Accelerated Blood Clearance and Altered Biodistribution of Repeated Injections of Sterically Stabilized Liposomes

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ABSTRACT
Sterically stabilized liposomes are considered promising carriers of therapeutic agents because they can facilitate controlled release of the drugs, thereby reducing drug-related toxicity and/or targeted delivery of drugs. Herein, we studied the pharmacokinetics and biodistribution of repeated injections of radiolabeled polyethyleneglycol (PEG) liposomes. Weekly injections of 99mTc-PEG liposomes dramatically influenced the circulatory half-life in rats. Biodistribution 4 h after the second dose showed a significantly reduced blood content (from 52.6 ± 3.7 to 0.6 ± 0.1% injected dose (ID), P < .01) accompanied by a highly increased uptake in the liver (from 8.1 ± 0.6 to 46.2 ± 0.8%ID, P < .01) and in the spleen (from 2.2 ± 0.2 to 5.3 ± 0.7%ID, P < .01). At subsequent injections the effect was less pronounced: after the fourth dose, the pharmacokinetics of the radiolabel had almost returned to normal. The same phenomenon was observed in a rhesus monkey, but not in mice. The enhanced blood clearance of the PEG liposomes also was observed in rats after transfusion of serum from rats that had received PEG liposomes 1 week earlier, indicating that the enhanced blood clearance was caused by a soluble serum factor. This serum factor was a heat-labile molecule that coeluted on a size exclusion column with a 150-kDa protein. In summary, i.v. administration of sterically stabilized PEG liposomes significantly altered the pharmacokinetic behavior of subsequently injected PEG liposomes in a time- and frequency-dependent manner. The observed phenomenon may have important implications for the repeated administration of sterically stabilized liposomes for targeted drug delivery.

The administration of liposome-associated chemotherapeutics has gained wide interest in the oncologic field as a means to improve the therapeutic index of anticancer drugs. Doxorubicin (DOX) in particular has been studied extensively in various liposomal formulations (Rahman et al., 1990, Vaage et al., 1994). Indeed, experimental as well as clinical studies have indicated that the cardiotoxicity of DOX can be reduced by liposomal formulation (Rahman et al., 1990, Treat et al., 1990). However, improved tumor targeting could not be demonstrated. In addition, major toxic effects of DOX liposomes on liver macrophages have been reported that may have been the result of uptake of DOX liposomes by Kupffer cells (Daemen et al., 1995). New insights in the recognition of liposomes by the mononuclear phagocyte system (MPS) has led to the development of MPS-avoiding liposomes. Surface coating of liposomes with hydrophilic polymers such as polyethyleneglycol (PEG) resulted in decreased recognition and subsequent phagocytosis by cells of the MPS (Woodle and Lasic, 1992). The development of these PEG liposomes appeared to be a major step forward toward clinical application. Experimental and clinical studies with DOX-encapsulated PEGylated liposomes not only showed reduced toxicity but also preferential localization at the tumor site (Papahadjopoulos et al., 1991; Gabizon, 1992; Huang et al., 1992). Recent phase I–II studies with Doxil (DOX in PEG-coated liposomes; SEQUUS Pharmaceuticals Inc., Menlo Park, CA) in patients with AIDS-related Kaposi’s sarcoma and other solid tumors have shown substantial therapeutic efficacy (Harrison et al., 1995; Goebel et al., 1996; Muggia et al., 1997; Northfelt et al., 1997), indicating that PEGylated liposomal formulations indeed could potentiate the therapeutic efficacy of chemotherapeutic agents. The long circulatory half-life of these PEG formulations seems to be crucial for their improved efficacy. Although pharmacokinetic studies in both animals and patients have established the prolonged blood residence time of PEG-coated liposomes, limited data are available on the influence of multiple injections on their pharmacokinetics.

ABBREVIATIONS: DOX, doxorubicin; MPS, mononuclear phagocyte system; PEG, polyethyleneglycol; PHEPC, partially hydrogenated egg-phosphatidylcholine; DSPE, distearoylphosphatidyl-ethanolamine; HYNIC, N-hydroxysuccinimidyl hydrazino nicotinate hydrochloride; ID, injected dose; ROI, region of interest.
pharmacokinetic behavior. Goins et al. (1998) demonstrated that the blood clearance profile of a second dose of PEG liposomes in rabbits was highly similar to the profile of the first dose given 6 weeks earlier. Recently, Oussoren and Storm (1999) injected rats with PEG liposomes with a 24- or 48-h dosing interval. In their study, the kinetic profile of each injection was virtually identical. Herein, we report on the major changes in pharmacokinetics of consecutive injections of radiolabeled PEG liposomes in animals. The long circulatory half-life of PEG liposomes appeared to decrease considerably at a second injection. The observed phenomenon was characterized and additional experiments were carried out to elucidate the underlying mechanism.

**Experimental Procedures**

**Materials.** Partially hydrogenated egg-phosphatidylcholine (PHEPC) with an iodine value of 35 was a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Distearoyl-phosphatidylethanolamine (DSPE) and the polyethylene-2000 (PEG) derivative of DSPE were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Triethylamine, cholesterol, and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). 1,2(3H)cholesterol and Na125I were purchased from Amersham Cygne (Eindhoven, the Netherlands). N-Hydroxysuccinimimidyl hydrazino nicotinate hydrochloride (S-HYNIC) was synthesized as described by Abrams et al. (1990) with minor modifications.

**Animals.** Randomly bred Wistar rats (HsdCpb:WU) and outbred Swiss mice (HsdCpb:SE) were obtained from Harlan Nederland (Zeist, the Netherlands). For the experiments, 6- to 8-week-old male rats, weighing 180 to 220 g, and 6- to 8-week-old female mice, weighing 20 to 25 g, were used. In one experiment, we used a male rhesus monkey (6 kg; China Scientific Instruments and Materials Cooperation, Beijing, China). 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 Liposomes.** The studies were performed with the following liposome types: 1) small PEG-DSPE:PHEPC:cholesterol:HYNIC-DSPE (molar ratio 0.15:1.85:1:0.07) (HYNIC-PEG liposomes) liposomes. HYNIC-DSPE is incorporated in the lipid bilayer as a chelator to facilitate their radiolabeling with technetium-99 m ([99mTc]HYNIC-PEG liposomes) liposomes. HYNIC-DSPE is incorporated in the lipid bilayer as a chelator to facilitate their radiolabeling with technetium-99 m ([99mTc]HYNIC-PEG liposomes) liposomes. HYNIC-DSPE is incorporated in the lipid bilayer as a chelator to facilitate their radiolabeling with technetium-99 m ([99mTc]HYNIC-PEG liposomes) liposomes. HYNIC-DSPE is incorporated in the lipid bilayer as a chelator to facilitate their radiolabeling with technetium-99 m ([99mTc]HYNIC-PEG liposomes) liposomes.

**Imaging and Tissue Distribution Studies.** Rats were divided into four groups. Each group received at least three i.v. injections of [99mTc]labeled HYNIC-PEG liposomes (10 MBq/rat). The interval between the injections was 1, 2, 3, and 4 weeks, respectively, for the successive groups. The in vivo distribution of the radiolabel was monitored by gamma camera imaging up to 4-h postinjection. At each imaging session, a control rat (not previously injected with liposomes) was included to exclude possible batch-to-batch differences of the radiopharmaceuticals. The animals were anesthetized with a mixture of enflurane (Ethane, 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. Rats were imaged at 5 min and 1 and 4 h after injection. Images (300,000 counts/image) were obtained with a symmetric 15% window at 140 keV and stored in a 256 × 256 matrix. After acquiring the last image, five rats of the imaged group were sacrificed with 30 mg of phenobarbital injected i.p., and dissected to determine the biodistribution of the radiolabel in the dissected tissues. Blood was obtained by cardiac puncture. The blood concentration of the liposomes was calculated assuming that the total blood weight was 6% of the body weight of rats (Baker et al., 1979). After cervical dislocation, liver, spleen, kidney, lung, and bone marrow were dissected, weighed, and their activity was measured in a shielded well-type gamma counter (Wizard; Pharmacia-LKB, Sweden). To correct for physical decay and to calculate uptake of the radiopharmaceuticals in each tissue sample as a fraction of the injected dose, aliquots of the injected dose were counted simultaneously. The results are expressed as a percentage of injected dose per organ (%ID). The effect of repeated injections with radiolabeled HYNIC-PEG liposomes also was studied scintigraphically in mice and in a rhesus monkey, both under general anesthesia as described above. In the monkey, the uptake in the liver was determined scintigraphically by drawing regions of interest (ROIs) over the liver on the anterior and posterior images. The percentage of injected dose in the liver at different time points was estimated from the ratio of the geometric mean of ROI counts in the liver divided by the geometric mean of ROI counts in an aliquot of the injected dose that had been counted simultaneously.

Additional experiments were carried out to evaluate the effect of the liposome composition of the first dose on the biodistribution of the second dose of PEG liposomes. Groups of five rats received an i.v. dose of either unlabeled small HYNIC-PEG liposomes, unlabeled large PEG liposomes, unlabeled non-PEG liposomes, or (nonliposomal) PEG-conjugated DSPE (2.5 μmol/kg). After 1 week, the rats were injected with 10 MBq [99mTc]labeled HYNIC-PEG liposomes. Imaging and tissue distribution studies were performed as described above.

**Pharmacokinetics.** To determine the circulatory half-life of the PEG liposomes at subsequent injections, two groups of six rats received 10 MBq [99mTc]labeled HYNIC-PEG liposomes i.v.. One group had been injected with unlabeled HYNIC-PEG liposomes 1 week previously (pretreated group). Imaging studies were performed under general anesthesia as described above. Dynamic views were obtained from the time of injection up to 15-min postinjection. Static
views were obtained at 30 min, and 1, 2, 4, 8, and 24 h postinjection. The blood clearance of $^{99m}$Tc-PEG liposomes was determined by drawing ROIs over the heart (representing blood pool activity) and the whole body. The activity in the heart measured at 2-min postinjection was denominated as 100%ID. For each subsequent image, the activity in the heart was determined relative to the first scan as percentage of injected dose, corrected for physical decay. In the monkey, the circulatory half-life of the radiolabeled HYNIC-PEG liposomes was determined by collecting multiple blood samples postinjection. The distribution half-life and elimination half-life were calculated by nonlinear least-square analysis of the data with a biexponential model.

Intrahepatic Distribution of PEG Liposomes. To monitor the intrahepatic distribution of the PEG liposomes at subsequent injections, two groups of three rats received 25 kBq $^{99m}$Tc-labeled PEG liposomes (10 $\mu$mol/kg i.v.). One group had been injected with unlabeled HYNIC-PEG liposomes 1 week previously. Eighteen hours postinjection, the rats were sacrificed. The livers were removed after perfusion via the portal vein with Gey’s balanced salt solution to remove blood and then for 3 min with 0.2% pronase (Boehringer, Mannheim, Germany). Isolation and fractionation of parenchymal and nonparenchymal cells was performed as described previously (Daemen et al., 1997). $^{3}$H content of aliquots of each cell fraction, containing a known number of cells, was determined by means of liquid scintillation counting. Radioactivity content of whole cell populations was calculated on the basis of 450 $\times$ 10$^6$ hepatocytes and 194 $\times$ 10$^6$ nonparenchymal cells/100 g b.wt. (Scherphof et al., 1983).

Measurement of Complement Activity. Additional experiments were carried out to investigate the role of the complement system in the biodistribution and pharmacokinetics of repeated injections with PEG liposomes. Rats (five rats per group) received two i.v. injections of HYNIC-PEG liposomes or an equal volume of 145 mM NaCl solution with a 1-week interval. Blood samples (1 ml) were obtained by retro-orbital puncture under ether anesthesia 1 h before and 1 h after each injection and centrifuged at 400 g for 15 min at 4°C. Serum samples were aliquoted and stored at -80°C until use. As a measure of serum complement activity, the CH-50 was determined according to the method of Kabat and Mayer (Mayer, 1971) with sheep erythrocytes sensitized with monoclonal anti-sheep IgM antibody.

Measurement of Mast Cell Activation. In a separate experiment, we investigated whether activation of mast cells contributed to the different pharmacokinetic behavior of PEG liposomes at repeated injections. Under general anesthesia, two groups of five rats were injected with HYNIC-PEG liposomes and one group of five rats received an equal volume of 145 mM NaCl solution i.v.. One of the groups receiving HYNIC-PEG liposomes had been injected with unlabeled HYNIC-PEG liposomes 1 week previously. The animals were observed for signs of respiratory distress. Electrocardiograms were recorded before the injection up to 30 min after injection. Urine samples were collected during 24-h postinjection. The urinary excretions of the two major histamine metabolites, N'-methylhistamine and N'-methylimidazoleacetic acid were measured as described by Kors et al. (1996).

Measurement of Vascular Permeability. To investigate whether the observed kinetic changes could be due to enhanced vascular permeability, two groups of five rats were injected simultaneously with $^{99m}$Tc-labeled HYNIC-PEG liposomes (10 MBq/rat) and with $^{125}$I-labeled BSA (0.4 MBq/rat). One group had been injected with unlabeled HYNIC-PEG liposomes the week before. Blood clearance of albumin, normally <4%/h, can be used to determine enhanced transcapillary leakage (Peters and Lewis, 1995). Blood samples were obtained 5 min and 4 h p.i. by retro-orbital puncture under ether anesthesia. Radioactivity in the samples was measured as described previously.

Serum Transfusion. The role of a humoral factor in the enhanced blood clearance of PEG liposomes was studied in a transfusion experiment. Rats that had received unlabeled HYNIC-PEG liposomes 1 week previously (pretreated) were bled under ether anesthesia by canulation of the abdominal aorta. Pooled blood samples were centrifuged at 400 g for 15 min to obtain serum. Fresh whole-blood samples of nonpretreated rats were used as control. Groups of five nonpretreated rats were transfused with either 3 ml of pretreated blood, 3 ml of pretreated serum, or 3 ml of control blood. Immediately after transfusion, 10 MBq $^{99m}$Tc-labeled HYNIC-PEG liposomes was administered i.v. Imaging and tissue distribution studies were performed as described previously.

The experiment was performed with fractionated serum samples. Serum samples of pretreated rats were pooled and fractionated with centrifugal Centriprep concentrators (Amicon Inc., Beverly, MA) with different molecular mass cutoff values. Ten-milliliter serum samples underwent three cycles of concentration and dilution. Four different fractions were obtained: >3 kDa, >10 kDa, >30 kDa, and >50 kDa. Unfractionated serum samples of pretreated rats and of nonpretreated rats served as positive, respectively, negative control in the experiment. All serum samples were stored overnight at 4°C. The next day, groups of three rats were i.v. injected with 2 ml of each fraction, immediately followed by i.v. injection of 10 MBq $^{99m}$Tc-labeled HYNIC-PEG liposomes.

Statistical Analysis. Values are shown as means ± S.E. The values were analyzed with a two-tailed unpaired t test (experiments comparing two groups of animals) and a one-way ANOVA (three or more groups). Nonparametric tests (Mann-Whitney and Kruskal-Wallis) were used if data were not normally distributed. Tukey tests were applied to correct for multiple comparisons. A corrected P value of <.05 was considered significant.

Results

Effect of Consecutive Injections on Biodistribution and Pharmacokinetics of PEG Liposomes

Weekly injections of $^{99m}$Tc-labeled HYNIC-PEG liposomes dramatically influenced the circulatory half-life in rats. Four hours after the second injection, the cardiac pool, representing the blood level of the liposomes, was no longer visible on the images (Fig. 1). In addition, enhanced uptake in liver and spleen was noted. Scintigraphic analysis of the images re-
revealed that the half-life of the distribution phase \( (t_{1/2a}) \) decreased from 2.4 h at the first injection to 0.1 h at the second injection (Fig. 2). The biodistribution of the radiolabel in the dissected tissues 4 h after the second injection was in accordance with the scintigraphic findings, showing a significantly decreased blood content (from 52.5 to 64.6% ID, \( P < .01 \)) and highly increased uptake in the liver (from 8.06 to 46.4% ID, \( P < .01 \)) and to a lesser extent, the spleen (Table 1). The uptake of the radiolabel in other tissues (bone marrow, lung, kidney) was significantly lower at the second injection compared with uptake at the first injection, presumably due to the lower blood levels. At subsequent injections, the enhanced blood clearance became less pronounced: after the fourth injection (3 weeks after the first injection), the liver uptake of \( {^{99m}Tc}\)-labeled PEG liposomes was still slightly increased but the blood content (4 h p.i.) had normalized (Table 1). In contrast, enhanced blood clearance was still observed when the second dose of \( {^{99m}Tc}\)-labeled PEG liposomes was given 3 weeks or even 4 weeks after the first injection (Fig. 3). However, a fourth dose injected 3 weeks after the first injection, did not show the accelerated pharmacokinetics, although the liver uptake (4 h p.i.) was still elevated. The observed phenomenon was found to be independent of size, surface characteristics, and radiolabel of the first dose of liposomes: unlabeled small (85 nm) or large (400 nm) PEG liposomes or small (100 nm) non-PEG liposomes all elicited enhanced blood clearance of the subsequent injection with \( {^{99m}Tc}\)-labeled HYNIC-PEG liposomes in a similar fashion. Administration of PEG-conjugated DSPE (nonpolar), however, did not influence the circulatory half-life of subsequently administered \( {^{99m}Tc}\)-labeled HYNIC-PEG liposomes.

To further investigate the effect of the time interval between liposome injections on the biodistribution of PEG liposomes, groups of rats received two successive injections of \( {^{99m}Tc}\)-labeled HYNIC-PEG liposomes at fixed intervals varying from 1 to 7 days. Surprisingly, no influence on the circulatory half-life of the PEG liposomes was noted when the interval between the first and second injection was reduced to 5 days or less, as determined by gamma camera imaging as well as biodistribution studies (data not shown). A separate group of rats was injected daily with \( {^{99m}Tc}\)-labeled HYNIC-PEG liposomes for 3 weeks or even 4 weeks after the first injection of liposomes: unlabeled small (85 nm) or large (400 nm) PEG liposomes or small (100 nm) non-PEG liposomes all elicited enhanced blood clearance of the subsequent injection with \( {^{99m}Tc}\)-labeled HYNIC-PEG liposomes in a similar fashion. Administration of PEG-conjugated DSPE (nonpolar), however, did not influence the circulatory half-life of subsequently administered \( {^{99m}Tc}\)-labeled HYNIC-PEG liposomes.

![Fig. 2. Blood clearance of \( {^{99m}Tc}\)-labeled HYNIC-PEG liposomes in rats after the first (○) or the second injection (●). The blood pool activity was determined by quantitative analysis of the scintigraphic images, by drawing regions of interest over the heart region. Blood pool activity at 2-min postinjection was set at 100%. Each point represents the mean values of six rats ± S.E.](image)

**Table 1**

<table>
<thead>
<tr>
<th>Organ</th>
<th>1st Injection</th>
<th>2nd Injection</th>
<th>3rd Injection</th>
<th>4th Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>52.5 ± 3.68</td>
<td>0.55 ± 0.05**</td>
<td>8.50 ± 2.93**</td>
<td>51.04 ± 7.30NS</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.19 ± 0.17</td>
<td>6.25 ± 0.36</td>
<td>5.31 ± 0.69**</td>
<td>2.29 ± 0.50NS</td>
</tr>
<tr>
<td>Liver</td>
<td>8.06 ± 0.79</td>
<td>46.41 ± 3.20**</td>
<td>41.52 ± 1.60**</td>
<td>14.82 ± 5.84NS</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.01 ± 2.26</td>
<td>0.92 ± 0.02</td>
<td>1.58 ± 0.40**</td>
<td>6.25 ± 0.96NS</td>
</tr>
<tr>
<td>Bone</td>
<td>7.82 ± 0.08</td>
<td>3.73 ± 0.88**</td>
<td>3.68 ± 1.41**</td>
<td>5.70 ± 0.90NS</td>
</tr>
<tr>
<td>Lung</td>
<td>0.73 ± 0.08</td>
<td>0.13 ± 0.02**</td>
<td>0.26 ± 0.08**</td>
<td>0.66 ± 0.13NS</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.05 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.01 ± 0.00NS</td>
<td>0.02 ± 0.00NS</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.52 ± 0.10</td>
<td>0.39 ± 0.04**</td>
<td>0.55 ± 0.07**</td>
<td>1.09 ± 0.43NS</td>
</tr>
</tbody>
</table>

NS, not significant. \( * P < .05; ** P < .01 \). Depicted \( P \) values (two-tailed unpaired \( t \) test) refer to differences between the first injection and, respectively, the second, third, and fourth injection.

**Table 2**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Pretreated Serum</th>
<th>Pretreated Blood</th>
<th>Control Blood</th>
<th>Heated Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>16.0 ± 5.71**</td>
<td>22.31 ± 6.14**</td>
<td>52.48 ± 2.58</td>
<td>45.52 ± 1.04NS</td>
</tr>
<tr>
<td>Spleen</td>
<td>8.82 ± 0.71*</td>
<td>9.36 ± 0.70**</td>
<td>2.15 ± 0.15</td>
<td>2.71 ± 0.17NS</td>
</tr>
<tr>
<td>Liver</td>
<td>44.87 ± 4.39**</td>
<td>48.91 ± 6.89**</td>
<td>10.59 ± 0.29</td>
<td>15.22 ± 1.32NS</td>
</tr>
</tbody>
</table>

NS, not significant. \( * P < .05; ** P < .01 \). Only \( P \) values (one-way ANOVA) applying to differences between the respective pretreated groups and the control group are depicted.

![Fig. 3. Biodistribution of \( {^{99m}Tc}\)-labeled HYNIC-PEG liposomes in rats, 4 h after a second injection administered 7, 14, 21, and 28 days after the first injection. Rats that received only one injection with \( {^{99m}Tc}\)-labeled HYNIC-PEG liposomes served as control. \( P \) values (nonparametric unpaired \( t \) test) apply to differences between the control rats and rats receiving, respectively, the second, third, and fourth injection. \( * P < .05, \quad ** P < .01 \).](image)
increased from 17.6 to 41.2%. As in rats, the pharmacokinetic changes in the monkey diminished with subsequent injections (Fig. 4).

**Intrahepatic Distribution of PEG Liposomes.** To further investigate the increased liposome uptake in the liver, the distribution of $^3$H-labeled PEG liposomes in isolated Kupffer and parenchymal liver cells was investigated. After the first injection, uptake of the liposomes in parenchymal and nonparenchymal cells was low (6.6%ID) and was equally distributed between Kupffer cells (3.1%ID) and hepatocytes (3.5%ID). In contrast, after the second injection the liposomes were mainly located in the Kupffer cells (88–95% of radioactivity in the liver).

**Release of Vasoactive Mediators**

In view of the extremely rapid blood clearance of the second dose of PEG liposomes, we investigated whether administration of PEG liposomes induced release of vasoactive mediators, resulting in increased vascular permeability. It has been suggested that reported side effects of liposomal administration, such as flushing, hypotension, and dyspnoe, could be due to release of vasoactive mediators such as arachidonic acid metabolites and/or histamine from mast cells or macrophages (Johnson et al., 1998). We found normal blood clearance of iodinated albumin in rats injected with 145 mM NaCl solution as well as in rats injected with a first or second dose of PEG liposomes (<4%/h). In addition, no differences were found between the groups with respect to the ECG recordings and urinary excretion of histamine metabolites (data not shown).

**Role of Serum Complement in Enhanced Blood Clearance of PEG Liposomes**

In several studies the role of the complement system in the blood clearance of liposomes by cells of the MPS has been demonstrated (Wassef and Alving, 1993; Devine and Marjan, 1997). We evaluated whether involvement of complement factors could be demonstrated in the enhanced blood clearance of a second dose of PEG liposomes. Total hemolytic complement in the rats injected with 145 mM NaCl solution measured 52 ± 1 U/ml at baseline and did not change with subsequent injections. In contrast, complement activity in rats injected with PEG liposomes, although displaying similar values at baseline as the control group, decreased significantly to a mean value of 21 ± 1 U/ml 1 h after the second injection ($P < .05$).

**Transfusion of Serum from Pretreated Rats**

The observation that the reduced blood residence time was associated with a sharp decline in complement activity suggested the presence of an opsonizing factor in the circulation before the second injection. We therefore investigated whether transfusion of blood or serum from pretreated rats could elicit the same pharmacokinetic changes in nonpretreated rats. As shown in Fig. 5, enhanced blood clearance could be evoked in normal rats by transfusion of blood as well

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**Fig. 4.** Scintigraphic images of a rhesus monkey 4 h after consecutive injections with $^{99m}$Tc-labeled HYNIC-PEG liposomes on day 0 (A), day 7 (B), day 21 (C), and day 35 (D). ← indicates the heart region, ⇒ indicates the liver region, and * indicates the spleen region.

**Fig. 5.** Scintigraphic images of rats 4 h after injection with $^{99m}$Tc-labeled HYNIC-PEG liposomes and transfusion with 3 ml of pretreated serum (A) or with 3 ml of pretreated blood (B) derived from rats that had been injected with PEG liposomes 1 week previously. Rats transfused with 3 ml of blood derived from nonpretreated rats served as controls (C). ← indicates the heart region, ⇒ indicates the liver region, and * indicates the spleen region.
as serum from pretreated rats. This phenomenon could be abolished by heating the serum at 56°C for 0.5 h before transfusion, indicating the involvement of a heat-labile serum component in the opsonisation process. The effect of transfusion appeared to be dose-dependent: reducing the amount of transfused serum diminished the changes in pharmacokinetic behavior of PEG liposomes (Fig. 6).

Preliminary Biochemical Characterization of Serum Factor

The transfusion experiments showed that a soluble serum factor could induce enhanced blood clearance of PEG liposomes. By fractionating serum from rats that had received PEG liposomes 1 week earlier with centrifugal concentrators, we further characterized this serum factor. In all groups injected with fractionated serum (>3 kDa, >10 kDa, >30 kDa, or >50 kDa) enhanced blood clearance was observed, suggesting that the serum factor had a molecular mass exceeding 50 kDa.

To further characterize this serum factor, serum from pretreated rats was concentrated and diluted five times in a centrifugal concentrator with a molecular mass cutoff value of 30 kDa to remove all serum proteins smaller than ~30 kDa. Subsequently, 5 ml of serum concentrated to 1 ml was applied on a Sephacryl S200HR gel filtration column (100 × 2.5 cm; Pharmacia Biotech, Uppsala, Sweden). The column was eluted with TEN-buffer (50 mM Tris-HCl, 1 mM EDTA, 145 mM NaCl, pH 7.4). The elution profile is shown in Fig. 7. Five fractions (A, B, C, D, and E) were collected and each fraction was concentrated five times with a centrifugal concentrator. Groups of three rats were i.v. injected with 2 ml of each fraction, and subsequently with 10 MBq 99mTc-labeled HYNIC-PEG liposomes. Only transfusion of fraction B elicited enhanced blood clearance in normal rats. In these rats, a blood level of 18.7 ± 2.7%ID was found 4 h p.i. (data not shown). Because each rat received the purified “clearance enhancer” purified from 3.3 ml of pretreated serum, the recovery of the gel filtration procedure can be calculated with the dose-response curve shown in Fig. 6. Based on the blood level found after injection of fraction B, the recovery of the purification procedure is 75%. Based on the liver uptake found after injection of fraction B, the recovery of the purification procedure is 60%. The protein composition of fractions A to E from the Sephacryl S200HR column were analyzed by SDS-polyacrylamide gel electrophoresis. Analysis on a 10% acrylamide gel indicated that the major component of fraction B had an apparent molecular mass of 150 kDa (Fig. 8).

Because the immunoglobulins IgG and IgM have both been implicated in the opsonisation of liposomes (Alving, 1984, Wassef et al., 1989), we evaluated whether the transfusible serum factor that caused the enhanced clearance of the PEG liposomes could be an IgG or (the monomeric form of) an IgM molecule. Pretreated rat serum was IgG-depleted by elution on a Protein G column (Pierce, Rockford, IL). The effluent of the Protein G column (IgG-depleted serum), as well as the Protein G-bound fraction (IgG fraction) were isolated. IgG depletion of the serum was confirmed by SDS-polyacrylamide gel electrophoresis. Both the IgG-depleted serum as well as the IgG-fraction from 10 ml of serum were injected in groups of three rats, immediately followed by an i.v. injection of 10 MBq 99mTc-labeled HYNIC-PEG liposomes. Transfusion of the IgG-fraction did not evoke any changes in pharmacokinetic behavior of the 99mTc-labeled HYNIC-PEG liposomes, whereas rats that received the IgG-depleted serum fraction did show enhanced clearance. IgM-depleted serum was prepared by eluting serum from pretreated rats on an anti-rat-IgM-Sepharose affinity column. Spiking of the rat serum with a trace of 125I-labeled rat IgM (ICN Biomedicals Inc., Costa Mesa, CA) indicated that >80% of the IgM was adsorbed to the column. Transfusion of the IgM fraction did not evoke any changes in pharmacokinetics of the 99mTc-labeled HYNIC-PEG liposomes, whereas rats that received the IgM-depleted serum fraction did show enhanced clearance. These experiments indicated that the enhanced blood clearance of a second dose of HYNIC-PEG liposomes was not Ig-mediated.

Discussion

The development of liposomal formulations with long circulating characteristics such as PEG liposomes has broad-
ened the therapeutic potential of anticancer drugs. Experimental and clinical studies have shown increased tumor targeting of Doxil compared with conventional liposomal DOX formulations (Gabizon, 1992; Huang et al., 1992; Gabizon et al., 1994). Apparently, the enhanced circulatory half-life of the PEG liposomal formulation enables preferential accumulation of the drug at the tumor site, resulting in improved antitumor activity. Nowadays, Doxil is administered with 4- to 6-week intervals to patients with solid tumors and Kaposi’s sarcoma, to improve the therapeutic index of doxorubicin. The present study shows remarkable pharmacokinetics of repeated injections of PEG liposomes in rats and rhesus monkey: the circulatory half-life of a second dose of radiolabeled PEG liposomes dramatically decreased when given 5 days up to 4 weeks after a first injection. Although subsequent injections attenuated this effect, enhanced uptake of PEG liposomes in the liver could still be noted at the fourth weekly injection. The pharmacokinetic changes were observed in rats and in rhesus monkey, but were not apparent in mice.

The PEG coating of the liposomes of the first injection apparently was not crucial in the development of the observed phenomenon, nor was the radiolabel or the size of the liposomes. We did not find evidence for increased transcapillary leakage, indicating that the enhanced blood clearance of liposomes at a second injection was due to rapid elimination of these particles. The experiments with isolated liver cells fit with uptake by macrophages. These observations suggested the presence of an opsonizing factor in the circulation before the second injection, resulting in opsonization and rapid phagocytosis of the second dose of liposomes. The results of our transfusion experiments supported this hypothesis. Transfusion of pretreated serum in nontreated rats indeed elicited enhanced blood clearance of a first dose of PEG liposomes, whereas heating the serum before transfusion at 56°C for 30 min abolished the effect. In addition, the enhanced blood clearance of PEG liposomes from the blood was directly related to the amount of pretreated serum administered. Finally, we showed that the observed phenomenon could be attributed to a serum factor. This serum factor coeluted on a size exclusion column with a 150-kDa protein and was not an antibody molecule.

Recently, two other studies focused on the pharmacokinetics of serial injections of PEG liposomes. Goins et al. (1998) reported similar pharmacokinetics of two subsequent injections of PEG liposomes in rabbits when given in a 6-week interval. These results are in line with our observation that the enhanced clearance is only observed at relatively short time intervals (<4 weeks). Oussoren and Storm (1999) administered four doses of PEG liposomes to rats with 1- or 2-day intervals and also reported unchanged pharmacokinetics. These results do not contradict our present results because our experiments indicate that the second injection only shows accelerated blood clearance when given at least 1 week after the first injection. Still, it cannot be excluded that the phenomenon described in the present study is dependent on the formulation of the liposomes used. Further studies

Fig. 8. SDS-polyacrylamide gel (10%) showing the protein composition of fractions A to E (lanes 4–8). Fraction B (lane 5) induced accelerated blood clearance of PEG liposomes. The major component of fraction B was a protein with an $M_r$ of 150 kDa. Lane 1, molecular mass markers; lane 2, total rat serum; and lane 3, IgG.
should demonstrate whether this phenomenon also applies for other liposomal formulations.

The concept of a serum opsonin triggering the uptake of liposomes by the MPS has been explored in several animal models. Liu et al. (1995a) demonstrated that hepatic uptake of liposomes in rats depended on serum opsonins, whereas in mice hepatic uptake did not involve specific serum opsonins. Their findings could explain our observation that in mice repeated injections with PEG liposomes did not evoke any pharmacokinetic changes.

What is known about the factor(s) that could have initiated the observed pharmacokinetic changes? The observation that PEG liposomes could elicit the production of a serum factor influencing their pharmacokinetics on subsequent injections has not been reported before. In general, because of their reduced interaction with plasma proteins, PEG liposomes have been considered to be nonimmunogenic. In contrast, the interaction of non-PEG liposomes with the immune system has long been recognized (Devine and Marjan, 1997). Depending on their physiochemical properties and lipid composition, liposomes can activate complement by binding antibodies or by binding complement, sometimes facilitated by serum proteins (Szebeni, 1998). Naturally occurring antibodies against phospholipids can be found in the blood of most species (Alving, 1984), and have indeed been shown to induce complement-mediated opsonisation of liposomes (Wassef et al., 1989). The observation in the present study that a minimum time-interval of 5 days between the first and the second injection was required to evoke rapid blood clearance argues against a role for autoantibodies, but suggests the development of a newly formed serum factor that may opsonize the PEG liposomes. Our transfusion experiments showed that this serum factor was not an Ig molecule.

Several reports have stressed the role of complement, and more specifically C3b, as serum opsonin, mediating the binding and phagocytosis of liposomes by cells of the MPS (Harrashima et al., 1994; Liu et al., 1995b, 1998; Hu and Liu, 1996). Clearly, the acute side effects in patients at liposomal administration, although probably originating from complement-activation and the subsequent release of anaphylacto- toxins, do not resemble the deferred effect of successive injections. Still, a complement factor seems to be a good candidate to explain our experimental results because heat treatment of pretreated serum abolished the opsonizing serum activity. The mitigating effect of successive injections could then perhaps be the result of consumption of complement factors. However, once the blood residence time of the PEG liposomes had normalized, one would have expected increased blood clearance of the liposomes on the following injection. This was not observed in our experiments. Additional transfusion experiments with serum depleted of specific complement factors will be needed to clarify the role of complement in the initiation of enhanced blood clearance of a second injection with PEG liposomes.

The preferential localization of the second dose of 3H-labeled liposomes in the Kupffer cells suggested the selective phagocytosis by these liver macrophages. Furthermore, the decrease in serum complement activity and our findings that the enhanced clearance of the PEG liposomes could be induced by a heat-labile, high-molecular serum factor suggest that opsonic phagocytosis of the liposomes was mediated by complement. Data in the literature support such a role for complement (Szebeni, 1998).

Our results have potential implications for the clinical application of PEG liposomal formulations. Obviously, unexpected changes in pharmacokinetic behavior of a drug are highly undesirable. Enhanced blood clearance of PEG liposomal drug formulations could compromise their therapeutic efficacy. In addition, if these PEG liposomes contain toxic drugs such as Doxil, increased uptake in the liver could cause enhanced liver toxicity (Daemen et al., 1995). Furthermore, the possible involvement of complement factors in the initiation and effectuation of rapid blood clearance of PEG liposomes constitutes a risk of potentially adverse biological reactions due to release of anaphylacto- toxins. However, predicting clinical outcome on the basis of animal studies is cumbersome. Still, the composition, size, and lipid dose of the small PEG liposomes in this study were very similar to many of the PEG liposomal drugs currently under clinical investigation.

The dosing frequencies at which the pharmacokinetic changes were most distinct (1–3 weeks) were somewhat higher than those currently used in clinical practice (3–6 weeks). This could explain that thus far, no enhanced hepatoxic side effects of PEG liposomal anticancer drugs have been reported in clinical trials, with the exception of a case of fulminating hepatic necrosis in a patient receiving biweekly cycles of PEG liposomal doxorubicin (Hengge et al., 1993). Similarly, aggravation of side effects on successive injections has not been reported yet. In our opinion, this lack of clinical evidence of pharmacokinetic changes on successive injections does not invalidate the clinical relevance of our findings. The assessment of serum enzymes, as a routine measure for liver toxicity, does not provide information on possible impairment of macrophage function. In addition, reports on infusion-related side effects of Doxil have led to the routine coadministration of antiallergic or anti-inflammatory drugs. Furthermore, the lack of direct comparative studies between PEG liposomal anticancer drugs and the conventional preparation precludes reliable assessment of uncompromised therapeutic efficacy during the course of the treatment. Last, the low injection frequency of PEG liposomal doxorubicin (3–6 weeks) was chosen to prevent the occurrence of cutaneous toxicity (Muggia et al., 1997). Promising results have been obtained with pyridoxine to prevent this cutaneous toxicity (Vail et al., 1998) and this agent may allow more frequent dosing in the near future.

In summary, this report describes some very intriguing aspects of the pharmacokinetic behavior of successive injections with PEG liposomes, which could have important repercussions on their application for clinical use. Our results warrant further clinical studies on the biodistribution and pharmacokinetics of PEG liposomal drug formulations.

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References


References


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