Effects of Liposome Dose and the Presence of Lymphosarcoma Cells on Blood Clearance and Tissue Distribution of Large Unilamellar Liposomes in Mice

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ABSTRACT

Large unilamellar liposomes (50 to 500 µmol of lipid per kg) were injected i.v. or i.p. into normal and lymphosarcoma-bearing mice. The percentage of the dose remaining in the blood and that accumulated in liver, spleen, and various other organs was measured 4 hr after injection. The results indicate that liposomes cause a dose-dependent saturation of the hepatic and splenic clearance capacities. One day after injection of 10^6 lymphosarcoma cells, the capacity of the tumor-bearing mice to eliminate liposomes from the blood (in a 4-hr period) was inhibited 30 to 50%. This could be ascribed to a decreased activity of the reticuloendothelial system caused by the tumor cells, as was indicated by the simultaneous inhibition of carbon clearance. Six days after injection of the lymphosarcoma cells, the elimination of liposomes from the blood in tumor-bearing mice was restored to the value in normal mice. The possible involvement of tumor cells in the uptake of liposomes by the liver was investigated morphologically after i.v. injection of peroxidase-containing liposomes into lymphosarcoma-bearing mice. Liposome-entrapped peroxidase activity was never observed in the tumor cells. The results presented here indicate that the lymphosarcoma cells do not directly participate in the hepatic uptake of liposomes, although their mere presence may have significant indirect effects on the elimination of liposomes from the blood and on their tissue distribution.

INTRODUCTION

The potential clinical usefulness of liposomes as a system for the controlled delivery of therapeutic agents has been discussed in a number of recent reviews (15, 17, 23). After i.v. or i.p. injection, a substantial proportion of the liposomes is found to accumulate in the liver and spleen. Liposomes can be taken up by tissue macrophages (mainly located in liver and spleen) as has been demonstrated for Kupffer cells in the liver (27, 37), but relatively little is known about any factors which may affect such uptake. In cancer chemotherapy, only a few successful applications of liposomes as a drug carrier system have been reported (11, 14, 19, 21, 25), and the presently available evidence supports the hypothesis that the therapeutic effects from a liposome-encapsulated cytotoxic drug are due to a delayed elimination from the blood and a sustained release of the drug from the liposomes, rather than to a selective uptake of the encapsulated drug by the tumor cells (16, 29).

Depression of reticuloendothelial activity has been suggested to serve as a means to reduce liver and spleen uptake of liposomes and to prolong the circulation time of the vesicles (1, 12, 13). The rate of disappearance of liposomes from the blood decreases with increasing dose (33), indicating that the clearance mechanism is a saturable process. Concomitantly, one would expect the proportion of liposomes taken up by the liver to decrease with increasing dose, but conflicting results have been reported on this subject (12, 33). Tumor cells may influence reticuloendothelial activity (3), but thus far little attention has been paid to the possible effects of the presence of tumor cells on the elimination of liposomes from the blood.

We confirmed that large liposome doses result in prolonged circulation time in normal as well as in tumor-bearing mice, after both i.v. and i.p. administration. During this study, we observed, however, a profound influence of the presence of lymphosarcoma cells in the animals on blood clearance and organ distribution of the liposomes. In addition, we obtained direct evidence of the lack of in vivo liposome uptake by lymphosarcoma cells situated in the liver, despite their direct accessibility from the bloodstream.

MATERIALS AND METHODS

Liposome Preparation. Sphingomyelin (from bovine brain) and cholesterol were obtained from Sigma Chemical Co., St. Louis, Mo. Phosphatidylserine, purified from bovine brain as described by Papahadjopoulos and Miller (24), was a gift from Dr. J. C. Wilschut of our laboratory. 125I-Labeled PVP2 (specific activity, 38 µCi/µg; M, 30,000 to 40,000) was purchased from the Radiochemical Centre, Amersham, England. Unipore polycarbonate filters were from Bio-Rad Laboratories, Richmond, Calif. Sepharose CL-2B and Ficoll (M, 70,000) were obtained from Pharmacia Fine Chemicals, Piscataway, N. J. Horseradish peroxidase Grade II 3,3'-diaminobenzidine tetrahydrochloride Grade II were from Sigma. The diammonium salt of 2,2'-azinodi(3-ethylbenzthiazolin)sulfonic acid (NADH) was from Boehringer/Mannheim, Mannheim, Germany.

REV were prepared essentially as described by Szoka and Papahadjopoulos (35). Sphingomyelin, cholesterol, and phosphatidylserine (molar ratio, 4:5:1) were dispersed in diethyl ether to give a concentration of 20 µmol of total lipid per ml (the diethyl ether was distilled in the presence of sodium bisulfite immediately before use). The aqueous phase (150 mM NaCl, 5 mM Tris-HCl, pH 7.4) contained 125I-labeled PVP (2.6 mg/ml with varying specific activity). Per ml of the organic phase, 0.3 ml of the aqueous phase was added, and the mixture was sonicated under nitrogen in a bath-type sonicator (Branson). After formation of a homogeneous suspension of inverted micelles, the organic phase was removed on a rotary evaporator, and the vesicles thus formed were extruded through a polycarbonate membrane with 0.4-µm pores (36).

1 This research was supported by the Netherlands Cancer Foundation Koningin Wilhelmina Fonds, Project GUKC llx.

2 To whom requests for reprints should be addressed.
Nonencapsulated 125I-labeled PVP was separated from encapsulated material by gel filtration on Sepharose CL-2B. Void volume fractions containing the liposomes were pooled and, if necessary, concentrated in an Amicon concentration cell (XM-300 filter). Vesicle lipid concentrations were determined by lipid phosphorus assay according to the method of Bartlett (4), as modified by Bottcher et al. (5). Liposomes prepared this way had an encapsulated volume of 4 to 5 liters per mol of phospholipid.

For encapsulation of peroxidase, a solution of 30 mg of horseradish peroxidase per ml of 150 mM NaCl:5 mM Tris-HCl, pH 7.4, was used as the aqueous phase. Peroxidase-containing liposomes were separated from nonentrapped enzyme by gel chromatography on Sepharose CL-2B. Peroxidase activity associated with the liposomes was measured by a modification of the method of Steinman and Cohn (34), as described by Roerdink et al. (28). The diammonium salt of 2,2′-azinomdi(3-ethylbenzthiazolin sulfonic acid) was used as a chromogen. Latency of the entrapped enzyme was defined as:

\[
\frac{\text{Total enzyme activity} - \text{Free enzyme activity}}{\text{Total enzyme activity}} \times 100\%
\]

Total and free enzyme activities refer to the activities in the presence and absence of 1% Triton X-100, respectively. Latency for the vesicles used in this study was 94%.

Animals. Three-month-old female C57BL X Gr F1 mice were obtained locally. A B-lymphosarcoma, which originated spontaneously in a C57BL mouse (7), was transplanted weekly by i.p. injection of 106 tumor cells. Seven days after inoculation, the spleen was significantly enlarged (from 100 to approximately 800 mg) and consisted almost entirely of tumor cells. The lymphosarcomatous spleen was excised and squeezed through nylon gauze in 10 parts of ice-cold 0.9% NaCl solution. The single-cell suspension thus obtained was used for tumor transplantation.

At the third day after i.p. injection, tumor cells were present in liver and spleen, as observed by light microscopy. In the liver, the tumor cells were located predominantly in the sinusoids, i.e., in direct contact with the blood compartment. In the spleen, they were mainly found in the marginal zone of the follicles of the white pulp. During the tumor development, the tumor cells in the spleen concentrically invaded the white pulp, obliterating the red pulp. At 7 days after inoculation, a diffuse tumor cell proliferative pattern was found in the spleen, and massive infiltrations were found in the liver, lungs, bone marrow, and kidneys. Macroscopically, no tumor growth or ascites production was observed in the peritoneal cavity at any day. Tumor-bearing mice died 8 or 9 days after i.p. inoculation of 106 lymphosarcoma cells.

Tissue Distribution Experiments. Normal or tumor-bearing mice (either 1 or 6 days after inoculation of 106 lymphosarcoma cells) were given i.p. or i.v. (lateral tail vein) injections of various doses of liposomes. Four hr after injection, the animals were anesthetized with ether, and the peritoneal cavities of mice that received an i.p. liposome injection were rinsed with 2 ml of 150 mM NaCl:5 mM Tris-HCl, pH 7.4, in order to recover the liposomes. A blood sample (0.2 ml) was taken from the venae cavae, and the liver was briefly perfused with 150 mM NaCl:5 mM Tris-HCl, pH 7.4, to remove the blood. Various organs were excised, rinsed in buffer, blotted on paper, and weighed. Radioactivity was measured in whole organs. For calculation of the proportion of the dose present in whole organs, radioactivity between blood and various organs was determined following an i.p. injection of PVP-containing liposomes. Only very small amounts of radioactive material were free or cell associated (results not shown). These findings are compatible with our earlier observations of rats (9), showing that in both blood and the peritoneal cavity, 125I-labeled PVP remained liposome associated for at least 6 hr following an i.p. injection of PVP-containing liposomes. Only very small amounts of radioactive material were free or cell associated (results not shown). These findings are compatible with our earlier contention that circulating macrophages are not extensively involved in the transport of liposomes from peritoneal cavity to blood.

Vesicles i.v. Injected. Vesicles containing 125I-labeled PVP were injected i.v. into normal and tumor-bearing mice in doses of 50 to 500 µmol of liposomal lipid per kg of body weight. The mice were sacrificed 4 hr after injection, and the distribution of radioactivity between blood and various organs was determined (Table 1). In all cases, 70 to 80% of the injected dose was recovered in blood, liver, and spleen. With increasing liposome dose, larger proportions remained in the circulation after 4 hr and, concomitantly, smaller fractions were found associated with liver and spleen.

One day after injection of tumor cells, the mice were clearly inhibited in their capacity to eliminate liposomes from the blood. The increase in the level of circulating liposomes in the tumor-bearing mice corresponded to a decrease in hepatic uptake. It is interesting to note that, in the tumor-bearing mice, the splenic component of blood clearance became of relatively more importance than in normal mice. In tumor-bearing mice, the amount of...
Liposomes in Lymphosarcoma-bearing Mice

Table 1

Vesicles injected i.v. in normal and tumor-bearing mice, dose-dependent blood elimination, and organ distribution

| Vesicles containing 125I-labeled PVP were injected i.v. at doses varying from 50 to 500 µmol/kg into normal mice or into lymphosarcomatous mice 23 hr or 6 days after inoculation of tumor cells. In one set of experiments, mice received 0.4 µmol of La(NO3)3 i.v. 24 hr prior to injection of liposomes.

<table>
<thead>
<tr>
<th>Mean % of injected dose per whole organ(s)</th>
<th>Normal mice</th>
<th>1-day tumor mice</th>
<th>La3+-treated mice</th>
<th>6-Day tumor mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dose</strong></td>
<td>125 µmol/kg</td>
<td>500 µmol/kg</td>
<td>125 µmol/kg</td>
<td>250 µmol/kg</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td>0.8 ± 0.2b</td>
<td>23.9 ± 10.1</td>
<td>3.6 ± 2.7</td>
<td>29.3 ± 8.1</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>55.6 ± 7.7</td>
<td>34.7 ± 5.5</td>
<td>32.4 ± 6.8</td>
<td>21.8 ± 4.6</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td>19.7 ± 1.9</td>
<td>13.6 ± 1.6</td>
<td>40.8 ± 5.8</td>
<td>30.0 ± 4.2</td>
</tr>
<tr>
<td><strong>Lungs</strong></td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.3</td>
<td>4.2 ± 1.5</td>
<td>2.7 ± 0.7</td>
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<tr>
<td><strong>Heart</strong></td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
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<tr>
<td><strong>Kidneys</strong></td>
<td>0.1 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>0.5 ± 0.3</td>
<td>1.0 ± 0.2</td>
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* Numbers in parentheses, number of mice given injections.

The increased circulation time of the liposomes in mice with a 1-day tumor and the concomitantly decreased hepatic uptake are indicative of a decreased activity of the reticuloendothelial system in such animals. This was confirmed by a direct measurement of the phagocytic index (by carbon clearance) in normal and tumor-bearing mice (Table 2). The phagocytic index of 1-day tumor mice was decreased compared to that of control mice. Six days after inoculation with tumor cells, the animals had recovered from the depression of reticuloendothelial activity. The carbon clearance was even substantially increased in those animals. La(NO3)3, known to depress the phagocytic activity of Kupffer cells in mice (20), caused a change in the tissue distribution in normal mice very similar to that in the 1-day tumor mice (Table 1).

**Vesicles i.p. Injected.** When the liposomes were injected i.p., the results were essentially similar to those obtained with i.v.-injected vesicles (Table 3). However, only in 1-day tumor mice was the percentage of the dose remaining in the blood 4 hr after injection clearly dose dependent. Also, uptake in the liver and spleen of these 1-day tumor mice was inhibited compared to that of normal mice. Six days after inoculation with tumor cells, blood levels and tissue distribution were almost restored to control values.

The different doses of vesicles were injected in the same total volume (0.45 ml) and apparently were transported to the blood at a high rate since, even with the high dose, less than 10% was recovered from the peritoneal cavity after 4 hr. The recovery of the vesicles tended to be lower after i.p. than after i.v. injection. It is conceivable that a fraction of the vesicles in the peritoneal cavity is not easily removed by washing and/or is taken up by lymph nodes.
Microscopic Visualization of Liposome-entrapped Horseradish Peroxidase in the Liver. Peroxidase-containing REV were injected into normal or lymphosarcoma-bearing mice. The distribution of the peroxidase reaction product, as seen in the light microscope 2 hr after injection of the liposomes into a normal mouse, is shown in Fig. 1. The reaction product was prominent in the Kupffer cells but could also be detected in endothelial cells and hepatocytes. When a similar dose of free peroxidase was injected, mixed with empty liposomes, peroxidase reaction product was detected only in endothelial cells, and no reaction product was found in Kupffer cells or hepatocytes (not shown). The endogenous peroxidase activity of Kupffer cells could easily be distinguished from the exogenous enzyme by virtue of the essentially different localization (nuclear envelope and endoplasmic reticulum) of the endogenous enzyme.

Six days after injection of the tumor cells, the distribution of peroxidase reaction product in the liver was identical to that observed in normal mice (Fig. 2). The presence of tumor cells in the sinusoids was conspicuous, but they did not contain any peroxidase reaction product. Apparently, the tumor cells did not take up liposome-encapsulated or free peroxidase whereas, in Kupffer cells, located in close proximity to the tumor cells, peroxidase reaction product was abundantly present. In Figs. 3 and 4, electron micrographs of the livers of a normal mouse and a 6-day tumor mouse that received peroxidase-containing liposomes are shown. Peroxidase activity was located in membrane-bound vacuoles in the cytoplasm of Kupffer cells. In agreement with our light microscopic observations, no peroxidase activity was detected in the tumor cells. Light and electron microscopic observations on the localization of the peroxidase reaction product in the livers of 1-day tumor mice (results not shown) were the same as in normal and 6-day tumor mice.

**DISCUSSION**

Our results confirm those of others (2, 12, 13, 32) in that i.v.-

Table 3

<table>
<thead>
<tr>
<th>Vesicles injected i.p. and organ distribution</th>
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<tr>
<td>Vesicles containing 125I-labeled PVP were injected i.p. at 125 and 500 μmol/kg into normal mice, 1-day tumor mice, and 6-day tumor mice. Four hr after injection, the peritoneal cavity was rinsed with 150 mM NaCl:5 mM Tris-HCl (pH 7.4), a blood sample was taken from the vena cava, and liver and spleen were removed and weighed. Radioactivity was determined in the whole organs.</td>
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<tr>
<td>Tissue radioactivity (% of injected dose per whole organ)</td>
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<tr>
<td>Dose (μmol/kg)</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>Normal mice</td>
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<tr>
<td>125</td>
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<td>1-Day tumor mice</td>
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<tr>
<td>6-Day tumor mice</td>
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<td>125</td>
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*a Mean ± S.D. of 5 mice.

Fig. 1. Micrograph of normal mouse liver 2 hr after i.v. injection of liposomes with entrapped peroxidase (2.8 μmol of liposomal lipid containing 0.13 mg of peroxidase). Peroxidase reaction product is conspicuously present in Kupffer cells (kc) but can also be detected in endothelial cells (ec) and in hepatocytes (bold arrows). Bar, 10 μm.

Fig. 2. Micrograph of the liver of a 6-day tumor mouse 2 hr after i.v. injection of liposomes with entrapped peroxidase (2.8 μmol of liposomal lipid containing 0.13 mg of peroxidase). Peroxidase reaction product is present in Kupffer cells (kc) but can also be detected in endothelial cells (ec) and in hepatocytes. Tumor cells (tc) are devoid of peroxidase activity. Bar, 10 μm.
injected liposomes are eliminated from the blood compartment in a dose-dependent way. Higher doses are cleared more slowly, indicating that the clearance mechanism is saturable, a well-known phenomenon for the clearance of large particles (6, 32). After i.p. injection, the elimination was less clearly dose dependent. The delaying effect of the transport from the peritoneal cavity to the blood, although small as compared to our previous observations of rats (9), may be responsible for this phenomenon. Only in the 1-day tumor rats, in which reticuloendothelial activity was depressed, was dose-dependent blood elimination of i.p.-administered liposomes manifest.

The slower clearance of high doses was paralleled by decreased hepatic and splenic uptake, as might be expected. Souhami et al. (33), on the other hand, did not observe decreased liver uptake concomitant with decreased blood elimination, which led these authors to suggest that the observed saturation of blood clearance capacity was related to an extrahepatic site of uptake. On the other hand, the size of the vesicles used by these authors (average diameter, 58 nm) may have allowed a larger proportion of the liposomes to gain access to the hepatocytes through the open endothelial fenestrations (38). Rahman et al. (26) recently presented indirect evidence that small unilamellar vesicles preferentially associate with liver parenchymal cells. Experiments from our own laboratory provided direct evidence of high uptake of both lipid and entrapped solute of small unilamellar vesicles in hepatocytes and low uptake in nonparenchymal cells. In the present study, we used liposomes with a mean diameter larger than 100 nm (36), so that most of the liposomes are likely to be prevented access from and thus association with the parenchymal cells of the liver. It is conceivable that differences in the distribution between the cell types in the liver are responsible for the discrepancy between the results of Souhami et al. and the data presented in this paper.

Clearance and organ distribution of i.v. injected liposomes were very similar in 1-day tumor mice and in lanthanum-treated mice. Both conditions probably involve a depression of Kupffer cell activity, as measured by carbon clearance in the tumor animals. A change in the reticuloendothelial function following tumor cell challenge has been reported by various investigators (22, 31) and may be due to tumor components in the serum or to altered plasma opsonin levels. It is not likely that the temporary depression in blood elimination of liposomes and of carbon particles following the injection of tumor cells is caused by direct interaction of the tumor cells with the Kupffer cells. In our morphological studies, no evidence was found of the presence of tumor cells in the liver before the third day after administration. In 6-day tumor mice, elimination from the blood and hepatic uptake of liposomes were comparable to those in control animals. Apparently, the tumor mice had recovered from the reticuloendothelial depression. Surprisingly, in the 6-day tumor mice, carbon clearance was increased to an even higher value than that found in control animals. At present, we do not know what causes this phenomenon. Saba and Antikatzides (31) reported that Walker 256 tumor cell-induced changes of reticuloendothelial carbon clearance in rats correlated well with changes in plasma opsonin levels. Thus, increased levels of opsonic protein in the plasma may be involved in the increased carbon clearance, as observed in our experiments. It may be noted that, for the large liposome dose, splenic uptake of liposomes in 6-day tumor mice also prevailed over control values (Chart 1). The high phagocytic index for carbon in these animals may, similarly, be caused by splenic macrophages whereas, in control animals, mostly the Kupffer cells will be involved in carbon clearance.

The depression of Kupffer cell activity in the 1-day tumor mice causes a shift of liposome accumulation towards the spleen, especially at the lower lipid doses. Apparently, there is an overflow of liposomes to the spleen, which in turn tends to become saturated at the higher lipid doses. There is a small but significant increase of liposome accumulation in the lungs of 1-day tumor mice. It remains to be seen whether these liposomes are only physically trapped or are associated with macrophages outside the vascular bed.

Morphological examination of the livers of mice that received liposome-entrapped peroxidase revealed that the peroxidase reaction product was concentrated in the Kupffer cells but was also present in endothelial cells and hepatocytes. Comparison with the adequate controls showed that the presence of peroxidase in the Kupffer cells and presumably also in the hepatocytes must be attributed to uptake of liposome-entrapped enzyme since at this dose free peroxidase is seen only in endothelial cells. The extrusion procedure used for the preparation of the liposomes yields a preparation in which a fraction of the liposomes will have a diameter smaller than 100 nm (35, 36). It may be this fraction that is allowed access to the parenchymal cells through the fenestrations in the endothelial sieve plates. Reaction product in the endothelial cells could be due to the uptake of free peroxidase that had leaked from the liposomes, as was also suggested by Roerdink et al. (28).

Our study suggests that if liposomes are to be used as a carrier system in the administration of cytostatic drugs, their advantage is to be found in their depot function, either intracellularly in cells such as Kupffer cells or extracellularly during prolonged presence in the circulation, rather than in their uptake by tumour cells per se. In our model system, absolutely no uptake was observed of liposomal contents by tumor cells localized in the liver sinusoids in direct contact with the blood compartment. Modest uptake by hepatocytes and, more abundantly, by Kupffer cells in close proximity to the tumor cells was at the same time clearly manifest. When considering liposomes as a circulating drug depot, one should recognize that attempts to prolong liposomal circulation time by means of blocking reticuloendothelial system activity may invoke adverse side effects. Although we and others have observed that even such drastic blockade as caused by lanthanides is transient and does not seem to cause any permanent damage (20, 27), too little is known about the functioning tissue macrophages to assume that such treatment is essentially harmless.

Finally, our results call for caution in tumor model studies with liposomes, because the administration of a moderate dose of tumor cells may profoundly influence in vivo clearance kinetics and disposition of liposomes.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 3. Electron micrograph of the liver of a normal mouse given an injection of liposome-encapsulated peroxidase. Details as in Fig. 1. Peroxidase reaction product is present in vacuoles in the cytoplasm of the Kupffer cell (kc). h, hepatocyte; s, sinusoid. Bar, 2.5 μm.
Fig. 4. Electron micrograph of the liver of a 6-day tumor mouse. Details as in Fig. 2. Peroxidase reaction product is present in vacuoles in the cytoplasm of the Kupffer cell (kc). Tumor cells (tc) are devoid of peroxidase activity. Bar, 2.5 μm.
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