Novel Method of Greatly Enhanced Delivery of Liposomes to Lymph Nodes

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ABSTRACT
Intravenously administered liposomes are versatile carriers for drugs, contrast agents, biologics, and DNA. Liposomes and other colloidal particles are currently under investigation as lymph node delivery vehicles. After s.c. injection, conventional liposomes move into the lymphatic vessels, but are poorly retained in each draining lymph node (<2% injected dose). In this report, we describe a novel method for greatly enhancing the retention of liposomes in the lymph nodes. This system is comprised of a s.c. injection of biotin-coated liposomes in an area where lymph node targeting is desired, followed by an adjacent s.c. injection of avidin. As the avidin moves through the lymphatic vessels, it causes aggregation of biotin-coated liposomes that are also in the process of migrating through lymphatic vessels. These aggregated liposomes become trapped in the next encountered lymph node. In the present study, experimental rabbits were s.c. administered biotin-coated liposomes in both hind feet, followed by an adjacent injection of avidin, whereas control rabbits were administered biotin-coated liposomes in both hind feet without the avidin. At 24 h, rabbits receiving avidin retained 13.7% of the injected liposomes in popliteal nodes and 2.3% in iliac nodes, whereas control rabbits retained only 1.7% of the liposomes in popliteal nodes and 0.3% in iliac nodes. Blood and liver uptake of the biotin-coated liposomes was greatly decreased in the experimental rabbits receiving avidin. This novel liposome delivery system may prove useful for the delivery of chemotherapeutic drugs, vaccine antigens, and biologic agents to lymph nodes.

The development of carrier systems for the targeted delivery of agents to lymph nodes has a wide variety of potential medical applications, including the treatment of viral and bacterial infections, treatment or prevention of tumor metastasis, and as a delivery vehicle for vaccine antigens (Moghimi and Rajabi-Siahboomi, 1996; Oussoren et al., 1997; Porter, 1999). Colloidal particles, which are injected either s.c., i.m., or i.p., are cleared through the lymphatic system and accumulate to varying degrees in the lymph nodes.

Liposomes, spontaneously forming lipid spheres, are one class of colloidal particle, which is proving to be a versatile carrier for a wide variety of i.v. administered agents, including drugs, contrast agents, biologics, and DNA (Bangham, 1992; Tilcock et al., 1992; Torchilin, 1996; Barron et al., 1999; Phillips, 1999). Liposomes are currently under investigation as lymph node delivery vehicles when administered through a s.c. or i.m. route (Velinova et al., 1996; Oussoren and Storm, 1997). After a s.c. injection, approximately 50% of the injected dose of liposomes clears from the site of injection by drainage through the lymphatics, while the other 50% is retained for >24 h at the initial site of injection. The effect of liposome size, lipid composition, and liposome surface modification on the deposition of liposomes in lymph nodes has been previously studied (Oussoren et al., 1997). Although 50% of a s.c. injected dose of liposomes is cleared from the injection site into the lymphatic vessels, only a small fraction of these liposomes are retained in each draining lymph node (1–2%) (Oussoren and Storm, 1997). Most of the liposomes that are cleared from the site of injection pass through the lymph nodes and enter the systemic circulation. Attempts to increase the deposition of liposomes in lymph nodes have focused on either increasing the percentage of an injected dose of liposomes that clears from the site of injection or increasing the retention of liposomes in the lymph nodes.

Several liposome modifications have resulted in modest increases in the deposition of s.c. injected liposomes in the lymph node. Oussoren et al. (1997) found that liposomes containing positively charged lipids had approximately 3 times more lymph node localization (3.6% of the injected dose) as liposomes containing neutral or negatively charged lipids (1.2% of the injected dose). Coating liposomes with IgG increased lymph node localization of liposomes to 4.5% of the injected dose at 1 h, but this level decreased to 3% by 24 h (Mangat and Patel, 1985). It has also been reported that

ABBREVIATIONS: GSH, glutathione; IgG, immunoglobulin G; 99mTc, technetium-99m; HMPAO, hexamethylpropyleneamine oxime; %ID, percentage of injected dose.
attaching mannose to the surface of a liposome increases lymph node uptake by 3-fold compared with control liposomes (Wu et al., 1981). None of these previously mentioned modifications has resulted in large increases in the percentage of liposomes deposited in the draining lymph nodes.

In the present study, we describe a novel method of increasing the retention and overall localization of liposomes in the primary lymph nodes. This method utilizes the high affinity ligands, biotin and avidin. Biotin-coated liposomes are injected s.c., followed by an adjacent s.c. injection of avidin. The polyvalent avidin drains into the lymphatic vessels where it causes aggregation of the biotin-coated liposomes that are also in the process of migrating through the lymphatic vessels. These aggregated liposomes become trapped in the next encountered lymph node. This process is illustrated in Fig. 1. The kinetics and deposition of these liposome aggregates are monitored by labeling the liposomes with technetium-99m (99mTc) and imaging the animals after injection.

**Materials and Methods**

**Liposome Manufacture and Characterization.** To follow the uptake of the biotin-coated liposomes in lymph nodes using gamma scintigraphy, the liposomes were labeled with 99mTc (Phillips et al., 1992). For this procedure, glutathione (GSH) in PBS, pH 6.3, and sucrose was encapsulated in the liposome before 99mTc labeling (Goins et al., 1998). The lipid composition was modified from previous 99mTc-liposome studies by our laboratory to include a phosphoethanolamine (Northern Lipids, Vancouver, Canada):cholesterol (Calbiochem, San Diego, CA):N-biotinoyldistearylphosphoethanolamine (Northern Lipids, Vancouver, Canada):α-tocopherol (Aldrich, Milwaukee, WI). Liposomes were prepared in a laminar flow hood using aseptic conditions. A dried lipid film was rehydrated in 300 mM sucrose (Sigma Chemical Co., St. Louis, MO) in sterile water at a total lipid concentration of 120 μmol/ml and lyophilized overnight. The resultant lyophilized powder was then rehydrated with 200 mM GSH (Sigma) in Dulbecco’s PBS, pH 6.3, at a final total lipid concentration of 120 μmol/ml. Immediately before extrusion, the lipid suspension was diluted to 40 μmol/ml with 100 mM GSH in PBS (pH 6.3) containing 150 mM sucrose and extruded through a series of polycarbonate filters (Lipex, Vancouver, Canada) at 55°C. Extruded liposomes were washed three times in PBS, pH 6.3, containing 75 mM sucrose and centrifuged at 45,000 rpm for 45 min in an ultracentrifuge (Ti-60 rotor, Beckman, Fullerton, CA) to remove any unencapsulated sucrose and GSH. The final liposome pellet was reconstituted in 300 mM sucrose/PBS to a total lipid concentration of approximately 60 μmol/ml and stored at 4°C until needed.

The diameter of the final liposome product was determined to be 136 nm using particle size analysis (Brookhaven Instruments, Holtsville, NY). The phospholipid concentration of the final product was 39 μmol/ml (Stewart, 1980).

**Liposome Labeling.** Liposomes were labeled with 99mTc as previously described (Phillips et al., 1992). A commercial kit of the lipophilic chelator, hexamethylpropyleneamine oxime (HMPAO, Cere tec, Nycomed-Amersham, Arlington Heights, IL), was reconstituted with 5 ml of 0.9% saline containing 10 mCi (370 MBq) of 99mTc-sodium pertechnetate. The kits were checked for the percentage of lipophilic HMPAO using the 3-step paper chromatography system as outlined in the package insert. An aliquot (1 ml) of 99mTc-HMPAO was added to a concentrated suspension of liposomes encapsulating GSH (2 ml) and incubated at room temperature for 30 min. Labeling efficiencies were determined by measuring the 99mTc activity associated with the 99mTc-liposome fraction before and after Sephadex G-25 (PD-10, Amersham Pharmacia Biotech, Piscataway, NJ) column separation using a dose calibrator (Radix Model Mark 5, Houston, TX).

**Imaging Studies.** 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) were anesthetized by injecting a ketamine:xylazine (both from Vedeo, St. Joseph, MO) (50:10 mg/kg, iv) cocktail in the brachial muscle. The rabbits were divided into two groups. The experimental rabbits (n = 4 rabbits and n = 8 hind feet) received an aliquot (0.3 ml; 1.5 mg of phospholipid/kg) of 99mTc-biotin-coated liposomes, which was injected s.c. into a shaved area on the dorsum of each hind foot, followed by a s.c. injection of 5 mg of avidin (Sigma) in 0.3 ml of saline into each rabbit hind foot at a location 2 cm proximal to the site of the 99mTc-biotin-coated liposome injection. The control rabbits (n = 4 rabbits and n = 8 hind feet) received a s.c. injection (0.3 ml; 1.5 mg of phospholipid/kg) of 99mTc-biotin-coated liposomes in each hind foot in the exact same manner as the experimental rabbits, except no avidin was administered. The dose of avidin chosen for this study was determined from pilot experiments conducted in the same manner as described above, except rabbits received either 0.25, 0.5, 1, 2, or 5 mg of avidin. Dynamic (1 min) scintigraphic images were acquired in a 64 × 64 Word Image Matrix using a Picker (Cleveland, OH) Dyna 4 gamma camera interfaced to a Medasys Pinnacle computer (Miami, FL). After obtaining the first dynamic 1-min image, the s.c. 99mTc-biotin-coated liposome blebs were massaged gently for 5 min. At T = 30, 40, 50, and 60 min postinjection, running leg movement was performed for 1 min by manual manipulation of both legs. Immediately after

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Fig. 1. This illustration demonstrates the proposed mechanism of liposome accumulation in the primary lymph node using the novel method described in this manuscript. Liposomes (1) coated with biotin on their surfaces are injected s.c. into the dorsum of rabbit foot. Multivalent avidin (2) is injected s.c. proximal to the site of liposome injection. As the biotin-coated liposomes and avidin migrate through the lymphatic vessels, the avidin encounters the biotin-coated liposomes and causes liposome aggregation. The liposome aggregates then become trapped in the next lymph node encountered (3).
completion of the leg movement at 60 min postinjection, dynamic acquisition was halted. Two static anterior images (1 min) were then acquired with one image of the legs and lower abdomen and the second image of the mid portion of the body. Animals were allowed to recover from anesthesia and housed for the night. At 24 h, animals were again anesthetized and two static images (10 min) were acquired depicting either the legs and lower abdomen or the mid portion of the body.

Biodistribution Studies. After imaging, the rabbits were euthanized with pentobarbital (Fort Dodge Laboratories, Fort Dodge, IA) (100 mg/kg, i.v.). Tissues were harvested, weighed, and counted for radioactivity (Canberra Multichannel Analyzer, Meridian, CT). The percentage of the injected dose (%ID) per organ was calculated by comparison with a standard aliquot of the $^{99m}$Tc-biotin-coated liposomes.

Image Analysis. Images acquired at 1, 30, and 60 min, and at 24 h were corrected for background activity and then analyzed. Regions of interest were drawn around the initial sites of s.c. injection for both hind feet in the baseline images acquired at 1 min postinjection. The $^{99m}$Tc-activity associated with both injection sites determined from the baseline image was considered to be 100% of the ID of liposomes. At 30 and 60 min, and at 24 h, regions of interest were drawn around the initial sites of injection and the popliteal nodes. Counts associated with these regions were corrected for decay. The percentage of initial baseline activity at the injection site and popliteal nodes was calculated at 30 and 60 min, and at 24 h. The estimated percentage of radiolabeled material that passed through the lymph node at 30 and 60 min, and at 24 h, was calculated from image analysis data using the following formula: percentage of nodal pass-through = ($\text{activity of injection site at baseline} - \text{activity of popliteal node at time } X$)/($\text{activity of injection site at baseline}$). The estimated nodal retention efficiency was calculated as: nodal retention efficiency = activity at the popliteal node at time $X$/($\text{activity of injection site at baseline} - \text{activity of the injection site at time } X$).

Statistical Analysis. Values are reported as mean ± S.E. Statistical analysis was performed using Excel (Microsoft, Redmond, WA) software for a Macintosh computer (Apple, Cupertino, CA). The following region of interest image data were analyzed for statistical differences between experimental rabbits receiving avidin and control rabbits at a given time by using an unpaired Student's $t$ test: %ID at the injection site, %ID at the popliteal node, percentage of nodal pass-through and nodal retention efficiency. The %ID located in the popliteal node as determined by scintillation well counting of tissue samples collected at 24 h postinjection for the experimental and control groups was also statistically analyzed. The acceptable probability for a significant difference between means was $P < .05$.

Results

Of the various avidin doses tested, the 5-mg avidin dose resulted in the best liposome retention in the popliteal node. It was the only dose of avidin in which the percentage of the injected dose in the popliteal lymph node did not significantly decrease from 30 to 60 min and continued to increase over the 24-h period (Fig. 2). Although less effective than the 5-mg dose, both the 1- and 2-mg avidin doses appeared to increase the lymph node targeting compared with the control situation when no avidin was injected. The 0.25- and 0.5-mg doses of avidin were not different from the control.

The increased popliteal node uptake in the experimental rabbits given the 5-mg dose of avidin compared with the control animals can clearly be observed in a representative scintigraphic image of the legs and lower abdomen by 60 min postinjection (Fig. 3). By 60 min there was also marked accumulation of liposomes in the iliac node. The uptake in both the popliteal and iliac nodes was sustained at 24 h. Representative images (Fig. 4) of the mid portion of the body also show the greatly decreased accumulation of $^{99m}$Tc-liposomes in the heart and liver of experimental animals compared with control animals.

The region of interest distribution data obtained from images acquired at 30 min, 60 min, and 24 h for the experimental and control rabbits are shown in Table 1. The biodistribution results obtained from tissue sampling at 24 h are shown in Table 2. Both the image analysis data and the tissue biodistribution results demonstrate that the experimental animals had an approximately 6-fold increase in liposome localization in the popliteal nodes compared with control rabbits. This increased uptake was highly significant for both the image analysis data ($P = .0001$) and for the tissue biodistribution data ($P = .0003$). A trend ($P = .1$) of increased uptake in the iliac nodes was observed in the experimental group ($2.3 \pm 0.8\%$) compared with the control

![Figure 2](https://via.placeholder.com/150)

**Fig. 2.** Percentage of injected dose of $^{99m}$Tc-biotin-coated liposomes in the popliteal node of rabbits as a function of amount of avidin injected. Values were determined from region of interest analysis of images acquired at 30 min, 60 min, or 24 h postinjection.

![Figure 3](https://via.placeholder.com/150)

**Fig. 3.** Characteristic scintigraphic images of the legs and lower abdomen of rabbits acquired at 30 min, 60 min, and 24 h after s.c. injection of $^{99m}$Tc-biotin-coated liposomes. Images of an experimental rabbit (+avidin) are depicted (top panel) compared with a control rabbit (bottom panel). The increased uptake by the popliteal nodes in the experimental animal is clearly visualized in the images acquired at 60 min and 24 h.}

![Figure 4](https://via.placeholder.com/150)

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**Table 1.** Biodistribution Results for Avidin Doses and Liposome Localization in Popliteal Nodes.

<table>
<thead>
<tr>
<th>Amount of Avidin Injected (mg)</th>
<th>%ID at Injection Site</th>
<th>%ID at Popliteal Node</th>
<th>Percentage of Nodal Pass-Through</th>
<th>Nodal Retention Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.5</td>
<td>2.5</td>
<td>80</td>
<td>70</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
<td>3.0</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>1.0</td>
<td>2.0</td>
<td>4.0</td>
<td>150</td>
<td>120</td>
</tr>
<tr>
<td>2.0</td>
<td>3.0</td>
<td>5.0</td>
<td>200</td>
<td>160</td>
</tr>
<tr>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

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**Table 2.** Tissue Biodistribution Data for Avidin Doses and Liposome Localization in Popliteal Nodes.

<table>
<thead>
<tr>
<th>Time Postinjection (min)</th>
<th>%ID at Injection Site</th>
<th>%ID at Popliteal Node</th>
<th>Percentage of Nodal Pass-Through</th>
<th>Nodal Retention Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.1</td>
<td>0.1</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>60</td>
<td>0.2</td>
<td>0.2</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>24</td>
<td>0.3</td>
<td>0.3</td>
<td>80</td>
<td>80</td>
</tr>
</tbody>
</table>
group (0.3 ± 0.0%) based on tissue biodistribution. There was statistically less uptake of $^{99m}$Tc-biotin-coated liposomes in the blood for the experimental group (0.6 ± 0.1%) compared with the control group (7.7 ± 1.0%) ($P = .03$). Likewise, the experimental group had statistically less accumulation in the liver than in the control group [2.7 ± 1.1% versus 16.9 ± 0.5% ($P = .01$)].

The percentage of nodal pass-through for the popliteal nodes and the nodal retention efficiency were determined at 30 min, 60 min, and 24 h using the formulae outlined under Materials and Methods (data not shown). The following significant differences were observed. At 60 min, the nodal retention efficiency for the experimental group was significantly greater than for the control group [(32.5 ± 6.3%) versus 7.8 ± 1.1% ($P < .01$)]. At 24 h, the percentage of nodal pass-through for the popliteal nodes in the experimental group (23.4 ± 3.1%) was approximately 50% of the value determined for the control group (41.8 ± 3.1%) ($P < .001$). At 24 h, the nodal retention efficiency for the experimental group (33.7 ± 3.8%) was significantly higher ($P < .001$) than for the control group (4.2 ± 0.5%).

**Discussion**

This novel biotin-liposome/avidin method for increasing the deposition of liposomes in lymph nodes may prove useful in a variety of applications such as vaccine antigen delivery and the treatment of viral or bacterial infections (Morein et al., 1996; Gregoriadis et al., 1997; Dufresne et al., 1999). Another potential application for this lymph node delivery system would be for the therapy of tumor metastasis or the prophylactic prevention of future tumor metastasis (Moghimi and Rajabi-Siahboomi, 1996; Hagiwara et al., 1997; Hirnle, 1997; Porter, 1997). Although the basic principle of biotin-colloid/avidin targeting of lymph nodes is particularly useful with liposomes, because they are poorly retained by lymph nodes, it may also be useful with other colloidal delivery systems such as polylactic acid nanocapsules (Couvreur et al., 1997; Barratt et al., 1984).

Scintigraphic imaging of $^{99m}$Tc-labeled liposomes appears to be a very useful tool for studying the delivery of liposome-encapsulated drugs to lymph nodes. Image analysis of the scintigraphic images acquired during these studies allows for easy estimation of a variety of factors, including: 1) the percentage of activity that clears from the injection site, 2) calculation of the percentage of liposomes that pass through the popliteal node, and 3) determination of the nodal retention efficiency.

Liposomes appear to be ideal carriers for lymph node drug delivery. After comparing a large number of lipid-based lymphatic drug carriers, including suspensions, emulsions, solutions, and liposomes, Hirnle found that large unilamellar liposomes administered intralymphatically had the best lymphatic delivery characteristics (Hirnle, 1997). This result was based on the fact that the administration of liposomes was much safer compared with emulsions or suspensions, because the liposomes did not deposit in the lungs after “spilling over” into the systemic circulation but were taken up by the liver and the spleen.

A major advantage of using the biotin-liposome/avidin sys-

**TABLE 1**

Lymph node accumulation of $^{99m}$Tc-biotin-coated liposomes after s.c. injection into the hind feet of rabbits

<table>
<thead>
<tr>
<th>Region</th>
<th>Experimental (+Avidin) $^a$</th>
<th>Control (-Avidin) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>60 min</td>
</tr>
<tr>
<td>Iliac node ($n = 4$)</td>
<td>1.0 ± 0.3</td>
<td>2.7 ± 1.0</td>
</tr>
<tr>
<td>Popliteal nodes ($n = 8$)</td>
<td>7.0 ± 1.6</td>
<td>6.3 ± 1.2**</td>
</tr>
<tr>
<td>Injection site ($n = 8$)</td>
<td>83.6 ± 3.7</td>
<td>79.6 ± 2.8</td>
</tr>
</tbody>
</table>

$^a$ Four animals were studied in each group. Both hind feet were injected with liposomes providing eight injection sites and eight popliteal nodes in which to study.

$^b$ Values are mean ± S.E.

$^c$ The data were determined from region of interest analysis of the gamma camera images acquired at various times postinjection.

**TABLE 2**

Biodistribution of $^{99m}$Tc-biotin-coated liposomes at 24 h after s.c. injection into the hind feet of rabbits

<table>
<thead>
<tr>
<th>Organ</th>
<th>Experimental (+Avidin) ($n = 4$)</th>
<th>Control (-Avidin) ($n = 4$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left popliteal node</td>
<td>11.4 ± 2.0</td>
<td>1.7 ± 0.8</td>
</tr>
<tr>
<td>Right popliteal node</td>
<td>15.9 ± 3.5</td>
<td>2.6 ± 1.2</td>
</tr>
<tr>
<td>Left + right popliteal nodes</td>
<td>13.7 ± 2.0**</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td>Iliac node</td>
<td>2.3 ± 0.8</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Blood</td>
<td>0.6 ± 0.1*</td>
<td>7.7 ± 1.0</td>
</tr>
<tr>
<td>Liver</td>
<td>2.7 ± 1.1**</td>
<td>16.9 ± 0.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.2 ± 0.06</td>
<td>0.4 ± 0.03</td>
</tr>
</tbody>
</table>

$^*$ Values are mean ± S.E.

$^\ast P < .05$, $^\ast\ast P < .01$, $^\ast\ast\ast P < .001$. Depicted $P$ values (two-tailed unpaired $t$ test) refer to differences between the experimental and control group.
tem described here is the low percentage of the injected dose that reaches the systemic circulation. In the animals administered avidin, less than 4.5% of the injected dose of liposomes distributed to the spleen, liver, urine, and blood, while the percentage of the injected dose that reached these tissues in the control animals was much greater, 29.4%. It also appears that the iliac node and subsequent secondary nodes in the experimental animals have increased nodal retention efficiencies, thus resulting in the delivery of liposomes to a chain of draining lymph nodes and decreased delivery into the systemic circulation. This decreased delivery of liposome-encapsulated drugs into the systemic circulation should result in greatly reduced toxicity of liposome-encapsulated drugs to organs such as the liver, spleen, and kidney, while allowing increased delivery of therapeutic agents to the targeted lymph nodes. It has previously been shown that the absolute amount of a liposome-encapsulated drug that passes completely through the lymphatic system and enters the systemic circulation through the thoracic duct is the dose-limiting factor for liposome-encapsulated cytotoxic drugs (Hirnle, 1997).

Future studies will need to investigate the effectiveness of this targeting system in various clinically related disease models. It will be important to determine how targeting to the lymph node is affected by the presence of tumors or infections within the lymph node. It will also be important to determine whether this targeting system is effective with repeat administrations in the same individual. In summary, this delivery system offers a new approach for the increased targeting of liposome-based therapeutic agents to lymph nodes.

References