SHORT NOTE

Interactions of Conventional or Photopolymerized Liposomes with Platelets in vitro

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Summary. We have examined the effects of liposomes on in vitro platelet aggregation. Liposomes were prepared from various conventional lipids and from a novel photopolymerizable phosphatidylcholine derivative (DPL, *bis*[1,2-(methacryloyloxy)dodecanoyl]-L-alpha-phosphatidylcholine). None of the liposome preparations studied caused marked platelet aggregation in either plasma or buffer solution. However, positively charged vesicles impaired the ability of platelets in plasma to aggregate in response to ADP, whereas negatively charged vesicles impaired the ability of platelets in buffer to aggregate in response to thrombin. DPL vesicles had only modest effects on platelets in plasma or buffer.

Lipid vesicles (liposomes) are currently being investigated as a system for the controlled delivery of drugs [1, 2]. However, the utilization of liposomes in this context requires that these particles enter the bloodstream where they may interact with blood cells, with the vascular endothelium as well as with plasma proteins. There is considerable information concerning the interaction of liposomes with plasma proteins including studies of the role of negatively charged lipids in the humoral clotting cascade [3, 4]. However, only limited information exists on the interaction of liposomes with the cellular elements of blood. Thus Weissmann and his colleagues have reported on interaction of liposomes with human leukocytes [5], whereas Ellens et al. [6] have reported that the injection of large doses of negatively charged vesicles does not reduce platelet counts in mice. Other workers have extensively investigated the interactions of platelets with surfaces composed of lipophilic polymers [7], but these were so unlike phospholipids in their properties that comparisons are difficult. To augment knowledge of liposome–blood cell interactions, we undertook a study of the effects of various types of lipid vesicles on platelet aggregation behaviour.

Multilamellar (MLV) or small unilamellar (SUV) liposomes were added to platelet suspensions in an aggregometer. Both direct effects of liposomes on platelet aggregation and effects on the ability of the platelets to respond to well-known triggers of aggregation (ADP or thrombin) were monitored [8].

In addition to testing the effects of conventional lipid vesicles on platelets we also tested the effects of liposomes composed of photopolymerizable phosphatidylcholine analogues [9, 10]. These analogues have been shown to form vesicles circumscribed by bimolecular membranes; the permeability characteristics of these membranes are similar to those of conventional liposomes, but the photopolymerized membranes are much more stable [11]. Recently it has been shown that photopolymerized lipid films can be coated on to glass or plastic backings [12, 13]. If photopolymerized lipid surfaces should prove to be non-thrombogenic, these coatings may provide the basis for novel forms of biomaterials that

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might be used, in vivo or ex vivo, in biomedical devices which are in contact with blood. In a preliminary fashion, we have tested the blood cell compatibility of photopolymer lipid surfaces by assessing the ability of photopolymerized vesicles to affect platelet aggregation.

Methods

Lipids including egg phosphatidyl choline (egg PC), dipalmitoyl and dimyristoyl phosphatidyl choline (DPPC, DMPC), phosphatidyl glycerol (PG) (a negatively charged lipid) and cholesterol were obtained from Avanti Polar Lipids, Birmingham, Ala. and were checked for purity by thin-layer chromatography. Stearylamine (SA) (a positively charged lipid) was from K & K Laboratories.

Liposomes were prepared as described previously [14]. Briefly, MLVs containing 10 mg total lipid/ml were prepared by vortex dispersion of a dried lipid film in 0.9% NaCl–10 mM Tris buffer (pH 7.5). Small unilamellar vesicles (SUVs) (10 mg/ml) were prepared by sonic dispersion (Heat Systems 375 W) in Tris-buffer under a nitrogen atmosphere; the SUVs were sonicated until the suspension appeared clear to the eye. Temperature was maintained at 45°C during these procedures. Polymerized lipid vesicles were prepared essentially as described by Regen et al. [9]; thus suspensions of MLV or SUVs composed of a dipolymerizable lipid (DPL) (*bis*-[12-(methacryloyloxy)dodecanoyl]-L-alpha-phosphatidyl choline-compound 4 [9]) were exposed to high intensity UV illumination (Rayonet Photochemical Reactor) for 15 min in a nitrogen atmosphere; temperature was maintained at 25°C with an external coil from a Lauda circulator. The completeness of polymerization of the liposomes was assessed by TLC on silica gel (the polymerized material remains at the origin while the monomer migrates). For SUVs lipid content can be converted to surface area based on the assumptions [15] that 1 μmole phospholipid corresponds to 2 100 cm^2 of bilayer surface area, and that the average molecular weight of the lipids used was about 750. This corresponds to 2.8 $cm^2/\mu g$ of lipid. For MLVs it is not possible to make this calculation, since the number of lamellae is not known. Lipid content of final samples of SUVs or MLVs was verified by analyses of organic phosphorus [16].

Platelet-rich plasma comprised of 200 000 platelets/ml in acid-citrate dextrose-diluted plasma was prepared by standard hematological methods [17]. Washed platelets were prepared in albumin-containing Tyrode buffer using the mustard procedure [18]. Platelet aggregation at 37°C was monitored in 0.5 cm^3 samples using a Biodata Aggregometer of the type normally used for clinical laboratory assay of platelet function. Liposomes were added to the aggregometer cuvette as small aliquots (0–100 μl) in NaCl-Tris. ADP (20 μl of a 100 mM stock) and thrombin (5 μl) were used to stimulate aggregation in platelet-rich plasma and in washed platelet suspensions respectively.

Results

None of the liposome compositions tested (with the possible exception of negatively charged lipids acting on washed platelets) showed any clear sign of directly causing platelet aggregation. However, several types of liposomes caused profound impairment of the ability of platelets to aggregate in response to ADP or thrombin. For example, in fig. 1*a* we see that uncharged SUVs did not induce aggregation in platelet-rich plasma, nor did they affect the ability of ADP to mediate aggregation. The same lack of effect was noted for negatively charged vesicles (egg PC/PG 9:1). By contrast, positively charged SUVs, while not initiating aggregation themselves, effectively inhibited ADP-mediated platelet aggregation. As seen in fig. 1*b*, the inhibitory effect of positive liposomes is sharply dose-dependent with effects first manifest at about 100–200 μg lipid/0.5 ml platelets and the effect fully developed at about 360 μg/0.5 ml platelets. It is unlikely that this effect is due to binding of ADP by liposomes, since the addition of ADP in the presence of liposomes results in the usual initial deflection of the aggregometer trace (shape change), but not in the full aggregation response.

In the case of washed platelets, negatively, rather than positively charged liposomes had a marked effect on platelet function. As seen in fig. 2, both neutral

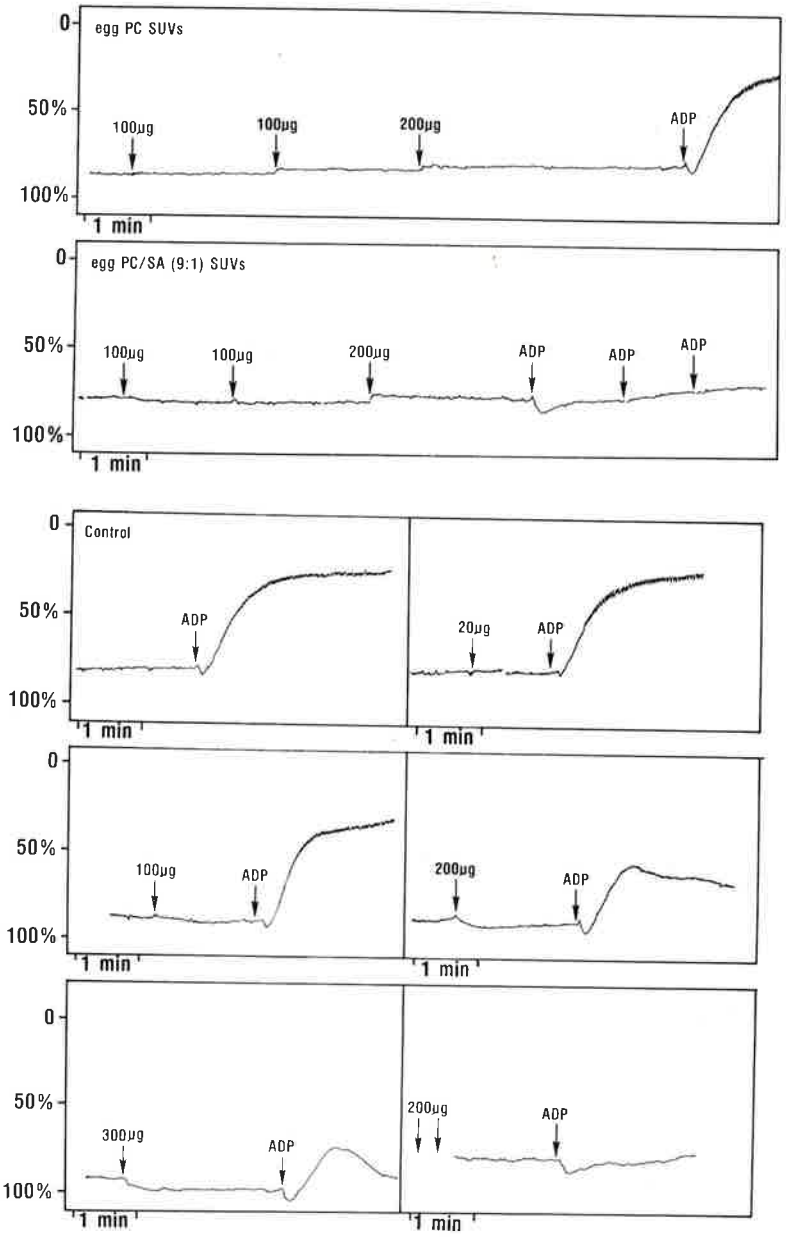

Fig. 1. (*a*) Effects of uncharged and positively charged SUVs on platelet aggregation in plasma. Platelet-rich plasma (0.5 ml at 200 000/ml with acid-citrate-dextrose anticoagulant was monitored in an aggregometer. At the indicated points small aliquots (0–20 µl) of egg PC SUVs (neutral charge) or egg PC/SA SUVs (positive charge) were added. After further incubation an aliquot (10 µl) of 100 mM ADP was added to induce aggregation. *Ordinate*: Light scatter (arbitrary scale: 0 indicates maximum aggregation. (*b*) Dose effects of positively charged SUVs on platelet aggregation. The protocol was the same as for (*a*).

and positively charged SUVs failed to cause aggregation of washed platelets and failed to inhibit thrombin-induced platelet aggregation. Negatively charged SUVs induced a modest positive slope of the aggregometer trace indicating that some platelet aggregation was occurring; more strikingly, negatively charged vesicles totally ablated the ability of platelets to undergo thrombin-induced aggregation. In fig. 3 we compare effects of negatively charged SUVs and of DPL SUVs (i.e., photopolymerized vesicles) on thrombin-induced aggregation of washed platelets. It is readily seen that low doses (20–50 µg) of negative SUVs markedly

Fig. 1. (*a*) Effects of uncharged and positively charged SUVs on platelet aggregation in plasma. Platelet-rich plasma (0.5 ml at 200 000/ml with acid-citrate-dextrose anticoagulant was monitored in an aggregometer. At the indicated points small aliquots (0–20 µl) of egg PC SUVs (neutral charge) or egg PC/SA SUVs (positive charge) were added. After further incubation an aliquot (10 µl) of 100 mM ADP was added to induce aggregation. *Ordinate*: Light scatter (arbitrary scale: 0 indicates maximum aggregation. (*b*) Dose effects of positively charged SUVs on platelet aggregation. The protocol was the same as for (*a*).

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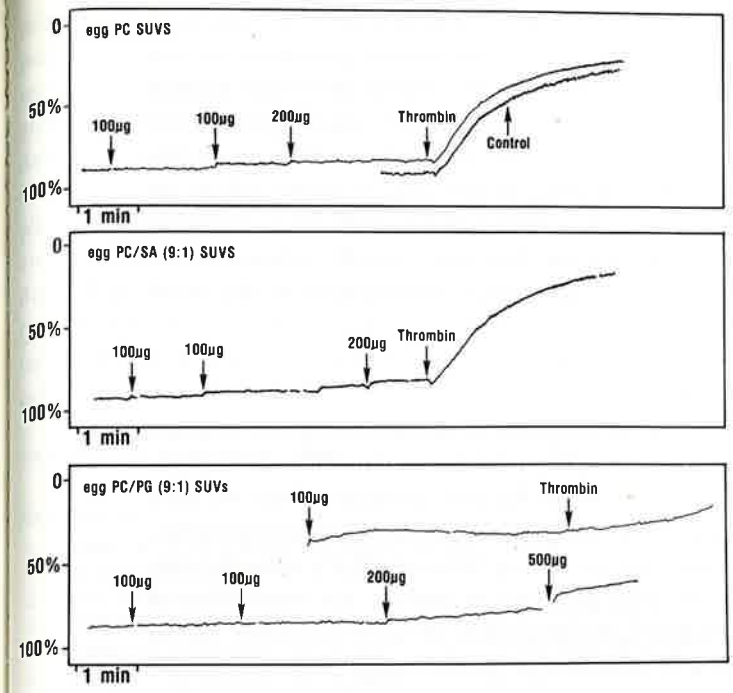

Fig. 2. Effects of neutral and charged SUVs on aggregation of washed platelets. The protocol was essentially the same as for (*a*) except that washed platelets suspended in albumin-Tyrode solution were used. Thrombin (5 µl) was used to stimulate aggregation. Egg PC SUVs (neutral), egg PC/SA SUVs (positively charged) and egg PC/PG SUVs (negatively charged) were compared.

impair the platelet response, whereas much larger quantities (50–100 µg) of DPL SUVs fail to do so; very high doses of DPL (250 µg equivalent to 700 cm^2 surface area) do cause changes in the shape of the aggregation response.

It is apparent that positively charged vesicles strongly affect platelet behaviour in plasma, but not in buffer solution, suggesting that the effect of the positive

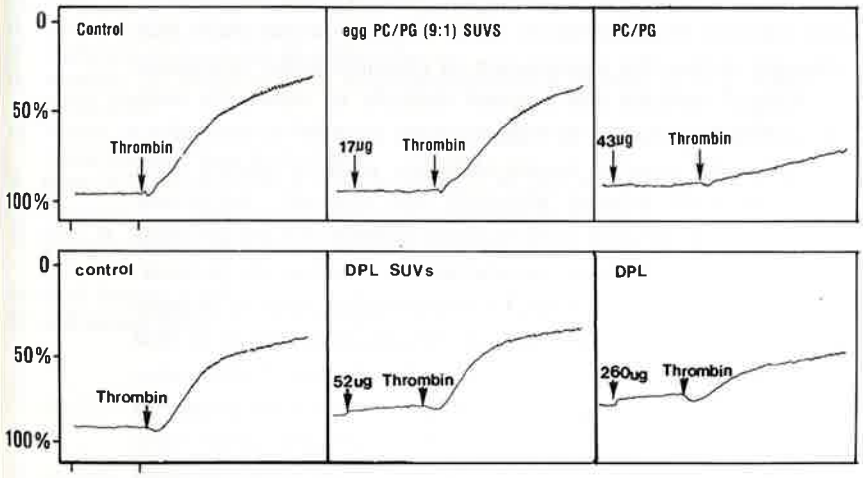

Fig. 3. A comparison of effects of negatively charged SUVs and neutral photopolymerized SUVs on platelet aggregation. The protocol was similar to that in (*a*). Various doses of egg PC/PG SUVs (negatively charged) or DPL SUVs (neutral photopolymerized lipid) were used. Washed platelets in albumin-Tyrode solution were employed and thrombin was used to stimulate aggregation.

vesicles is mediated via a plasma factor, possibly fibrinogen [20]. By contrast, negatively charged vesicles seem able to interact with washed platelets, but not with platelets in plasma. As we have suggested previously [19], the protein coating acquired by liposomes in plasma may be an important determinant of the subsequent interaction of the vesicles with cells. Neutral vesicles, including vesicles made from photopolymerizable lecithin, do not exhibit strong effects on platelet function. In this report we have not undertaken a systematic exploration of the role of vesicle charge, size or fluidity, but have merely compared the effects of a few commonly used types of liposomes with effects of the novel photopolymerizable lipids.

Discussion

Questions about the mechanisms and significance of liposome–platelet interactions in vitro are likely to be complex, and we must be aware of the limitations of the relatively simple approaches used here. In this communication we have explored three types of situations with the intent of searching for major liposome–platelet interactions; these situations are (*a*) interactions of MLVs with platelets in plasma; (*b*) interaction of SUVs with platelets in plasma; (*c*) interaction of SUVs with platelets in albumin–buffer solutions; each of these situations has its inherent problems. With MLVs it is impossible to determine with reasonable accuracy the surface area of the liposomes available for platelet interaction, since this depends on the number of lamellae and may change with different lipid composition. With SUVs in plasma there is a problem of vesicle instability due to possible interactions with plasma lipoproteins [3]. With SUVs interacting with washed platelets there is little problem with vesicle breakdown and the experimental situation is simpler, but it is also far from physiological reality. In addition to problems relating to the liposomes, the functional status of the platelets and the complexity of the mechanism of platelet aggregation [8] must be kept in mind.

Despite these limitations, the experiments reported here provide preliminary information of liposome–platelet interactions in vitro. First, it seems clear that lipid vesicles do not strongly induce the aggregation of platelets either in plasma or in buffer. Second, charged vesicles can interact directly or indirectly with platelets to impair their ability to respond to triggers such as ADP or thrombin. The effect of positively charged vesicles seems indirect, since it occurs with platelet-rich plasma but not with washed platelets. By contrast, negatively charged vesicles seem to interact directly with washed platelets to impair their response to thrombin. The nature of this interaction is unclear at present; possibly the vesicles bind to the platelet and block critical surface sites or impede membrane rearrangements necessary for aggregation. Finally, it is also clear that polymerized vesicles prepared from photo-activatable phosphatidylcholine analogues have only small effects on platelet aggregation, as do vesicles prepared solely from conventional phosphatidylcholines. Photopolymerizable lipids have been synthesized by several groups [9, 21, 22]. Membranes of photopolymerized lipid represent a novel hybrid material with some of the characteristics of a biological membrane (extreme thinness, low permeability) and some of the characteristics of a synthetic polymer membrane (chemical and physical stability). In

this report we demonstrate that surfaces composed of polymerized lipids (in the polydisperse form of liposomes) do not interact strongly with platelets and thus may prove to be non-thrombogenic. This finding suggests the possibility of using polymeric lipids to provide innocuous, non-thrombogenic coatings for biomedical devices which are in contact with blood. However, additional investigations are necessary, including study of the interactions of lipid polymer surfaces with platelets under flow conditions, and the long-term behaviour of these surfaces in vivo. In summary, lipid vesicles can interact with platelets in diverse ways depending on the physical characteristics of the vesicle surface and the availability of plasma components. However, those vesicles most commonly used in vivo (neutral or negatively charged) are unlikely to provoke substantial platelet aggregation.

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