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Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors

(phospholipid vesicles/drug delivery systems/cancer therapy/glycolipids)

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ABSTRACT **The rapid clearance of circulating liposomes from the bloodstream, coupled with their high uptake by liver and spleen, has thus far been an obstacle to any attempts at targeting to tumors. We have assessed the impact of liposome composition on their clearance from the circulation in normal and tumor-bearing mice and on their uptake by tumors and various normal tissues. By selective changes in lipid composition, while maintaining a mean particle diameter of ≈100 nm, we have achieved up to a 60-fold increase in the fraction of recovered dose present in blood 24 hr after i.v. injection. Concomitantly, there was a decrease by a factor of 4 of the recovered dose localizing in the liver and spleen, the major organs of the reticuloendothelial system. Parallel experiments in tumor-bearing mice demonstrated a 25-fold increase of the liposome concentration in the tumor when formulations with long and short blood residence time were compared. The most favorable results were obtained with liposomes containing a small molar fraction of a negatively charged glycolipid, such as monosialoganglioside or phosphatidylinositol, and a solid-phase neutral phospholipid as the bulk component. The biodistribution of such formulations is of considerable therapeutic potential in cancer for increasing the concentration of cytotoxic agents in tumors while minimizing the likelihood of toxicity to the reticuloendothelial system.**

Liposome encapsulation has been proven useful for reducing the toxicity of certain drugs (1–6) as well as for enhancing the efficacy of macrophage-activating factors (7, 8) and drugs directed against parasites residing within the reticuloendothelial system (RES) (9–12). The propensity of the RES to remove liposomes from the circulation has thus far limited the prospect of targeting liposomes to tissues other than liver, spleen, and lung (13–15).

Designing liposomes with prolonged circulation time would require a reduction of the rate of their clearance by the RES and of the leakage of liposome contents in the bloodstream. Our strategy to achieve these goals was based on the following prior observations. (*i*) The inclusion of cholesterol (Chol) and solid-phase phospholipids such as sphingomyelin and distearoyl phosphatidylcholine has been shown to increase liposome stability in plasma as determined by the degree of retention of various liposome-encapsulated markers *in vitro* (16–18). (*ii*) The same solid-phase phospholipids (sphingomyelin or distearoyl phosphatidylcholine) in the form of small sonicated liposomes are cleared more slowly after intravenous injection than those made with fluid phospholipids such as egg yolk phosphatidylcholine (PtdCho) (19–22). (*iii*) The inclusion of certain gangliosides, which confer to the liposome surface a negative charge and increased hydrophilicity, synergizes with Chol to enhance liposome stability in plasma (23) and prolongs liposome half-life in blood with a concomitant decrease in liver and spleen uptake (24). In addition, inclusion of phosphatidylinositol (PtdIns) in liposomes (25), or hydrophilic coating of polystyrene particles with polymeric glycols (26) results in significantly enhanced blood half-lives. (*iv*) Small unilamellar liposomes exhibit a slower clearance from the circulation when compared to larger multilamellar ones of the same composition (22, 27).

In an earlier communication (28), we reported preliminary findings on the ability of monosialoganglioside (GM1), sulfatides (galactocerebroside sulfate), and PtdIns to prolong the circulation time of liposomes composed of egg PtdCho, as compared to a commonly used acidic phospholipid, phosphatidylglycerol (PtdGro). In this study, we report on a pronounced increase in blood residence time with a parallel decrease in uptake by liver and spleen for liposomes composed of certain glycolipids in combination with solid-phase phospholipids and Chol. Our results also reveal a strong correlation between residence time in blood and uptake by implanted tumors in mice.

MATERIALS AND METHODS

The sources of materials used in this study are as follows: Egg PtdCho and PtdGro, bovine brain phosphatidylserine (PtdSer) and sphingomyelin, bovine liver PtdIns, hydrogenated soybean PtdIns, dipalmitoyl PtdGro, dioleoyl PtdCho, and distearoyl phosphatidylcholine were all from Avanti Polar Lipids; *dl*-α-tocopherol, trisialoganglioside (GT1), and globosides (GL4) were from Supelco (Bellefonte, PA); Chol, cholesterolsulfate, and 8-hydroxyquinoline (oxine) sulfate were from Sigma; bovine brain sulfatides and GM1 were from either Supelco or Sigma; hydrogenated soy PtdCho was from Nattermann Chemie (Cologne, F.R.G.) through American Lecithin (Atlanta); ^{67}Ga citrate was from New England Nuclear; deferoxamine mesylate was from CIBA–Geigy; and the acetate form of AG1X2 resin was from Bio-Rad.

Liposome Preparation. Liposomes were prepared by thin lipid film hydration (29) with an isotonic solution of NaCl (pH range, 6.0–7.0) containing deferoxamine mesylate (25–35 mM). The ratio of neutral phospholipid to Chol was 2:1, and, when present, the negatively charged lipid was 10–11% of the neutral phospholipid. α-Tocopherol was included at a ratio of 1% of neutral phospholipid. Multilamellar vesicles were formed by Vortex mixing the lipid dispersions at room temperature or, in the case of high-phase-transition lipids, at 55°C for ≈30 min. Liposomes with homogenous size distribution were obtained by extrusion (29) of the Vortex mixed preparation through double polycarbonate membranes of 0.08- or 0.05-μm pore size (Nucleopore), using an extruder device from Lipex Biomembranes (Vancouver, BC, Can-

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Abbreviations: Chol, cholesterol; PtdGro, phosphatidylglycerol; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; RES, reticuloendothelial system.

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ada). When high-phase-transition phospholipids were used, the extruder device was preheated to 55°C.

Liposome size was determined by dynamic light scattering with a submicron particle analyzer (Coulter Electronics, Hialeah, FL, model N4; or Nicomp model 200, Hiac/Royco, Silver Spring, MD). The mean size obtained was in the range of 70–120 nm, with a standard deviation not larger than 30% of the Gaussian mean. In cases in which a second vesicle population of larger size was present, or the Gaussian mean was 130 nm or greater, the liposomes were repeatedly reextruded and/or spun for 20 min in a microcentrifuge at 10,000 $\times$ g. This procedure was effective for eliminating any vesicles of 0.5 μm diameter or larger and for achieving the desired size distribution. Unencapsulated deferoxamine was removed by gel filtration of the liposome suspension through Sephadex G-50 or G-75 (Sigma). Phospholipid concentration was determined by a phosphate assay (30). Liposomes were stored either under argon or in vacuum-sealed tubes at 5°C and tested within 1 week after preparation.

Labeling Procedure. The method of labeling preformed liposomes with ^{67}Ga-labeled deferoxamine has been described in detail (31). Briefly, between 100 and 200 μCi of ^{67}Ga citrate was adjusted to a vol of 0.5–1.0 ml with a solution (1 mg/ml) of oxine sulfate in 0.9% NaCl. The solution was incubated for 1 hr at 50°C. Liposome suspensions were incubated overnight at 4°C with 1–2 μCi of ^{67}Ga-labeled oxine per μmol of phospholipid. This results in formation of ^{67}Ga-labeled deferoxamine complex in the aqueous interior of liposomes (31). Removal of unencapsulated ^{67}Ga-labeled oxine was achieved by passing the liposome suspension through an anion-exchange resin (AG1X2, acetate form, 200–400 mesh).

Biodistribution Studies in Normal Mice. Two- to 3-month-old Swiss–Webster (SW) female mice (Simonsen Laboratories, Gilroy, CA) were injected i.v. with liposomes containing 1 μmol of phospholipid per mouse. Each liposome formulation was tested in three to six mice for each time point. Retroorbital bleeding (≈1 ml) under ether anesthesia, sacrifice by cervical dislocation, and complete animal dissection followed 1, 4, and 24 hr after injection. Encapsulated ^{67}Ga-labeled deferoxamine served as a marker to determine the distribution of liposomes in the various tissues. All tissues were weighed and their radioactivity was quantitated in a γ-counter (Gamma 8000, Beckman) using integral counting above 10 keV. Blood volume and correction factors for the blood content of various tissues were determined in age- and sex-matched SW mice by examining the tissue distribution of ^{111}In-oxine-labeled syngeneic erythrocytes 10–15 min after i.v. injection. Labeling of erythrocytes was performed as described (32). Total recovered dose and percentages of recovered and injected dose per organ and per g of tissue were calculated.

Biodistribution Studies in Tumor-Bearing Mice. Two- to 3-month-old BALB/c female mice (Simonsen) were inoculated i.m. into the hind leg with 10^6 tumor cells of the J6456 lymphoma (33). Mice were tested 2–3 weeks after tumor inoculation when the local tumor implants weighed between 0.5 and 2 g. The liver weights were within the normal range, although microscopic dissemination of the tumor to the liver is often present at this advanced stage (33). Phospholipid dose, route of injection, and other details of the biodistribution analysis were as for normal mice. Blood volume and correction factors for blood content of tissues, including the tumor, were obtained in female BALB/c mice bearing J6456 tumors of approximately the same size and following the same procedure as for normal mice.

RESULTS

Tissue Distribution in Normal Mice. To facilitate a comprehensive analysis of liposome distribution, we have presented the tissue distribution data of the following three compartments: (*i*) blood; (*ii*) liver and spleen (used as an approximation of the RES); (*iii*) carcass and skin (consisting of all skeletal muscles, bones, skin, and appendages). The fate of

Table 1. Liposome distribution in blood, liver and spleen (RES), and carcass and skin (C + S), 24 hr after injection

Liposome composition, molar ratio	Tissue uptake ratio		% injected dose (SD)			
	RES/ blood	RES/ C + S	Blood	RES	C+S	Total body
GTI–PtdCho–Chol (1:9:5)	205.4	9.2	0.3 (0.0)	51.3 (7.7)	5.6 (0.9)	58.2 (8.8)
PtdSer–PtdCho–Chol (1:10:5)	188.0	9.0	0.2 (0.0)	37.6 (1.2)	4.2 (1.1)	43.3 (0.6)
GL4–PtdCho–Chol (1:9:5)	127.0	3.7	0.2 (0.1)	25.4 (4.1)	6.8 (4.7)	34.3 (9.2)
PtdGro–PtdCho–Chol (1:9:5)	123.3	3.6	0.3 (0.1)	37.0 (3.6)	10.2 (2.9)	49.8 (3.9)
Distearoyl phosphatidylcholine–Chol (10:5)	68.1	3.3	0.7 (0.4)	47.7 (4.9)	14.1 (0.9)	67.6 (4.2)
Sulf–PtdCho–Chol (1:10:5)	47.0	2.6	0.5 (0.3)	23.5 (1.1)	9.0 (1.9)	41.6 (2.5)
Sphingomyelin–PtdCho–Chol (8:2:5)	33.0	2.2	0.4 (0.1)	13.2 (3.3)	5.9 (3.2)	21.9 (1.4)
HPtdIns–dioleyl phosphatidylcholine–Chol (1:10:5)	15.5	3.4	2.0 (0.5)	31.0 (3.5)	9.0 (1.4)	46.3 (1.8)
CS–distearoyl phosphatidylcholine–Chol (1:10:5)	10.9	2.9	3.2 (1.8)	34.9 (1.9)	12.0 (3.6)	55.6 (6.0)
PtdCho–Chol (10:5)	8.6	2.2	3.0 (0.7)	25.8 (1.3)	11.5 (2.3)	44.4 (2.9)
GM1–sphingomyelin–PtdCho–Chol (1:8:2:5)	7.4	1.7	1.0 (0.2)	7.4 (1.9)	4.3 (0.7)	14.4 (1.9)
Dipalmitoyl phosphatidylglycerol–distearoyl phosphatidylcholine–Chol (1:10:5)	4.8	2.1	6.5 (0.8)	31.2 (3.2)	15.2 (0.9)	59.9 (5.5)
PtdGro–distearoyl phosphatidylcholine–Chol (1:10:5)	4.5	1.2	1.2 (0.8)	5.4 (0.6)	4.4 (0.9)	12.9 (1.4)
PtdIns–PtdCho–Chol (1:9:5)	3.6	1.3	4.1 (1.3)	14.8 (3.4)	11.8 (2.3)	37.4 (6.3)
Sulf–distearoyl phosphatidylcholine–Chol (1:10:5)	3.3	1.5	7.6 (3.2)	24.7 (4.4)	16.5 (2.0)	57.5 (3.7)
GM1–PtdCho–Chol (1:9:5)	3.0	1.5	6.0 (0.4)	17.9 (4.4)	12.1 (1.7)	42.3 (1.5)
HPtdIns–distearoyl phosphatidylcholine–Chol (1:10:5)	2.2	1.8	12.2 (3.4)	27.0 (3.5)	15.4 (3.7)	61.6 (4.0)
GM1–spingomyelin–distearoyl phosphatidylcholine–Chol (1:8:2:5)	2.1	1.3	7.1 (1.6)	15.1 (7.1)	11.6 (1.8)	36.9 (5.3)
HPtdIns–HPtdCho–Chol (1:10:5)	1.9	1.1	9.8 (0.9)	18.3 (5.9)	16.3 (2.9)	51.1 (6.7)
GM1–distearoyl phosphatidylcholine–Chol (1:10:5)	1.0	0.7	16.0 (3.8)	16.5 (1.4)	23.0 (1.7)	66.3 (4.2)

Sulf, bovine brain sulfatide; HPtdIns, hydrogenated soybean PtdIns; HPtdCho, hydrogenated soybean PtdCho; CS, Chol sulfate. Ratio values were calculated by dividing the percent of injected dose in RES (liver and spleen) by the percent of injected dose in blood or C + S (carcass and skin) obtained 24 hr after i.v. injection of 1 μmol of phospholipid per mouse (SW female). Values for the average uptake by all other organs can be obtained by adding blood, RES, and carcass and skin and subtracting from the total body figures.

liposome contents *in vivo* was followed by using a high-affinity ^{67}Ga-labeled deferoxamine complex. In a previous report (31), we showed that this label is very appropriate for tracing liposomes, given its fast renal excretion rate and the low likelihood of metal translocation to transferrin if the liposome contents are released in the extracellular space.

The relationship between blood clearance and uptake of liposomes by various tissues is shown in Table 1. The results of liposome distribution, obtained with 20 different formulations at 24 hr after injection, are expressed by the RES/blood and RES/carcass and skin ratios, together with the respective percentages of injected dose and total body recoveries. Clearly, even small changes in formulation have a dramatic impact on the *in vivo* behavior of liposomes, resulting in a >200-fold difference in the RES/blood ratio. The RES/carcass and skin ratio was also considerably reduced by changes in liposome composition, in close parallel to the decrease in the RES/blood ratio. The pattern emerging with all formulations tested indicates that as the uptake by the RES decreases, there is a concomitant increase in the liposome blood pool and in the fraction of dose accumulating in carcass and skin. The recovered dose in the blood was ≈60-fold higher for GM1–distearoyl phosphatidylcholine–Chol liposomes compared to those containing GT1 (or PtdSer)–PtdCho–Chol, while the dose recovered in the RES compartment was lower by a factor of ≈4, and that in the carcass and skin compartment increased 3.6-fold.

Table 1 also shows that use of lipids conferring a rigid bilayer, as in the cases of distearoyl phosphatidylcholine and hydrogenated soybean PtdIns, enhances liposome circulation time and that a small molar fraction of negatively charged lipid can have very different effects depending on the chemical composition. Among the fluid formulations, GM1- and PtdIns-containing liposomes gave relatively low ratios. Among the solid formulations, GM1 and hydrogenated soybean PtdIns-containing liposomes gave the lowest ratios, with nearly as much of the dose recovered in blood as in liver and spleen.¶

The comparative distribution of four representative liposome compositions at different times after injection is shown in Fig. 1. The GM1–distearoyl phosphatidylcholine composition exhibits much higher levels in blood at all time points when compared to the other three compositions (Fig. 1*A*). By contrast, uptake of GM1–distearoyl phosphatidylcholine by liver and spleen is diminished when compared to the other formulations (Fig. 1*B*). In carcass and skin (Fig. 1*C*), as well as in other organs such as gut and kidneys (data not shown), the GM1–distearoyl phosphatidylcholine composition shows a gradually increasing uptake with time and reaches a significantly higher level than the other formulations at the 24-hr time point. Fig. 1 also shows the following points: The differences in the blood levels between the various formulations were mainly established within the first hour after injection (Fig. 1*A*); the localization of GM1–distearoyl phosphatidylcholine liposomes in carcass and skin (Fig. 1*C*) is a late phenomenon occurring mainly between 4 and 24 hr.

To determine whether a significant fraction of liposomes circulate in association with blood cells, we separated plasma from blood cells by centrifugation at various time points after liposome injection. With the two formulations examined

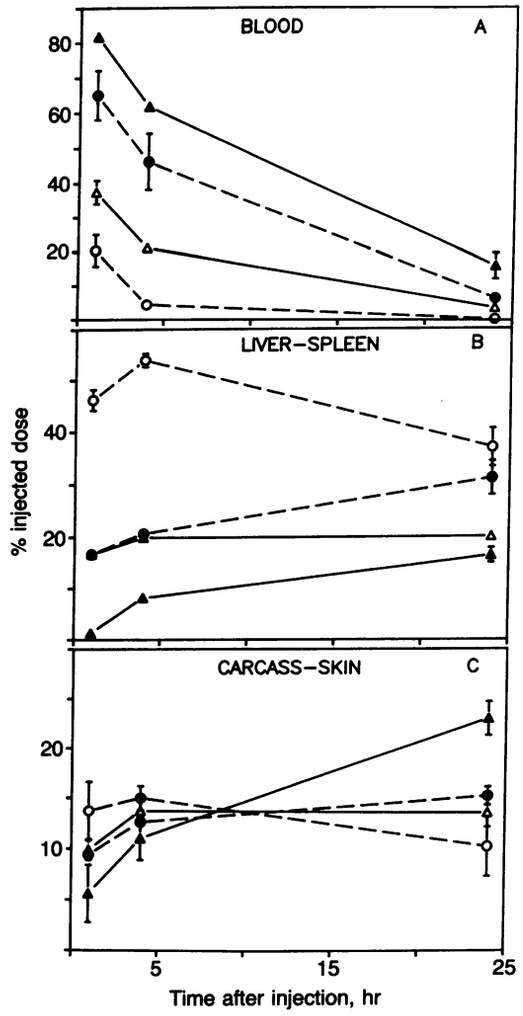

FIG. 1. Compartmental distribution of liposomes versus time after injection: a study with four representative liposome compositions. *A*, *B*, and *C* represent different tissue compartments as indicated. ○, PtdGro–PtdCho–Chol; △, GM1–PtdCho–Chol; ●, dipalmitoyl PtdGro–distearoyl phosphatidylcholine–Chol; ▲, GM1–distearoyl phosphatidylcholine–Chol.

(GM1–distearoyl phosphatidylcholine–Chol and hydrogenated soybean PtdIns–distearoyl phosphatidylcholine–Chol), >90% of the blood radioactivity was recovered in plasma, indicating that passive transport of liposomes by blood cells does not contribute significantly to the long circulation time in blood.

Tissue Distribution of Liposomes in Tumor-Bearing Mice. Some of the liposome formulations described above were tested in tumor-bearing mice, using the J6456 intramuscular tumor model. A remarkable increase in tumor uptake (up to 25-fold) was observed as liposome formulations selected for longer circulation times in normal mice were tested (Table 2). Uptake of the optimal liposome formulation (GM1–distearoyl phosphatidylcholine–Chol) reached values >5% of injected dose per g of tumor and 10% of the injected dose in the whole tumor (Fig. 2). In contrast, when ^{67}Ga-labeled deferoxamine was injected in free form, the uptake by tumors at 24 hr postinjection was <0.1% of injected dose per g. The increased liposome concentration in tumors was not related to the tumor vascular blood pool, since the values shown were obtained after correcting for the blood content of tumors of similar size. Table 2 also reveals a significant decrease in the liver/tumor ratio as liposome concentration in the tumor increases. Moreover, the comparison of the tumor/carcass

¶As also shown in Table 1, total body recovery of the liposome label varied from 12.9% to 67.6% among the different formulations, with no identifiable relationship to RES values, blood values, or their ratios. However, there was a clear association between low recoveries and the presence of phospholipids with widely different phase-transition temperatures in the same formulation (sphingomyelin and PtdCho, PtdGro and distearoyl phosphatidylcholine). This could be due to a phase separation between lipids with different phase transitions, which would produce an increase in leakage rates (34).

Table 2. Uptake of liposomes in tumor-bearing mice: Effect of liposome composition

Liposome composition	% injected dose per g of tissue (SD)			Tissue uptake ratio	
	Tumor	Liver	Carcass	Liver/tumor	Tumor/carcass
PtdGro–PtdCho–Chol	0.2 (0.0)	36.4 (6.3)	0.5 (0.1)	182.0	0.4
Sulf–PtdCho–Chol	0.8 (0.1)	13.6 (1.8)	0.5 (0.0)	17.0	1.6
Distearoyl phosphatidylcholine–Chol	2.1 (0.3)	36.5 (7.5)	0.4 (0.1)	17.4	5.3
Sulf–distearoyl phosphatidylcholine–Chol	2.1 (0.3)	32.1 (4.5)	0.4 (0.1)	15.3	5.3
CS–distearoyl phosphatidylcholine–Chol	2.5 (0.1)	29.7 (1.4)	0.3 (0.0)	11.9	8.3
GM1–PtdCho–Chol	3.5 (0.6)	20.7 (1.0)	0.5 (0.0)	5.9	7.0
Dipalmitoyl–PtdGro–distearoyl phosphatidylcholine–Chol	4.1 (1.6)	38.3 (0.5)	0.3 (0.0)	9.3	13.7
HPtdIns–distearoyl phosphatidylcholine–Chol	4.1 (1.1)	37.8 (0.4)	0.4 (0.1)	9.2	10.3
GM1–distearoyl phosphatidylcholine–Chol	5.3 (0.9)	31.7 (1.4)	0.6 (0.0)	6.0	8.8

Sulf, bovine brain sulfatide; CS, Chol sulfate; HPtdIns, hydrogenated soybean PtdIns. BALB/c female mice were inoculated with 10^6 J6456 tumor cells in the hind leg. Two to 3 weeks later, mice received one of the indicated formulations at a dose of 1 μmol of phospholipid per mouse and were sacrified 24 hr later. Average tumor weight ranged from 0.5 to 2.0 g. For molar ratio of components, see Table 1.

ratios, showing a >20-fold increase, indicates that liposome accumulation was preferentially enhanced in the tumor as opposed to a nonspecific enhancement in all body tissues.

Fig. 2 presents the compartmental distribution of GM1–distearoyl phosphatidylcholine–Chol liposomes between 4 and 48 hr after injection in mice bearing the J6456 lymphoma. Liver and spleen and tumor continued to accumulate liposomes between 4 and 24 hr, reaching a peak at 24 hr. This was followed by a slow disappearance of the label, which was slowest in the case of the tumor. At 48 hr, the tumor still retains ≈10% of the injected dose, while blood is close to the zero level. These observations indicate that a high concentration of liposomes recirculating for at least several hours after injection is required for their accumulation in tumors.

DISCUSSION

Analysis of the current literature suggests that liposomes are removed rapidly from the circulation by the phagocytic cells of the RES (13, 19, 27). This has led to the view that liposome targeting to tissues outside the RES has only limited prospects if liposomes are injected into the general circulation (35, 36). Our study shows that the uptake of liposomes by the RES can be greatly diminished, and, in such cases, other body compartments, such as implanted tumors and carcass and skin, are much more accessible to liposomes. The vesicle size range used in this study (≈100 nm) was chosen because it may theoretically allow passage through large fenestrations, such as those of sinusoidal capillaries (37), and at the same time provide a greater carrying capacity than smaller sonicated liposomes (19–21). A change in liposome composition from the most widely used (PtdGro–PtdCho–Chol) to the best one studied so far (GM1–distearoyl phosphatidylcholine–Chol) shows a 25-fold increase in liposome concentration in the tumor, as well as a 20-fold increase of the tumor/carcass ratio. Although the liver uptake was still ≈6-fold higher than that of the tumor, this ratio may be therapeutically acceptable, since the liver is the usual metabolizing organ for most chemotherapeutic agents (38). Also, it is likely that liposome concentration in the tumor could be further increased in tumors of smaller size than those used in this study because of better vascularization. An earlier study with sonicated distearoyl phosphatidylcholine–Chol liposomes showed remarkable accumulation in small implanted tumors (21), although the use of ^{111}In–nitrilotriacetic acid chelation complex as a marker makes the interpretation difficult because of the much higher background uptake of the marker itself by the tumor as compared to the ^{67}Ga-labeled deferoxamine complex used here. The differences in labeling methods and in the tumor models preclude a direct comparison of tumor uptake between earlier studies (21, 39) and those reported here.

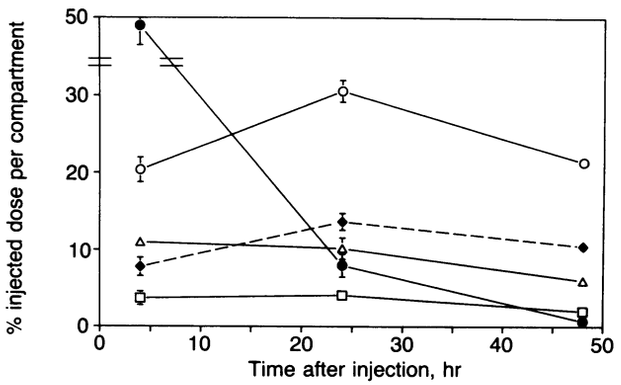

FIG. 2. Compartmental distribution of GM1–distearoyl phosphatidylcholine–Chol liposomes versus time after injection in BALB/c female mice bearing the J6456 lymphoma. ●, Blood; ○, liver and spleen; △, carcass and skin; □, other organs; ◆, tumor. In addition to the compartments described for normal mice, tumor was examined as a separate compartment. For additional details, see legend to Table 2.

The mechanism of liposome accumulation in implanted tumors and their detailed localization remains unclear at present. The permeability of tumor vasculature is generally increased as compared to normal tissues (40), although this varies among tumors. The extravasation of macromolecules occurs predominantly by convective transport. The efficiency and kinetics of this process are determined, among other factors, by the tissue capillary and basement membrane permeability and the size or molecular weight of the particle in question (41). Liposomes could extravasate through leaky endothelium by passive convective transport phenomena. Obviously, higher concentration and longer blood residence time of liposomes would result in greater efficiency of extravasation per unit volume of convective transport. It is still possible, however, that the accumulation in tumors, as well as in the carcass, is due to endocytic uptake or binding of liposomes by capillary endothelial cells. Morphological studies should clarify this point.

The results presented here define some of the parameters that prolong liposome blood residence time and reduce uptake by the RES, indicating synergistic effects between a rigid bilayer, carbohydrate-containing lipids, and a negative surface charge. These results shed new light on the mechanisms by which the last two parameters affect liposome recognition and clearance from blood. Negative surface charge has long been considered a factor contributing to decreased liposome circulation times and enhanced uptake by the RES *in vivo* (26, 42) and by cells *in vitro* (43, 44). This view may need to be reassessed since the highest levels in blood (Table 1) were obtained with GM1, followed by

hydrogenated soybean PtdIns, and, to a lesser extent, sulfatide, which all bear a negative charge.

We have considered the following physical explanation for differences in tissue distribution between liposomes containing GM1, PtdIns, and sulfatide, and most other negatively charged lipids studied so far. The various negatively charged lipids can be divided in two broad categories. PtdSer, PtdGro, and other phospholipids commonly used, such as phosphatidic acid and dicetyl phosphate, have negatively charged groups that are "exposed" to the aqueous environment. The exposed configuration may allow direct interaction between the negatively charged group on the liposome surface and either plasma proteins or cell-surface proteins, thus accelerating liposome clearance. On the other hand, in both GM1 and PtdIns, the negative charge is "shielded" by a bulky hydrophilic group. In the case of GM1, evidence on head-group conformation indicates that the carboxyl group of the sialic residue and some OH groups of the terminal galactose form an internal chelation complex (45). Therefore, the carboxyl group is oriented toward the terminal galactose residue (46) and may thus be unavailable for interactions with opsonizing proteins. Other gangliosides either lack the terminal sugar residue or contain additional molecules of sialic acid in terminal, exposed positions. Although the negatively charged sulfate group of sulfatides is not shielded by an additional sugar residue, its position on the equatorially oriented 3-OH group of galactose may be sterically hindered because of the perpendicular orientation of the sugar moieties of all glycosphingolipids to the axis of the ceramide (46). One of the possible contributions of a shielded negative charge to liposome half-life is the prevention of vesicle aggregates during preparation and storage and, perhaps, also immediately after injection. The presence of aggregates would result in rapid RES clearance, overriding other properties of the bilayer.

The experiments presented in this report indicate that long-circulating liposomes offer potential applications for drug targeting to tumors and other tissues, hitherto not feasible with commonly used liposome preparations. The most significant immediate application of the present studies is that of increasing the concentration of anticancer agents in tumors with concomitant reduction of RES toxicity. If the observations reported here can be extended to other tumor models, then this method may have the advantage of general applicability and simplicity compared with other approaches relying on recognition of specific antigens expressed by tumor cells, such as antibody conjugation to toxins, drugs, or radionuclides (47, 48).

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