Microscopic Localization of Sterically Stabilized Liposomes in Colon Carcinoma-bearing Mice

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ABSTRACT

Using light and electron microscopy, we investigated the in vivo distribution of liposomes sterically stabilized by specific lipids which prolong their circulation in blood. Tissue distribution of sterically stabilized liposomes composed of distearoyl phosphatidylcholine: cholesterol:monosialoganglioside GMI (10:5:1)-encapsulated 67Ga-Desferal indicates that more than 30% of liposomes still remain in the blood at 24 h after tail vein injection. Moreover, such liposomes accumulated in tumors (C-26 colon carcinoma cells implanted s.c.), reaching almost the same level of uptake as liver (~20% injected dose/g tissue). The microscopic localization of liposomes labeled with encapsulated colloidal gold or rhodamine-labeled dextran coincided well with the tissue distribution. To evaluate circulation parameters, two sizes of gold-containing egg phosphatidylcholine:cholesterol:distearoyl phosphatidyethanolamine (derivatized at its amino position with a 1900 molecular weight segment of polyethylene glycol) (10:5:6:8) liposomes were injected. The plasma was examined by electron microscopy of negative-stained preparations at 0.5, 4, and 24 h after liposome injection. It was found that the ratio of small (<100 nm diameter) to large (>100 nm) liposomes increased with time, indicating a much faster clearance of the larger liposomes. To detect the localization of liposomes in various tissues, appropriate samples were fixed 24 h after the injection of gold-containing liposomes (between 80 and 100 nm in diameter) composed of egg phosphatidylcholine:cholesterol:monosialoganglioside GMI (10:5:1) or egg phosphatidylcholine:cholesterol:derivatized distearoyl phosphatidyethanolamine. The tissues examined for this study included normal liver, bone marrow, and implanted neoplasms. Silver-enhanced colloidal gold was found predominantly within Kupffer cells in the normal liver and within macrophages in the bone marrow. Rarely were any silver-enhanced gold particles detected in hepatocytes. In all preparations, electron microscopy revealed the presence of gold in endosomes and lysosomes of fixed sinusoidal lining macrophages in the liver and bone marrow. Peripheral to the implanted tumors, silver enhancement revealed gold in small blood vessels and focally beyond the vessel boundaries in extracellular spaces around tumor cells. Gold particles were not observed within the tumor cell cytoplasm. At the tumor border, nonenhanced gold was occasionally seen by electron microscopy in cells of the mononuclear phagocyte system. We obtained the same localization pattern as with silver enhancement by using an alternative aqueous content marker, rhodamine B isothiocyanate-dextran. We conclude that liposomes of specific composition, which have the ability to remain in circulation with a half-life of 12–24 h, are also able to traverse the endothelium of small blood vessels, including those in tumors, and extravasate into extracellular spaces. The persistence of such liposomes in the circulation and their ability to reach tumor cells within a solid carcinoma make them highly attractive vehicles for chemotherapeutic agents.

INTRODUCTION

Recent pharmacokinetic and therapeutic studies with tumor-bearing mice revealed that sterically stabilized liposomes have considerable potential as drug carriers (1). Liposomes composed of phosphatidylcholine and cholesterol mixed with a small amount of either PEG-DSPE3 or GMI have a prolonged circulation time in blood and reduced uptake by liver and spleen (1–3). Most importantly, increased accumulation has been demonstrated in solid tumors (2, 4). Results from recent studies on the administration of liposome-encapsulated doxorubicin as a chemotherapeutic agent have demonstrated increased therapeutic efficacy and reduced toxicity compared to the administration of the free drug (1).

Despite extensive studies on tissue distribution (1, 3, 5), and with the exception of the liver, it is still not known where liposomes are localized at the cellular level in various tissues following i.v. injection. The localization of liposomes in the liver has been examined by various markers, including 5-Br, 4Cl,3-indolyolphosphate (6), single or paired enzymes such as glucose oxidase and horseradish peroxidase (7), and colloidal gold (8). All of these studies have shown that in the liver, liposomes were internalized by Kupffer cells of the mononuclear phagocyte system, more commonly referred to as the reticuloendothelial system (6, 9–11). Other tissues where liposomes have been localized are spleen (12); bone marrow for small liposomes (13); lungs for large liposomes (14); and implanted tumors for sterically stabilized liposomes (2). However, there are several important long-standing key questions in the liposome field that need to be answered: By what cells are liposomes taken up into other tissues, including tumors? Are there any tissues or pathologic conditions where the liposomes can cross blood vessel endothelium and basal lamina? Are intact liposomes present in extravascular spaces?

To understand better the mechanism of liposome uptake in various tissues, we have developed a liposome preparation with encapsulated colloidal gold particles and applied silver enhancement of the colloidal gold to monitor the in vivo fate of liposomes by optical and electron microscopy (15). We chose sterically stabilized liposomes of well-defined particle size, which had been shown by earlier studies to give superior accumulation in implanted tumors (1, 2).

MATERIALS AND METHODS

Materials. Egg PC and DSPC were purchased from Avanti Polar Lipids, Inc. (Pelham, AL). PEG-DSPE synthesized as described earlier (16) was from Liposome Technology, Inc. (Menlo Park, CA). Cholesterol (highly purified) was from Calbiochem (La Jolla, CA). Monosialoganglioside GMI, morpholino-ethane-sulfonic acid, 4-morpholinepropanesulfonic acid, 8-hydroxyquinoline sulfate (oxine), and RITC-Dex (M, 9000) were from Sigma Chemical Co. (St. Louis, MO). 67Ga citrate was from New England Nuclear (Boston, MA); Desferal was from CIBA-Geigy (Summit, NJ); Dowex resin 50x4–400 was from Aldrich (Milwaukee, WI); and the acetate form of AG IX2 resin was

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3 The abbreviations used are: PEG-DSPE, distearoyl phosphatidylethanolamine derivatized at its amino position with a 1900 molecular weight segment of polyethylene glycol; PC, egg phosphatidylcholine; DSPC, distearoyl phosphatidylcholine; GMI, monosialoganglioside GMI; RITC-Dex, rhodamine B isothiocyanate-dextran; Desferal, defereroxamine mesylate.

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from Bio-Rad (Richmond, CA). All organic solvents used were reagent grade or high-pressure liquid chromatography grade. All other materials were as previously described (1).

Preparation of 67Ga-labeled Liposomes. Liposomes composed of DSPC:cholesterol:GM1 (molar ratio, 10:5:1) or PC:cholesterol:PEG-DSPE (molar ratio, 10:5:0.8) were prepared by shaking thin lipid films with an isotonic solution of morpholinio-ethane-sulfonic acid (0.05 mm) and 4-morpholinopropanesulfonic acid (0.05 mm) containing dextranamine mesylate at 55°C for 30 min. After four cycles of freezing (−56°C) and thawing (55°C), the liposomes were extruded under pressure (300 psi) (18) through a stack of two Nucleopore membranes (Pleasanton, CA) (0.2-mm pore size two times; 0.1-mm pore size two times; and 0.05-μm pore size seven times). The extruder device was preheated to 55°C, due to the high-phase-transition phospholipids used. To obtain homogeneous size, liposomes were spun in a microcentrifuge at 10,000 × g for 5 min. The precipitate containing any large liposomes was discarded. The average diameter of liposomes was ~90 nm, as measured with a Malvern dynamic laser scattering machine (Malvern Instruments, Ltd., Malvern, England) and based on mass distribution. Unencapsulated Desferal was removed by passage twice through Dowex dry columns at 3000 × g for 15 min. The labelling procedure used was as before (2). One hundred μCi of 67Ga citrate was incubated with 1 mg oxine sulfate in 1 ml 0.9% saline for 1 h at 50°C. Liposome suspensions were incubated overnight at 4°C with 2 μCi of 67Ga-labeled oxine/μmol of phospholipid. 67Ga-labeled Desferal was entrapped in the aqueous interior of liposomes. Unencapsulated 67Ga-labeled oxine and excess oxine sulfate were removed by passing the liposome suspension through an anion-exchange resin (AG 1X2, acetate form, 200-400 mesh) and a Dowex dry column. Preparation of Colloidal Gold-Liposomes. A solution of citric acid (120 mm) and K2CO3 (30 mm) was freshly prepared and mixed with gold tetrachloride HAuCl4 (12.72 mm) in a ratio of 1:1 (pH 3.4). Liposomes composed of either PC:cholesterol:GM1 (molar ratio, 10:5:1) or PC:cholesterol:PEG-DSPE (molar ratio, 10:5:0.8) were prepared by reverse-phase evaporation (17) with gold chloride:citrate in the aqueous phase (15). A thin lipid film (10 μmol phospholipid) was dissolved in 1 ml of diethyl ether and mixed with 0.5 ml gold chloride:citrate. The mixture was emulsified for 3 min in a bath sonicator, and diethyl ether was removed under vacuum at room temperature. The liposomes underwent three cycles of freezing (−56°C) and thawing (55°C) and then were extruded under pressure (18) through Nucleopore membranes, twice through pore size 0.1 μm and five times through 0.05 μm. Immediately after final extrusion, the pH of the liposome suspension was raised to 6 by adding NaOH. It was then incubated at 55°C for 30 min. The color of liposome suspension turned pink-purple, which indicated an appropriate particle size. After gold particles had formed, unencapsulated free gold and excess citrate were removed by passing the liposome suspension through a column (1 × 15 cm) of Sephacryl S-500 (Pharmacia, Piscataway, NJ). The average diameter of the liposomes was 80–100 nm, as determined by electron microscopy. The percentage of liposomes containing gold as determined by negative-stain electron microscopy (15) was between 60 and 90%, varying among preparations. Most of them contained more than one gold particle. The gold-liposomes were stable during 2 weeks of storage under argon at 4°C. In vivo, gold-containing liposomes remain intact in the bloodstream; the relative ratio of gold-containing to plain liposomes recovered in plasma at 24 h after i.v. injection in mice was almost the same as before injection (15).

Animals and Tumor Models. Female BALB/c mice, 4–5 weeks old (West Seneca Laboratory, West Seneca, NY), were inoculated with a single-cell suspension of C-26 mouse colon carcinoma (106 cells) directly into the liver or subcutaneous tissue (19). Mice were tested 2 weeks after tumor cell seeding. Tumor diameters were from 5 to 10 mm. After 0.5, 4, and 24 h, the heparinized blood was diluted with 1 volume of phosphate-buffered saline and left at 4°C for 4 h without centrifuging. The liposomes in plasma were stained with 2% ammonium molybdate for electron microscopy. The relative ratio of small to large colloidal gold-liposomes was determined by a random count of 100 liposomes in a random electron microscopic field.
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Tissue Collection and Fixation for Microscopy. Colloidal gold-containing liposomes (0.25 ml, 2 μmol phospholipid) were injected into mice via the tail vein. The mice were sacrificed 24 h after liposome injection. Tissues were collected following perfusion with heparinized phosphate-buffered saline and fixative (1.5% glutaraldehyde, 0.1 M sodium cacodylate, 1% sucrose, pH 7.4).

Light Microscopy following Silver Enhancement of Colloidal Gold. The tissue specimens were embedded in water-soluble JB-4 resin (21) from Polysciences, Inc. (Warrington, PA). All procedures involving tissue handling were performed at 4°C. Sections were cut from embedded specimens with a Sorvall JB-4 microtome at a thickness of 2.5 μm.

Electron Microscopy. Injection and tissue collection of colloidal gold-liposomes were performed as described for light microscopy. The specimens were further fixed, dehydrated in graded series of alcohols, and embedded in Epox 812 resin (Ernest F. Fullam, Inc., Latham, NY), and thin sections were examined with a JEOL 100CX transmission electron microscope operating at 80 kV.

Fluorescence Microscopy. Liposomes composed of PC:cholesterol:GM1 were prepared according to the method described above, but with aqueous contents of 100 mg/ml RITC-Dex in 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer. This fluorescent liposome marker has a molecular weight of 9000, with excitation and emission wavelengths of 546 and 590 nm, respectively. The final solution was adjusted to 300 mOsm with NaCl (pH 7.4). Unencapsulated RITC-Dex was removed by G150 Sephadex gel filtration and by passage through an Amicon concentration unit (Amicon, Beverly, MA) with a 30,000 molecular weight cutoff filter membrane. Liposomes with encapsulated RITC-Dex (0.25 ml, 2 μmol phospholipid) were injected into the tail vein, and mice were sacrificed 24 h later. Tissues were collected following perfusion with heparinized phosphate-buffered saline and fixed in 4% paraformaldehyde. Frozen sections (5 μm thickness) of the specimens were examined by fluorescence microscopy.

RESULTS

Tissue Distribution of Liposome-encapsulated 67Ga-Desferal. 67Ga-Desferal complex as a marker for aqueous contents was encapsulated in liposomes composed of DSPC:cholesterol:GM1 (molar ratio, 10:5:1). 67Ga-labeled liposomes were injected into the tail vein of mice bearing C-26 colon carcinoma implanted s.c. The level of liposomes remaining in blood and their uptake by liver, muscle, and tumor at different times are shown in Fig. 1. GM1-liposomes showed their sterically stabilized property in the blood circulation, where 66% of the injected dose was present in 1 ml (approximately 1 g) blood 1 h after injection. Fig. 3. Localization of liposomes in liver. Liver tissue was collected 24 h after colloidal gold liposome injection, a, light micrograph of overall view of lipidosome localization. Silver-enhanced gold particles (arrows) heavily label Kupffer cells. Rare particles may be seen in hepatocytes. K, electron micrograph of a Kupffer cell directly exposed in a sinusoid of mouse liver. Colloidal gold particles (arrows) are observed in the Kupffer cell only. In c, the portion of Kupffer cell (inset) at higher magnification (b) shows accumulated colloidal gold particles in secondary lysosomes. K, Kupffer cell; H, hepatocyte; E, endothelial cell.
Fig. 4. Localization of liposomes in tumor. Thick sections show C-26 colon carcinoma implanted s.c. (a, c, d, e, f), and directly in the liver (b). A mast cell shows red due to metachromatic staining (a, arrowhead). Silver-enhanced gold particles (arrow) infiltrate into the extracellular space of the poorly differentiated tumor cells. c and d, various sizes of penetrating blood vessels show dense accumulations of silver-enhanced colloidal gold markers surrounding blood vessels. d, silver-enhanced...
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Fig. 5. Electron micrographs showing colloidal gold (arrows) in the intracellular vesicles of a typical mononuclear phagocyte. The colloidal gold often can be found within the endosomes (lower inset) and secondary lysosomes (upper inset) of macrophages at the border of the liver-implanted tumor.

injection. Approximately one-third of injected liposomes remained in circulation at 24 h after injection. For tissue distribution within 1 h following injection, liposome uptake by tumor was lower compared with the uptake by liver. However, liposomes appeared to accumulate rapidly at increased concentrations in the tumor, reaching almost the same level as in the liver (more than 20% of injected dose/g) at 24 h after injection. In contrast, the liposome uptake level of normal tissue (skeletal muscle) was still lower than 0.5% of injected dose/g 24 h after injection. These results confirm earlier observations obtained with other tumors (2, 4) that sterically stabilized liposomes not only have a prolonged circulation time but also exhibit a favorable tumor uptake:liver uptake ratio.

Selective Clearance of Liposomes in Blood Circulation. A mixture of two size populations of gold-labeled liposomes composed of PC:cholesterol:PEG-DSPE was examined by electron microscopy before injection. Most of them had diameters between 50 and 500 nm. Liposomes greater than 100 nm [182 ± 58 (SD) nm] were termed large liposomes, and those under 100 nm [80 ± 12 (SD) nm], small liposomes. Forty-four % of liposomes were greater than 100 nm, and 56% were less than 100 nm, in a random count of 100. The mixture was injected into the tail veins of mice. Micrographs of large and small liposomes in plasma are shown in Fig. 2, a and b, respectively. Half an hour after injection, fewer large liposomes remained in the bloodstream, and the ratio of small to large liposomes is improved. This selective clearance of large liposomes also correlates with the findings in Table 1.

H & E. a, b, d, and e × 1500; c and f × 600.

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We investigated the localization of liposomes in mouse colon carcinoma (C-26) implanted s.c. and in the liver (19). Tumors implanted s.c. formed ovoid nodules and could be easily separated from surrounding tissues. The tumor implanted directly into the liver formed a solitary solid tumor without satellite nodules or metastases. Although the interface between normal liver and tumor slightly interdigitated, it was possible to separate the tumor (white) from liver (red) tissue. Poorly differentiated tumor cells can be seen in the thick sections of tumors implanted s.c. (Fig. 4a) and in the liver (Fig. 4b). The cell morphology and architecture of the tumor in these two locations are the same. Mitoses are abnormally frequent. In some regions, silver-enhanced colloidal gold particles are seen to be predominantly scattered in the extracellular space between tumor cells (Fig. 4a, a and b). Silver was rarely found in tumor cell cytoplasm. It is interesting to note that metachromatic staining mast cells, an important source of vasoactive amines, were also present among the tumor cells (Fig. 4a).

Blood vessels penetrated into the tumor mass of the C-26 colon carcinomas. The tumor exhibited numerous vessels of various diameters (Fig. 4, c and d). Dense, silver-enhanced colloidal gold often surrounded blood vessels. In these areas, the silver particles often could be seen in streams connected to blood vessels (Fig. 4c). In some areas close to small blood vessels, silver-enhanced gold was clearly localized on the parenchymal side of the endothelial cells (Fig. 4d). In addition, silver-enhanced gold particles could be observed to cross the blood vessel endothelium, extensively penetrating into the extravascular, interstitial space between tumor cells (Fig. 4d). In other areas, silver-enhanced colloidal gold was scattered around nonendothelial-bound streams of erythrocytes, possibly in a region of angiogenesis (Fig. 4e). However, not all of the observed silver-enhanced colloidal gold particles observed traversed blood vessels (Fig. 4f).

By electron microscopy, gold particles were seen to be ingested by macrophages and found in their endosomes and secondary lysosomes (Fig. 5), including mononuclear phagocytes at the border of the tumor implanted in the liver (Fig. 5, upper inset). Gold particles could also be seen, but less frequently in the extracellular space and occasionally within endothelial cells. Quite likely, it is much more difficult to observe single particles in the extracellular space, because of the limited width of the thin sections used for electron microscopy, compared to the much thicker sections used for optical microscopy (15).

Liposome localization monitored by a second marker, RITC-Dex, revealed a pattern of localization similar to that resulting from silver enhancement of colloidal gold. Fig. 6 shows a phase-contrast view of a 5-μm frozen section of subcutaneous tumor (a) and its corresponding fluorescence image (b). The fluorescence signals inside the tumor mass are focal, mostly surrounding the vasculature, with a gradual decrease away from them. Injection of free-RITC-Dex under similar conditions did not show any fluorescence in the tumor region. These results indicate that the fluorescence signals were derived from RITC-Dex encapsulated in liposomes.

Localization of Liposome-encapsulated Marker in Bone Marrow. Silver-enhanced gold particles were seen exclusively in the resident macrophages of bone marrow (Fig. 7), although we cannot discount the possibility of some particles being present in the extracellular space. All hematopoietic cells, megakaryocytes, and developing myeloid and erythroid cells were unlabeled. As in other tissues, electron micrographs revealed colloidal gold in dense bodies within macrophages (Fig. 5).

**DISCUSSION**

In this study, we used liposomes partially composed of PEG-derivatized phospholipid or Gm1 glycolipid. Both types of liposomes evade the rapid uptake by the reticuloendothelial system and thereby remain longer in circulation (1–3). This increase in circulation time is probably due to their bulky hydrophilic head groups and has been termed “steric stabilization” (1, 22). Our comparative pharmacokinetic studies indicate that at 24 h after
liposome injection, $G_{M1}$-containing liposomes (80–100 nm diameter) have a 4-fold higher plasma concentration, one-fourth less uptake in the liver, and a 4-fold increase within a C-26 subcutaneous tumor compared to commonly used liposomes composed of DSPC and cholesterol without any glycolipid. PEG-containing liposomes also showed effects similar to those of $G_{M1}$-containing liposomes when compared with conventional DSPC:cholesterol liposomes (1). These ratios are even much higher when liposomes sterically stabilized with $G_{M1}$ or PEG-DSPE are compared with conventional liposomes composed of phosphatidylglycerol:PC:cholesterol (2–4). Most of the experiments referred to above were carried out by using encapsulated $^{67}$Ga-Desferal. $^{67}$Ga labeling has been applied as a quantitative method to measure liposome distribution in various tissues. The results shown in Fig. 1 indicate liposome retention as intact liposomes because the free $^{67}$Ga-Desferal complex remains in the circulation only for a very short time (1, 2). However, the pharmacokinetics studies provide no definitive information on the cellular or intracellular localization of liposomes. In this investigation, in addition to Ga-Desferal liposomes, we used liposomes of similar composition and size containing colloidal gold and a fluorescent marker in order to clarify their localization at the cellular and subcellular level, in C-26 colon carcinoma and other tissues.

Many studies have shown that the size of liposomes is one of the most important determinants of their longevity in the bloodstream (23, 24). Small unilamellar liposomes (<100 nm diameter) remain significantly longer in the circulation than large liposomes (25, 26). Our observations that large liposomes (>100 nm) disappear from plasma much faster than small ones (<100 nm) confirm this finding. Furthermore, it has been shown in our previous investigations (15) that the ratio of colloidal gold-liposomes and plain liposomes in plasma was almost unchanged for 24 h after injection. These results indicate that gold-labeled liposomes stay intact in the blood without losing their contents.

Much early work, including our pharmacokinetics studies, has shown that liposome uptake by liver is increased sharply within 1-4 hours after injection. Hepatic sinusoids connect the arterial and venous circulation. It is widely accepted that the liver sinusoidal lining is composed of interdigitating cells. One cell type is the typical endothelial cell, the other is the fixed macrophage called the Kupffer cell (27). Kupffer cells are directly exposed to the sinusoid (Fig. 3b), giving them the opportunity to ingest circulating particles such as liposomes. That the large liposomes were cleared faster than small liposomes may be due to two reasons. First, large liposomes may be more easily entrapped in sinusoids. Second, their larger surface may promote faster endocytosis. In most other tissues, whether the blood vessels and capillaries are continuous or fenestrated, circulating liposomes must cross the endothelial barrier to reach the extravascular space.

Many types of tumors are well vascularized, increasing the opportunity for liposome delivery. In general, vessels within tumors have been reported to have wide endothelial junctions, a large number of fenestrae, transendothelial channels formed by vesicles, and discontinuous or absent basement membranes (28). The C-26 carcinoma when implanted s.c. or in the liver is well vascularized, growing rapidly, but without obvious metastases. In the thick sections through the tumor, numerous penetrating vessels and branches of varying size could be seen. In order to penetrate into the tumor region, liposomes in the bloodstream must traverse the thin endothelial barrier. Our studies of liposome pharmacokinetics and uptake by tumors revealed that this process is not as fast as the uptake by Kupffer cells, which are directly exposed in the sinusoids of the liver. The maximum concentration of liposome-encapsulated Ga-Desferal in the subcutaneous tumor was observed 24 h after i.v. injection (Fig. 1). Since the $^{67}$Ga-Desferal complex itself does not accumulate in tumors (2), its presence in tumor indicates intact liposomes. In order to ascertain whether the presence of gold particles in the tumor mass unequivocally signifies the presence of intact liposomes, we used one alternative liposome marker, RITC-Dex. The results indicate a similar pattern of distribution. Moreover, in a previous study (15), when we injected i.v. free gold particles, which were retrieved from gold-labeled liposomes after they were disrupted by centrifugation, the silver-enhanced marker was found exclusively within Kupffer cells 24 h after injection, rather than in the tumor region. This demonstrated that the particles in the tumor region were most likely derived from the accumulation of gold-containing liposomes that penetrated the tumor, albeit slowly.

Initial studies with a fluorescent marker indicated liposome infiltration into the interstitial space between tumor cells (1). The data presented here provide definitive evidence for this in some instances, as well as clues to the possible mechanism by which liposomes escaped from blood vessels and extravasated into the tumor. We have considered the following three possibilities.

First, the liposomes may have escaped from discontinuous capillaries reported to be present in tumors of poorly differentiated mammary (29) and human renal carcinomas (30). Vessels supplying tumors may be lined by inherently leaky, fenestrated endothelium (31, 32). In our study, the dense silver-enhanced particles and fluorescence pattern always surrounded and localized to the penetrating vessels (Figs. 4, c and d; Fig. 5), consistent with the previous suggestion that macromolecular particles may cross gaps between endothelial cells (29–32). Mast cells, found among the poorly differentiated tumor cells in some regions (Fig. 4a), synthesize histamine and other vasoactive substances that may be released to cause extravasation of liposomes.
amines. Defects in tight junctions between venular endothelial cells could form in response to vasoactive mediators (27), permitting liposomes to flow into the interstitial space. Second, the silver-enhanced gold was not only scattered throughout the vessels in tumors, it was also heavily deposited in the endothelial basement membrane away from junctional clefts, most likely due to transendothelial vesicular transport (Fig. 4d). The occasional observation of gold particles within the endothelial cells indicates that vesicular transport may well be one of the pathways that transfer liposomes from blood vessels to the interstitial space. This was observed more frequently in some transgenic mice. Earlier studies reported vesicular transport of macromolecules (33) and even colloidal carbon particles 50 nm in diameter (34) in tumors. Our observations support this mechanism.

Third, tumor angiogenesis includes the formation of capillary sprouts (35). The sprouts are highly permeable because of gaps between adjacent endothelial cells and openings at the vessel termini, allowing virtually unlimited passage of materials, including erythrocytes, into the surrounding tissue (34). Scattered silver-enhanced gold can be seen in thick sections, along with erythrocytes in tumor areas that we interpret to be capillary sprouts (Fig. 4e). It is important to note that although liposomes could escape from tumor blood vessels by the above pathways in some areas, silver-enhanced gold was absent in many other areas of the tumor region, even where dense accumulation of silver-enhanced gold was found entrapped in small vessels (Fig. 4f).

Bone marrow was also considered as one of the major sites of interest for lipidosome localization because of possible drug toxicity and attendant leukopenia after i.v. injection. We observed the silver-enhanced gold exclusively in the resident macrophages. This may be advantageous in reducing the toxic effect of liposome-encapsulated drugs on the hematopoietic cells in the bone marrow, including megakaryocytes and developing myeloid and erythroid cells.

Sterically stabilized small liposomes containing PEG-DSPES or Gm1 ganglioside can remain in the circulation for a relatively long period of time. The present study provides definitive evidence that they are also able to traverse the endothelium of small blood vessels in tumors and extravasate into interstitial spaces. In liver and bone marrow they are endocytosed primarily by cells of the mononuclear phagocyte system. The persistence of such liposomes in the circulation and their ability to reach sites of a solid carcinoma make them highly attractive vehicles for chemotherapeutic agents. The evidence presented here provides a possible mechanism for the increased therapeutic efficacy of anthracyclines encapsulated in sterically stabilized liposomes against mouse colon carcinoma and lymphoma (1). It is quite likely that the encapsulated drug, being much more diffusible through the tumor mass than the macromolecular carrier itself, can reach distant tumor cells within the same area where liposomes have extravasated and exert its cytotoxic effect. In this respect, sterically stabilized liposomes provide a unique opportunity for increasing the therapeutic index of a variety of therapeutic agents against tumors that allow their extravasation beyond the endothelial barrier.

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