Possible Tumor Localization of Tc-99m-Labeled Liposomes: Effects of Lipid Composition, Charge, and Liposome Size

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The possible in vivo distribution of liposomes after they have been directly labeled with Tc-99m has been studied in rats bearing the Walker 256 carcinoma. The importance of lipid composition, charge, and size of liposome were studied with respect to possible tumor-localizing properties. Tumor uptake was best with small, fluid-membrane, negatively charged liposomes, as indicated by the distribution of the Tc-99m label. The uptake was visualized on scintigrams.

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Liposomes (phospholipid vesicles) have been studied widely as potential carriers of drugs (*1,2*) and emphasis has been laid particularly on antitumor substances. Their relative distributions between normal and neoplastic tissue is obviously of vital importance. It has been suggested that this may be favorably altered by coating the liposomes with antibody to specific target cells (*3*), but there are other, possibly simpler, ways of altering their properties in the hope of achieving tumor localization. For example, the charge (*4*) or charge density on the liposome surface may be altered; the fluidity of the membrane may be varied by using phospholipids with high or low transition temperatures, or by using different proportions of cholesterol in the membrane structure. Also, liposome size may be reduced by prolonging the time the liposomes are sonicated during their preparation (*4,5*). We have studied the effects of some of these changes using a technetium-99m label attached to the liposome, and the tagging has enabled us to follow visually the possible distribution of liposomally associated label in vivo.

MATERIALS AND METHODS

Liposomes were formed from the following lipid compositions (molar ratios in brackets): at 37°C, phosphatidylcholine:cholesterol:dicetylphosphate (7:2:1), (77:22:1), and (5:5:1); phosphatidylcholine:cholesterol:phosphatidic acid (7:2:1); phosphatidylcholine:cholesterol:stearylamine (7:2:1); phosphatidylcholine:cholesterol (8:2); and (above 50°C) dipalmitoyl-phosphatidylcholine:cholesterol:dicetylphosphate (7:2:1).

Liposomes were obtained after addition of aqueous phase to membrane films formed after rotary evaporation of chloroform:methanol (1:1 v/v) solutions of the lipid mixtures at the specified temperatures, under reduced pressure. Initially they were shaken by hand in 0.9% NaCl solution to give a 20% (w/v) lipid suspension. (The hand-shaken liposomes are referred to in Fig. 3.) Subsequently, later batches were sonicated, surrounded by an ice bath, using a 160-watt sonicator and a 1-cm-diameter titanium probe for 20 bursts (6 μm peak to peak) of 30 sec, with 30 sec cooling between. In one case a shorter sonication time was used (see Fig. 3).

The liposomes were labeled with technetium-99m using a stannous chloride labeling method (*4*). Liposomes (600 mg lipid) in 3 ml were mixed with 0.5 ml of a sterile solution of 3 m*M* $SnCl_2$, previously prepared in oxygen-free water and stored under an atmosphere of nitrogen at 4°C. Five to ten mCi of sodium pertechnetate in 2.5 ml of 0.9% NaCl solution was added immediately, vigorously shaken, and left to stand for at least 15 min before use. The

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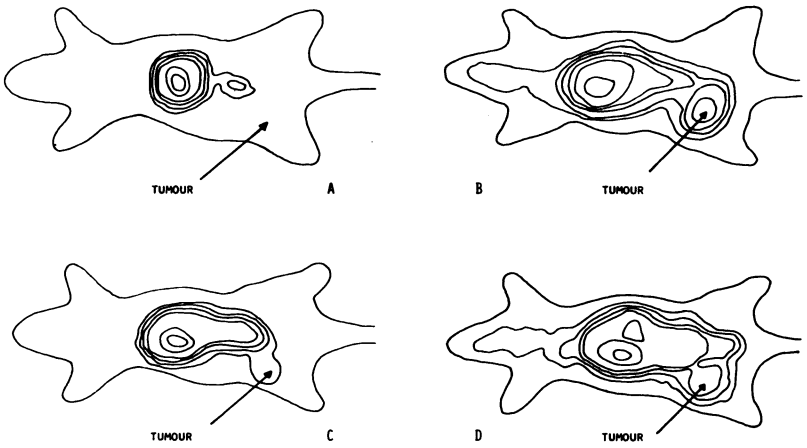

FIG. 1. Computer-drawn contour distribution pattern of Tc-99m label associated with (A) stannous colloid, (B) anionic, (C) neutral and (D) cationic liposomes 26 hr after i.v. injection into rats bearing the Walker 256 carcinoma, showing visually the tumor localization of Tc-99m label. Anionic lipid composition—phosphatidylcholine:cholesterol:dicetylphosphate (7:2:1). Neutral lipid composition—phosphatidylcholine:cholesterol (8:2). Cationic lipid composition — phosphatidylcholine:cholesterol:stearylamine (7:2:1). Dose of lipid is 80 mg, carrying about 1 mCi of technetium per rat. Liposomes were sonicated for a total of 10 min as described in the text. Contour lines are comparable.

labeling efficiency was greater than 97%, as shown by dialysis. Electron-microscope observation of negatively stained preparations showed that there was no contamination with technetium stannous colloid, which would normally be formed under these conditions in the *absence* of the lipid (*6*). Prolonged dialysis (30 hr at 37°C) showed that the attached label was firmly bound to the liposomes. Because of the low contamination of the preparations with free technetium, no attempt was made to remove it. Brief reports of the distribution of such Tc-99m-labeled liposomes in normal and tumor-bearing rats have been made (*4,7*).

Tumors for these studies were obtained by subcutaneously implanting 4 million viable Walker 256 carcinoma cells into the right inguinal region of Wistar rats (200–250 g). These cells were obtained from an ascites form of the tumor. Six days after implantation, Tc-99m-labeled liposomes (80 mg lipid, or 20 mg where stated, in 0.8 ml) were injected through the tail vein of each rat. Groups of animals were also injected with either free sodium pertechnetate or technetium stannous colloid, prepared from stannous chloride and sodium pertechnetate.

At various times between 0 and 48 hr after injection, animals were anesthetized and the distribution of radioactivity determined scintigraphically by placing them individually on the parallel-hole collimator of a gamma camera, whose output was linked to a computerized data-processing unit. Animals were then killed by cervical dislocation, and tissues and organs removed, washed, weighed, and radioassayed using an automatic scintillation counter.

Unless otherwise noted, measurements of tissue radioactivity distribution were usually made at approximately 24 hr after injection of liposomes. In the great majority of animals, scanning was carried out immediately before removal of tissue.

RESULTS

The effect of liposome charge on possible tumor localization of the Tc-99m-labeled liposomes can be seen in the isocontour pictures from four rats (Fig. 1). With all liposome preparations and the stannous colloid, most of the radioactivity is concentrated in the liver, which is readily visible in the scans, but the tumor is clearly seen in rats injected with anionic liposomes (B) and is also discernible in rats receiving cationic liposomes (D). By comparison,

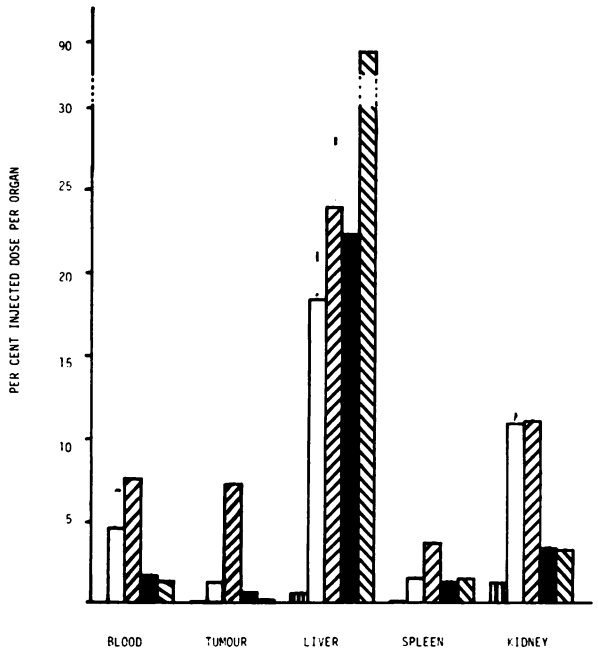

FIG. 2. Organ distribution of radioactivity at 26–28 hr after i.v. injection of Tc-99m carried in: ▥ free sodium pertechnetate (two rats); ■ neutral liposomes (four rats, 80 mg of lipid each); ▨ anionic liposomes (12 rats, 80 mg of lipid each); □ cationic liposomes (nine rats, 80 mg of lipid each); ▧ stannous colloid (four rats). Liposome composition as described in Fig. 1. Means given. Blood values are for total blood volume. Mean size of tumors ± one standard deviation = 2.8 g ± 1.5 g.

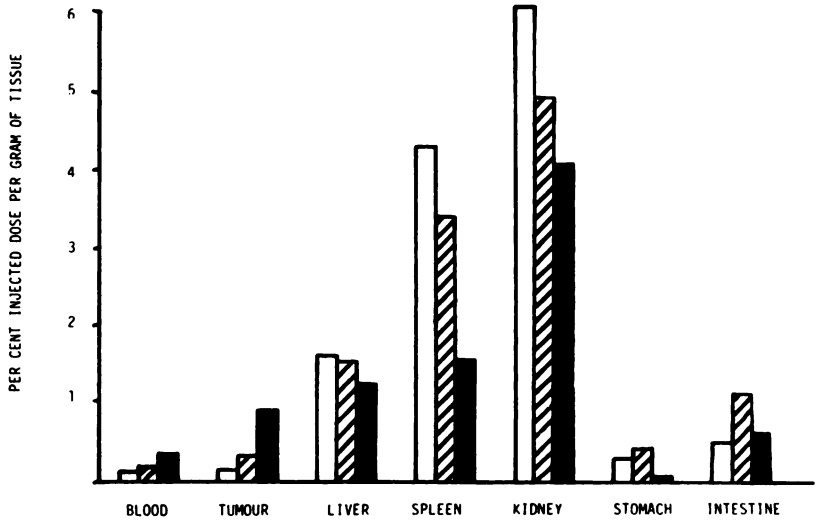

FIG. 3. Effect of sonication (liposome size) on uptake of Tc-99m label associated with phosphatidylcholine:cholesterol: dicetylphosphate (7:2:1) liposomes (anionic) 24 hr after administration. □ Hand shaken; ▨ 2 min (4 × 30 sec) sonication; ■ 10 min (20 × 30 sec) sonication. Average of two rats. Dose of lipid 20 mg and approximately 1 mCi of technetium per rat. Mean size of tumors ± one standard deviation 3.0 g ± 0.7 g.

tumor uptake is poor with neutral liposomes (C), and essentially absent with the Tc-stannous colloid (A).

The tissue distribution indicated by radioassay of individual organs and tissues is shown in more detail in Fig. 2. The figures for distribution of Tc-99m label agree with the computer-drawn pictures. The radioactivity is mainly concentrated in the liver, whereas in the tumor, radioactivity associated with anionic liposome was found to accumulate to a greater extent than that observed when cationic liposomes were used: 7.29 ± 4.63 compared with 1.29 ± 0.83, expressed as a percentage of injected radioactivity per total tumor mass. Technetium-99m-labeled neutral liposomes showed poor concentration in the tumor, and labeled stannous colloid was even worse (0.28 ± 0.002%). It is well known that free pertechnetate normally localizes in the thyroid, stomach, and kidney after i.v. administration and is excreted rapidly in the urine (*8*), but it is not known to localize in this tumor to any marked extent. At 24 hr we found negligible quantities of free pertechnetate in blood, liver, spleen, and tumor (see Fig. 2).

For the anionic liposomes the effect of increased sonication time (reduction in liposome size) on tissue distribution of radioactivity can be seen in Fig. 3. Compared with the larger sizes, smaller liposomes show greater persistence of radioactivity in the blood, with decreased liver and spleen radioactivity and a marked reduction in the kidney. Most noticeably, "well-sonicated" anionic liposomes show a sevenfold tumor uptake as compared with the "hand-shaken" product (Fig. 3). This effect was also seen with cationic liposomes, but was much less marked (data not shown). For small anionic liposomes, the blood clearance of the technetium label can be seen in Table 1. These figures represent a fall from 100% to 1–3% over 24 hr. The tumor localization of the label is also recorded in Table 1. This takes place within a few hours after injection and persists at a relatively constant value over 24 hr; then by 48 hr there is very little radioactivity in the tumor. The tumor-to-blood ratio shows the greatest observed difference at 24 hr (Table 1).

Table 2 summarizes the investigations of the tumor-localizing properties of technetium associated with well-sonicated anionic liposomes of five different lipid compositions.

Preparation (a) showed greatest tumor uptake of the label, and other preparations can be compared with it.

Preparation (b), with approximately twice the molar proportion of cholesterol, gave decreased tumor uptake.

Preparation (c), with one-tenth the charge density, showed some reduction in tumor uptake.

Preparation (d), in which a lipid of high transition temperature (dipalmitoylphosphatidylcholine) was substituted for one that was more fluid (egg phosphatidylcholine), showed almost no tumor uptake.

Preparation (e)—in which the negatively charged dicetylphosphate was replaced with a lipid of similar charge, phosphatidic acid—showed the same level of tumor uptake as preparation (a).

Preparations (f), (g), and (h) give comparable results for cationic liposomes, neutral liposomes, and technetium-stannous colloid.

DISCUSSION

The use of gamma-emitting nuclides to follow liposomal distribution has been the subject of several papers (*4,5,7,9,10*), but in only four has the

TABLE 1. TIME COURSE SHOWING BLOOD CLEARANCE, TUMOR LOCALIZATION AND TUMOR-TO-BLOOD RATIO OF Tc-99m (RADIOACTIVITY/g) FOLLOWING INJECTION OF Tc-99m-LABELED LIPOSOMES

Time after injection	% injected dose of radioactivity per gram of tissue mean (and range)			
	Blood	Tumor	Tumor-corrected‡	Tumor / blood ratio
*15 min	11.83	1.83	0	0.15
30 min	7.54	1.13	0	0.15
1 hr	5.48	2.27	1.42	0.41
2 hr	4.54	2.64	1.94	0.58
24 hr	0.30	1.65	1.60	5.50
48 hr	0.13	0.33	0.31	2.54
†5 min	16.94 (16.45–17.70)	0.35 (0.33–0.37)	0	0.02 (0.019– 0.022)
4 hr	5.36 (1.46– 9.18)	1.10 (0.36–1.56)	1.00 (0.33–1.55)	0.28 (0.14 – 0.31)
10 hr	1.59 (1.11– 2.06)	1.50 (1.25–1.77)	1.47 (1.21–1.76)	1.10 (0.58 – 1.61)
24 hr	0.13 (0.10– 0.15)	1.33 (0.97–1.46)	1.24 (0.97–1.46)	10.02 (6.93 –14.60)

Anionic liposomes (phosphatidylcholine:cholesterol:phosphatidic acid, 7:2:1 molar ratio); 20 mg lipid and a dose of 200–1000 μCi per rat. Sonication time for liposomes 10 min, as described in Materials and Methods.

* Experiment 1—six rats.

† Experiment 2—four groups of three rats; values = mean and range.

‡ An assumption was made that at early time intervals (5 min† or 15 min*) radioactivity associated with tumor was entirely due to blood content and no concentration by tumor was occurring.

Mean weight of tumors ± s.d. 2.8 ± 2.1 g* 9.6 ± 2.4 g†.

influence of liposome charge and size been studied with respect to their possible tumor localization (*4,5,7,10*). We find that liposome composition and structure can profoundly alter tumor concentration of radioactivity when using Tc-99m-labeled liposomes. With liposomes of some lipid compositions the concentration was sufficient to allow imaging of the experimental tumors with a gamma camera. In terms of tumor uptake of technetium, the best lipid composition we used here—preparation (a) (Table 1)—had a net anionic charge and a high charge density (10% molar proportion of dicetylphosphate), but the particular lipid producing the charge seemed in these studies to be unimportant (e.g., replacement of dicetylphosphate by phosphatidic acid). Only very small Tc-99m-labeled anionic liposomes resulted in uptake of label to any marked extent in the tumor, and it may be that those with

TABLE 2. LOCALIZATION OF Tc-99m LABEL ASSOCIATED WITH LIPOSOMES OF DIFFERENT LIPID COMPOSITIONS IN THE WALKER 256 CARCINOMA 24 HR AFTER I.V. INJECTION

Preparation	Lipid composition (molar ratio)	% injected dose per gram of tumor (mean and range)	No. of rats (p values)
(a)	Anionic PC:C:DCP* (7:2:1)	1.32 (0.54–1.81)	12 (—)
(b)	Anionic PC:C:DCP (5:5:1)	0.42 (0.20–0.36)	2
(c)	Anionic (PC:C:DCP (77:22:1)	0.69 (0.68–0.70)	2
(d)	Anionic DPPC:C:DCP (7:2:1)	0.17 (0.12–0.22)	2
(e)	Anionic PC:C:PA (7:2:1)	1.32 (0.92–1.65)	6 (NS)
(f)	Cationic PC:C:ST (7:2:1)	0.42 (0.35–0.47)	12 (0.001)
(g)	Neutral PC:C (8:2)	0.29 (0.21–0.36)	4
(h)	Stannous colloid	0.11 (0.07–0.13)	4

Means and ranges given, statistical data, Student's t test, comparing samples with preparation (a). Liposomes sonicated for total of 10 min as described in text. Dose as in Fig. 1. Size of tumors (grams) = 2.8 ± 1.4 (mean ± s.d.).

* PC—Phosphatidylcholine; C—Cholesterol; DCP—Dicetylphosphate; DPPC—Dipalmitoylphosphatidylcholine; PA—Phosphatidic acid; SA—Stearylamine; NS—Not significant.

a unilamellar structure will prove to be most suitable for tumor localization.

High cholesterol content and decreased fluidity of the liposomal membrane appeared to discourage uptake of the label. It may be that these observations are related because increased cholesterol content also reduces membrane fluidity of certain lipid species (*11*).

In the work reported here, most observations were made at approximately 24 hr as a compromise between maximum concentration in the tumor (which occurred at approximately 10 hr) and the relatively stable, low levels of radioactivity in the blood at the later time. We have previously shown that at 24 hr approximately 40–50% of the liposomal label has been excreted (*4*).

Figure 2 shows in histogram form the proportion of radioactivity concentrated in the experimental tumor. The greatest concentration of radioactivity was obtained with Tc-99m-labeled anionic liposomes [preparation (a), Fig. 2]. This preparation also had the highest levels of radioactivity in the blood (Fig. 2). We considered the possible contribution of blood-to-tumor radioactivity in the rats. In early gamma camera pictures of the rats (up to 3 hr) when most of the radioactivity was in the blood, the tumor was not discernible, but in later pictures (24 hr) tumor imaging was greatly improved, since then the blood levels had fallen to about 1.0% of the initial postinjection value (*7*) (see Table 1). It appears that blood constitutes some 2–15% by weight of the tumor tissue, depending on the size of the tumor. Experiment 1 (Table 1) demonstrates that the tumor has a blood content of 15%, assessed at the 15-min time interval, while Experiment 2 (Table 1) shows a blood content of 2% at 5 min. These differences may indicate the greater proportion of necrotic tissue in the larger tumors of Experiment 2. The latter experiment also shows a 500-fold change in tumor-to-blood ratio in 24 hr (Table 1), and it is then that this ratio reaches maximum and the tumor may be best observed scintigraphically in our experiments.

Figure 2 shows the relatively high levels of radioactivity associated with the kidneys. Since the free pertechnetate ion is normally excreted through the kidneys (*8*), it is possible that the kidney radioactivity is not associated with liposomes; this seems all the more likely since by 24 hr the rats have lost 50% of the administered radioactivity (*4*).

We have no information about the process by which the liposomal label becomes associated with the experimental tumor. The only other papers dealing with possible tumor localization of liposomes in animal models (*4,5,7,10*) likewise provide no information on this point. It is difficult to explain how the differences in lipid composition, charge, and size of liposome influence tumor localization of the label. The importance of liposome charge on the eventual concentration of Tc-99m in the tumors may be related to properties of the tumor-cell surface (*12,13*), or to the effects of liposomal interactions with plasma components (*14*) before localization. Decreased membrane fluidity and increased cholesterol content of the liposomes appear to reduce tumor uptake of label, and this may be related to the observation of Johnson (*15*) that macrophages are unable to ingest liposomes containing a high molar ratio of cholesterol.

The effects of liposome size on tumor localization of label (*4,5,7*) may be related to the method of interaction of liposome with tumor cells. Small, unilamellar, anionic liposomes have been reported to fuse with cells in culture (*11*) and may, because of their size, be pinocytosed rather than phagocytosed. The greater uptake of anionic liposome label may also be a measure of the increased half-time of such liposomes in the blood. This is supported by the sonication studies, which show that increased uptake of Tc-99m is found with small, well-sonicated liposomes.

Although the technetium label can be identified in the tumor, it is not absolutely clear whether this represents liposome localization or whether the technetium is associated in some other way with the tumor. Our data, however, are in total agreement with those of Neerunjun et al. (*5*) who used liposomes labeled with In-111 bleomycin. They too found that well-sonicated, negatively charged liposomes are best localized in the animal tumors they studied.

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