Is half-life of circulating liposomes determined by changes in their permeability?

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Membrane permeability *Liposome half-life* *Cholesterol* *Phospholipid* *Bilayer stability*

1. INTRODUCTION

Understanding of factors related to the clearance of liposomes from the blood is of central importance in their use as a drug delivery system [1]. For instance, efficient interaction of drug-containing liposomes with target cells in the intravascular space or access to extravascular cells is likely to be promoted by the carrier's prolonged presence in the circulation. To this end, there has been variable success through reduction of vesicle size [2], adjustment of surface charge [3], or phospholipid composition [4,5], the use of phospholipase-resistant phospholipids [6] and, indirectly, by blocking the reticuloendothelial system [3,7,8]. However, an important prerequisite for retarded liposome clearance is resistance to the detrimental effect of blood on bilayer stability [9,10]. Recent studies have shown that excess cholesterol in certain types of liposomes greatly improves bilayer stability in the presence of blood and reduces their permeability [11–16]. This has led to the preparation of small unilamellar vesicles that, while in the circulation, remain relatively intact and retain entrapped solutes quantitatively [11,13]. In work with such liposomes it was observed [4,5] that rate of clearance varied widely according to their phospholipid component. Here we present evidence that liposomal half-life in the circulation is determined by the extent to which phospholipids, alone or in association with cholesterol, prevent plasma-induced bilayer permeability.

Abbreviatons: PC, egg phosphatidylcholine; SM, sphingomyelin; DSPC, distearoyl phosphatidylcholine; CF, carboxyfluorescein; 4-APP, 4-aminopyrazolo-(3,4d)-pyrimidine.

2. MATERIALS AND METHODS

Sources and grades of PC SM, DSPC, cholesterol, [^{3}H]PC, [^{14}C]SM and CF have been described [5,17]. CF was used without further purification since previous work [5] with both the purchased (unpurified) and purified CF from the same batch showed that the minor proportions of impurities in this particular batch (lot C4C) did not interfere significantly with the use of the dye as a marker of liposomal permeability. The ultraviolet and visible absorption spectra of unpurified and purified CF (5 μM solution) were practically identical [5] and very similar to those obtained by others [18]. 4-APP (95% pure) was purchased from Sigma Chemical Company. All other reagents were of analytical grade.

2.1. *Preparation of liposomes*

Small unilamellar liposomes (30–60 nm average diameter) of neutral charge containing quenched CF (0.2 M) were prepared from 20 μmol PC, 20 μmol SM or combinations of the two phospholipids (hybrid liposomes; 20 μmol total phospholipid) mixed with tracer [^{3}H]PC and/or [^{14}C]SM and 20 μmol of cholesterol. Sonication [5] was carried out at 4°C for PC and 37°C for SM and hybrid liposomes. The extent of phospholipid incorporation (83.0–94.5% of the amount used) was estimated from ^{3}H and/or ^{14}C assays [5,17] in the

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preparations. In the hybrids, PC content was 23%, 53% and 77% of total phospholipid. In some experiments small unilammellar liposomes were prepared from DSPC alone (20 μmol) or DSPC (20 μmol) and equimolar cholesterol with the sonication step carried out at about 65°C. They were allowed to anneal at the same temperature. Formation of small unilammellar liposomes (all types) and retention of size to the time of their use were judged as before [5]. In a few instances, liposomes composed of equimolar DSPC and cholesterol failed to form [5] and these preparations were discarded. Others [19] have observed similar difficulties with preparing liposomes from higher melting phospholipids. Quenched CF (latency) values (95.5–93.3% of total CF) in all liposomal preparations immediately before use were estimated [4] from 100 $(Dye_t - Dye_f)/Dye_t$ where t and f denote total CF measured in the presence of Triton X-100 (1% final concentration) and free CF respectively. CF entrapment values in the preparations were 0.5–0.8% of total used per ml liposomes.

2.2. *In vitro experiments*

Fresh plasma samples (0.5 ml) from T.O. male mice were preheated to 37°C, mixed with 0.1 ml CF-containing liposomes (0.9–1.2 μmol phospholipid and 94–120 μg CF) and incubated at the same temperature. At time intervals 10 μl of the mixtures were assayed [4,5] for CF latency as above. Released CF (% of total entrapped) was derived from differences in CF latency values before and after incubation.

2.3. *In vivo experiments*

T.O. male mice weighing 20–25 g were injected into the tail vein with 0.2 ml CF-containing liposomes ([^{3}H]PC and/or [^{14}C]SM-labelled) (1.8–2.4 μmol phospholipid, 188–240 μg CF and 9.1×10^4 -2.0×10^5 dpm ^{3}H and/or 1.0×10^4–3.0×10^4 dpm ^{14}C). At time intervals 25 μl blood plasma samples were assayed [4,5] for latent CF. In other experiments, T.O. male mice were made lipoprotein deficient [20] by daily intraperitioneal injections of 60 mg 4-APP/kg body weight. 4-APP was dissolved in 0.5 ml of 0.01 M sodium phosphate adjusted to pH 2 with 1 N HCl before use. In similarly treated control mice 4-APP was omitted from the injected solution. 24 h after the last injection, 4-APP-treated and control mice were tested [21] for total cholesterol in blood plasma and then given intravenously 0.2 ml CF-containing liposomes (2.5 μmol phospholipid) composed of equimolar PC and cholesterol. They were bled at time intervals and blood plasma samples assayed for latent CF as above. Total cholesterol levels (mean ± SD) in control and 4-APP-treated mice were 2.26 ± 0.42 (5 mice) and 0.19 ± 0.13 mmol/l plasma (10 mice) respectively.

3. RESULTS AND DISCUSSION

Recent work [4,5] with small unilamellar cholesterol-rich liposomes of varying phospholipid composition has revealed a half-life range in mice of 0.1–16 h. Half-lives in these experiments [4,5] were derived from the clearance patterns of quenched CF entrapped in the aqueous phase of liposomes and were, therefore, likely to represent those of intact vesicles: quenched CF can be measured only after the disruption of liposomes with Triton X-100 and ensuing dilution of the dye to

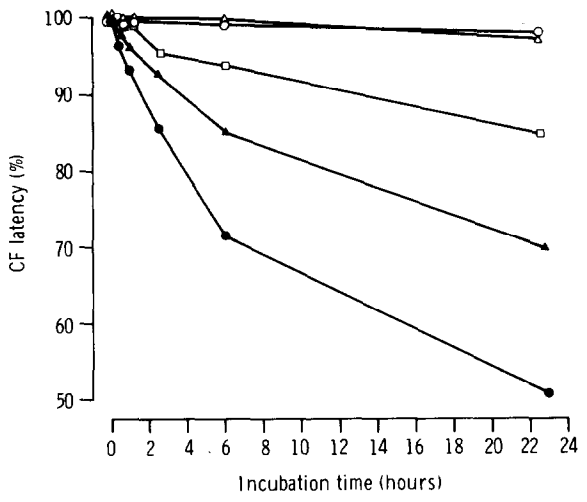

Fig.1. The effect of phospholipid composition of liposomes on their permeability in plasma. In a typical experiment shown here, small unilamellar liposomes containing quenched CF and composed of SM (○), 77% SM, 23% PC (△), 47% SM, 53% PC (□), 23% SM, 77% PC (▲) and PC (●) were incubated in the presence of mouse plasma at 37°C. All liposomal preparations contained cholesterol, equimolar to the total phospholipid. CF latency values at time intervals are % of total CF present. For other details see Materials and Methods.

Table 1

In vivo and in vitro behaviour of liposomal CF

Liposomes	Half life in vivo (h)		CF released in vitro (% of total present)[d]	Quenched CF removed in vivo (% of injected)[f]
	quenched CF[a]	[^{14}C]SM[b]		
SM	11.0	10.0	1.0[e]	–
77% SM, 23% PC	11.0	10.7	1.0[e]	2.0
47% SM, 53% PC	7.0	7.0	6.0	15.9
23% SM, 77% PC	4.5	3.6	12.3	32.0
PC	2.1	2.3[c]	11.5	34.5

[a] Half-lives estimated from fig.2

[b] Half-lives estimated from clearance patterns (not shown) of liposomes labelled with [^{14}C]SM

[c] Estimated from clearance patterns (not shown) of liposomes labelled with [^{3}H]PC

[d] Estimated as difference in CF latency before and after incubation with plasma for periods of time equivalent to half-lives of quenched CF for the respective preparations

[e] Values were $< 2\%$ after 29 h incubation

[f] Estimated from fig.2 by subtracting quenched CF present in the circulation at times equivalent to the (quenched CF-based) half-lives of the respective preparations from quenched CF in SM liposomes present at the corresponding periods. For details see the text

Cholesterol-rich small unilamellar liposomes incubated with mouse plasma or injected into mice contained quenched CF and were composed of SM only, PC only or of combinations of the two phospholipids in the proportions shown. Molar ratio of total phospholipid to cholesterol was 1.0

concentrations that will allow it to fluoresce [4]. Since such variations in liposome clearance cannot be explained on the basis of differences in vesicle surface charge or size (all preparations used were neutral and of similar average vesicle size [30–60 nm] [4,5,13]), we have investigated one attribute of phospholipids that may influence liposome clearance namely their varying resistance to removal by plasma HDL [16] with bilayer permeability affected accordingly [5,16]. In the quenched CF-containing liposomal preparations used in this study, the phospholipid component SM was replaced by increasing amounts of PC and, as shown in fig.1 permeability (to CF) in the presence of mouse plasma increases accordingly. As expected [4,5,22], after intravenous injection of mice with SM liposomes (fig.2) the rate of clearance of quenched CF is slow and remains so upon modest (23%) substitution of liposomal SM with PC. However, rate of clearance is augmented gradually with 53%, 77% and 100% substitution. Half-lives (table 1) as estimated from clearance patterns of quenched CF decreased from 11 (SM and 77% SM, 23% PC liposomes) to 2.1 h (PC liposomes) with intermediate values (7 h and 4.5 h) for the hybrid preparations (47% SM, 53% PC and 23% SM, 77% PC) reflecting the effect of PC concentration. Similar half-life values were obtained when radiolabelled phospholipids were used as markers of the liposomal lipid phase (table 1).

In spite of this apparently causal relationship between liposome permeability in vitro and clearance in vivo (figs.1 and 2), it could, however, be argued that since increasing amounts of PC in SM liposomes render these increasingly vulnerable to HDL attack [16], corresponding CF leakage from the circulating vesicles may be such that the remainder of entrapped CF is still of high enough concentration to self-quench and thus account for the reduced vesicle clearance rates. It may there-

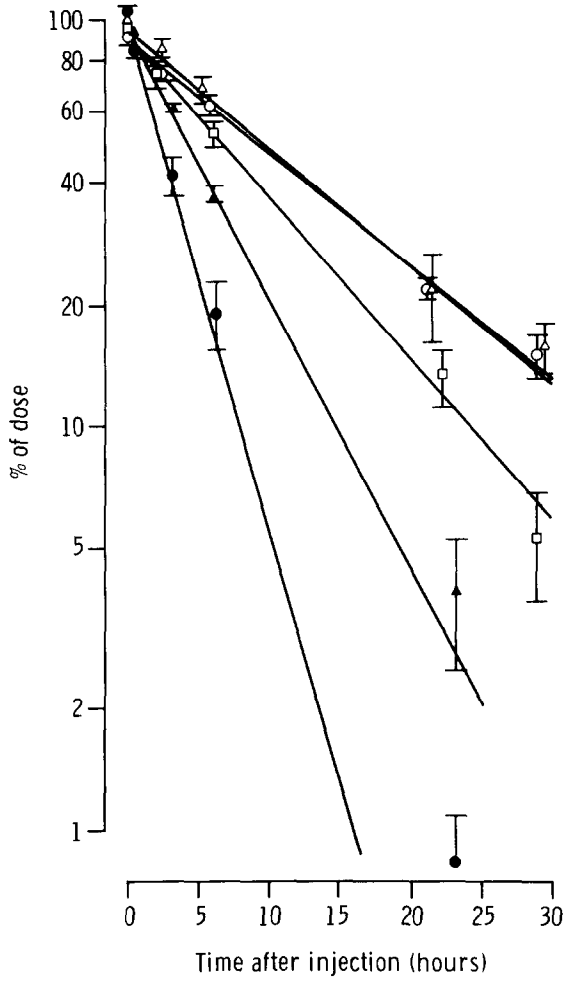

Fig.2. The effect of phospholipid composition of liposomes on their clearance from the circulation. Liposomes as described in fig.1 were injected intravenously into mice. Latent CF values (mean ± SD; 3–6 animals) at time intervals are % of injected latent CF per total mouse plasma [4]. For other details see legend to fig.1 and Materials and Methods.

fore be that liposomes, regardless of phospholipid composition, are removed from the circulation at comparable rates and that differences observed are artefacts reflecting degrees of CF leakage. This possibility, not supported by the similarity in half-life values as measured with the quenched CF and phospholipid markers (table 1), was nonetheless examined: according to fig.2, 50% of the quenched CF administered in PC liposomes is removed from the circulation within 2.1 h (assumed half-life) after injection whereas only 18% of quenched CF in SM liposomes is removed during the same time. If PC and SM liposomes are indeed cleared at the same rate, the difference (34.5%; table 1) between SM and PC liposomal quenched CF removal during the 2.1 h period should then reflect CF leakage from PC liposomes to that extent. As free (leaked) CF is removed from the circulation very rapidly [13] and its quantitation is therefore difficult if at all possible, CF leakage was estimated from fig.1 where the volume ratio of plasma to liposomes simulates that attained in the circulation of mice injected as in fig.2. We have already shown [5,13] that mouse whole blood affects liposomal permeability similarly to or even less than plasma and, it is thus probable that CF release caused by plasma in vitro is comparable to that occurring in the intravascular space in vivo. According to table 1 and legend there is very little release (< 2%) of CF from SM liposomes upon extended (over 29 h) incubation with plasma and, assuming that this is true in vivo as well, the half-life (11 h) of quenched CF in SM liposomes must be a close approximation of the clearance rate of the carrier itself. On the other hand, after incubation of PC liposomes for 2.1 h (the period of time needed for the removal of 50% of quenched CF from the circulation) 11.5% of the entrapped CF is released. It would thus appear that only a fraction of the difference (34.5%) between SM and PC liposomal quenched CF removal in vivo at 2.1 h (tentatively assumed to represent CF leakage) could be justified in these terms. In fact, because of the continuous removal of liposomes from the circulation, the total amount of leaked CF during the 2.1 h period must be considerably smaller than the total (11.5%) leaked in vitro and has probably no significant influence on the overall quenched CF clearance rate. Similar conclusions drawn from observations with the hybrid preparations (table 1) strongly suggest that small unilamellar liposomes are cleared from the blood with half-lives which are inversely related to permeability changes in the bilayer effected by blood components, presumably HDL. This is supported by the finding that in lipoprotein deficient mice, half-life of PC liposomes was increased from 2 h (control mice) to 4.5 h (see Materials and Methods).

An alternative explanation for the relationship

between liposomal clearance and phospholipid component may be that the latter, through structural characteristics expressed on the liposomal surface, directs vesicle removal from the circulation, for instance by way of association with opsonins or other proteins and/or recognition by the tissues. If so, liposomal preparations sharing the same phospholipid component should exhibit similar rates of clearance. This was tested with small unilamellar cholesterol-free and cholesterol-rich DSPC liposomes. The choice of DSPC enables us to use a cholesterol-free preparation that, in contrast to similar preparations made of PC, SM or other phospholipids [4,5,16], is reasonably stable [4] in the presence of blood and thus suitable for clearance studies. Figure 3b shows that half-life values for cholesterol-free and cholesterol-rich DSPC liposomes in mice (1.3 h and 20 h respectively) do not support the above proposition and are probably too diverse to be attributed to different phospholipid density on the bilayer. Interestingly, however, half-lives correspond to partial permeability and complete impermeability of the respective liposomes in the presence of plasma (fig.3a). Further, using the same approach as earlier to estimate contribution of CF leakage to the clearance pattern of the cholesterol-free DSPC liposomes, it can be seen (fig.3b) that the difference (50%) in quenched CF removal between cholesterol-rich and cholesterol-free DSPC liposomes 1.3 h after injection compares with only 20% leakage in vitro for the same period (estimated from fig.3a). Again, in view of the continuous clearance of liposomes from the circulation, total leakage in vivo must have been even less and therefore account for only a fraction of removed quenched CF. It thus seems likely that the widely different half-lives reflect differences in bilayer permeability.

The present results raise a number of questions. For instance, assuming that liposomal half-life is determined by the extent of plasma-promoted bilayer porosity, is a half-life value of 20 h (to our knowledge the longest recorded so far) for the completely impermeable (to CF) cholesterol-rich DSPC liposomes an upper limit? Our experience with SM liposomes is that half-life values also depend on the amount of liposomal lipid injected (e.g., 16 h and 10 h for 3 mg and 1 mg injected SM) [4,5] and it is conceivable that a dose of DSPC

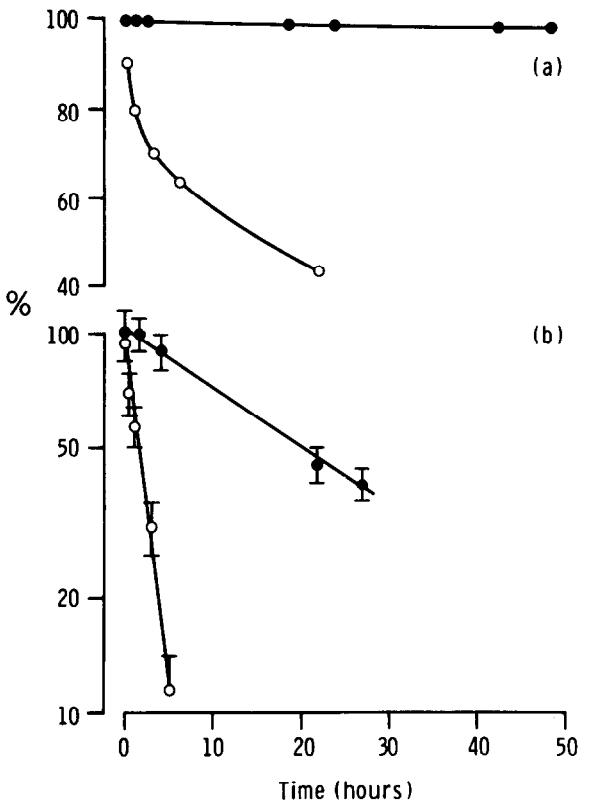

Fig.3. Permeability of DSPC liposomes in mouse plasma and clearance from the circulation. Small unilamellar liposomes containing quenched CF and composed of DSPC (○) or equimolar DSPC and cholesterol (●) were incubated in the presence of mouse plasma at 37°C (a) or injected intravenously into 4 mice (b). Values at time intervals are % latent CF of total CF present or % (mean ± SD) latent CF of injected latent CF per total mouse plasma [4]. For other details see Materials and Methods.

higher than that (1.35 mg) used in fig.3b will exhibit a greater half-life. Since a half-life upper limit for stable liposome must exist, presumably to approach that shown by a tissue saturation dose, will it be different in another animal species (e.g., man) when comparable amounts of lipid per kg body weight are injected? Furthermore, what is the mechanism underlying the removal of intact small unilamellar liposomes from the circulation and how does it relate to the acceleration of such removal when bilayers become leaky? Finally, will it be possible to apply knowledge acquired with

small liposomes to augment the half-lives of larger vesicles with higher drug trapping efficiency? Answers to such questions should contribute toward the rational application of liposomes in drug delivery.

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REFERENCES

[1] Gregoriadis, G. (1981) Lancet 2, 241–247.
[2] Juliano, R.L. and Stamp, D. (1975) Biochem. Biophys. Res. Commun. 63, 651–658.
[3] Gregoriadis, G. and Neerunjun, E.D. (1974) Eur. J. Biochem. 47, 179–185.
[4] Gregoriadis, G. and Senior, J. (1980) FEBS Lett. 119, 43–46.
[5] Senior, J. and Gregoriadis, G. (1982) Life Sci. 30, 2123–2136.
[6] Deshmukh, D.S., Bear, W.D., Wisniewski, H.M. and Brockerhoff, H. (1978) Biochem. Biophys. Res. Commun. 82, 328–334.
[7] Suhami, R.L., Patel, H.M. and Ryman, B.E. (1981) Biochim. Biophys. Acta 674, 354–371.
[8] Abra, R.M. and Hunt, C.A. (1981) Biochim. Biophys. Acta 666, 493–503.
[9] Krupp, L., Chobanian, A.V. and Brecher, P.I. (1976) Biochem. Biophys. Res. Commun. 72, 1251–1258.
[10] Scherphof, G., Roerdink, F., Waite, M. and Parks, J. (1978) Biochim. Biophys. Acta 542, 296–307.
[11] Gregoriadis, G. and Davis, C. (1979) Biochem. Biophys. Res. Commun. 89, 1287–1293.
[12] Finkelstein, M. and Weissmann, G. (1979) Biochim. Biophys. Acta 587, 202–216.
[13] Kirby, C., Clarke, J. and Gregoriadis, G. (1980) Biochem. J. 186, 591–598.
[14] Damen, J., Morselt, H. and Scherphof, G. (1981) Biochim. Biophys. Acta 665, 538–545.
[15] Guo, L.S.S., Hamilton, R.L., Goerke, J., Weinstein, J.N. and Havel, R.J. (1980) J. Lipid Res. 21, 993–1003.
[16] Allen, T.M. (1981) Biochim. Biophys. Acta 640, 385–397.
[17] Kirby, C. and Gregoriadis, G. (1980) Life Sci. 27, 2223–2230.
[18] Ralston, E., Hjelmeland, L.M., Klansner, R.D., Weinstein, J.N. and Blumenthal, R. (1981) Biochim. Biophys. Acta. 649, 133–137.
[19] Weinstein, J.N., Klausner, R.D., Innerarity, T., Ralston, E. and Blumenthal, R. (1981) Biochim. Biophys. Acta. 647, 270–284.
[20] Councell, R.E., Schappa, L.W., Korn, N. and Huler, R.J. (1980) J. Nucl. Med. 21, 852–858.
[21] Richmond, W. (1973) Clin. Chem. 19, 1350–1356.
[22] Hwang, K.J., Luk, K-F.S., and Beaumier, P.L. (1980) Proc. Natl. Acad. Sci. USA 77, 4030–4034.