Plasma-induced release of solutes from small unilamellar liposomes is associated with pore formation in the bilayers

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(Received 5 May 1981/Accepted 1 July 1981)

Understanding of the behaviour of liposomes within the biological milieu is essential to their rational design and effective use in drug targeting (Gregoriadis, 1979). One line of related research concerns the effect of blood on liposomal stability (Gregoriadis & Davis, 1979; Finkelstein & Weissmann, 1979; Allen, 1981). Previous work (Gregoriadis, 1973) revealed that, after intravenous injection, circulating liposomes lose much of their solute content. This was subsequently attributed (Krupp *et al.*, 1976; Scherphof *et al.*, 1978) to the destabilizing action of plasma high-density lipoproteins, which remove phospholipid molecules from the bilayers. It has been suggested (Scherphof *et al.*, 1981) that, for small unilamellar liposomes, such destabilization is synonymous with their total destruction. Recently we (Kirby *et al.*, 1980a,b; Kirby & Gregoriadis, 1980; Gregoriadis & Senior, 1980) have shown both *in vitro* and *in vivo* that the presence of cholesterol in small unilamellar liposomes decreases, in proportion to its concentration, the loss of phospholipid molecules to plasma high-density lipoproteins and ensuing solute release.

In the present paper we have examined the nature of changes in the structure of small unilamellar liposomes effected by plasma. Our results show that plasma induces the formation of pores in the liposomal membrane, the size of which is inversely proportional to the amount of cholesterol present. We have found no evidence of major bilayer breakdown, even for liposomes that are devoid of cholesterol.

Materials and methods

The sources and grades of egg phosphatidylcholine, cholesterol, carboxyfluorescein, Sepharose CL-6B and Ultrogel AcA 34 have been described previously (Kirby *et al.*, 1980a,b). [6,6'-(n)-³H]-Sucrose (sp. radioactivity 1–5 Ci/mmol), [³H]inulin (sp. radioactivity >500 Ci/mol) and ¹²⁵I-labelled polyvinylpyrrolidone (Povidon, 1-vinylpyrrolidin-2-one polymers; 20–60 µCi/mg; average mol.wt. 30 000–40 000) were purchased from The Radio-

chemical Centre, Amersham, Bucks., U.K. All other reagents were of analytical grade.

Small unilamellar liposomes containing radiolabelled sucrose, inulin and polyvinylpyrrolidone were prepared (Kirby *et al.*, 1980a,b) from a chloroform solution of 40 µmol of egg phosphatidylcholine and, when appropriate, 11.4 µmol (cholesterol-poor liposomes) or 40 µmol of cholesterol (cholesterol-rich liposomes) (7:2 and 7:7 molar ratios respectively). The solvent was evaporated under a stream of O₂-free N₂ and the dried lipids were subsequently disrupted with 2 ml of 0.1 M-sodium phosphate buffer supplemented with 0.8% NaCl and 0.02% KCl (phosphate buffer, pH 7.4) and containing 0.2 M-carboxyfluorescein and tracers of [³H]sucrose (1.25 × 10⁸ d.p.m.), [³H]inulin (1.28 × 10⁸ d.p.m.) or radioiodinated polyvinylpyrrolidone (8.5 × 10⁷ c.p.m.). The suspension was sonicated (Kirby *et al.*, 1980a,b) for 10 min (1 min sonication with 30 s cooling periods) at 4°C and then centrifuged at 100 000 g (*r*_{av}, 8.6) for 60 min to remove debris and multilamellar structures. Separation of radiolabelled marker-containing small unilamellar liposomes in the supernatant from untrapped material was carried out on a Sepharose CL-6B column (0.8 cm × 20 cm) equilibrated with phosphate buffer and liposomes were dialysed against the same buffer until their use (at the most within 24 h after the gel-filtration step). ³H and ¹²⁵I radioactivity in liposomal samples was assayed as described previously (Gregoriadis & Ryman, 1972). Entrapment values for the three solutes varied between 1.2 and 5.0%. In some experiments 'empty' (buffer-loaded) small unilamellar liposomes were prepared as described above but in the absence of radiolabelled markers. The diameter of all liposomes, as measured by electron microscopy (Kirby *et al.*, 1980a), was 30–60 nm.

Fresh blood plasma (1.0 ml) from T.O mice or phosphate buffer (1.0 ml) was incubated with 0.25 ml of liposomes containing each of the three radiolabelled markers at 37°C for 30 min, and subsequently chromatographed through an Ultrogel AcA 34 column (1 cm × 25 cm). ³H and ¹²⁵I labels were then measured in the fractions obtained. In

other experiments, 1.0 ml of mouse plasma or 1.0 ml of phosphate buffer both containing carboxyfluorescein (0.2 M final concentration) were incubated at 37°C for 30 min with 0.25 ml of buffer-loaded liposomes and then chromatographed as described above. Carboxyfluorescein in the fractions obtained was assayed (Kirby *et al.*, 1980a,b) in the absence (free dye) and presence (total dye) of Triton X-100 (1% final concentration). The minimum amount of carboxyfluorescein that could be measured accurately was 4 ng/ml. Latent dye serving as an index of liposomal stability (Weinstein *et al.*, 1977; Kirby *et al.*, 1980a,b) was estimated (Kirby *et al.*, 1980b) from the expression:

$$100 (\text{Dye}_t - \text{Dye}_f) / \text{Dye}_t$$

where t and f denote total and free dye respectively.

Results and discussion

We have previously found (Kirby *et al.*, 1980a,b) that plasma-induced destabilization of cholesterol-free small unilamellar liposomes is very rapid and associated with nearly total release of entrapped solutes. This would be compatible with both total breakdown of the vesicles (Scherphof *et al.*, 1981) or formation in otherwise intact vesicles of pores through which only solutes below a certain size can pass freely (Gregoriadis & Davis, 1979). Our present attempt to establish which of the two events occurs has been 2-fold. (a) We have entrapped in liposomes radiolabelled solutes of various molecular weights and then measured release from the vesicles in the presence of plasma. If liposomes do indeed break down, release of contents into the medium would be expected to be independent of solute size. On the other hand, preferential release of solutes favouring those of smaller size could be accounted for by pore formation; (b) we have incubated buffer-loaded liposomes with plasma containing 0.2 M-carboxyfluorescein. At this concentration carboxyfluorescein is fully quenched and if pores are formed in the vesicles, there should be diffusion of quenched carboxyfluorescein into their interior. Liposomes, on re-isolation, should then contain self-quenched dye.

After incubation of liposomal sucrose, inulin and polyvinylpyrrolidone in the presence of phosphate buffer at 37°C and chromatography, 94.5–99% of the three solutes applied to the Ultrogel columns are recovered with liposomes (see the legend to Fig. 1). On the other hand, elution profiles of the solutes after incubation in the presence of plasma indicate liposomal membrane destabilization and solute release. As expected (Kirby *et al.*, 1980b) portions of markers that are still entrapped were eluted together with liposomes (fractions 1–3), whereas those that have been released were eluted according to the molecular weight of the solute (fractions 7–13 for

Fig. 1. *Ultrogel chromatography of small unilamellar liposomes containing solutes of various molecular weight after incubation with plasma*

Cholesterol-free (a), cholesterol-poor (b) and cholesterol-rich (c) small unilamellar liposomes containing radiolabelled sucrose (---), inulin (—) or polyvinylpyrrolidone (····) were incubated in the presence of mouse plasma at 37°C for 30 min. Samples were subsequently chromatographed through an Ultrogel AcA 34 column (see the Materials and methods section). Radioactivity measured in the fractions obtained is expressed as a percentage of the total recovered. Recoveries were 93.2–97.8% of radioactivity applied to the columns. After chromatography of liposomes incubated in the presence of phosphate buffer at 37°C for 30 min, 94.5–99.0% of the radioactive labels applied was recovered with liposomes in fractions 1–3.

polyvinylpyrrolidone, 11–16 for inulin and 14–17 for sucrose). The small amount of freed inulin recovered in fractions 7–11 was probably bound to a plasma component. Furthermore, Fig. 1 confirms our previous finding (Kirby *et al.*, 1980a,b) that solute release from liposomes in the presence of plasma is inversely proportional to their cholesterol content. Quantification of the elution profiles from three separate experiments (Table 1) indicates that release of solutes is dependent on their size. For instance, with cholesterol-free liposomes, 80.6, 68.9 and 26.9% of sucrose, inulin and polyvinylpyrrolidone are released respectively. These results, showing preferential loss of the smaller sucrose and inulin molecules, argue against breakdown of

Table 1. *Release of solutes from liposomes in the presence of plasma*

Small unilamellar liposomes containing radiolabelled sucrose, inulin and polyvinylpyrrolidone were incubated in the presence of mouse plasma at 37°C for 30 min. Samples were then chromatographed on Ultrogel AcA 34 columns. Radioactivity released from liposomes and eluted in corresponding fractions (see Fig. 1) was pooled and expressed as a percentage of the total applied. For other details see the Materials and methods section and the legend to Fig. 1. Results are means $\pm$ S.D. for the numbers of experiments indicated in parentheses.

Liposomes	Released radioactivity (%)		
	Sucrose	Inulin	Polyvinylpyrrolidone
Cholesterol-free	80.6 $\pm$ 10.4 (3)	68.9 $\pm$ 6.9 (3)	26.9 $\pm$ 3.7 (3)
Cholesterol-poor	42.2 $\pm$ 2.8 (3)	31.1 $\pm$ 7.2 (3)	26.1 $\pm$ 3.0 (3)
Cholesterol-rich	4.1 $\pm$ 2.1 (3)	7.7 $\pm$ 0.9 (3)	6.6, 7.7

liposomes and are consistent with the maintenance of a closed, albeit leaky, vesicle structure. Since the extent of loss of the two smaller solutes is similar, pores formed are likely to be large enough to allow relatively free passage of sucrose and inulin and small enough to restrict exit of polyvinylpyrrolidone. Incorporation of some cholesterol in liposomes (7:2, phospholipid/cholesterol molar ratio) appears to reduce the size of pores so that inulin loss (31.1%) becomes similar to that of polyvinylpyrrolidone (26.1%), whereas sucrose loss (42.2%) is still considerably higher (there was no overlapping between inulin and sucrose values). This implies that the size of pores in such liposomes is now small enough to restrict the passage of inulin and polyvinylpyrrolidone and to a lesser extent that of sucrose. When, on the other hand, phospholipid and cholesterol are equimolar, the amount released is low (4.1–7.7%) for all three solutes and any pores present must be too small, even for sucrose (Table 1).

Results from the reverse experiment i.e. incubation of buffer-loaded liposomes with plasma containing quenched carboxyfluorescein are shown in Table 2. On chromatography of the incubation mixtures, fractions expected to contain liposomes also contained latent dye, indicating carboxyfluorescein entry into the vesicles (in spite of their plasma-induced destabilization), presumably through formed pores. Carboxyfluorescein latency for cholesterol-free liposomes (56.2%) was lower than for cholesterol-poor (75.9%) and cholesterol-rich (89.7%) liposomes. This is probably because in the latter cases it is more difficult for the dye, once taken up, to leak out of liposomes during chromatography or on standing (Kirby *et al.*, 1980a,b). Interestingly, a similar pattern of increasing carboxyfluorescein latency was observed in liposomes incubated with phosphate buffer (Table 2).

Our recent work has shown that after incubation of cholesterol-free small unilamellar liposomes with

Table 2. *Entry of carboxyfluorescein into liposomes in the absence and presence of plasma*

Buffer-loaded small unilamellar liposomes were incubated in phosphate buffer or mouse plasma both containing 0.2 M-carboxyfluorescein at 37°C 30 min. Samples were then chromatographed on Ultrogel AcA 34 columns and fractions expected to contain liposomes were pooled and assayed for latent carboxyfluorescein. For other details see the Materials and methods section. Results are means $\pm$ S.D. for the numbers of experiments indicated in parentheses.

Liposomes	Carboxyfluorescein latency (%)	
	Phosphate buffer	Mouse plasma
Cholesterol-free	40.8 $\pm$ 2.2 (3)	56.2 $\pm$ 4.4 (3)
Cholesterol-poor	63.0 $\pm$ 3.1 (3)	75.9 $\pm$ 2.3 (3)
Cholesterol-rich	74.1 $\pm$ 0.7 (3)	89.7 $\pm$ 2.7 (3)

plasma, as much as 67% of the egg phosphatidylcholine is removed by plasma high-density lipoproteins (Kirby *et al.*, 1980b). The present data (Fig. 1 and Tables 1 and 2) indicate that, in spite of this heavy loss of phospholipid, liposomes retain their closed vesicle structure. However, some replenishment with phospholipid from plasma high-density lipoproteins cannot be ruled out. Although the location (random or selective), number, size and size homogeneity of pores formed in the vesicles remain at present unknown, it is safe to assume that the pores are not wide enough to allow free passage of solutes as large as polyvinylpyrrolidone. On the other hand, after incorporation of increasing amounts of cholesterol, leakage of solutes is reduced to an extent that even molecules as small as sucrose can be restrained quantitatively within the vesicles (Table 1). This is presumably the result of the condensing effect (Ladbrooke *et al.*, 1968; Demel & Kruyff, 1976) of the sterol on the acyl chains of the phospholipids, which in turn reduces their loss to

high-density lipoprotein (Kirby *et al.*, 1980b) and the size of formed pores. Since in the present experiments the liposome/plasma ratio (1:4) in the incubation mixtures simulates the initial dilution of liposomes in the plasma of intravenously injected animals (Kirby *et al.*, 1980a; Kirby & Gregoriadis, 1980) the effect of plasma on the liposome structure is likely to be similar *in vivo*. It is anticipated that control of pore formation in liposomes *in vivo* will, in conjunction with control of clearance rates from the circulation (Gregoriadis & Senior, 1980), lead to the design of vesicles that can release entrapped solutes of various sizes at predetermined rates.

We thank Mrs. M. Moriarty for excellent secretarial assistance. This work was supported in part by an NIH National Cancer Institute contract (NO1-CM-87171).

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