THE PHOSPHOLIPID COMPONENT OF SMALL UNILAMELLAR LIPOSOMES CONTROLS THE RATE OF CLEARANCE OF ENTRAPPED SOLUTES FROM THE CIRCULATION

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Received 11 July 1980

1. Introduction

Successful use of liposomes as a drug carrier in vivo is often dependent on their prolonged presence in the blood [1,2]. This is expected to reduce uptake of liposomes by the reticuloendothelial system and allow them to deliver drugs to alternative cell targets [1]. To this end, there has been modest success through the manipulation of liposomal surface charge [3] and size [4]. However, with liposomes that have little or no cholesterol, entrapped solutes leak out rapidly in the presence of blood. Solute leakage is effected through the loss of phospholipid molecules to plasma high density lipoproteins and ensuing bilayer destabilization [5–7]. We have found that the presence of excess cholesterol in the bilayers greatly diminishes such phospholipid movement, thereby preserving their stability [7–9]. Thus, solutes carried by neutral or positively charged small unilamellar cholesterol-rich liposomes composed of egg phosphatidylcholine can attain a half-life in rodents (~2 h) which is similar to that exhibited by the carrier [4,7].

Here, we have investigated conditions that will allow circulating liposomes to extend their presence in the blood considerably and at the same time enable them to retain solutes quantitatively. Our data show that, for small unilamellar neutral liposomes, the half-life of their solute content in the circulation of mice can be controlled by the appropriate choice of the phospholipid component and the addition of excess cholesterol or its omission. Extended half-life ($\leqslant$16 h) of the solute is reflected in its reduced uptake by the liver and spleen.

Abbreviations: egg PC, egg phosphatidylcholine; CF, carboxyfluorescein, DLPC, dilauroyl phosphatidylcholine, DMPC, dimyristoyl phosphatidylcholine; DSPC, distearoyl phosphatidylcholine, DOPC, dioleoyl phosphatidylcholine

2. Materials and methods

The sources and grades of egg PC, cholesterol, CF and ^{111}In-labelled bleomycin (>10 mCi/μg In) have been described [7,9,10]. DLPC, DMPC, DSPC, DOPC and sphingomyelin were purchased from Sigma Chemical Co. All other reagents were of analytical grade.

Small unilamellar liposomes (30–60 nm diam.) with entrapped CF or ^{111}In-labelled bleomycin were prepared [7] from 30 μmol phospholipid alone or supplemented with 30 μmol cholesterol (1:1 molar ratio): after evaporation of the solvent $CHCl_3$ under a stream of O_2-free nitrogen, the dry lipid film was disrupted with 0.25 M CF or a tracer of ^{111}In-labelled bleomycin dissolved in 1.5 ml 0.1 M sodium phosphate containing 0.8% NaCl and 0.02% KCl (phosphate buffer, pH 7.4). The suspension was sonicated [7] for 10 min (1 min sonication with 30 s cooling periods) at 4°C (at 65°C when DSPC was used) and then centrifuged at 100 000 × *g* for 60 min. Separation of CF- or ^{111}In-labelled bleomycin-containing small unilamellar liposomes in the supernatant from the untrapped materials was carried out on a Sepharose CL-6B (Pharmacia) column (1 × 25 cm) equilibrated with phosphate buffer and liposomes dialysed against the same buffer until their use, at most within 24 h following the gel filtration step. ^{111}In in liposomes was measured as in [10] and CF in suitably diluted samples assayed [9] in the absence (free dye) and presence (total dye) of Triton X-100 (1% final conc.) on a Perkin-Elmer fluorimeter using excitation and emission wavelengths of 490 and 520 nm, respectively. The minimum amount of the dye that could be measured accurately was 4 ng/ml [7]. Latent CF serving as an index of liposomal stability [7,10,11] was estimated [9] from

100 ($Dye_t - Dye_f$)/Dye_t, where t and f denote total and free dye respectively.

In in vitro experiments, 1.0 ml mouse (TO strain) heparinized fresh blood was incubated at 37°C with 0.1 ml CF-containing liposomes. At time intervals, blood samples were centrifuged at 3000 rev./min for 10 min and plasma assayed for CF latency [9]. In other experiments, TO mice weighing 25–30 g were injected into the tail vein with 0.2 ml CF-containing liposomes and bled at time intervals. Plasma obtained from heparinized blood samples was then assayed [9] for total, free and latent CF. Mice injected as above with liposomes containing ^{111}In-labelled bleomycin were killed at 72 h and radioactivity was measured [10] in blood, liver, spleen, lungs, kidney, brain and skeletal muscle.

3. Results and discussion

We have studied the effect of phospholipid composition of small unilamellar liposomes on the rate of clearance of their solute contents from the blood of injected animals. To distinguish between the portion of solute in the circulation that is still entrapped from the portion that may have leaked out of liposomes, CF was chosen as a model solute: as CF quenches fully when entrapped at a high concentration (e.g., 0.05–0.25 M) and fluoresces only upon its leakage and subsequent dilution in the surrounding medium [11], assay of quenched (latent) dye in the blood will allow accurate monitoring of liposomal CF levels. If CF leakage in the circulation is nil or minimal, its clearance rates should then be identical or, at least, similar to that of its carrier. Therefore, attempts have been made to reduce permeability of liposomes to entrapped solutes by the incorporation of excess cholesterol into their structure. As discussed in [7–9] this approach effectively reduces the blood-induced permeability of liposomes made of egg PC to a number of solutes. Data in table 1 show that cholesterol acts similarly for liposomes composed of a variety of other phospholipids although for those made of DLPC the effect is less pronounced (49.4% latency, table 1). Indeed, the latency (86.5%) of liposomes made of DSPC alone is actually reduced (to 32.4%) by the incorporation of cholesterol, probably because of an increase in their fluidity [2] (table 1).

Fig.1 shows the rates of clearance of entrapped CF from the circulation of mice injected with liposomes

Table 1
The effect of the cholesterol content of liposomes on the latency of entrapped CF in the presence of blood

Lipid composition	CF latency (%)
Dilauroyl phosphatidylcholine	Nil (58.0)
Dimyristoyl phosphatidylcholine	Nil (66.8)
Distearoyl phosphatidylcholine	86.5 (93.5)
Dioleoyl phosphatidylcholine	Nil (90.1)
Egg phosphatidylcholine	Nil (81.1)
Sphingomyelin	27.5 (96.6)
Dilauroyl phosphatidylcholine, cholesterol	49.4 (96.7)
Dimyristoyl phosphatidylcholine, cholesterol	94.7[a] (99.2)
Distearoyl phosphatidylcholine, cholesterol	32.4[c] (98.9)
Dioleoyl phosphatidylcholine, cholesterol	88.4 (97.5)
Egg phosphatidylcholine, cholesterol	86.3 (97.0)
Sphingomyelin, cholesterol	97.6[b] (98.8)

[a] Incubated for 6 h; [b] incubated for 12 h; [c] 1:0.25 phospholipid:cholesterol molar ratio

Small unilamellar liposomes (2 μmol phospholipid) composed of various phospholipids without or with cholesterol (1:1 molar ratio) and containing 0.25 M CF, were incubated with 10 vol. mouse heparinized fresh blood at 37°C for 1 h unless otherwise stated. Typical CF latency values shown here are means of duplicate readings and are expressed as % of the latencies (shown in parentheses) of the respective liposomal preparations

exhibiting minimum CF leakage in the presence of blood (see table 1). Half-lives of the dye range from a minimum of 0.1 h to a maximum of 16 h for liposomes made of DLPC and sphingomyelin, respectively and, in the latter case, this corresponds to an ~800-fold increase over the half-life (<1.2 min) [7] of the free CF. Since there is inevitably some leakage of CF into the circulation, half-lives of the entrapped dye are likely to be, especially for liposomes made of DLPC, underestimates of those for the respective liposomal preparations. Interestingly, there is no obvious relationship between clearance rates of the liposomal dye and physical characteristics of the component phospholipids. For instance, DPLC, DMPC and DSPC are all saturated phospholipids with fatty acid esters of increasing length yet, respective half-lives of liposomal CF are 0.1, 6 and 1.5 h. Similarly, although both DOPC and egg PC are unsaturated phospholipids, half-lives (1 and 2 h, respectively) of liposomal CF overlap with those observed

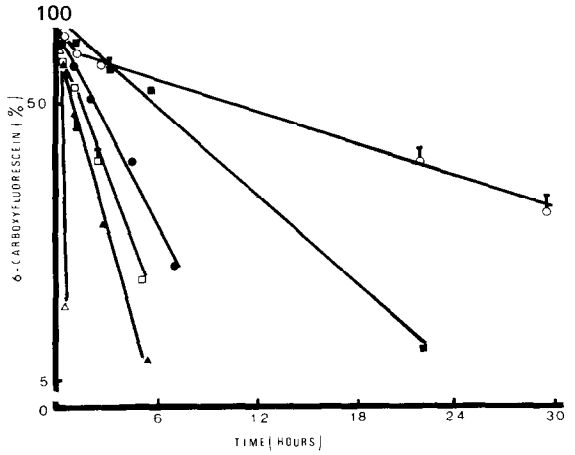

Fig.1. The effect of the phospholipid component of liposomes on the clearance of entrapped CF from the blood of injected mice. Mice were bled at time intervals after intravenous injection of CF entrapped in small unilamellar liposomes (3 mg phospholipid) composed of dilauroyl phosphatidylcholine (△), dioleoyl phosphatidylcholine (▲), distearoyl phosphatidylcholine (□), egg phosphatidylcholine (●), dimyristoyl phosphatidylcholine (■) or sphingomyelin (○). With the exception of liposomes made of distearoyl phosphatidylcholine, all preparations contained cholesterol (1:1 molar ratio). Latent CF values (means from 3 animals) in total mouse plasma are expressed as % of latent CF injected (SE ≥3% are shown). Half-lives estimated from slopes were DLPC, 0.1; DOPC, 1; DSPC, 1.5; egg PC, 2; DMPC, 6; sphingomyelin, 16 h.

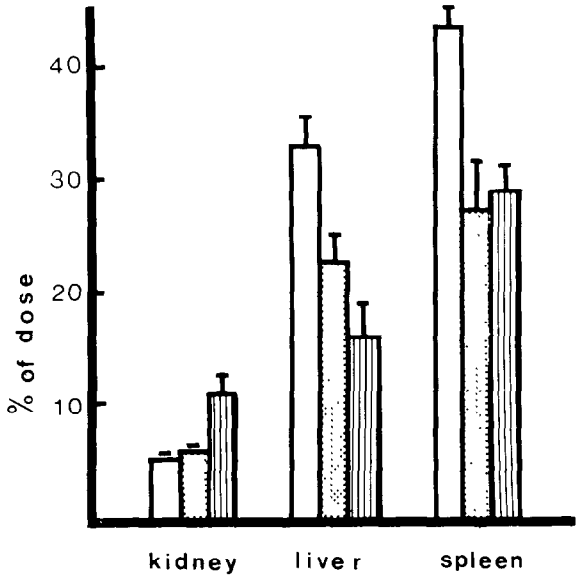

Fig.2. Effect of the phospholipid component of liposomes on the uptake of entrapped ^{111}In-labelled bleomycin by tissues of injected mice. Mice were killed 72 h after the intravenous injection of ^{111}In-labelled bleomycin (10^5–2×10^5 cpm) entrapped in small unilamellar liposomes (3 mg phospholipid) composed of egg phosphatidylcholine (open bars), dimyristoyl phosphatidylcholine (dotted bars) or sphingomyelin (shaded bars). All preparations contained cholesterol (1:1 molar ratio). ^{111}In radioactivity values (means from 3 animals) are expressed as % ±SE of the injected dose/g tissue. Organ weights in 12 mice were 0.53 ± 0.01 (kidney), 1.7 ± 0.05 (liver) and 0.12 ± 0.01 g (spleen). Radioactivity levels in the total plasma of mice at death were <4% and in brain, lungs and skeletal muscle <0.5, 4.0 and 1.0% of the injected dose/g tissue.

for saturated DSPC and DMPC (1.5 and 6 h, respectively).

Differences in liposomal solute survival in the circulation, effected by the nature of the phospholipid component of liposomes, are expected to reflect the extent of solute uptake by the liver and spleen. In studies (fig.2) on tissue distribution of liposomal solutes, we have used as a model solute ^{111}In-labelled bleomycin. ^{111}In, unlike CF (unpublished) persists in tissues for several days [10] thus allowing examination of their radioactivity levels at a time when blood is devoid of circulating liposomes. The total amount of the solute delivered to tissues by a given liposomal preparation can therefore be measured. From fig.2, for liposomal preparations which progressively increase the half-life of the solute they carry (e.g., those made of egg PC, DMPC and sphingomyelin; fig.1), transport of their radioactivity to the liver is accordingly decreased. Thus, hepatic uptake of ^{111}In is reduced from ~32% (egg PC liposomes) to 23% (DMPC liposomes) and to 16% for sphingomyelin liposomes. Considerable reduction of radioactivity uptake for the latter preparation is also observed in the case of spleen (fig.2). In contrast, uptake of ^{111}In by kidney is greater when the long-lived sphingomyelin liposomes are given, probably because of the prolonged, albeit minor, leakage of radioactivity in the blood and its subsequent excretion into the urine [10].

Whatever the surface properties of liposomes that enable some preparations to persist in the blood longer than others, these must be considered in conjunction with the mechanism(s) responsible for the recognition and uptake of liposomes by the liver and spleen. It has been shown, for instance, that plasma components such as α_1-macroglobulin [12] and other proteins [13] coat liposomes upon contact and it may be that this opsonizes them for phagocytosis by

the reticuloendothelial system. It is conceivable that the efficiency of this process is reduced by certain phospholipids and increased by others. Whilst events controlling liposome clearance from the circulation await elucidation, it is apparent from this and other studies [7–9,14–17] that appropriate choice of loposomal size, surface charge and composition can contribute to the design of liposomes suitable for realistic attempts at therapy.

Acknowledgement

This work was supported in part by a NIH National Cancer Institute contract (NO1-CM-87171).

References

[1] Gregoriadis, G. (1979) in: Drug Carriers in Biology and Medicine (Gregoriadis, G. ed) pp. 287–341, Academic Press, London, New York.
[2] Papahadjopoulos, D. ed (1978) Ann. NY Acad. Sci. 308, 1–462.
[3] Gregoriadis, G. and Neerunjun, E. D. (1974) Eur. J. Biochem. 47, 179–185.
[4] Juliano, R. L. and Stamp, D. (1975) Biochem. Biophys. Res. Commun. 63, 651–658.
[5] Scherphof, G., Roerdink, F., Waite, M. and Parks, J. (1978) Biochim. Biophys. Acta 542, 296–307.
[6] Chobanian, J. V., Tall, A. R. and Brecher, P. I. (1979) Biochemistry 18, 180–187.
[7] Kirby, C., Clarke, J. and Gregoriadis, G. (1980) Biochem. J. 186, 591–598.
[8] Gregoriadis, G. and Davis, C. (1979) Biochem. Biophys. Res. Commun. 89, 1287–1293.
[9] Kirby, C., Clarke, J. and Gregoriadis, G. (1980) FEBS Lett. 111, 324–328.
[10] Gregoriadis, G., Neerunjun, E. D. and Hunt, R. (1977) Life Sci. 21, 357–369.
[11] Weinstein, J. N., Yoshikani, S., Henkart, P., Blumenthal, R. and Hagins, W. A. (1977) Science 195, 489–492.
[12] Black, C. D. V. and Gregoriadis, G. (1976) Biochem. Soc. Trans. 4, 253–256.
[13] Tyrrell, D. A., Richardson, V. J. and Ryman, B. E. (1977) Biochim. Biophys. Acta 497, 469–480.
[14] Mayhew, E., Rustum, Y. M., Szoka, F. and Papahadjopoulos, D. P. (1979) Cancer Treat. Rep. 63, 1923–1928.
[15] Ganepathi, R., Krishan, A., Wodinsky, I., Zubrod, C. G. and Lesko, L. J. (1980) Cancer Res. 40, 630–633.
[16] Scherphof, G., Roerdink, F., Hoekstra, D., Zborowski, J. and Wisse, E. (1980) in: Liposomes in Biological Systems (Gregoriadis, G. and Allison, A. C. eds) pp. 179–209, Wiley, Chichester, New York.
[17] Weinstein, J. N., Magin, R. L., Yatvin, M. B. and Zaharko, D. S. (1979) Science 204, 188–191.