor is responsible for the enhanced clearance of a second PEG-liposome injection, given 5 to 21 days after the first injection. The hypothesis of the presence of a limited pool of opsonins has also been discussed in other studies (Oja et al., 1996; Laverman et al., 2000).

In our previous study, we showed that after a second injection of PEG-liposomes, 46% of the dose cleared to the liver within minutes. Of the liposomes localized in the liver, 88 to 95% were taken up by the Kupffer cells (Dams et al., 2000), indicating that the liver macrophages are at least partly responsible for the effectuation of the enhanced clearance effect. In the present in vivo studies, we show that the macrophages in the liver and spleen also play a crucial role in the induction of the enhanced clearance effect. When macrophages were depleted before the first injection, PEG-liposomes showed after a second injection the usual long circulation time. This reveals that the presence of macrophages is required for the enhanced clearance of a second liposome dose. It is tempting to speculate that macrophages that have

![Fig. 4. Biodistribution of 99mTc-PEG-liposomes in rats, 4 h after a second injection administered 2 weeks after the first injection. Liver and splenic macrophages were depleted either before the first injection or before the second injection by injection with clodronate liposomes. Rats of the control group did not receive clodronate liposomes. Values are expressed as %ID per organ. Error bars represent standard deviation (n = 3/group).](image)

![Fig. 5. Scintigraphic images of rats, 4 h after injection of 111In-labeled PEG-liposomes containing doxorubicin (Caelyx). Rats of group A were injected with Caelyx 1 week earlier. Rats of group B received empty PEG-liposomes of the same composition as Caelyx, without containing doxorubicin, 1 week before. Rats of group C served as negative control and received PBS 1 week before the injection with 111In-labeled Caelyx.](image)

![Fig. 6. Biodistribution of 111In-labeled PEG-liposomes containing doxorubicin (Caelyx) in rats, 4 h after injection. Rats received either Caelyx, empty PEG-liposomes, or PBS (negative control) 1 week before. Values are expressed as %ID per organ. Error bars represent standard deviation (n = 5/group).](image)
taken up the first dose of liposomes are responsible for the production of the proposed opsonin(s) (Dams et al., 2000).

Long-circulating liposomes are used in clinical practice as a carrier for chemotherapeutic drugs. Therefore, we investigated whether the commercial PEG-liposome formulation containing doxorubicin (Caelyx or Doxil) also show the enhanced clearance effect upon repeated injection. Caelyx was not able to induce enhanced clearance of Caelyx given 1 week later. However, a first injection with empty PEG-liposomes (i.e., similar liposomal formulation without doxorubicin) did induce rapid clearance of subsequently administered Caelyx. This strongly suggests that the presence of doxorubicin inside the PEG-liposomes prevents induction of the enhanced clearance effect. This may relate to the study by Daemen et al. (1997) showing that a single injection of doxorubicin containing PEG-liposomes strongly reduced the phagocytic activity of the liver macrophages. Adverse effects of Caelyx on the hepatosplenic macrophages may have impaired the role of these cells in the induction phase of the enhanced clearance effect.

Tardi and colleagues studied the pharmacokinetics of ovalbumin-coated PEG-liposomes upon repeated injections (Tardi et al., 1997). They observed a normal pharmacokinetic behavior after weekly injections of control PEG-liposomes (without coated ovalbumin) (Tardi et al., 1997). This was most likely due to the high lipid doses of 60 μmol/kg used in their studies, similar to our findings that the effect diminishes with an increasing dose used in the second injection. At a dose level of 50 μmol/kg, the pharmacokinetic behavior of the PEG-liposomes in the case of repeated injection is not altered.

The exact mechanism underlying the enhanced clearance effect in the case of repeated administration of liposomes is not clear. The liver and spleen macrophages are probably involved in both the induction and the effectuation of the effect. We speculate that the induction most likely relates to the production and excretion of an opsonic serum factor (or factors) by the liver and spleen macrophages. The liposomes are probably opsonized by the produced serum factor(s) and subsequently recognized and phagocytosed by the (possibly) “activated” hepatosplenic macrophages. The observed enhanced clearance effect diminishes with time, indicating that the factor is either produced and circulating in sufficient amounts for only a limited period of time and/or produced by only one generation of macrophages. Interestingly, the attenuation of the enhanced clearance effect correlates with the half-life of rat Kupffer cells, which is approximately 12 days (Wacker et al., 1986).

In summary, our results indicate that the change in pharmacokinetic behavior upon repeated injection is a general characteristic of liposomes, unrelated to the presence of PEG. Most likely, Kupffer cells and spleen macrophages are involved in the induction and effectuation of this process, since the presence of viable macrophages in liver and spleen is mandatory. The induction of the effect does not depend on liposome size and long-circulating property, but it is affected by the lipid dose. PEG-liposomes containing doxorubicin could not induce enhanced clearance of a second dose of the same formulation. Since we showed that the enhanced clearance effect is not a characteristic caused by the PEGylation of the liposomes, our findings may have considerable impact on the clinical application of several liposomal formulations that are administered repeatedly.

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