Analysis of the Fate of Systemically Administered Liposomes and Implications for Their Use in Drug Delivery

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INTRODUCTION

The ability to selectively "target" cytotoxic drugs to tumor cells in vivo has been a long-cherished goal in cancer therapy. In the last few years, liposomes have attracted considerable interest as potential candidates for accomplishing this task (for reviews, see Refs. 13 and 19). Recent in vitro studies have demonstrated that liposomes bearing cell-specific antibodies react with antigen-bearing target cells with much greater efficiency than do liposomes without surface antibodies (14, 15, 22). Although encouraging, similar targeting to specific cells in vivo has not been successful. Clearly, many obstacles must be overcome to allow liposomes to interact with the desired target cell. For example, to treat solid tumors or their metastases that proliferate in the extravascular compartments of major organs, liposomes containing drugs would be injected into the circulation and thus would have to traverse capillaries to reach the tumor cells (4). If transcapillary passage of intact liposomes cannot occur, then the effort of constructing liposomes with sophisticated ligands that "recognize" tumor cells would be futile because the mechanical barrier imposed by the microcirculation would prevent liposomes from reaching the intended target cell. Information on the transcapillary transport capabilities of liposomes is thus crucial for critical evaluation of the potential use of liposomes as carriers of cytotoxic drugs in cancer therapy.

In this study, we have examined the ability of liposomes of differing size to cross anatomically different classes of capillaries. Our results indicate that limited transcapillary transport of liposomes following i.v. injection occurs in open sinusoidal capillaries (liver) but does not occur in organs with continuous capillaries (lung). Moreover, we demonstrated the efficient uptake of liposomes by circulating phagocytic cells and those cells belonging to the RE system. This localization pattern agrees with the well-documented role of the RE system in the clearance of particulate materials from the circulation.

MATERIALS AND METHODS

Animals. Specific-pathogen-free C57BL/6N mice were obtained from the Frederick Cancer Research Center's Animal Production Area and the Laboratory Animal Services Division, Smith Kline and French Laboratories.

Cell Cultures. The B16-BV8 invasive variant line of the C57BL/6N melanoma (25) was maintained in Eagle's minimal essential medium supplemented with 5% bovine serum, vitamin solution, sodium pyruvate, nonessential amino acids, and L-glutamine as described previously (26). All components of this medium were obtained from Flow Laboratories, Rockville, Md. The medium was endotoxin free as determined by the Limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, Mass.). All cultures were incubated at 37° in a humidified atmosphere containing 5% CO2 and were free of Mycoplasma, reovirus type 3, pneumonia virus of mice, K virus, Theiler's encephalitis virus, Sendai virus, minute virus of mice, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by MA Bioproducts, Walkersville, Md.).

Preparation and Purification of AM. AM were harvested by pulmonary lavage using a technique slightly modified from that described by Holt (17). Lungs were lavaged from prewarmed endotoxin-free media containing 1 x 10^-4 M dibucaine hydrochloride (Sigma Chemical Co.,"

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Preparation and Purification of Liver Cell Populations. The term "nonparenchymal cells" will be used to describe all cells present in the liver except for parenchymal cells (hepatocytes). The nonparenchymal cell fraction in this paper is the total cell population recovered from the liver after Pronase digestion of parenchymal cells (see below). The nonparenchymal cell fraction includes sinusoidal cells (Kupffer cells, endothelial cells), bile duct cells, connective tissue cells, lymphocytes, fat cells, and any other Pronase-resistant cells (e.g., small numbers of plasma cells, neutrophilic granulocytes, mast cells).

C57BL/6N mice given i.v. injections in the tail vein of radiolabeled SUV or MLV (phosphatidylcholine:phosphatidylserine, 3:7 mol ratio; 2 µmol/mouse in 0.2 ml phosphate-buffered saline [KCI (0.2 g/liter), NaCl (8.0 g/liter), KH2PO4 (0.2 g/liter), and Na2HPO4·7H2O (2.18 g/liter)]. Livers were removed at the indicated intervals, and purified fractions of parenchymal and nonparenchymal cells were prepared (see below). Localization of liposome-associated radioactivity in each cell fraction was determined as described elsewhere (9) and was expressed as a percentage of the total radioactivity in the liver before fractionation. In experiments with liposomes bearing 2 radiolabels (membrane-associated [3H]DPPC and encapsulated 51Cr-EDTA), the ratio of the 2 radioactivities is normalized by the definition of the 2 radioactivities in the original liposome preparation as 1.0, so that ratios are calculated by simply dividing total radioactivity of one isotope by the total radioactivity of the second isotope.

Nonparenchymal cells were isolated by Pronase digestion (21). Typical cell yields of 1.5 x 10^6 cells/g liver were obtained (liver weight, 1.46 ± 0.22 (S.D.) g; N = 60). This compares favorably with that reported previously by others (21). Different cell types within the harvested nonparenchymal cells were identified by enzyme cytochemistry and/or cell surface markers. Kupffer cells were identified by their rapid adherence to glass, positive staining for peroxidase and nonspecific esterase, presence of surface Fc and C3 receptors, and their capacity to phagocytize 51Cr-labeled sheep RBC or latex beads. Kupffer cells identified by these criteria were found to represent between 45 and 55% of the total nonparenchymal cell population. Endothelial cells represent the other major cell type in the nonparenchymal fraction (25 to 35%) and were identified by positive esterase staining and a negative peroxidatic reaction. In certain experiments, purified Kupffer cell and endothelial cell populations were isolated from heterogeneous nonparenchymal cell fractions by centrifugal elutriation (21). Cells were introduced into the rotor at a flow rate of 11.0 ml/min at 4°, and 150-ml fractions were collected at flow rates of 13.5, 20, 22, 25, 31, 37, 40, and 50 ml/min using a rotor speed of 2500 rpm. Kupffer cells were identified by their ability to phagocytose latex beads or sheep RBC and to stain positively for peroxidase and acid phosphatase. Kupffer cells were recovered primarily in the eluted fraction collected at a flow rate of 40 ml/min with a smaller number of Kupffer cells being recovered at a flow rate of 25 ml/min. Both fractions were composed of pure Kupffer cells. Typical yields of 7 to 8 x 10^6 Kupffer cells/g liver were obtained with a purity of >90% and a viability of >95%.

Parenchymal cells were isolated by the method of Van Berkel (36). Yields of 5 x 10^7 cells/g liver with a viability of >95% were obtained routinely. The parenchymal origin of the recovered cells was confirmed by measurement of 2 hepatocyte-specific enzymes, glutamine synthetase and arginase. The specific activities of both enzymes (millimoles/mg liver protein) measured in isolated parenchymal cells was consistently between 80 and 90% of the activities found per mg crude liver homogenates. The absence of Kupffer cell contamination was confirmed by the failure to detect significant numbers of Fc-positive, esterase-positive phagocytic cells in the preparation.

In all of the procedures described above, no significant differences in cell yield were found between liposome-treated and untreated control mice.

Lymphokines. Cell-free supernatants were harvested from mitogen-stimulated rat lymphocytes and from unstimulated control lymphocytes as described previously (25).

Lipids and Preparation of Liposomes. Chromatographically pure egg phosphatidylcholine and beef brain phosphatidylserine were purchased from Avanti Biochemicals, Birmingham, Ala. Sonicated SUV and large MLV were prepared from phosphatidylserine and phosphatidylcholine (3:7 mol ratio) as described elsewhere (9). Liposome preparations were sized as described in Ref. 9. In typical preparations, more than 90% of the liposomes within any given preparation were within the size range of 300 to 800 Å and 0.5 to 10 µm for SUV and MLV, respectively. Liposomes containing [3H]DPPC were prepared as described previously (9) by addition of [3H]DPPC (specific activity, 10 Ci/mmol; Applied Science Labs, Inc., State College, Pa.) to the original phospholipid mixture to give a final specific activity of 0.3 µCi/mg. Encapsulation of lymphokines (26) and 51Cr-EDTA (9) within liposomes was achieved as described previously. Liposome preparations always were used within 4 hr. The volume of lymphokine suspension encapsulated within liposomes was determined from measurements of the aqueous internal space in liposomes as described elsewhere (26).

Activation of AM Following i.v. Injection of Free and/or Liposome-encapsulated Materials. Mice were given i.v. injections of MLV (2.5 or 5 µmol phospholipid) containing lymphokines as described elsewhere (8, 32).

Macrophage-mediated Cytotoxicity Assay. Macrophage-mediated cytotoxicity was assessed by a radioactivity release assay as described previously (26). AM cultures were washed with media, and 5 x 10^5 target cells labeled with 51I-labeled 2'-deoxyuridine were added to each well in 0.2 ml Eagle's minimal essential medium supplemented with 5% fetal bovine serum, vitamin solution, sodium pyruvate, nonessential amino acids, and L-glutamine. Target cells alone were always plated as an additional control. Twenty-four hr after the addition of target cells, the cultures were washed and refed to remove nonplated cells. Adherent target cells were lysed with 0.1 ml of 0.2 N NaOH at 24 or 72 hr after plating. The lyse was adsorbed on cotton swabs, placed directly into 12- x 75-mm tubes, and monitored for radioactivity in a gamma counter. The percentage of cytotoxicity in the macrophage assays was computed by the following formula:

\[
\text{% of cytotoxicity} = \frac{\text{cpm of target cells with normal macrophages} - \text{cpm of target cells with activated macrophages}}{\text{cpm of target cells with normal macrophages}} \times 100
\]

The statistical significance of differences between groups was determined by Student's 2-tailed t test.

X-irradiation and Bone Marrow Reconstitution. Anesthetized mice were irradiated with 800 rads (source-to-skin distance, 40 cm; dose rate, 70 rads/min). X-iradiation was given to the whole body, to the whole body with the thorax and head shielded with 4-mm lead shields, or to the thorax and head with the remainder of the body shielded. For reconstitution experiments, bone marrow cells (10^7) from syngeneic donors were injected i.v. in 0.2 ml Hank's balanced salt solution at 4 and 24 hr after X-irradiation.

Electron Microscopy. Samples of lung, liver, and spleen were excised from mice killed 10 min, 1 hr, 4 hr, and 24 hr after being given i.v. injections of MLV as described previously (9). Tissues were cut into small (≥1-cm) pieces and fixed in 0.1 M cacodylate buffer (pH 7.3) containing 3% glutaraldehyde and 2% paraformaldehyde for 1 hr. The samples were transferred to a fixative containing 2% glutaraldehyde.
and 8% tannic acid in cacodylate buffer for 1 hr, rinsed thoroughly with cacodylate buffer, fixed with 1% buffered osmium tetroxide for 1 hr, rinsed with cacodylate buffer and then with distilled water, and stained en bloc with 1% uranyl acetate for 1 hr. Samples were then dehydrated with a graded series of ethanol. Samples were kept on ice throughout the fixing and dehydration procedures until the last alcohol changes, at which time the samples were transferred to room temperature. The samples were embedded in Spurr’s low viscosity medium and cut with glass knives on a LKB Ultratome III ultramicrotome. Sections were stained with Reynold’s lead citrate for 1 min, dried, and examined in a Hitachi HU-12A transmission electron microscope at an accelerating voltage of 75 V.

RESULTS

Fate of i.v. Injected Liposomes in Organs with Discontinuous Capillaries (Liver and Spleen). Problems with fixing and extraction of lipids by solvents during tissue preparation have hindered detection of liposomes in vivo (24, 27). These problems have been overcome by using a tannic acid: glutaraldehyde fixation procedure (20, 38) which enables liposomes to be reliably identified within blood vessels and in extravascular tissues. However, even when well fixed, SUV (300 to 800 Å in diameter) are extremely difficult to identify in vivo. For this reason, all of the ultrastructural observations presented here have been derived from experiments using large MLV (0.5 to 10.0 μm diameter). It is important to note that glutaraldehyde fixation of cells could lead to the appearance of myelin figures which conceivably may resemble liposomes. There are 2 reasons to rule out such a potential artifact: (a) MLV are much larger than myelin figures; (b) MLV are also observed in freeze fracture of unfixed macrophages that engulfed liposomes.

Within 10 min after i.v. injection of MLV, electron microscopic observations revealed liposomes of various sizes within the vascular sinusoids of the liver and the spleen. At this time, no uptake of liposomes into cells was seen (Figs. 1A and 2A). By 1 hr, extensive uptake of liposomes by RE cells lining the sinusoids was observed in both the liver (Fig. 1B) and the spleen (Fig. 2B), but no uptake of MLV by hepatic parenchymal cells and non-RE cells in the spleen was observed. This pattern remains unchanged even at 4 hr after liposome injection (Figs. 1, C and D and 2C). Although 99% of the injected liposomes have been cleared from the circulation by this time (9), no evidence of liposome uptake by parenchymal cells was detected in either the liver or the spleen.

These ultrastructural observations are supported by studies on the distribution of radiolabeled liposomes in parenchymal (hepatocytes) and Kupffer cell fractions isolated from mouse liver. As shown in Table 1, both SUV and MLV injected i.v. localize initially in the nonparenchymal cell fraction but, with time, radioactivity is detected in the parenchymal cell fraction. Further separation of the nonparenchymal cell fraction by centrifugal elutriation (see “Materials and Methods”) revealed that the liposome-derived radioactivity detected in this fraction localized almost exclusively (>90%) in Kupffer cells (not shown). The fraction of the liposome inoculum recovered in association with hepatocytes is higher for SUV than for MLV. Studies using liposomes bearing 2 radiolabels revealed additional differences in the interaction of SUV and MLV with liver cells. The ratio of membrane-associated to encapsulated radiolabeled liposomes in the nonparenchymal cell fraction was similar to that in the original inoculum (1.0) for both SUV and MLV, indicating that the liposomes were structurally intact. In contrast, the ratio in the parenchymal fraction was increased, particularly for MLV. This indicates that loss of liposomal contents has occurred. In the case of MLV, the marked increase in the ratio and the failure to detect liposomes in hepatocytes by electron microscopy suggest that transfer of radiolabeled phospholipid to these cells may occur by an exchange process. These results could not be attributed to an artifact in the cell isolation procedure since the samples for the MLV and SUV studies were prepared in an identical manner and both the SUV and MLV were prepared from identical phospholipids. Thus, intact liposomes do not penetrate the sinusoidal lining to interact with hepatocytes on any significant scale.

Fate of i.v.-injected Liposomes in Organs with Continuous Capillaries (Lung). In contrast to the discontinuous capillaries in liver and spleen, the capillaries in other major organs such as the lung possess a continuous endothelium and basal lamina (42). To study whether liposomes can successfully traverse the wall of continuous capillaries, we examined the behavior of i.v.-injected liposomes in the lung capillary bed. The lung was chosen not only because of the structure of its capillaries but also because of our interest in using liposomes as carriers for delivering immunomodulators to the lung to stimulate antitumor activities in AM (8–10). Previous studies from our laboratories have shown that, following i.v. injection of MLV, liposomes are present in AM recovered from the alveoli by lavage (8, 10, 32). Whether the uptake of MLV by AM occurs in the alveolar space following extravasation of liposomes or whether MLV were engulfed by blood monocytes which then migrated to the alveoli to become AM was investigated.

The arrest of MLV in the pulmonary capillary bed after i.v. injection (Fig. 3, A and B) is followed by their uptake into blood monocytes (Fig. 3, C and D) or into polymorphonuclear cells (Fig. 4). By 4 hr after injection, mononuclear cells containing liposomes were also detected in the alveolar spaces (Fig. 3E).

<table>
<thead>
<tr>
<th>Time</th>
<th>SUV Paren-</th>
<th>SUV Paren-</th>
<th>MLV Paren-</th>
<th>MLV Paren-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min</td>
<td>ND*</td>
<td>81.3</td>
<td>ND</td>
<td>82.4</td>
</tr>
<tr>
<td>1 hr</td>
<td>14.5</td>
<td>65.8</td>
<td>5.6</td>
<td>76.3</td>
</tr>
<tr>
<td>4 hr</td>
<td>30.7</td>
<td>55.9</td>
<td>11.4</td>
<td>69.3</td>
</tr>
<tr>
<td>8 hr</td>
<td>31.3</td>
<td>45.4</td>
<td>14.1</td>
<td>61.6</td>
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</tbody>
</table>

* ND, not detected.
AM containing engulfed MLV can be reliably distinguished from type II pneumocytes. The lipid bilayers of MLV appear as distinct circular or elliptical membranes, whereas inclusions of surfactant in type II pneumocytes contain stacks of lipid lamellae rather than concentric whorls (Fig. 3F). The presence of liposomes free in the alveolar tissues, or in any other location, suggesting transcapillary transfer of free liposomes was never observed. On the other hand, the high frequency of liposomes within blood monocytes and/or polymorphonuclear cells suggests that liposomes reach the extravascular compartment by passive transfer inside these cells during their migration from the bloodstream into the alveoli.

Additional evidence that liposomes are unable to cross lung capillaries and that they reach the alveoli only when transported inside migrating cells has been obtained from our experiments on the recruitment of AM to the lung. In the normal lung, AM are derived from blood monocytes of bone marrow origin (2, 3, 12, 33, 37, 38). Whole-body X-irradiation suppresses circulating monocytes and, by damaging precursors in the bone marrow, X-irradiation also prevents the recruitment of monocytes to the lung (2, 37). Reconstitution of X-irradiated animals with bone marrow cells results in reappearance of AM in lung lavage fluids within 7 to 10 days (37). In contrast, localized X-irradiation of the chest has been shown to damage preexisting AM, but this can be rapidly compensated by recruitment of blood monocytes into the alveoli (37). We have exploited these conditions to determine the origin of tumoral AM recovered from mice given i.v. injections of liposomes containing encapsulated lymphokines. As reported in our previous work (8, 32), AM harvested from normal rodents (mice or rats) do not exhibit tumoricidal activity. The i.v. injection of liposome-encapsulated lymphokines results in the activation of AM to become cytotoxic against neoplastic cells (Table 2). Whole-body X-irradiation of mice 24 hr before the i.v. injection of liposomes containing lymphokines abolished the in vivo activation of AM (Table 2).

This experiment was complicated, however, by the fact that following whole-body X-irradiation very few viable cells can be recovered from the lung. Lavage fluids from X-irradiated mice (800 rads) primarily contained dead cells and debris. Mice in which the thorax was shielded during irradiation with 800 rads of X-radiation showed a similar loss of responsiveness by AM to in situ activation by i.v.-injected liposomes (Table 2). The yield of lavaged AM from these mice was similar to that recovered from untreated controls (data not shown). We do not attribute the unresponsiveness of these AM to i.v.-injected MLV containing lymphokines to their inability to respond to the activation stimulus. Data shown in Table 2 demonstrate that AM recovered from X-irradiated mice can be activated by incubation in vitro with liposomes containing lymphokines. Shielding of the thorax during irradiation protects the AM but damages circulating monocytes and macrophage precursors in the bone marrow (37). The failure of the protected AM to respond to liposome-encapsulated lymphokines in vivo suggests that liposomes are simply not reaching these cells in vivo. In contrast, mice subjected to local X-irradiation of the chest before the i.v. injection of liposome-encapsulated lymphokines yielded AM with a significant tumoricidal activity (Table 2). X-irradiation of the thorax destroyed macrophages in the alveoli but had little effect on circulating monocytes or their recruitment to the lung. These data strengthen the hypothesis that AM are derived from blood monocytes that interact with liposomes within the microcirculation and then migrate into the alveoli. The recovery of the response of AM to liposome-encapsulated lymphokines in mice given whole-body X-irradiation (800 rads) following reconstitution with bone marrow cells also supports this interpretation. AM from mice treated with whole-body X-irradiation are unresponsive to activation by liposomes containing lymphokines injected 1 day after recon-

### Table 2

<table>
<thead>
<tr>
<th>Treatment of macrophage donors</th>
<th>% of destruction of B16 melanoma cells by AM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>In vivo activation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>In vitro incubation with&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MLV</td>
<td>SUV</td>
</tr>
<tr>
<td>X-irradiation (800 rads)</td>
<td>Time of i.v. injection of liposomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>24 hr before harvest of AM</td>
<td>39</td>
<td>31</td>
</tr>
<tr>
<td>Whole-body</td>
<td>24 hr after X-irradiation</td>
<td>0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Whole-body X-irradiated</td>
<td>24 hr after X-irradiation</td>
<td>8&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thorax</td>
<td>24 hr after X-irradiation</td>
<td>32</td>
<td>Not done</td>
</tr>
<tr>
<td>Whole-body bone marrow cell</td>
<td>24 hr after reconstitution</td>
<td>0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Not done</td>
</tr>
<tr>
<td>reconstituted&lt;sup&gt;1&lt;/sup&gt;</td>
<td>7 days after reconstitution</td>
<td>30</td>
<td>Not done</td>
</tr>
</tbody>
</table>

<sup>a</sup> AM (1 x 10<sup>5</sup>) were plated into 38-sq mm wells. The AM were thoroughly washed before the addition of 5-<sup>32</sup>P-labeled B16 melanoma cells. Percentage of cytotoxicity was calculated by comparison to untreated AM. Means of triplicate cultures terminated after 72 hr of cocultivation. This is a representative of several experiments.

<sup>b</sup> Mice were given i.v. injections of 5 μmol phospholipids (MLV or SUV).

<sup>c</sup> AM harvested from the various treatment groups were incubated in vitro with SUV, with MLV containing lymphokines, or with media alone (controls) for 24 hr. The AM cultures were then thoroughly washed, and their tumoricidal activity was monitored against the radiolabeled B16 target cells.

<sup>d</sup> No viable cells were recovered.

<sup>e</sup> Decrease in tumoricidal properties differed significantly from the appropriate control groups (p < 0.001).

<sup>1</sup> Bone marrow cells (10<sup>5</sup>/mouse) were injected i.v. at the indicated time.

Additional evidence that liposomes are unable to cross lung capillaries and that they reach the alveoli only when transported inside migrating cells has been obtained from our experiments on the recruitment of AM to the lung. In the normal lung, AM are derived from blood monocytes of bone marrow origin (2, 3, 12, 33, 37, 38). Whole-body X-irradiation suppresses circulating monocytes and, by damaging precursors in the bone marrow, X-irradiation also prevents the recruitment of monocytes to the lung (2, 37). Reconstitution of X-irradiated animals with bone marrow cells results in reappearance of AM in lung lavage fluids within 7 to 10 days (37). In contrast, localized X-irradiation of the chest has been shown to damage preexisting AM, but this can be rapidly compensated by recruitment of blood monocytes into the alveoli (37). We have exploited these conditions to determine the origin of tumoral AM recovered from mice given i.v. injections of liposomes containing encapsulated lymphokines. As reported in our previous work (8, 32), AM harvested from normal rodents (mice or rats) do not exhibit tumoricidal activity. The i.v. injection of liposome-encapsulated lymphokines results in the activation of AM to become cytotoxic against neoplastic cells (Table 2). Whole-body X-irradiation of mice 24 hr before the i.v. injection of liposomes containing lymphokines abolished the in vivo activation of AM (Table 2).
stition with bone marrow cells. However, significant tumoricidal response of AM is observed following the i.v. injection into mice of MLV containing lymphokines reconstituted with bone marrow cells 7 days previously (Table 2).

**DISCUSSION**

To date, morphological information on the intravascular behavior and fate of i.v.-injected liposomes has been limited to ultrastructural observations showing that components from MLV could be taken up by both Kupffer cells and hepatocytes (7, 29, 30, 44). Substantial technical difficulties in preserving liposomal structure for electron microscopy have hampered investigation of the fate of liposomes after their arrest in the capillary beds of other major organs. The tannic acid:glutaraldehyde procedure used here provides both excellent fixing of lipids and resistance to solvent extraction (20). This technique is therefore far superior to the usual method of stabilizing liposome structure by fixing with osmium. Tannic acid:glutaraldehyde also increases the electron density of the surface membrane of capillary endothelial cells (38), thus facilitating the study of liposome-endothelial interactions. Finally, the excellent lipid fixing achieved with this method makes it possible to distinguish liposomes incorporated into AM from the lamellar inclusions of surfactant present in type II pneumocytes (Fig. 3F).

The ultrastructural experiments described here have failed to detect any evidence of transcapillary passage of MLV to the extravascular compartments in the liver, spleen, or lung. Mechanically, opportunities for transcapillary transfer of circulating liposomes would be expected to be greatest in the sinusoids of the liver and spleen. In these organs, the endothelium is discontinuous and is interrupted by large gaps. Moreover, the subendothelial basement membrane in these capillaries is absent (42). The mean diameter of endothelial openings in the sinusoidal wall is 0.1 μm, and particles of larger diameter fail to gain access to hepatocytes (16, 43). The elastic deformability of liposomes (11) may permit liposomes marginally larger than 0.1 μm to penetrate endothelial gaps to interact subsequently with hepatocytes. However, it is extremely unlikely that intact, large MLV (0.5 to 10 μm) could penetrate through the endothelial barrier.

Our analysis of liposome localization in nonparenchymal, sinusoidal, and parenchymal liver cells reveals that liposomes do indeed gain access to hepatocytes. The kinetics of liposome association with hepatocytes is slower than with the nonparenchymal (RE) cell fraction. SUV are more efficient than are MLV in interacting with hepatocytes. This may simply reflect the ability of SUV to penetrate endothelial gaps. Furthermore, our experiments with the double-labeled liposomes indicate that, whereas SUV recovered in association with hepatocytes are apparently intact, MLV retained in the liver show preferential transfer of liposomal lipid to hepatocytes without the concomitant transfer of liposomal contents. Similar results have been reported recently by Freise et al. (11).

In contrast to the transfer, albeit limited, of liposomal components across hepatic sinusoids, our results indicate that lung capillaries are completely impermeable to either SUV or MLV. This finding is perhaps not surprising when interpreted in terms of our current knowledge of capillary structure. Lung capillaries possess an uninterrupted lining of endothelial cells with tight junctions between adjacent cells which, in turn, overlie a continuous basal lamina. Studies using tracer molecules of defined size indicate that endothelial junctional permeability is restricted to particles smaller than 20 Å in diameter (31). The system of endothelial vesicles, which are believed to represent the so-called "large-pore" permeability channel in continuous capillaries (4), do not exceed 800 Å in diameter and would not accommodate the MLV (0.5 to 10 μm) observed in AM in this study. Moreover, recent studies indicate that endothelial vesicles may not be a dynamic system in which vesicles are continually formed but may represent a permanent structural channel crossing the endothelial cell (4). If this is the case, then the limited diameter (20 Å) of the "neck" of vesicles opening to the luminal surface would be far too small to accommodate even SUV.

Even if arrest of liposomes in the pulmonary microcirculation were somehow to lead to dissolution of endothelial cell junctions and to permit the liposomes to penetrate between adjacent cells, liposomes must still cross the subendothelial basal lamina and the adventitial connective tissue and penetrate between alveolar epithelial cells to eventually reach the alveolar space. For this reason, it is likely that continuous capillaries of the kind found in the lung and in the central nervous system muscle, connective tissue, exocrine pancreas, and gonads (42) present an impenetrable mechanical barrier to the movement of circulating liposomes into the extravascular tissues. The impenetrability of brain capillaries to liposomes has been demonstrated by the failure of potent centrally acting neurotropic agents encapsulated in liposomes to alter brain function after i.v. injection, even under conditions in which permeability of the blood-brain barrier to serum albumin is markedly enhanced (35). Reports that SUV administered i.v. increase brain glucose and the release of catecholamine (24) do not establish that liposomes can cross brain capillaries. What is more likely is that these actions are attributable to the well-documented ability of exogenous phospholipids to alter cerebral metabolism and catecholamine turnover (5, 6).

The studies presented here on the recruitment and transcapillary migration of blood monocytes into the alveolar space indicate that liposomes can be transferred passively across capillaries by being carried inside migratory cells. These observations are also pertinent to the mechanisms by which liposome-encapsulated macrophage activators stimulate macrophage-mediated tumoricidal reactions in the lung (8–10, 32). Our results suggest that the population of tumoricidal AM recovered from alveoli after i.v. injection of liposomes containing macrophage activators are derived from circulating monocytes that engulf liposomes within pulmonary capillaries and subsequently migrate to the extravascular alveolar tissue.

The interaction of liposomes with a third class of capillaries, the fenestrated capillaries, has yet to be investigated in detail. However, preliminary observations from our laboratory on the transfer of liposomes across fenestrated capillaries in the intact isolated perfused cat salivary gland have failed to detect significant transfer of either SUV or MLV.

Ever since the first reports describing the use of liposomes as drug carriers were published, investigators have discussed the possibility of using liposomes to target drugs to specific cell

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types in vivo. Unfortunately, our findings that circulating liposomes localize in phagocytic mononuclear cells and that liposomes have limited ability to traverse capillaries pose major obstacles to the targeting of liposomes to organ parenchymal cells situated outside the circulation. Unless methods can be devised to render capillaries selectively permeable to liposomes without impairing their permeability to blood cells or other materials, the data presented here suggest that the probability of liposomes reaching extravascular target cells will be low, even in tumors vascularized by capillaries with structural defects (23).

Although the present findings raise serious doubts about the feasibility of targeting liposomes to target cells in the extravascular compartment, we consider that targeting of liposome-encapsulated materials within the confines of the intravascular compartment still offers exciting therapeutic opportunities. For example, it should be possible to direct ligand-bearing liposomes to specific blood cells (e.g., lymphoid cell subsets) and perhaps also to target liposomes to specific regions of the vascular system using ligands directed against regional and/or organ-specific determinants on the vascular lumen (28). The ability to achieve high concentrations of drugs in selected organs by such an approach could be of substantial therapeutic value. This approach might also be combined profitably with the use of the recently described novel classes of liposomes which break down and release their contents in response to changes in the physicochemical properties of the local tissue environment, such as temperature and pH (41, 45).

In conclusion, the mechanical barrier of the capillary wall is a major hindrance to the targeting of liposomes to many cells. In contrast, the localization of liposomes in circulating and fixed phagocytic cells offers a viable mechanism for targeting liposome-associated materials to RE cells and monocytes. This strategy has already been used successfully to deliver materials which stimulate macrophage-mediated defense reactions against parasites (1) and tumors (9, 10).

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Fig. 1. Electron micrographs of mouse liver after the i.v. injection of MLV. In A, liposomes (arrows) are visible in liver sinususes 10 min after being injected i.v. In B, small MLV are seen phagocytosed by Kupffer cells 1 hr after being injected i.v. C, MLV in liver sinus adjacent to RBC. Liver hepatocytes (H) are indicated at the periphery of the sinus. D, MLV phagocytosed by Kupffer cells. Portions of liver hepatocytes (H) are also shown.

Fig. 2. Electron micrographs of mouse spleens after i.v. injection of MLV. A, sample taken 10 min after i.v. injection shows numerous liposomes (arrows) in the blood sinus. B, sample taken 1 hr after i.v. injection. C, sample taken 4 hr after i.v. injection. Note the uptake of liposomes by splenic phagocytes.

Fig. 3. Electron micrographs of mouse lung after i.v. injection of MLV. In A, liposomes (arrows) of various sizes are visible in the lung capillary 10 min after i.v. injection. B, large liposomes (arrows) visible inside the capillary 1 hr after i.v. injection. C, large MLV in the capillary 60 min after i.v. injection. P, polymorphonuclear cell; M, monocyte. D, monocyte with internalized MLV 60 min after i.v. injection. Note the presence of RBC in the lumen. E, AM with internalized liposome. The liposome is adjacent to the nucleus (N) of the macrophage. The capillary lumen is indicated by arrows. F, pneumocyte type II from untreated mouse. Note the well-preserved ultrastructure of the surfactant (arrows) accentuated by the use of the tannic acid/glutaraldehyde fixing technique.
Fig. 4. Uptake of liposomes by circulating polymorphonuclear cells. Leukocytes were isolated from the blood of mice given i.v. injections of MLV 60 min earlier. Arrows, liposomes.
Analysis of the Fate of Systemically Administered Liposomes and Implications for Their Use in Drug Delivery

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