Use of ^{99m}Tc-Labeled Liposomes Encapsulating Blue Dye for Identification of the Sentinel Lymph Node

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Colloidal radiopharmaceuticals are commonly used in combination with blue dye for localization of the sentinel node. Liposomes are colloidal particles composed of spontaneously forming lipid spheres that can carry a wide variety of diagnostic and therapeutic agents. Conventional liposomes are poorly retained in lymph nodes (<2% of the subcutaneously injected dose). We have previously described a system for increasing the retention of liposomes in the lymph nodes by ~7-fold. This system is comprised of subcutaneously injected biotin-coated liposomes, followed by an adjacent injection of avidin. When the avidin moves into the lymphatic vessels, it causes aggregation of the biotin-coated liposomes that are also in the process of migrating through the lymphatic vessels. These aggregated liposomes become entrapped in the next encountered lymph node. In this study, we use this novel lymph node delivery system with liposomes that encapsulate blue dye, resulting in intense blue staining of the sentinel node. These liposomes can also be labeled with ^{99m}Tc, permitting scintigraphic imaging and radioguided probe localization of the sentinel node. **Methods:** Liposomes coated with biotin and coencapsulating blue dye and glutathione were labeled with ^{99m}Tc using hexamethylpropyleneamine oxime. Rabbits were subcutaneously administered 0.3 mL ^{99m}Tc-biotin-liposomes containing blue dye in both hind feet, followed by a subcutaneous injection (0.3 mL) of 5 mg avidin in only one hind foot (experimental). The other hind foot served as a control. **Results:** Labeling efficiencies (mean ± SEM) for liposomes encapsulating blue dye were 92.1% ± 1.9%. Necropsy at 24 h revealed that the popliteal node on the experimental leg receiving the avidin was intensely blue stained compared with virtually no blue coloration of the control node. Tissue counts of these nodes were 12.2 ± 1.5 percentage injected dose (%ID) in the experimental node compared with 1.2 ± 0.1 %ID in the control nodes ($P < 0.0001$). **Conclusion:** Biotin-liposomes encapsulating blue dye can be successfully labeled with ^{99m}Tc, providing a convenient option for the visualization and radiolocalization of the sentinel node. This biotin-liposome/avidin system may also have potential for the delivery of therapeutic drugs and radiopharmaceuticals to lymph nodes.

Key Words: liposomes; sentinel node; ^{99m}Tc; blue dye; avidin

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Although sentinel node localization for the staging and risk stratification of patients with cancer is proving to be an effective tool, improvement of the technology and refinement of the protocols are still needed (*1*). A review of the literature indicates that most investigators are reporting success rates of 80%–95% for identification of the sentinel lymph node, and it is generally believed that the false-negative rate can be kept to <5% (*2*).

Colloidal radiopharmaceuticals currently used to detect sentinel nodes were originally developed for imaging the reticuloendothelial system without consideration of their particular application to sentinel node detection. In most locations, these colloidal radiopharmaceuticals are used in combination with blue dye for identification of the sentinel lymph node. It is generally believed that the combination of blue dye and a colloidal radiopharmaceutical for sentinel node detection is additive (*3,4*). When Albertini et al. (*4*) compared the sentinel node detection efficiency of ^{99m}Tc–sulfur colloid with that of vital blue dye mapping, they found that blue dye mapping identified 69.5% of the sentinel nodes, whereas ^{99m}Tc–sulfur colloid detection identified 83.5%. In combination, these two techniques detected 96% of the sentinel lymph nodes.

Each agent has its advantages and disadvantages for sentinel node localization. Radiopharmaceuticals are useful for revealing the general location of the body for initiation of the surgical incision and for probe-guided definitive identification of the sentinel node during surgery. On the other hand, the use of blue dye allows for visualization of the sentinel node during surgery, which facilitates performance of the surgery. One significant disadvantage of blue dye is that it must be injected just before surgery because it moves rapidly into the lymphatic vessels and is poorly retained by the sentinel node. When blue dye alone is used, a secondary lymph node may be misidentified as the sentinel node because of rapid passage of all dye through the sentinel node.

One possible solution to the problem of the rapid passage of blue dye through the sentinel node would be the development of a blue-staining colloid. Although a blue colloid

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would appear to be useful for sentinel node detection, we are unaware of any previous reports using a blue colloid for this application. In this article, we describe the development of a blue colloid radiopharmaceutical composed of liposomes encapsulating patent blue dye that can be labeled with ^{99m}Tc.

Liposomes, a type of colloidal particle, are spontaneously forming spheres composed of lipid bilayers that can encapsulate a wide range of hydrophilic agents (dyes, drugs, radionuclides) within their aqueous interior (*5*). Encapsulation protects the drug from degradation and increases its pharmacokinetic profile. These characteristics have lead researchers to determine the lymphatic delivery of liposomes after their subcutaneous or intradermal injection (*6*). Liposomes containing only blue dye have been described as possible lymph node markers when administered by direct lymphatic vessel catheterization (*7*). These catheterizations required infusion of a high dose (several milliliters) of liposomes because the liposomes were poorly retained by the primary lymph nodes.

This poor retention of liposomes has been noted by our group (*8*) and other investigators (*9*). Only approximately 1%–2% of a subcutaneously injected dose of liposomes is retained in the primary lymph node. We have described a method of increasing by 7-fold the retention of liposomes by the primary lymph nodes draining from a body region (*10*). This method uses the high-affinity ligands, biotin and avidin. With this method, liposomes coated with biotin on their surface are injected subcutaneously; this is followed by an adjacent subcutaneous injection of avidin. When the biotin-liposomes that are in the process of migrating through the lymphatic vessels meet with the avidin, which is also moving into the lymphatic vessels, the association of the biotin-liposomes with avidin results in liposome aggregates. These liposome aggregates then become trapped in the first encountered lymph node. In this article, we describe a single sentinel node detection agent that has the combined attributes of being a colloidal carrier and containing blue dye and a radiolabel. This agent is composed of biotin-liposomes encapsulating patent blue dye and glutathione. These blue-biotin-liposomes are easily labeled with ^{99m}Tc using the hexamethylpropyleneamine oxime (HMPAO)-glutathione labeling method (*11*).

MATERIALS AND METHODS

Liposome Manufacture and Characterization

Liposomes were comprised of a 58:39:1:2 molar ratio (total lipid) of distearoyl phosphatidylcholine (Avanti Polar Lipids, Pelham, AL), cholesterol (Calbiochem, San Diego, CA), *N*-biotinoyl distearoyl phosphoethanolamine (Northern Lipids, Vancouver, Canada), and α-tocopherol (Aldrich, Milwaukee, WI). Liposomes were prepared in a laminar flow hood using aseptic conditions. A dried film was formed by mixing the lipid ingredients in chloroform and then removing the chloroform by rotary evaporation and vacuum desiccation for at least 4 h. The dried lipid film was rehydrated in 300 mmol/L sucrose (Sigma, 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 mmol/L reduced glutathione (GSH) (Sigma) and 10 mg/mL patent blue violet dye (CI 42045; Sigma) in Dulbecco's phosphate-buffered saline (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 mmol/L GSH and 10 mg/mL blue dye in PBS (pH 6.3) containing 150 mmol/L sucrose and extruded through a series (2 μm, two passes; 400 nm, two passes; 100 nm, five passes) of polycarbonate filters (Lipex, Vancouver, Canada) at 55°C. Extruded liposomes were washed three times in PBS, pH 6.3, containing 75 mmol/L sucrose and centrifuged at 45,000 rpm for 45 min in an ultracentrifuge (Ti60 rotor; Beckman, Fullerton, CA) to remove any unencapsulated sucrose, GSH, and blue dye. The final liposome pellet was reconstituted in 300 mmol/L sucrose/PBS to a total lipid concentration of ~60 μmol/mL and stored at 4°C until needed.

The average diameter of the liposomes was determined to be 130 nm using particle size analysis of two sample runs (Brookhaven Instruments, Holtsville, NY). The size distribution was monomodal and ranged from 115.3 to 148.9 nm. Phospholipid concentration was determined to be 29 mmol/L using the Stewart assay (*12*). The intraliposomal concentration of blue dye was determined spectrophotometrically at 635 nm after release of the dye from the liposome after chloroform extraction (*13,14*).

Liposome Labeling

Liposomes were labeled with ^{99m}Tc as described (*11*). A commercial kit of the lipophilic chelator, HMPAO (Ceretec; Nycomed-Amersham, Arlington Heights, IL), was reconstituted with 5 mL saline containing 370 MBq ^{99m}Tc-pertechnetate. The kits were checked for the percentage of lipophilic HMPAO using the three-step paper chromatography system outlined in the package insert. An aliquot (1 mL) of ^{99m}Tc-HMPAO was added to a concentrated suspension of liposomes encapsulating GSH and blue dye (2 mL; phospholipid concentration, 29 mmol/L) and incubated at room temperature for 30 min. Labeling efficiencies were determined from the ^{99m}Tc activity associated with the ^{99m}Tc-liposomes before and after Sephadex G-25 column separation with a dose calibrator (Mark 5; Radix, Houston, TX). Chromatography of before- and after-column aliquots of ^{99m}Tc-blue-biotin-liposomes was also performed on paper (no. 589; Schleicher & Schuell, Keene, NH) developed in 0.9% saline. With this technique, the liposomes remain at the origin while free ^{99m}Tc and ^{99m}Tc-HMPAO move with the solvent front.

Imaging Studies

Animal experiments were performed under the National Institutes of Health animal use and care guidelines and approved by our institutional animal committee. Two male New Zealand White rabbits (2.5–3.0 kg) were studied per day. Rabbits (n = 6) were anesthetized with a ketamine (50 mg/kg)/xylazine (10 mg/kg) cocktail in the brachial muscle. An aliquot (0.3 mL; 12 ± 0.9 MBq; 1.5 mg phospholipid/kg body weight) of ^{99m}Tc-blue-biotin-liposomes was injected subcutaneously in a shaved area on the dorsum of each hind foot. This liposome injection was immediately followed with a subcutaneous injection (0.3 mL) of 5 mg avidin (Sigma) in saline in only the right hind foot (experimental). This avidin injection was located 2 cm proximal to the injection of ^{99m}Tc-blue-biotin-liposomes. The left hind foot in each rabbit served as a control. Dynamic (1 min) scintigraphic images were

acquired in a 64 × 64 Word image matrix using a Dyna 4 gamma camera (Picker International, Cleveland, OH) interfaced to a Pinnacle computer (Medasys, Miami, FL). The right and left subcutaneous liposome blebs were massaged gently for 5 min. At 30, 40, 50, and 60 min after injection, running leg movement was performed for 1 min by manual leg manipulation of both legs. Immediately after completion of the leg movement at 60 min after injection, dynamic acquisition was halted and a 1-min static anterior image was acquired of the legs and lower abdomen. This was followed by acquisition of a 1-min static anterior image of the middle portion of the rabbit's body extending from above the heart to slightly below the iliac node. Animals were allowed to recover from anesthesia and housed overnight. At 24 h, animals were again anesthetized and static images were acquired.

Biodistribution Studies

After the imaging study, rabbits were killed with intravenous pentobarbital (100 mg/kg). Tissues were harvested, weighed, and counted for radioactivity (Multichannel Analyzer; Canberra, Meridian, CT). The percentage injected dose (%ID) per organ was calculated by comparison with a standard aliquot of ^{99m}Tc-blue-biotin-liposomes.

Image Analysis

Images acquired at 1, 30, and 60 min and at 24 h were analyzed. Regions of interest were drawn around the initial sites of subcutaneous injection in both hind feet for the baseline images acquired at 1 min after injection. The activity of the baseline injection site at 1 min 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 and for background activity. 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:

$$\text{\% Nodal pass-through} = \frac{\begin{array}{l}(\text{activity of injection site at baseline} \\ \quad - \text{activity of injection site at time x}) \\ \quad - \text{activity of popliteal node at time x}\end{array}}{\text{activity of injection site at baseline}}.$$

The estimated nodal retention efficiency was calculated as:

$$\text{Nodal retention efficiency} = \frac{\text{activity at node at time x}}{\begin{array}{l}\text{activity of injection site at baseline} \\ - \text{activity of injection site at time x}\end{array}}.$$

Statistical Analysis

Values are reported as mean ± SEM. Statistical analysis was performed using Excel software (Microsoft, Redmond, WA) for the Macintosh computer (Apple Computers, Cupertino, CA). The Student unpaired t test was used to compare the %ID at the injection site, %ID at the popliteal node, percentage nodal pass-through, and nodal retention efficiency at a given time. Statistical differences in the %ID located in the popliteal node determined by tissue sampling at 24 h were also compared for the two groups. Acceptable probability for a significant difference between means was $P < 0.05$.

RESULTS

Liposome Characterization

The intraliposomal concentration of blue dye in the final liposome product was 0.15 mg/mL. The encapsulation efficiency was 1.5%. After three high-speed centrifugation washings, the resuspended liposome suspension was intensely blue in color as shown in Figure 1.

In Vitro Labeling

Blue-biotin-liposomes labeled with ^{99m}Tc with high efficiency (92.1% ± 1.9% for six separate labeling experiments over 3 mo) using the HMPAO-glutathione labeling method indicated that neither the biotin surface coating nor the coencapsulated blue dye interfered with the ^{99m}Tc-HMPAO-glutathione labeling process. Paper chromatography of the before- and after-column fractions revealed that virtually all blue coloration remained at the origin with the liposomes.

Image Analysis and Tissue Biodistribution Data

Figure 2 depicts anterior 1-min static images showing the greatly increased deposition of ^{99m}Tc-blue-biotin-liposomes in the left popliteal node (right side of images) that received

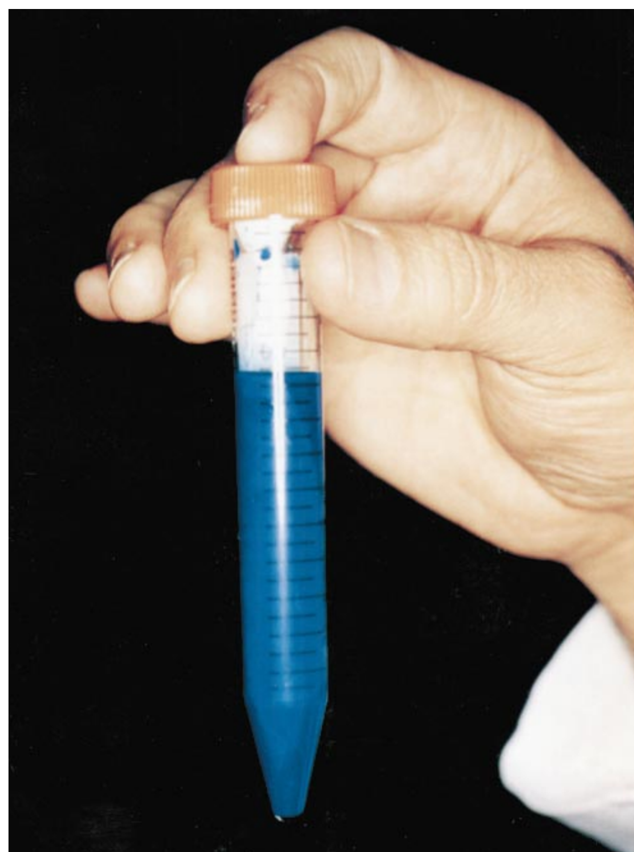

FIGURE 1. Blue-biotin-liposomes. Liposomes remained intense blue color even after three centrifugation washes before resuspension.

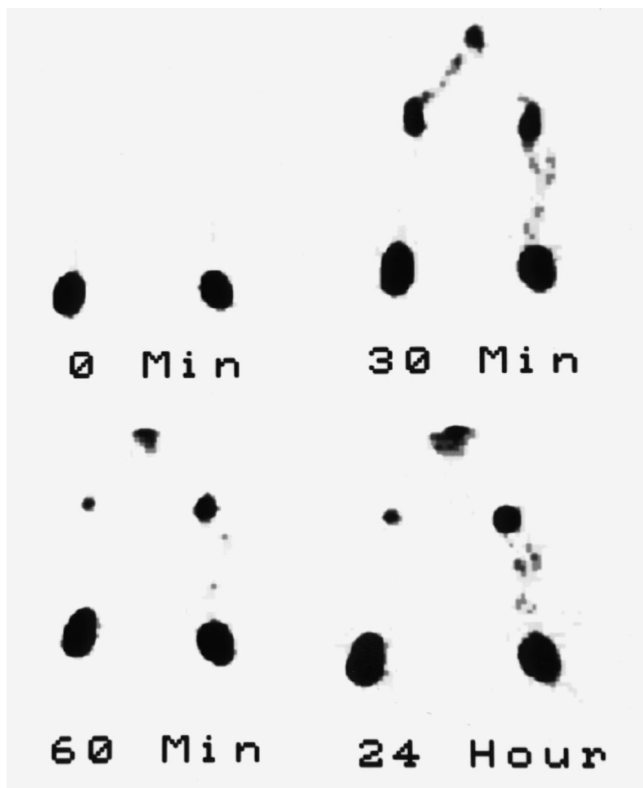

FIGURE 2. Scintigrams show anterior views of legs and lower abdomen of rabbit at 0 min (baseline), 30 min, 60 min, and 24 h after subcutaneous injection of ^{99m}Tc-labeled blue-biotin-liposomes in both hind feet; this was followed by subcutaneous injection of avidin in only left hind foot. Increased retention of ^{99m}Tc activity in left popliteal node compared with right popliteal node is easily visualized at 60 min and 24 h.

TABLE 2
Tissue Biodistribution of ^{99m}Tc-Blue-Biotin-Liposomes in Rabbits at 24 Hours After Injection

Organ	%ID/organ	%ID/g	Weight (g)
Popliteal node			
Experimental (with avidin)	12.2 ± 1.5*	71.4 ± 12.4†	0.212 ± 0.03
Control (without avidin)	1.2 ± 0.1*	7.4 ± 1.8†	0.196 ± 0.04

*$P < 0.0001$.
†$P < 0.01$.
Data are expressed as mean ± SEM.

the avidin compared with the right control node (left side of images). Although ^{99m}Tc-blue-biotin-liposome localization in the left and right popliteal lymph nodes is similar at 30 min, the difference in retention is obvious at 60 min and 24 h. It is visually apparent from these images that most of the liposomes that had accumulated in the control popliteal node at 30 min had moved out of the node after leg movement at 60 min after injection. This displacement was greatly decreased from the experimental popliteal node, which received the avidin.

The image analysis distribution data are shown in Table 1. These results show that the experimental popliteal node had significantly increased liposome deposition compared with the control popliteal node (8.5% vs. 1.0%; $P < 0.001$) at 24 h. The difference was also highly significant at 60 min (6.5% vs. 1.7%; $P < 0.001$). Tissue biodistribution results depicted in Table 2 also showed a highly significant increase in liposome deposition in the experimental popliteal node compared with the control popliteal node (12.2 vs. 1.2 %ID/popliteal node; $P < 0.0001$). The differences were also significant on a %ID/g basis (71.4 vs. 7.4 %ID/g; $P < 0.01$). There was also a significant difference in the calculated %ID at the injection site between the control and experimental leg at 30 and 60 min ($P < 0.05$).

The percentage pass-through and the nodal retention efficiencies for the popliteal node are shown in Table 3. Nodal retention efficiency was significantly increased on the experimental leg compared with that of the control leg at 60 min (20.1% ± 2.4% with avidin vs. 8.5% ± 1.4% in controls) ($P < 0.01$) and at 24 h (17.0% ± 1.3% with avidin vs. 2.5% ± 0.4% in controls) ($P < 0.0001$).

The greatly increased retention of the ^{99m}Tc-blue-biotin-liposomes in the experimental popliteal node (right side of

TABLE 1
Accumulation of ^{99m}Tc-Blue-Biotin-Liposomes in Rabbits Determined by Image Analysis

	Experimental (with avidin; n = 6)			Control (without avidin; n = 6)		
Region	30 min	60 min	24 h	30 min	60 min	24 h
Popliteal nodes	9.8 ± 1.6	6.5 ± 1.0*	8.5 ± 0.5†	7.4 ± 0.6	1.7 ± 0.2*	1.0 ± 0.2†
Injection site	73.9 ± 4.0‡	67.3 ± 3.3§	49.4 ± 3.7	84.6 ± 1.1‡	79.3 ± 1.9§	58.0 ± 1.3

*$P < 0.001$.
†$P < 0.001$.
‡$P < 0.05$.
§$P < 0.05$.
Data are expressed as mean ± SEM.

TABLE 3
Derived Lymphatic Functions of ^{99m}Tc-Blue-Biotin-Liposomes in Rabbits

Function	Experimental (with avidin; $n = 6$) 30 min	Experimental 60 min	Experimental 24 h	Control (without avidin; $n = 6$) 30 min	Control 60 min	Control 24 h
% Pass-through	16.2 ± 2.7*	26.2 ± 2.8	42.1 ± 3.5	8.0 ± 0.6*	19.0 ± 1.9	41.0 ± 1.3
Nodal retention efficiency	37.9 ± 3.0†	20.1 ± 2.4‡	17.0 ± 1.3§	48.1 ± 2.0†	8.5 ± 1.4‡	2.5 ± 0.4§

*$P < 0.05$.
†$P < 0.05$.
‡$P < 0.01$.
§$P < 0.0001$.
Data are expressed as mean ± SEM.

image) is readily observed visually during necropsy at 24 h (Fig. 3A). The experimental popliteal node (right side of image) is stained a deep blue color as visualized after excision from the body, whereas the control popliteal node (left side of image) has only minimal blue coloration (Fig. 3B). An additional important visual observation was that the lymphatic vessels leading to the popliteal node were stained blue. The staining of these lymphatic vessels at 24 h would indicate that the endothelial lining of the lymphatic vessels appears to take up at least a small portion of the ^{99m}Tc-blue-biotin-liposomes, although the lymphatic tract was not apparent scintigraphically at 24 h.

DISCUSSION

This biotin-liposome/avidin delivery system is clearly effective in a rabbit model for the prolonged blue staining of lymph nodes while simultaneously allowing radiotracer localization of the primary lymph node. This lymph node delivery system has several potential advantages over the current sentinel node protocols using both blue dye and radiopharmaceutical colloids: (a) the requirement of only one procedure instead of two separate procedures; (b) definitive verification from scintigraphic imaging that the blue dye has migrated from the injection site to the sentinel node; (c) the potential for decreasing patient time in the operating room because the blue dye localization procedure would not need to be performed just before surgery while the patient is in the operating room; (d) flexibility in the timing of the injection of the agent, which could be performed on the day before the surgery; and (e) the possibility that ^{99m}Tc-blue-biotin-liposomes will prove to have increased sensitivity for the diagnosis of sentinel lymph nodes.

B

FIGURE 3. (A) Posterior aspect of hind legs of rabbit show exposed popliteal nodes at necropsy at 24 h. Feet of rabbit are located superior to picture. Blue coloration of popliteal node on experimental side (right side of image) that received avidin is readily observed. (B) Excised experimental (right side of image) and control (left side of image) popliteal nodes.

Although image analysis reveals that nodal retention efficiency with this biotin-liposome/avidin technique is ~8.5 times greater in experimental animals that were administered avidin than that with the controls, there still is room for optimization to achieve increased lymph node retention. Estimation of the percentage nodal pass-through and nodal retention efficiency appear to be useful parameters for characterization of sentinel node–localizing agents. From these parameters it can be seen that only 1 in 20 liposomes that are cleared from the injection site is retained by the popliteal node on the control leg at 24 h, whereas nearly 1 in 5 liposomes is retained in the popliteal node on the experimental side. Because 4 of 5 liposomes still pass through the popliteal node, it is likely that this biotin-liposome/avidin system could be further optimized by appropriate dosing of avidin to enable retention of nearly all of the dose in the first lymph node encountered. Further modification of the size of the liposome and the concentration of biotin on the surface of the liposome could also increase lymph node retention. Further research is necessary to assess the importance of these various factors.

The differences we found between the clearance of liposomes from the injection site of the experimental and control leg are unexplained. These differences were not observed in a previous study with biotin-liposomes that did not contain blue dye (*10*).

In addition to its diagnostic applications, this biotin-liposome/avidin system has obvious potential applications as a lymph node delivery vehicle. Instead of being used to deposit blue dye in the lymph node, a therapeutic drug or therapeutic radionuclide could be encapsulated in the liposomes, resulting in a slow release of drug from a depot site in the lymph node.

Several questions remain, including whether these animal experiments will extrapolate to human clinical studies. In addition, it will be important to determine whether the avidin component of this delivery system will produce an immune response on repeated administration, leading to decreased effectiveness. These immunogenic aspects of avidin have been addressed by other researchers who are using intravenous avidin administration as a method to improve target-to-background ratios (*15–17*). In this regard, it should be possible to use recently described modified avidin molecules that are significantly less immunogenic (*18*).

CONCLUSION

^{99m}Tc-biotin-liposomes containing blue dye may be useful for sentinel node detection. This biotin-liposome/avidin system may also have applications for therapeutic drug or radionuclide delivery.

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