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Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues

Theresa M. Allen, Christian Hansen and Jennifer Rutledge

Pharmacology Department, University of Alberta, Edmonton (Canada)

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Many of the applications of liposomes drug-delivery systems have been limited by their short circulation half-lives as a result of rapid uptake into the reticuloendothelial (mononuclear phagocyte) system. We have recently described liposomes formulations with long circulation half-lives in mice (Allen, T.M. and Chonn, A. (1987) FEBS Lett. 223, 42–46). A study of the principal factors important to the attainment of liposomes with prolonged circulation half-lives is presented in this manuscript. Liposomes with the longest circulation half-lives, in mice, had compositions which mimicked the outer leaflet of red blood cell membranes (egg phosphatidylcholine/sphingomyelin/cholesterol/ganglioside G_{M1}, molar ratio 1:1:1:0.14). Several other gangliosides and glycolipids were examined, but none could substitute for G_{M1} in their ability to prolong circulation half-lives. However, other negatively charged lipids with bulky headgroups, i.e., sulfatides and phosphatidylinositol, had some effect in prolonging circulation half-lives, but G_{M1} was clearly superior in this regard. Bilayer rigidity, imparted by sphingomyelin or other high-phase-transition lipids, acted synergistically with the negatively charged components, especially G_{M1}, in extending circulation times. Circulation half-lives of liposomes increased with decreasing size, but even larger (0.2–0.4 μm) liposomes of the optimum formulations had significantly prolonged half-lives in circulation. Uptake of liposomes into tissues other than liver and spleen increased with increasing circulation times of the liposomes for i.v. and for i.p. injections. Liposomes appeared to move from the circulation into the carcass between 6 and 24 h post-injection. Our ability to achieve significant prolongation in circulation times of liposomes makes possible a number of therapeutic applications of liposomes which, until now, have not been achievable.

Introduction

Liposomes will achieve their maximum therapeutic utility only when we find ways to keep them out of the reticuloendothelial (RE) system, allowing them to be directed to other sites in vivo. A preliminary report from our laboratory [1] has indicated that it is now possible to reduce considerably the rate and extent of uptake of liposomes into the RE system and thereby achieve significantly prolonged circulation half-lives in vivo in mice.

Previous studies from our laboratory and from other laboratories (reviewed in Ref. 2) have demonstrated that larger liposomes of conventional formulations are rapidly removed from circulation following i.v. injection by uptake primarily into Kupffer cells of liver and fixed macrophages of spleen. Small liposomes, of less than 0.1 μm diameter, can pass through fenestrated endothelium or through regions of increased capillary permeability and gain access to liver parenchymal cells, and some tumor cells, by this means. However, liver and spleen still remain the major sites of localization even for small liposomes.

With regard to thereapeutic applications for liposomes, other than those which specifically involve the RE system, this presents some major problems. The

Abbreviations: PC, egg phosphatidylcholine; SM, bovine brain sphingomyelin; DSPC, distearoylphosphatidylcholine; PPI, plant phosphatidylinositol; HPPI, hydrogenated plant phosphatidylinositol; PA, phosphatidic acid; DPPC, dipalmitoylphosphatidylglycerol; PS, phosphatidylserine; HSPC, hydrogenated soy phosphatidylcholine; SO_4^-, bovine brain sulfatides; G_{M1}, II^3NeuAc-GgOse$_4$Cer; G_{M2}, II^3NeuAc-GgOse$_3$Cer; G_{M3}, II^3NeuAc-LacCer; G_{D1a}, IV^3NeuAc-II^3NeuAc-GgOse$_4$Cer; G_{T1b}, IV^3NeuAc,II^3(NeuAc)$_2$-GgOse$_4$Cer; ASG_{M1}, GgOse$_4$Cer, gangliotetraosylceramide; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; MLV, multilamellar vesicle; LUV, large unilamellar vesicle; HDL, high-density lipoprotein.

Correspondence: T.M. Allen, Pharmacology Department, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7.

pronounced tendency for liposomes to localize in the RE system raises concerns about RE impairment and its consequences, particularly during continued liposome administration (reviewed in Ref. 3). In addition, short circulation times severely limit the use of liposomes as microreservoir systems for the slow release of biologically active molecules which are normally degraded rapidly within the vasculature. Finally, rapid uptake of liposomes into liver and spleen greatly reduces the possibility of extravascularization of liposomes and substantially prevents the targeting of liposomes to cells within the vasculature or targeting to non-RE tissues.

In this paper we present a series of studies in mice designed to examine factors important to the achievement of liposomes with long circulation times. The role of glycolipids, negative charge, bilayer rigidity, liposome size and route of injection have been examined.

Materials and Methods

Egg PC, SM, DSPC, PPI, HPPI, PA, DPPG and PS were purchased from Avanti Biochemicals, Birmingham, AL. Gangliosides were purchased from Supelco, Bellefonte, PA (mixed ganglioside, G_{M1}, asialo G_{M1} (ASG_{M1}), G_{D1a}, G_{T1b}), from Calbiochem, San Diego, CA (G_{M1}, G_{M2}, G_{M3}, G_{D1a}, G_{T1b}) and from Makor Chemical, Jerusalem (G_{M1}). Ceramides, ceramide galactoside, cerebrosides (bovine), glucocerebrosides, monogalactosyldiacylglycerol, diglactosyldiacylglycerol, globosides, ceramide trihexoside, and bovine brain sulfatides (SO_4^-) were purchased from Supelco. Galactocerebrosides, glucocerebrosides and cholesterol (Chol) were purchased from Sigma Chemical, St. Louis, MO. HSPC was a gift from Liposome Technology, Menlo Park, CA. Sterile, pyrogen-free 0.9% saline (injection USP) was purchased from Travenol Canada. 1,2-di[^{14}C]palmitoyl-L-3-phosphatidylcholine ([^{14}C]-DPPC, 3.7–4.4 GBq/mmol) was purchased from Amersham Canada. Na^{125}I was purchased from Edmonton Radiopharmaceutical Center, Edmonton, AB and ^{111}In-oxine was purchased from Amersham Canada, Oakville, Ontario. Tyraminylinulin was synthesized and ^{125}I-tyraminylinulin was prepared according to the technique of Sommerman et al. [4].

Liposome preparation

Liposomes (LUV) were prepared according to the reverse-evaporation (REV) procedure of Szoka and Papahadjopoulos [5]. Gangliosides or glycolipids were dissolved in chloroform/methanol (2:1, v/v) and aliquots were added to appropriate lipid mixtures in chloroform prior to evaporation of organic solvent. Sterile pyrogen-free 0.9% saline buffered with 10 mM Tes (pH 7.4) (buffered saline) was used for all experiments. Buffered saline containing sufficient cpm of ^{125}I-tyraminylinulin was added during liposome preparation to result in 10^5–10^6 cpm/mouse of entrapped label. Liposomes containing lipids with high phase-transitions were prepared at higher temperatures by the REV method using chloroform/diethyl ether (1:1) instead of diethyl ether. Liposomes were extruded through appropriate sizes of Nucleopore filters according to Olsen et al. [6]. The liposome-entrapped volumes were determined and liposomes, in most cases, were measured using a Nicomp Instruments Laser particle sizer. For some liposome preparations, involving primarily those to be extruded through 0.1 μm or smaller pore sizes, liposomes were made by vortexing dried lipid preparations in buffered saline containing ^{125}I-tyraminylinulin (multilamellar vesicles, MLV). Lipid concentrations were normally 10 μmol/ml. Preparations of liposomes with lipids containing high-phase-transition lipids were heated to above their phase transition during formation and extrusion.

Animal experiments

Female ICR (outbred) mice in the weight range of 23–27 g were obtained from the Animal Breeding Unit of the University of Alberta and maintained in standard housing.

Mice (three per group) were injected in the tail vein with 0.5 mg phospholipid in 0.2–0.25 ml of liposomes, containing 10^5–10^6 cpm of entrapped ^{125}I-tyraminylinulin, suspended in buffered saline. Some (control) mice received free ^{125}I-tyraminylinulin suspended in buffered saline in order to determine the rate of elimination of free label from the body for each new preparation of radiolabelled compound. Some groups of mice were injected either intraperitoneally (i.p.) or subcutaneously (s.c.) in the footpad. At selected times post-injection, e.g., 0.5, 2, 6 and 24 h, the mice were killed and the tissues and organs were excised and counted for radiolabel in a Beckman 8000 gamma-counter. Normally, tissues and organs sampled included liver, spleen, lung, heart, kidney, thyroid, blood and carcass (which was the remainder of the animal after excision of the preceding organs). When more thorough studies of tissue distribution were performed, addition tissues included mesenteric and other lymph nodes, muscle, tail, blood cells (separated from plasma), skin, leg, gut and bone marrow. Bone marrow was sampled by extrusion of the femur, counting the numbers of resulting cells in a Coulter Counter (Model ZF) and then counting for radioactivity. A conversion factor of $4.6 \cdot 10^8$ cells per g bone marrow was determined and used to calculate the weight of bone marrow from the numbers of cells. All tissue counts were normalized to 10^6 cpm injected per animal. Blood-correction factors were determined [8] and applied to normally sampled tissues and carcass. Results were sometimes expressed as blood/RES ratio, which is the ratio of percent in-

jected counts present in blood to percent injected counts present in liver plus spleen at given times post-injection.

Leakage studies

The leakage of quenched calcein (40 mM, 280 mosM) from liposomes of various compositions was determined according to the technique of Allen and Cleland [7] by monitoring fluorescence increase accompanying leakage in a Perkin Elmer MPF-4 spectrofluorimeter at 37°C in 25% human plasma at a lipid concentration of 50 μm. 100% leakage was determined by lysing liposomes with the detergent $C_{12}E_8$.

Exchange / transfer with HDL

The ability of PC/Chol (2:1) 0.2 μm LUV to exchange [^{14}C]DPPC with, or transfer label to, human HDL was measured according to the technique of Allen [9]. Liposomes containing increasing mol% of G_{M1} were incubated for 2 or 16 h at 37°C with HDL followed by chromatography over a Sepharose CL-4B column and quantitation of the radiolabel associated with the HDL peak.

Results

We have previously reported that, of the several gangliosides tested (G_{M1}, G_{M2}, G_{M3}, G_{D1a}, G_{T1b}), only G_{M1} was capable of significantly increasing the circulation half-life of liposomes [1]. We have now tested several other glycolipids, either alone or in combination with G_{M1}, to see if they were capable of decreasing RE uptake of liposomes after i.v. injection (Table I). Only G_{M1} was effective in significantly increasing the blood/RES ratio in PC/Chol liposomes (Table I) or in

TABLE I

The effect of glycolipids and gangliosides on blood/RES ratios in mice

Conditions were: 2 h post-injection (three mice per group) for 0.1 μm liposomes composed of egg PC/Chol (2:1) containing 10 mol% glycolipid or ganglioside, or in some cases 10 mol% of each. Percentage remaining in vivo represents the percentage of injected counts remaining in sampled organs plus carcass 2 h post-injection.

Glycolipid	Blood/RES ratio	% remaining in vivo
None	0.68 ± 0.15	78.1 ± 0.4
Sulfatides	0.54 ± 0.22	63.3 ± 1.3
Glucosylceramide	0.72 ± 0.21	84.6 ± 12.1
Galactosylceramide	1.01 ± 0.63	8.7 ± 0.2
Monoglucosyldiacylglycerol	0.31 ± 0.08	67.4 + 3.2
Diglucosyldiacylglycerol	0.74 ± 0.44	43.2 ± 7.2
Globosides	0.08 ± 0.03	74.2 ± 0.21
Ceramide trihexoside	0.86 ± 0.44	64.2 ± 7.5
ASG_{M1}	0.46 ± 0.10	75.2 ± 8.1
G_{M1}	4.27 ± 1.07	75.9 ± 3.1
G_{M1} : glucosylceramide	3.98 ± 0.91	81.3 ± 3.3
G_{M1} : galactosylceramide	5.95 ± 1.42	56.8 ± 5.0

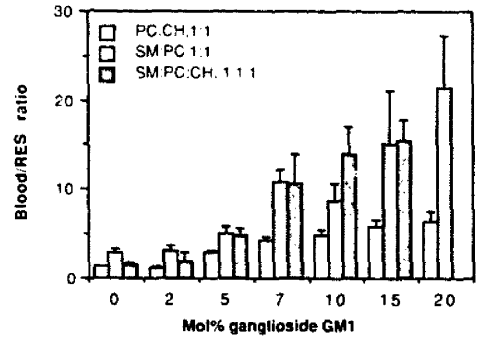

Fig. 1. Blood/RES ratios for three liposome compositions (0.5 mg, 0.1 μm MLV) with increasing G_{M1} concentrations. The G_{M1} concentration is represented by mol% of phospholipid. Results are expressed as means ± S.D. (n = 3). CH, cholesterol.

SM/PC (4:1 or 1:1) liposomes (not shown). Globosides increased liposome uptake, while other glycolipids were neutral, neither increasing nor decreasing blood/RES ratios when compared to similar liposomes lacking glycolipid. Liposomes containing galactosylceramide were very leaky, and liposomes containing diglucosyldiacylglycerol had somewhat increased leakage compared to other formulations. When 10 mol% glycolipid was incorporated into liposomes containing 10 mol% G_{M1}, the glycolipid behaved in a neutral fashion, with the non-recognition effect of G_{M1} predominating (Table I). Increasing the glucosylceramide content of liposomes in a stepwise fashion up to 40 mol% or decreasing the content to 5 mol% in the presence or absence of G_{M1} had no significant effect on their ability to avoid RE uptake (data not shown). Decreasing the G_{M1} content stepwise down to 2 mol%, while keeping the glucosylceramide content constant at 20 mol%, resulted in decreasing blood/RES ratios (data not shown).

The effect on blood/RES ratios, at 2 h post-injection, of increasing G_{M1} concentrations in liposomes (MLV) of three compositions extruded through 0.1 μm filters is shown in Fig. 1. For similar-sized liposomes, there was an increase in blood/RES ratios with increasing G_{M1} concentrations. Fluid liposomes (PC/Chol, 2:1) gave lower ratios than more rigid formulations containing sphingomyelin. The formulations resulting in the highest blood/RES ratios had lipid compositions similar to the outer monolayer of red blood cells (SM/PC, 1:1 and SM/PC/Chol, 1:1:1). G_{M1} concentrations of the region of 7–15 mol% appeared to be optimal. Higher G_{M1} concentrations were avoided in subsequent experiments because of the expense of the materials and the increased tendency of liposomes to leak contents at higher G_{M1} concentrations (data not shown). The increased blood/RES ratios at the higher G_{M1} concentrations at 2 h post-injection resulted from a decrease in liposome uptake into liver, spleen and carcass.

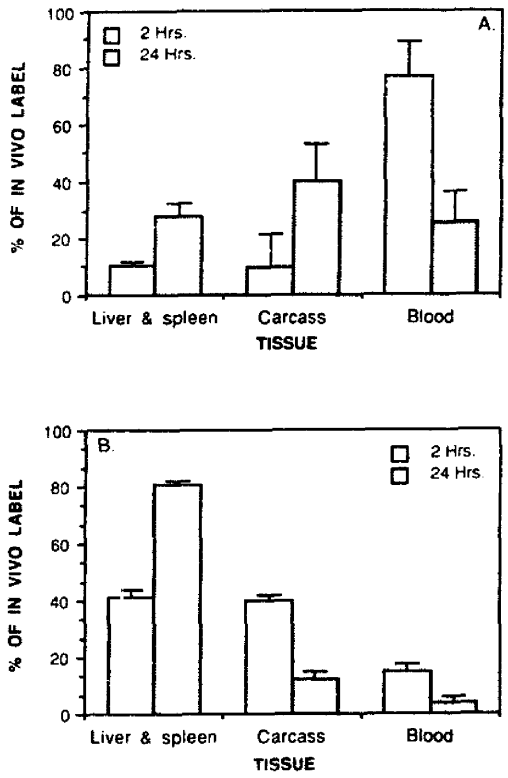

Fig. 2. Percentage of label remaining in vivo at 2 and 24 h post-injection in RE tissue (liver and spleen), carcass and blood for two liposome compositions (0.5 mg, 0.1 μm MLV). Results are expressed as means ± S.D. (A) SM/PC/G_{M1} (1 : 1 : 0.14), $n = 9$. (B) PC/Chol (2 : 1), $n = 3$.

At concentrations of G_{M1} between 7 and 15 mol%, in liposomes composed of SM/PC (1 : 1) or SM/PC/Chol (1 : 1 : 1), between 75 and 85% of the injected counts remaining in vivo were circulating in the blood at 2 h post-injection. The data pooled from three separate experiments (nine mice in total) on SM/PC/G_{M1} (1 : 1 : 0.14) 0.1 μm MLV are shown in Fig. 2a. Data from experiments with SM/PC/Chol/G_{M1} (1 : 1 : 1 : 0.14) 0.1 μm liposomes were not significantly different from the data presented in Fig. 2a. Between 2 and 24 h post-injection (i.v.) of long-circulation-time liposomes, there was a decrease in blood levels, an increase in liver, spleen levels and a large increase in carcass levels of entrapped label (Fig. 2a). The relatively low liver and spleen levels of label and the high carcass levels were in marked contrast to the low carcass levels and the high liver and spleen levels seen for formulations with short circulation-times (PC/Chol, 2 : 1) 24 h post-injection (Fig. 2b).

It should be noted that carcass also contains urinary bladder and since the free label is excreted via the kidney, carcass levels at earlier time-points (e.g., 0.5 or 2 h) may be elevated due to residual counts retained in bladder. Because PC/Chol liposomes are much more susceptible to breakdown in plasma than SM/PC/G_{M1} liposomes, the elevated carcass levels in Fig. 2B at 2 h post-injection may represent free label in the urinary tract.

In order to try to determine where liposomes were localizing in carcass by 24 h post-injection (i.v.) we dissected various tissues and determined tissue uptake of liposomal label as cpm/mg tissue. We observed that mice injected with long-circulation-time liposomes had higher tissue levels for many tissues examined at 24 h post-injection than at 2 h post-injection (Fig. 3a). This contrasts with the results for liposomes with short circulation-times, where tissue levels at 2 h post-injection, except for liver and spleen, were generally higher than at 24 h and at 24 h post-injection were very low, with the possible exception of lymph nodes (Fig. 3b). In

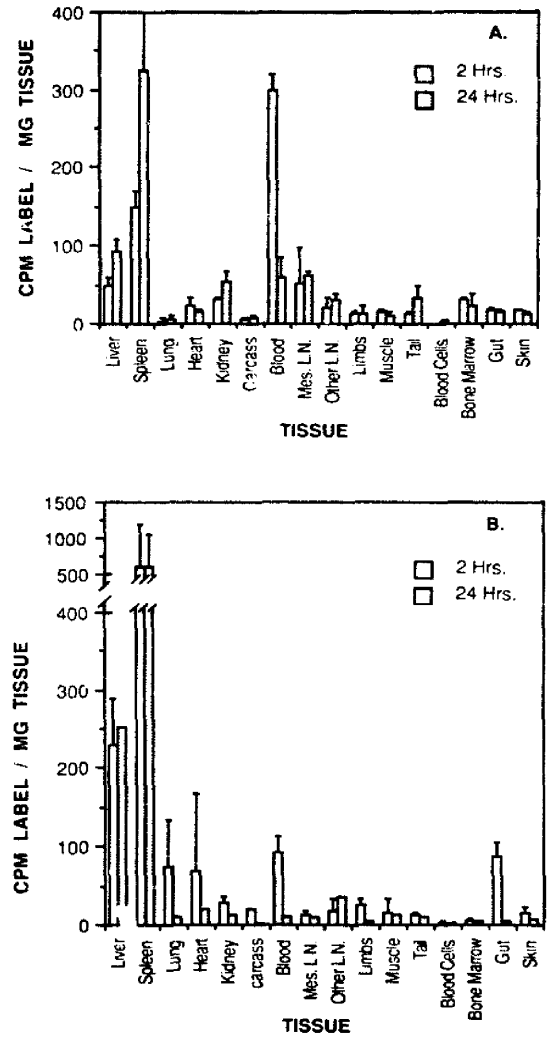

Fig. 3. Tissue distribution of two compositions of liposome (0.5 mg, 0.1 μm MLV) at 2 and 24 h post-injection. Results are expressed as cpm label per mg tissue ± S.D. ($n = 3$). (A) SM/PC/Chol/G_{M1} (1 : 1 : 1 : 0.14). (B) PC/Chol (2 : 1).

TABLE II

Tissue distribution (cpm / mg) for 6 days post-injection for ICR mice

The mice (three per group) were injected subcutaneously in the right foot on day 0 with liposomes (0.1 μm MLV) composed of SM/PC/Chol/G_{M1} (1 : 1 : 1 : 0.14). Results are expressed as means ± S.D.

Injection site	Day			
	1	2	3	6
L. foot	12.0 ± 6.3	8.0 ± 3.2	8.7 ± 3.1	6.0 ± 2.0
R. foot	1926 ± 461	2216 ± 359	1511 ± 390	778 ± 173
L. limb	3.7 ± 1.7	3.1 ± 1.1	2.7 ± 0.4	1.6 ± 0.3
R. limb	887.8 ± 18.8	111.1 ± 13.9	59.4 ± 5.6	45.7 ± 14.1

particular, long-circulation-time liposomes, following i.v. injection, achieved elevated levels in bone marrow, lymph nodes and kidney 24 h post-injection (Fig. 3a). At 2 h post-injection, some of the label in kidney may represent excretion of free label, but this is not expected to contribute substantially to kidney levels 24 h post-injection. No significant uptake of liposomes into blood cells was observed, with all counts present in blood being confined to the plasma component.

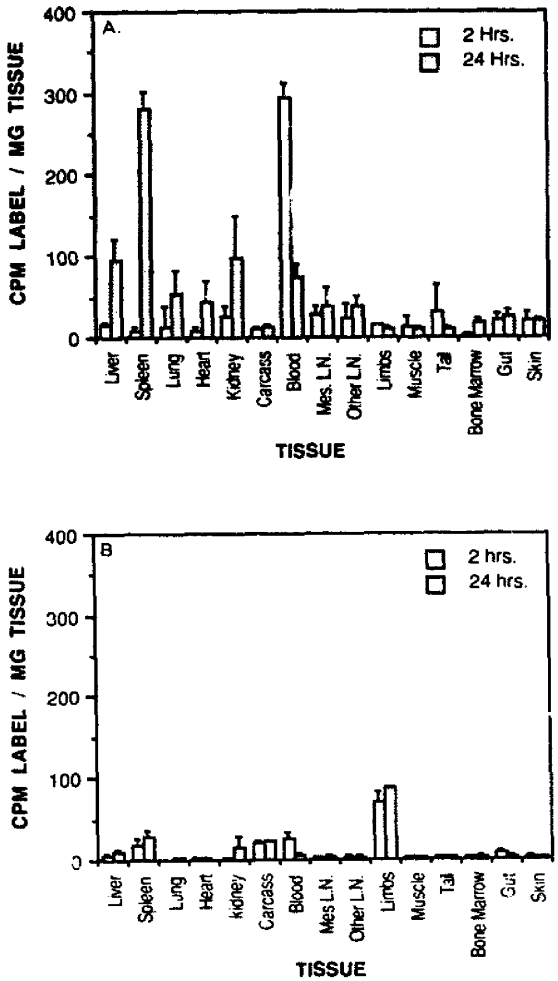

Fig. 4. Tissue distribution of SM/PC/Chol/G_{M1} (1 : 1 : 1 : 0.14) liposomes (0.5 mg, 0.1 μm MLV) as a function of site of injection. Results are expressed as cpm label per mg tissue ± S.D. ($n = 3$). (A) Intraperitoneal administration. (B) Subcutaneous (footpad) administration.

Tissue distribution was also examined at 2 h and 24 h following intraperitoneal (i.p.) and subcutaneous (s.c.) injections of long-circulation-time liposomes. Circulating levels of liposomes after i.p. injection were similar to that following i.v. injection (Fig. 4a). However, liver and spleen uptake were much lower at 2 h following i.p. injection. Therefore, we observed higher blood/RES ratios at early time-points following i.p. injection. Liposome levels 24 h post-injection were elevated in most tissues, particularly lung, heart and kidney as compared to i.v. injections.

Following s.c. injection, for long post-injection periods the liposomes were still localized mainly at the site of injection. We observed low blood levels, which were maximal at 2 h post-injection (approx. 8% of in vivo counts), and low liver and spleen levels, which were maximal 24 h post-injection (approx. 4% of in vivo counts) (Fig. 4b). There was no significant uptake into bone marrow or other tissues examined (Fig. 4b). Table II presents the data for the injected right foot and the right limb compared to the non-injected foot and limb for up to 6 days post-injection. The injected liposomes are cleared only slowly over this time, probably through drainage by the limb lymphatics which show elevated counts on the injected (right) side.

Blood/RES ratios were also determined as a function of liposome size for liposomes extruded ten times through Nucleopore filters of defined pore size. The results for some typical experiments are reported in Table III. Blood/RES ratios increased with decreasing liposome size down to 0.08 μm pore size. Liposomes extruded through 0.05 μm pore size frequently had increased liver uptake compared to slightly larger liposomes, possibly because of the ability of the smaller liposomes to penetrate liver sinusoids and gain access to heptocytes. Rigid liposomes of all compositions had higher blood/RES ratios than fluid liposomes. Some preparations of neutral, SM-containing liposomes showed great size heterogeneity after extrusion, which

TABLE III

Blood/RES ratios in mice (three per group) as a function of liposome size 2 h post-injection (i.v.) for various liposome compositions

Liposome composition	Pore size (μm)	Trapped volume L/M phospholipid	Blood/RES
PC/Chol, 2:1	0.8	8.1	0.03±0.01
	0.4	6.7	0.02±0.00
	0.2	5.6	0.16±0.07
	0.1	2.9	0.15±0.11
	0.08	2.6	0.47±0.29
	0.05	1.6	0.64±0.65
HSPC/Chol, 2:1	0.4	8.5	0.17±0.01
	0.2	6.6	1.2 ±0.2
	0.1	4.1	1.0 ±0.3
	0.08	4.0	1.1 ±0.3
	0.05	2.2	0.91±0.19
SM/PC/Chol, 1:1:1	0.4	19.6	0.17±0.06
	0.2	9.5	1.1 ±0.6
	0.1	8.6	1.5 ±0.3
	0.05	10.1	1.0 ±0.3
SM/PC/Chol/G_{M1}, 1:1:1:0.2	0.4	8.0	7.0 ±0.9
	0.2	5.2	13.2 ±8.8
	0.1	3.9	16.3 ±3.8
	0.08	2.9	24.6 ±1.5
	0.05	1.9	19.2 ±1.4
DSPC/Chol/PPI, 2:1:0.2	0.4	8.7	0.16±0.02
	0.2	4.9	0.76±0.18
	0.1	3.3	0.90±0.17
	0.08	2.5	1.7 ±0.1
	0.05	1.6	1.6 ±0.4
DSPC/Chol/G_{M1}, 2:1:0.2	0.4	6.1	1.1 ±0.2
	0.2	5.2	5.2 ±1.6
	0.1	4.5	7.4 ±1.3
	0.05	3.6	3.8 ±0.4
DSPC/Chol/HPPI, 2:1:0.2	0.4	6.3	0.26±0.04
	0.2	5.5	0.31±0.05
	0.1	2.8	0.51±0.04
	0.08	2.7	1.1 ±0.2
	0.05	2.2	0.84±0.27

tended to disappear when negative charge in the form of G_{M1} or other phospholipids was added to the liposomes. Addition of lipids with high phase-transitions to the liposomes increased blood/RES ratios by approximately one order of magnitude at all sizes. Addition of 7–10 mol% G_{M1} to the liposomes increased blood/RES ratios by approximately one more order of magnitude at all sizes (Table III and unpublished data). Maximum blood/RES ratios were found for liposomes extruded through 0.1 μm or 0.08 μm filters, however significantly elevated blood/RES ratios could be found even for larger liposomes containing G_{M1}.

Because G_{M1} is a molecule with a large negatively charged headgroup, we have also explored the effect of various phospholipids with negatively charged

TABLE IV

Effect of phospholipid headgroups

The effect of negatively charged phospholipid headgroups on blood/RES ratios in mice (three per group) 2 h post-injection (i.v.) for 0.1 μm LUV composed of DSPC/Chol (2:1) and containing 10 mol% of negative charge.

Phospholipid composition	Blood/RES ratio	Trapped volume (L/M)	% remaining in vivo
DSPC/Chol	0.25±0.12	6.9	93.4±11.4
DSPC/Chol/PA	0.01±0.00	4.8	88.6± 1.3
DSPC/Chol/PS	0.20±0.02	2.8	83.9± 3.7
DSPC/Chol/DPPG	0.37±0.04	4.9	88.8± 2.4
DSPC/Chol/SO_4^-	0.36±0.10	7.8	88.4± 1.0
DSPC/Chol/HPPI	0.51±0.04	2.8	88.0± 0.6
DSPC/Chol/PPI	0.90±0.17	3.3	79.3± 0.5
DSPC/Chol/G_{M1}	7.4 ±1.3	4.5	90.3± 1.3

headgroups for their effect on blood/RES ratios. The results are given in Table IV for DSPC/Chol, 2:1 liposomes containing 10 mol% negative charge. Although several sizes of liposomes were examined at both 2 and 24 h post-injection (i.v.), results are given in Table IV for only one liposome size (0.1 μm) at 2 h post-injection. Comparable trends were observed for other liposome sizes. Liposomes with 10 mol% of G_{M1} had the highest blood/RES ratios, but liposomes containing phosphatidylinositol had increased blood/RES ratios compared to liposomes with other negatively charged phospholipids (Table IV). Liposomes containing PA or PS were rapidly removed from circulation, while those containing DPPG and sulfatides had slightly higher blood/RES ratios than neutral liposomes (Table IV).

It was possible to obtain a good estimate of the degree of contents leakage from liposomes of various compositions by measuring the percentage of injected counts remaining in vivo at various time-points post-injection, since the free label was eliminated rapidly from the body via filtration by the kidney [1,4]. However, we have also made a few direct measurements of contents

TABLE V

Calcein leakage

Leakage of calcein (40 mM) from 0.2 μm liposomes of various compositions at 37°C in 25% human plasma.

Composition	% Remaining after 5 h
SM:PC:G_{M1}, 4:1:0.35	40.0
SM:PC:G_{M1}, 2:1:0.21	52.5
SM:PC:G_{M1}, 1:1:0.14	66.0, 81.6
SM:PC:G_{M1}, 1:3:0.28	65.5, 61.0
SM:G_{M1}, 1:0.7	27.9
SM:Chol:G_{M1}, 4:1:0.35	79.2
SM:Chol:G_{M1}, 2:1:0.21	95.9
SM:Chol:G_{M1}, 1:1:0.14	99.3

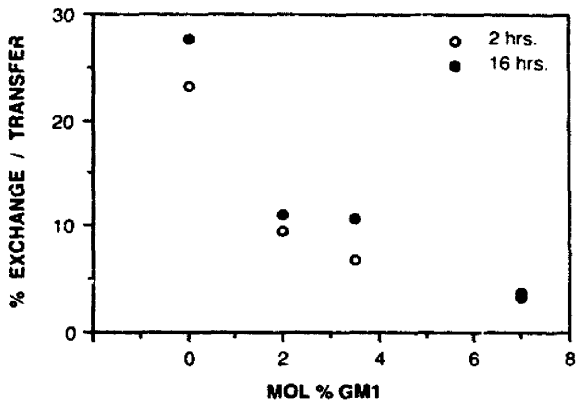

Fig. 5. Percentage of [^{14}C]DPPC label in PC/Chol (2:1) liposomes (0.2 μm LUV) associated with HDL following 2 or 16 h incubation at 37°C as a function of G_{M1} concentration. Liposomes were separated from HDL on a Sepharose CL-4B column. The ratio of liposome/HDL phospholipid was 2.0. Each point is the result of a single experiment.

leakage from liposomes containing G_{M1} using entrapped calcein as an aqueous space marker. The latency of contents after 5 h of incubation at 37°C in 25% human plasma is given in Table V. Increasing the sphingomyelin content of the liposomes decreased latency, while addition of cholesterol increased latency. All of these liposome compositions have high blood/RES ratios and these experiments show that it is possible to vary the leakage properties of long-circulation-time liposomes over a wide range.

We have previously noted that increasing liposomal circulation times were correlated with a decreased ability of the liposomes to exchange with or transfer phospholipid to high-density lipoproteins (HDL) [9]. We have therefore studied the effect of increasing concentrations of G_{M1} on the ability of PC:CH (2:1) liposomes (0.2 μm LUV) to exchange/transfer radiolabelled [^{14}C]DPPC with HDL. Increasing concentrations of G_{M1} in liposomes were correlated with a decreased ability of the liposomes to exchange with or transfer phospholipid to HDL (Fig. 5).

Discussion

G_{M1} is very effective in reducing reticuloendothelial uptake of liposomes, particularly when combined with somewhat rigid liposomal formulations. This observation has recently been confirmed by Gabizon and Papahadjopoulos [10]. Uptake of liposomes by the RE system is thought to occur as a result of opsonization of liposomes by plasma proteins, leading to binding and uptake by RE cells [11–13]. Previous experiments have demonstrated that rigid liposomes (e.g., those containing sphingomyelin, DSPC, cholesterol or other rigidifying phospholipids) are resistant to exchange of phospholipid with, or net transfer of phospholipid to, serum high-density lipoproteins [9]. In this paper, we show that fluid liposomes containing G_{M1} also have a dramatically decreased ability to exchange/transfer lipids with plasma HDL. This may occur because apolipoproteins involved in exchange/transfer are prevented from gaining access to, and insertion into the phospholipid bilayer, because of the presence of a surface glycocalyx.

We have also shown that gangliosides reduce the leakage of aqueous contents from liposomes, a phenomenom which is induced by plasma proteins [14] and a property which has been correlated with decreased RE uptake [15,16]. Therefore, G_{M1} may decrease RE uptake through an ability to reduce opsonization of liposomes by certain classes of plasma proteins. This possibility is currently under investigation.

It is also possible that an additional role of G_{M1} and/or a tightly packed bilayer may be to directly interfere with interactions between liposomes and the cell-surface proteins on target cells involved in the binding of liposomes to cells [17].

We have found that liposomes with the longest circulation times (SM/PC/Chol/G_{M1}, 1:1:1:0.2) have lipid compositions which simulate several of the surface characteristics of erythrocytes. The erythrocyte shares common characteristics of all mammalian cells in having a surface glycocalyx of glycoproteins and glycolipids containing large numbers of sialic acid residues. Erythrocytes have an asymmetric distribution of phospholipid with the outer monolayer containing primarily phosphatidylcholine and sphingomyelin in addition to cholesterol [18], a composition similar to long-circulation-time liposomes. When sialic acid is removed from the surface of erythrocytes, and from liposomes containing G_{M1}, by neuraminidase treatment, an increased RE uptake is apparent. Thus, the presence of surface sialic acid is clearly a determining factor in the avoidance of RE uptake by liposomes containing G_{M1}. However, other gangliosides contribute sialic acid and carbohydrate to the surface of liposomes and yet lack the ability to prolong circulation times.

Why G_{M1} appears to be unique in the ability to prolong the circulation half-lives of liposomes is not clear. The answer may lie, in part, in the molecular conformation of the molecule, the location of the negative charge relative to the phospholipid bilayer and the carbohydrate backbone, and the packing characteristics of G_{M1} in phospholipid bilayers. Gangliosides G_{M1}, in concentrations up to 14–20 mol%, is randomly distributed in membranes [19–21] under conditions where G_D and G_T appear to be phase separating into domains [21,22]. From X-ray diffraction measurements and electrokinetic properties, it appears that the thickness of the glycocalyx imparted by G_{M1} in PC bilayers is 2.5 nm from the bilayer and the fixed negative charges are in a plane 1 nm from the bilayer surface [23–25]. In the case

of G_{M1}, the negative charge is shielded from the surface by the presence of two neutral sugars [26], while in the case of G_D, G_T, G_{M2} and G_{M3} this is not the case. The presence of a screened negative charge may contribute to RE avoidance, perhaps by decreasing or preventing opsonization of the bilayers [10], or direct binding to target cell-surface proteins [17].

A critical minimum concentration of G_{M1} (7 mol%) is needed in the bilayers in order to observe the long-circulation effect, and this may be necessary in order to achieve one or more of the following: a minimum charge density, a minimum concentration necessary for the carbohydrate residues to form intermolecular hydrogen bonds [27], or the presence of a hydrophilic surface. It has been predicted that surface hydrophobicity may be a key factor in the phagocytosis of particulate matter [28].

Wynn [29] has predicted the conformation of G_{M1} by energy-minimization techniques and postulates a planar concentration of hydroxyl groups in G_{M1} which is not shared by G_{M2}. These planar hydroxyl groups may confer a sufficiently hydrophilic surface to the liposomes to prevent opsonization. Illum et al. [28] have shown that hydrophilic coatings decrease uptake of colloidal particles by the liver and by peritoneal macrophages.

This paper provided evidence that small liposomes (0.1 μm), when kept in circulation for longer times than could previously be achieved, effect elevated liposome levels in organs other than liver and spleen, including some tissues that are not of reticuloendothelial origin. This is apparent after both i.v. and i.p. administration of long-circulation-time liposomes. Gabizon and Papahadjopoulos have reported that similar liposomes achieve elevated tumor levels compared to conventional formulations [10]. These observations provide encouragement for the therapeutic applications of liposomes in non-RE tissues and suggest that targeting of smaller liposomes to specific tissues may be achievable.

Following s.c. administration of long-circulation liposome formulations, we have provided evidence that the liposomes remain at the site of injection in significant quantities for several days. It is possible that these formulations may be useful in depot applications where slow release of contents is desired over a long period of time.

We have also provided evidence that liposomes with compositions that avoid RE uptake can be formulated with a large range of contents leakage rates (this work and Ref. 1). This should prove useful in altering the rates of contents release for a wide variety of molecules in situations requiring applications of liposomes for controlled release within the vasculature.

In summary, a significant increase in circulatory half-lives has been made possible with liposomal formulations containing 7–15 mol% G_{M1} and this has led to increased uptake of liposomes into tissues where there is normally little or no evidence for liposome localization. These developments make possible a number of therapeutic applications of liposomes which have not previously been feasible.

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