ABSTRACT
A major obstacle in the development of red cell substitutes has been overcoming their short circulation persistence. In this study, distearoyl phosphoethanolamine polyethylene glycol 5000 (PEG-PE) (10 mol%) was added to the formulation of liposome-encapsulated hemoglobin (LEH) to decrease reticuloendothelial system uptake and prolong LEH circulation persistence. PEG-LEH was radiolabeled with technetium-99m, infused into rabbits (25% of blood pool at 1 ml/min) (n = 5), and monitored by scintigraphic imaging at various times out to 48 h. At 48 h, animals were sacrificed, and tissue samples were collected for counting in a scintillation well counter. Tissue distribution data at 48 h revealed that 51.3 ± 3.4% of the technetium-99m-PEG-LEH remained in circulation, a greater than 3-fold increase in the circulation half-life compared with circulation half-lives previously reported for non-PEG-containing LEH formulations. The liver had the greatest accumulation at 48 h (12.7 ± 0.7%), followed by bone marrow (6.2 ± 0.1%), whereas the spleen had only 1.4 ± 0.2%. The addition of PEG-PE to the LEH formulation greatly prolongs the circulation persistence of LEH and represents a significant step in the development of red cell substitutes with prolonged oxygen delivery.

Polyethylene Glycol-Modified Liposome-Encapsulated Hemoglobin: A Long Circulating Red Cell Substitute

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ABBRIDATIONS: LEH, liposome-encapsulated hemoglobin; PEG, polyethylene glycol; DSPE, distearoyl phosphoethanolamine; 99mTc, technetium-99m; HMPAO, hexamethylpropyleneamine oxime; % ID, percentage of injected dose; RES, reticuloendothelial system; PEG-LEH, polyethylene glycol-coated liposome-encapsulated hemoglobin; DMPG, dimyristoyl phosphatidylglycerol.
al., 1997). With large doses of lipid such as that given when LEH is administered, the RES could potentially become saturated so that PEG surface modification may provide only minimal extension of the circulation half-life compared with standard liposomes.

Another important factor controlling circulation half-life of a liposome-encapsulated agent is the precise mole percentage (mol%) of polyethylene glycol distearoyl phosphoethanolamine (PEG-DSPE) included in the liposome formulation. The mol% and the molecular weight of the PEG headgroup appear to be critical for successfully increasing the circulation persistence of LEH. Previous attempts to significantly prolong the circulation of LEH with 5 mol% PEG-PE were unsuccessful (Zheng et al., 1994). The present study was undertaken to determine whether the addition of a higher 10 mol% PEG-DSPE with a PEG headgroup of greater molecular weight (5000 versus 1900) would increase the circulation persistence and change the tissue biodistribution of LEH. To monitor this new PEG-LEH formulation after i.v. administration in rabbits, we used a previously developed method of labeling LEH with technetium-99m (99mTc) in a stable fashion combined with scintigraphic imaging to allow for noninvasive determination of changes in organ biodistribution over the course of the study. This report summarizes our findings that the addition of PEG-DSPE to the LEH formulation can increase circulation persistence.

Materials and Methods

PEG-LEH Preparation. PEG-LEH was produced using sterile technique by microfluidization after rehydration of a dried lipid film of distearoyl phosphatidylcholine (DSPC), cholesterol, distearoyl phosphoethanolamine-N-(polyethylene glycol 5000) (DSPE-PEG), and α-tocopherol (mole ratio 50:38:10:2) with αα-crosslinked hemoglobin (Bionetics, Rockville, MD) containing 30 mM reduced glutathione (Sigma Chemical Co., St. Louis, MO), 10 mg/ml human serum albumin, and 9% sucrose. After production, PEG-LEH was separated from unencapsulated hemoglobin by ultrafiltration through a 300-kDa polysulfone filter. The final PEG-LEH preparation was tested as previously described (Farmer and Gaber, 1987; Goins et al., 1994), and the characteristics of PEG-LEH are shown in Table 1.

PEG-LEH Labeling. PEG-LEH was labeled using a previously described and validated method for labeling liposomes with 99mTc (Rudolph et al., 1991; Phillips et al., 1992; Goins et al., 1993; Phillips and Goins, 1995). PEG-LEH (4 ml) was incubated for 30 min with 2 ml of 99mTc-hexamethylpropyleneamine oxime (HMPAO) (Ceretec; Amersham, Arlington Heights, IL) that had been previously incubated for 5 min with 10 mCi of 99mTc-sodium pertechnetate in 5 ml of 0.9% saline. After the labeling process, the liposomes were separated from free 99mTc by passage over a Sephadex G-25 column. Labeling efficiency was calculated by comparing precolumn and postcolumn values using a dose calibrator (Radex Model Mark J, Houston, TX). Labeling efficiency ranged from 57 to 65%.

Animal Experiments. Animal experiments were performed under the National Institutes of Health Animal Use and Care guidelines and were approved by the University of Texas Health Science Center at San Antonio Institutional Animal Care Committee. Male New Zealand White rabbits (2.5–3.0 kg), that had been fasted the night before the study, were anesthetized with 50 mg of ketamine-10 mg of xylazine/kg b.wt. i.m. One ear of the rabbit was catheterized with a 23-gauge venous line, and the other ear was catheterized with a 20-gauge arterial line. Blood samples were drawn from the arterial line, and 99mTc-PEG-LEH was infused in the venous line. Rabbits were then placed in the supine position under a Picker (Cleveland, OH) large-field-of-view gamma camera interfaced with a Pinnacle imaging computer (Medasys, Ann Arbor, MI), and image acquisition was begun with a low-energy all-purpose collimator as the 99mTc-PEG-LEH solution was introduced at 1.0 ml/min. The animals received a total dose of 5 mCi 99mTc-PEG-LEH (3.5 mCi 99mTc activity, 36.9 ml, 1.13 g phospholipid/kg b.wt., 0.16 g hemoglobin/kg b.wt.) equivalent to 25% of their circulating blood volume based on 59 ml/kg b.wt. (Kaplan and Timmons, 1979). One-minute dynamic 64 × 64 pixel scintigraphic images were acquired over a continuous period of 2.5 h. Blood was drawn into capillary tubes (50 µl) at various times after 99mTc-PEG-LEH injection to monitor circulation persistence. At both 24 and 48 h postinfusion, static images and blood samples were acquired. After 48 h, the animals were euthanized with an overdose of pentobarbital. Tissue samples were collected, weighed, and counted for radioactivity in a scintillation well counter (Canberra, Meridian, CT) for calculation of biodistribution. Calculations used to estimate the total percent of the injected dose (% ID) in blood, muscle, skin, and bone marrow were blood volume, 5.9 ml/100 g b.wt.; muscle, 45 g/100 g b.wt.; skin, 10 g/100 g b.wt., respectively. The % ID in the bone marrow was estimated to be 12 times the % ID in one femur (Dietz, 1944). Control studies with 99mTc-labeled red blood cells were performed for the blood pool contribution subtraction as follows. Briefly, heparinized blood (6.0 ml) was withdrawn from rabbits (n = 3) and added to a red blood cell labeling kit (Ultratag; Mallinkrodt, St. Louis, MO). After labeling with 99mTc, the blood was reinfused into each rabbit through an ear vein. After adequate equilibration of the 99mTc-labeled red blood cells (20 min), scintigraphic images were acquired and analyzed to determine the percent distribution of total acquired counts in the heart, liver, and spleen.

Image Analysis. Image analysis was performed using a nuclear medicine analysis workstation (Pinnacle computer; Medasys, Ann Arbor, MI). Whole body images were decay corrected, and then regions of interest were drawn over the liver, spleen, and heart in images acquired at various times after the infusion of 99mTc-PEG-LEH. A box was drawn around the whole animal in each image at the same time points to represent the counts from the total injected dose given during the study. The counts in each organ were converted to a percentage of total body counts. The distribution percentages in a given organ for each animal were averaged, and standard errors were calculated. Images were corrected for blood pool contribution using a previously described technique (Rudolph et al., 1991).

Blood Clearance Analysis. Blood samples were counted for radioactivity in a scintillation well counter. The radioactive counts were decay corrected to account for the half-life of 99mTc and plotted against the sampling time to generate blood clearance curves. Blood clearance curves were fitted to a two-compartment model to generate circulation half-lives using Scientist for Windows software with supplemental Pharmacokinetic Model Library (MicroMath, Salt Lake City, UT).

Results

An image of a rabbit acquired 2 h after the infusion of 99mTc-PEG-LEH is shown in Fig. 1A. The distribution of 99mTc activity visualized at this time is due to 99mTc activity in the heart and other vasculature. The 99mTc activity in the liver is primarily due to the vascularity of the liver, and no 99mTc activity can be detected in the spleen. This distribution

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td>Characteristics of PEG-LEH</td>
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<tr>
<td>Unimodal size distribution</td>
</tr>
<tr>
<td>Phospholipid content</td>
</tr>
<tr>
<td>Hemoglobin content</td>
</tr>
<tr>
<td>Endotoxin level</td>
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<tr>
<td>Sterility</td>
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Fig. 1. Gamma camera images of rabbits acquired after a 25% topload infusion of 99mTc-labeled PEG-LEH. The images were taken at 2 h (A), 24 h (C), and 48 h (D) after infusion of 99mTc-PEG-LEH. B, For comparative purposes, an image of 99mTc-labeled red blood cells (RBCs) acquired after equilibration at 20 min is shown. The 2-h image of a rabbit receiving 99mTc-PEG-LEH is very similar to the 99mTc-RBC image.

noted for 99mTc-PEG-LEH appears to be very similar to the distribution of 99mTc activity noted in the 99mTc-labeled red blood cell image demonstrated in Fig. 1B. The 24-h 99mTc-PEG-LEH image (Fig. 1C) continues to reveal a large amount of heart activity representing 99mTc-PEG-LEH still in circulation. The increased 99mTc activity visualized in the liver compared with the 2-h image indicates that the liver is the major site of PEG-LEH removal. Although continued 99mTc activity is visualized in the heart at 48 h, the trend of increasing liver 99mTc activity is apparent (Fig. 1D). It is also apparent that the 48-h image has a lower resolution because of the decreased 99mTc activity secondary to decay of the 99mTc radionuclide (half-life = 6.1 h).

Figure 2 illustrates the organ distribution of 99mTc-PEG-LEH as a percentage of injected dose (% ID) at various times postinfusion as estimated by image analysis. Figure 2A shows the distribution of the 99mTc activity in the various organs uncorrected for the 99mTc activity contributed by the blood associated with that organ. Figure 2B shows the blood background-corrected 99mTc activity corresponding to the estimated organ uptake of 99mTc-PEG-LEH over time. Most of the blood pool-corrected organ uptake of 99mTc-PEG-LEH is in the liver: 5% ID at the time of completion of the infusion. Liver uptake decreases to 2% ID by 2 h. This slight decrease in liver 99mTc activity is apparent by visual inspection of the images because the heart has more intensity in relation to the liver at 2 h than it does at 45 min. At 24 and 48 h, the uptake in the liver is 10% ID. The spleen demonstrated only measurable uptake after 24 h, and at 48 h, it had increased 99mTc activity to only 1.4% ID. The uptake of 99mTc-PEG-LEH in other organs was minimal, and image analysis was not possible.

A clearance profile (Fig. 3) for 99mTc-PEG-LEH was generated by monitoring 99mTc activity in blood samples and fitting the data to a two-compartment pharmacokinetic model. The distribution phase rate constant was 0.0005 h^{-1}, and the elimination phase half-life was 65.2 h. The volume of distribution was 173.9 ± 8.4 ml.

Table 2 shows the 48-h organ biodistribution for 99mTc-PEG-LEH both on a % ID/organ and % ID/g tissue basis obtained by tissue sampling at necropsy. The blood has 4 times more 99mTc-PEG-LEH (51.3 ± 3.4% ID) than the liver (12.7 ± 0.7% ID). Approximately 5% ID is found in the bone marrow, small bowel, and colon. Only minimal amounts are noted in the other organs listed. The spleen has the largest dose based on % ID/g tissue, followed by the blood and the liver.

Discussion

The PEG-LEH formulation in this study demonstrated prolonged circulation persistence and offers the potential of providing extended oxygen delivery. The slow rate of clearance from the blood pool also results in a decreased stress on the RES. The circulation half-life of 65 h for this PEG-LEH formulation is greater than 3 times longer than that previously reported for earlier non-PEG-containing LEH formulations (Farmer and Gaber, 1987; Rudolph et al., 1991; Zheng et al., 1994). In a previous study, after administering the same volume of a non-PEG-containing LEH formulation to rabbits in an identical fashion (Rudolph et al., 1991), we measured a circulation half-life of 18 h. The organ distribution pattern of this non-PEG-containing LEH formulation
samples in a scintillation well counter at 48 h (Allen et al., 1989). In the case of LEH, however, ganglioside increase the circulation persistence of a liposome formulation. Tissue biodistribution of 99mTc-PEG-LEH obtained from counting tissue samples over the first 2 h.

The use of 99mTc as a label allowed serial dynamic scintigraphic imaging and determination of changes in biodistribution at multiple time points without sacrifice of the animal until the end of the study. This 99mTc liposome label has been shown to be extremely stable, with in vitro studies showing <2% dissociation of 99mTc from the liposome label during incubation with plasma at 37°C for 90 h (Phillips et al., 1992). The 99mTc label also allowed determination of actual tissue biodistribution after sacrifice by counting tissue samples in a scintillation well counter. High quality statistics are possible out to 90 h due to the high sensitivity of the scintillation well counter for counting tissue samples. Generally, the tissue samples cannot be counted until at least 60 h after the start of a study so that sufficient time is allowed for the 99mTc to decay (>1 μCi of activity saturates the electronics of the well counter and produces excessive dead time).

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**TABLE 2**

Tissue biodistribution of 99mTc-PEG-LEH obtained from counting tissue samples in a scintillation well counter at 48 h

<table>
<thead>
<tr>
<th>Organ</th>
<th>% ID/Organ</th>
<th>% ID/g Tissue</th>
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<tbody>
<tr>
<td>Blood</td>
<td>51.27 ± 3.44</td>
<td>0.315 ± 0.022</td>
</tr>
<tr>
<td>Liver</td>
<td>12.69 ± 0.70</td>
<td>0.125 ± 0.016</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.42 ± 0.20</td>
<td>1.179 ± 0.064</td>
</tr>
<tr>
<td>Femur</td>
<td>6.18 ± 0.14</td>
<td>0.043 ± 0.002</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.09 ± 0.21</td>
<td>0.093 ± 0.012</td>
</tr>
<tr>
<td>Lung</td>
<td>0.85 ± 0.10</td>
<td>0.092 ± 0.012</td>
</tr>
<tr>
<td>Heart</td>
<td>0.11 ± 0.01</td>
<td>0.035 ± 0.014</td>
</tr>
<tr>
<td>Brain</td>
<td>0.05 ± 0.00</td>
<td>0.004 ± 0.000</td>
</tr>
<tr>
<td>Skin</td>
<td>1.61 ± 0.11</td>
<td>0.006 ± 0.001</td>
</tr>
<tr>
<td>Muscle</td>
<td>2.30 ± 0.28</td>
<td>0.002 ± 0.000</td>
</tr>
<tr>
<td>Urine</td>
<td>1.09 ± 0.26</td>
<td>0.027 ± 0.006</td>
</tr>
<tr>
<td>Bowel</td>
<td>5.25 ± 0.39</td>
<td>0.013 ± 0.002</td>
</tr>
<tr>
<td>Colon</td>
<td>5.34 ± 0.89</td>
<td>0.013 ± 0.002</td>
</tr>
</tbody>
</table>

Values represent mean ± S.E.M.
Future studies will need to address the oxygen-carrying capacity of PEG-LEH. For adequate oxygen transport, it obviously will be important that a blood substitute have an adequate hemoglobin concentration. The hemoglobin content of the current preparation is low (1.2 g/dl), and methods to increase its hemoglobin content need to be studied. Other methods of manufacturing liposomes may result in an LEH formulation with increased hemoglobin content. One such method, the dehydration-rehydration method, has been reported to have significantly increased hemoglobin content (Brandl and Gregoriadis, 1994). Researchers using this method have described hemoglobin contents of >10 g/dl.

It will also be important for the hemoglobin in LEH to remain in a functional state after infusion. Several animal studies have demonstrated that a substantial portion of both cross-linked hemoglobin and LEH are converted to met-hemoglobin after infusion (Boer et al., 1993; Phillips et al., 1997). Using the radionuclide, oxygen-15, we recently demonstrated that after initial administration, LEH carries the amount of oxygen that would be expected for its hemoglobin concentration. However, liposomes encapsulating only bovine hemoglobin gradually lost their ability to carry oxygen over a 24-h period, presumably due to met-hemoglobin conversion. This occurred even though approximately 50% of the encapsulated hemoglobin remained in circulation (Phillips et al., 1997). On the other hand, LEH-encapsulating human heme-lyosin, which contains naturally occurring antioxidants, retained hemoglobin in the oxyhemoglobin state over a 24-h period and only lost oxygen-carrying capacity due to its removal from the circulation by the RES (Phillips et al., 1997). These observations establish the important difference between physical half-life and functional half-life of red cell substitutes and suggest that methods to protect encapsulated hemoglobin from oxidative reactions may prolong the functional half-life of LEH. Recently, several approaches, such as the inclusion of artificial enzymatic reduction systems in LEH to decrease met-hemoglobin formation, have been reported (Ogata et al., 1996; Takeoka et al., 1996). The ability to coencapsulate hemoglobin protectant systems with encapsulated hemoglobin gives liposomes a significant advantage over other methods of prolonging hemoglobin circulation such as crosslinking hemoglobin or conjugating hemoglobin with PEG.

Another important approach for decreasing met-hemoglobin formation inside of liposomes may be to genetically engineer hemoglobin that is less susceptible to met-hemoglobin conversion (Vandegriff, 1995; Olson, 1996). Future research investigating various approaches of decreasing the rate of met-hemoglobin formation will be important, especially now that methods of significantly prolonging the circulation persistence of red cell substitutes are becoming available. The surface modification of LEH with PEG represents an initial step in the development of an encapsulated hemoglobin-based red cell substitute with prolonged oxygen-delivery capability.

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