Vol. 82, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1978 Pages 328-334

LONG-LIVING LIPOSOMES AS POTENTIAL DRUG CARRIERS

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Received March 27,1978

SUMMARY: Neutral, unilamellar liposomes (vesicles) composed of a dialkyl analog of phosphatidylcholine and cholesterol, and containing ^{14}C-maltose as entrapped marker, were administered intravenously to mice. After one and two days, radioactivity in blood and liver remained 3-4 times higher than after administration of liposomes of egg (diester) phosphatidylcholine and cholesterol. It appears that the vesicles were taken into liver cells by endocytosis, and that phospholipases are involved in the capture as well as in the breakdown of conventional liposomes. Liposomes that are semi-resistant to catabolic enzymes may become useful in the manipulation of drug delivery.

Phospholipids (or phospholipid-cholesterol mixtures) swollen in water form concentric, closed bilayers (liposomes) which can entrap dissolved molecules (1), antibiotics, anti-tumor drugs, enzymes, etc. (2,3). Entrapment of drugs by liposomes can, potentially, overcome such problems as drug resistance, premature breakdown of drugs, or immune reactions, make it possible to deliver non-permeating drugs into cells, and permit the homing of drugs to target tissue.

Intravenously injected multilamellar liposomes are rapidly (largely within an hour) removed from the circulating blood, taken up mainly by the liver. Liposomes with a positive surface charge survive somewhat longer in curculation (4), and positive or neutral sonicated, unilamellar liposomes (vesicles) much longer (5). Clearance from the blood is biphasic, first rapid, then slower and almost linear (4,5).

0006-291X/78/0821-0328$01.00/0

We speculate that capture or lysis of liposomes, or both, are mediated by the action of circulating or cellular phospholipases. If this is the case, then liposomes consisting of phospholipase-resisting phospholipids might have very long circulation and survival times. We have, therefore, prepared liposomes (vesicles) in which a phosphatidylcholine (esterPC) was replaced by its dialkyl analog (alkylPC). Maltose (^{14}C) was chosen as trapped marker because it permeates very slowly and is rapidly metabolized (oxidized) when free.

EXPERIMENTAL

Materials. Egg lecithin (esterPC) was prepared according to Singleton et al. (6); the most abundant species in this preparation is 1-palmitoyl-2-oleoyl-phosphatidylcholine, together with species having stearic acid in position 1 (∿20%) or linoleic acid in position 2 (∿20%). The dialkyl analog, D,L-2-(tetradecyl)octadec-cis11-ene-1-01-(0)phosphorylcholine, was prepared as reported (7). ^{14}C-maltose was bought from ICN (Irvine, CA); mice (CD/1) from Charles River Breeding Labs (Wilmington, MA).

Preparation of liposomes (vesicles): 80 µmoles of esterPC (egg lecithin) or alkylPC and 40 µmoles cholesterol in chloroform in a 25 ml round bottom flask were freed of solvent in a rotary evaporator at 50°C in high vacuum, then dispersed in 0.8 ml of Tris buffered saline (10 mM in 0.9% NaCl, pH 7.2) containing 80 µCi of ^{14}C-maltose by sonication in a bath type instrument. The dispersion was transferred to a conical tube, ultrasonically irradiated at 20 KHZ for 4 hours at 4°C (8). A Bronson sonifier (Model W140D) (∿40W) equipped with microtip was used. Water-saturated argon gas was flushed over the surface of the solution. Titanium fragments and any undispersed lipid were then removed by centrifugation in the cold at 105,000 x g for 60 min. The optically clear supernatant was subjected to gel filtration on a Sephadex-G-50 column (45 x 1 cm). Two radioactive elution peaks corresponded to liposomes (5-6% of maltose entrapped) and free maltose. The fractions containing liposomes were pooled and concentrated by ultrafiltration under nitrogen pressure to about 1 ml in a magnetically stirred Diaflo apparatus with membrane UM-10 (Amicon Corp., Lexington, MA). The liposomal preparations were sealed under nitrogen and stored at 4°C overnight.

Leakage of ^{14}C-maltose from liposomes. Samples of liposomal solution (esterPC-cholesterol) were left at 4°C for 60 hr, or at 37°C for 12 hr. Then the preparations were again chromatographed on Sephadex-G-50, and the radioactivity in liposome and free maltose fractions was determined. Leakage rates were 0.15% per hour at 4°C, 0.8% per hour at 37°C. Liposomes of the dialkyl analog (alkylPC) have been tested before in our laboratory for permeability against non-ionic solutes; at the given mole fraction of cholesterol, their permeability rates are equal to those of the esterPC-cholesterol liposomes (9).

Injections and sampling of liposomes; control. Liposomal preparations in buffered saline (50 µl) containing about 2 mg of the lipid were injected into the tail vein of mice (CD/1; average weight, 38 g). Up to 10 µl of blood were drawn from the vein in heparinized capillary pipettes at various time intervals. The blood drawn was weighed, dissolved in scintillation solvent (Radi Sol, Beckman) and analysed in a Packard Tri-Carb liquid scintillation counter. Counts were corrected with an appropriate standard quenching curve derived by mixing the liposomal preparations with different amounts of non-radioactive blood.

The total injected radioactivity was calculated from a sample taken 2 min after injection, assuming a total blood volume of 2.7 ml. For each experimental point, 4 to 7 animals were analyzed. Standard errors were around 10 percent, relative of the values measured. As control, one group of animals was injected intravenously with ^{14}C-maltose in saline.

RESULTS

Clearance of free and trapped maltose from blood. Clearance, in percent of radioactivity remaining in the blood, is shown in Fig. 1. It has been reported (4,5,10) that clearance is biphasic with a rapid phase (saturation of capture sites?) followed by a slower one; a logarithmic plot of Fig. 1 is in agreement with this view. Half-times of clearance taken from such a plot are shown in Table I. The rapid phase half-time (T_1) for esterPC (egg-cholesterol) vesicles is identical with that of dipalmitoylphosphatidylcholine-cholesterol vesicles in rats (5). It should be noted that T_1 of alkylPC is considerably larger than T_1 for esterPC.

Figure 1 and Table I show that, after 24 hours, over three times more alkylPC than esterPC is retained in circulation, and that it will take 4-5 days to reduce this level by 50 percent.

Liposomes in the liver. In Table II, 24 hr after injection, the liver retains three times more alkylPC than esterPC liposomes. On comparison with the radioactivity derived from free maltose it becomes apparent that many of the egg lecithin liposomes, but certainly most of the alkylPC liposomes, must still be intact. The half-life of alkylPC liposomes in the liver seems to be around 24 hours (Table II).

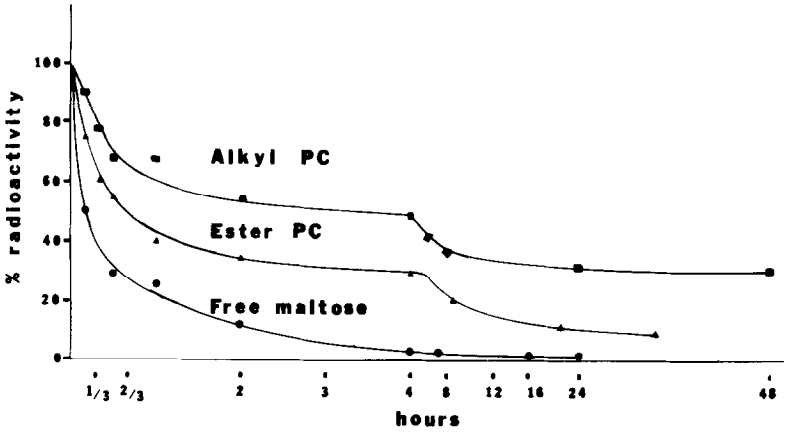

Fig. I. Clearance of injected free and encapsulated ^{14}C-maltose radioactivity from blood.

Table I. Half-life times of clearance from blood of free and entrapped ^{14}C-maltose. Rapid phase, T_1; slow phase, T_2.

	T_1 hours	T_2
Maltose	0.15	11
Egg-lecithin vesicles	0.3	16
AlkylPC vesicles	0.8	110

Table II. Uptake of radioactivity in livers of mice injected intravenously with free or liposome-encapsulated ^{14}C-maltose.

Injected form	Time in hours	Radioactivity % of initial (2 min.) amount in blood
Free maltose	24	3.4 ± 0.9
Egg-lecithin vesicles	24	9.4 ± 2.3
AlkylPC vesicles	24	29.0 ± 5.2
	48	13.7 ± 0.7

± SEM, 4 mice

DISCUSSION

The two phospholipids used in our study are nearly isosteric, and their bilayers show nearly identical permeation rates

for non-ionic solutes (9). The observed differences in clearance rates are therefore due to different rates of capture and lysis, not to different passive diffusion rates of the marker. This conclusion is supported by the fact that the clearance rates for the esterPC-cholesterol vesicles agree with those found for non-permeating markers (4,5).

The replacement of phosphatidylcholine with its dialkyl analog extends the life-time of liposomes considerably, in the circulating blood as well as in tissues. Significantly, it is the long-time survival in circulation (T_2) that is especially extended; in previous studies only the rapid phase (T_1) could be influenced by manipulating liposome charge or size (4,5). The potential practical advantages of an extension of circulating time are obvious; it should be stated, though, that a dialkyl phosphatidylcholine is not a promising candidate for clinical application because it may well be completely resistant to break-down and therefore show long-term toxicity. Phospholipids containing ether linkages might be only semi-resistant to catabolism and prove to be more suitable.

The long persistence of radioactivity in the liver (Table II) shows that the liposomes are still intact, and since they are monolamellar vesicles they cannot have been assimilated by liposome-plasma membrane fusion but must have been taken up by endocytosis (11).

Since the first-phase blood clearance rate, as expressed by half-life time T_1, is considerably slower for the enzyme-resistant alkylPC liposomes than for esterPC liposomes (Table I), it appears that phospholipases are involved not only in the lysis but also in the capture of the liposomes. The fast-captured fraction is still large even for alkylPC, around 60% in 60 minutes;

for future clinical long-term application it might be desirable to reduce the size of this fraction, perhaps by preinjection of empty liposomes; injection of carbon particles has not been successful (4). Our results do not support the hypothesis (5) that the first, rapid phase of clearance results from capture of larger multilamellar liposomes, since preparation under our conditions results in nearly quantitative formation of unilamellar vesicles (8). By the way, the clearance of free maltose is also biphasic.

One result of our study is rather puzzling. The clearance rate of liposomes from the liver, with a half-life of about 24 hours (Table II), does not agree very well with the blood clearance rate of 4 days. Since it is unlikely that such a large discrepancy is due to the inaccuracies of measurements, it appears that the circulating liposomes are not taken up by the liver in proportion to the decrease of liver liposomes; in other words, there is no simple equilibrium between both liposome levels. Rather, the data suggest that the blood liposome level is kept high by replenishment from the liver liposome pool. This would imply exocytosis of intact vesicles.

ACKNOWLEDGEMENT: Supported in part by research grant GM2185 of the National Institute of General Medical Sciences, U.S. Public Health Service.

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