Possible hemostatic effect of synthetic liposomes in experimental studies under flow conditions

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Background and Objectives. The possibility of developing synthetic platelet substitutes is a subject of current interest. We explored the possible hemostatic effect of synthetic phospholipid incorporated in multilamellar vesicles (MLVs) or intermediate unilamellar vesicles (IUVs) using a well-characterized experimental system with circulating human thrombocytopenic blood (10 min, 250 s⁻¹).

Design and Methods. The ability of the liposomes containing different combinations of dipalmitoylphosphatidylcholine (DPPC), phosphatidylethanolamine (PE) and dipalmitoylphosphatidylserine (DPPS) to promote fibrin formation (%F) on the damaged subendothelium was morphometrically evaluated. Generation of thrombin in the system was monitored through prothrombin fragment F1+2 determination.

Results. IUV liposomes containing DPPC, 1DPPS:9DPPC, 1DPPS:3DPPC, 1PE:1DPPC increased fibrin deposition on the subendothelium (53.87±11.0%; 39.76±6.75%; 40.69±10.54% and 32.22±7.35%, respectively vs. thrombocytopenic blood 11.5±1.2%; *p<0.05), while 9PE:1DPPS IUV liposome failed to promote a procoagulant effect. MLV liposomes containing DPPC alone, 1DPPS:3DPPC and 1PE:1DPPC showed a positive effect on fibrin deposition (85.50±5.95%, 59.86±11.55% and 43.73±7.84% respectively; *p<0.05). However, no effect was observed in those experiments performed with liposomes containing 3DPPS:1DPPC. After perfusion experiments, the coagulation system became activated, but differences were not statistically significant vs. control experiments, except for MLV liposomes containing DPPC alone (*p<0.05).

Interpretation and Conclusions. These results confirm that, at an experimental level, liposomes containing phospholipids could potentially be used to improve hemostasis in patients with quantitative or qualitative platelet disorders.

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In recent years, the demand for platelet concentrates has increased progressively.¹ New, more aggressive oncohematologic treatments, poorer recovery and the development of refractoriness after platelet transfusion have contributed to the rise in the need for platelet concentrates.¹,² All these reasons justify the search for new methods to prepare platelet products or the development of new platelet alternatives that are safer and hemostatically efficacious, and have a longer shelf-life than currently available platelet concentrates.¹,²

Hemorrhagic episodes are usually related to the severity of thrombocytopenia and can be prevented or controlled with platelet transfusions.³,⁴ Platelets contribute to the hemostatic process in two different ways. First, their adhesive and cohesive functions lead to the formation of a hemostatic plug.³ Second, they can activate coagulation mechanisms through the exposure of negatively charged phospholipid on their surface, acting as a catalytic site for the development of coagulation that will consolidate the initially constituted hemostatic plug.⁶

Different studies have suggested that platelet preparations with impaired metabolic or functional integrity still retain a certain degree of hemostatic capacity.⁷,⁸ Studies by our group have reported that non-viable platelets or synthetic phospholipid preparations could promote hemostatic effects, increasing fibrin deposition on damaged vascular surface.¹⁰,¹¹

The possibility of developing synthetic substitutes, which could be stored for longer periods while maintaining a low risk of biological contamination, is an appealing one. Previous studies, carried out using raw phospholipid preparations,¹¹ showed that such compositions improved hemostatic mechanisms. The present study attempts to develop elaborated phospholipid preparations, liposomes, of different compositions and sizes, which are well characterized and standarized and
which could be useful to promote local fibrin deposition on damaged vessels under thrombocytopenic conditions. For this purpose, we applied a modification of the original perfusion technique.\textsuperscript{10-12} Using this model we morphometrically evaluated the deposition of fibrin onto damaged vascular segments exposed to circulating thrombocytopenic blood in which we incorporated the different liposome preparations. These liposomes were characterized by particle size analysis as well as the distribution of the aminophospholipids, before and after their incorporation into the blood. Furthermore, prothrombin activation in the perfused blood was assessed by monitoring levels of prothrombin fragments 1+2 using an ELISA method.

**Design and Methods**

**Chemicals and buffers**

Dipalmitoyl-\textit{DL-\textalpha-} phosphatidylcholine (DPPC), phosphatidylethanolamine (PE) and dipalmitoyl-\textit{DL-\textalpha-} phosphatidyl-L-serine (DPPS) were purchased from Sigma (St. Louis, MO, USA). Tris-HCl buffer (pH 7.4) and borate buffer (pH 9.1) were made at 10 mM concentration. Tris-HCl buffer contained 145 mM NaCl. Triton X-100 was obtained from Sigma. Acetone was of spectroscopic grade (Merck, Germany).

**Preparation of multilamellar liposomes (MLV)**

Phospholipids (DPPC alone, DPPC with PE at a molar ratio of 50:50 or DPPC with DPPS at molar ratios of 75:25 and 25:75) were dissolved in chloroform in a round-bottomed flask and dried in a rotary evaporator under reduced pressure at 50°C to form a thin film on the flask. The film was hydrated with Tris buffer to give a lipid concentration of 145 mM NaCl. Multilamellar liposomes were formed by constant vortexing for 4 min on a vortex mixer and sonication in a bath (Elma, Transsonic Digitals, Germany) for 10 min.

**Preparation of unilamellar liposomes (IUV)**

MLV formed by DPPC, 1PE:1DPPC, 9PE:1DPPS, 1DPPS:3DPPC and 1DPPS:9DPPC were downsized to form intermediate unilamellar vesicles by extrusion (IUV) at 50°C in an Extruder device (Lexip Bio membranes, Canada) through polycarbonate membrane filters of variable pore size under nitrogen pressures of up to 55 $\times$ 10\textsuperscript{5} N.m\textsuperscript{-2}.\textsuperscript{13} Liposomes were extruded sequentially through polycarbonate filters (0.8, 0.4, 0.2 and 0.1 $\mu$m (Nucleopore, Cambridge, MA, USA) in order to obtain liposomes of a nominal size of about 150 nm.

**Blood collection and preparation of thrombocytopenic blood**

Blood was obtained from healthy volunteers who had not ingested drugs affecting platelets or coagulation mechanisms in the previous 10 days. Blood was anticoagulated with low molecular weight heparin (LM WH; Fragmin, Kabi Pharmacia, Stockholm, Sweden) at a concentration of 20 U/mL.\textsuperscript{10, 11,17} This concentration of LMWH allows thrombin generation when blood is exposed to a denuded vascular segment.\textsuperscript{10,11,14} Platelets and leukocytes were reduced from the blood by means of a filtration procedure\textsuperscript{13} using an RC 100 filter ( Pall Corp., Glen Cove, NY, USA). After filtration, platelet counts ranged from 2,000 to 6,000 platelets/\mu L and leukocyte counts were below 100 cells/\mu L.\textsuperscript{15}

**Preparation of vascular segments**

Rabbit abdominal aorta segments were obtained from New Zealand White rabbits that weighed 2.5-2.8 kg. Arteries for perfusion studies were prepared and processed as previously described.\textsuperscript{16,17} The subendothelium was exposed after enzymatic deendothelization with \textit{\alpha-} chymotrypsin.\textsuperscript{16} This procedure ensures a homogeneous exposure of subendothelial structures. Everted vascular segments (8-12 mm in length) were mounted in the plexiglass rod, which is inserted into the outer plexiglass shell of the annular chamber.\textsuperscript{18} The rod is fixed in its central position by an O-ring.

**Preparation of perfusates and perfusion studies**

Two milliliter aliquots of each liposome preparation were added to the thrombocytopenic blood. Control perfusion runs were carried out adding equivalent amounts of the solvent used in the phospholipid preparations. The perfusates, with a total volume of 20 mL, were always incubated at 37°C for 5 min before starting the perfusion. Perusions were performed in annular chambers according to the method described by Baumgartner.\textsuperscript{18} Blood was recirculated through the chamber for 10 min at 37°C at a shear rate of 250 sec\textsuperscript{-1} using a peristaltic pump, as previously described.\textsuperscript{18} This low shear rate facilitates fibrin deposition on the subendothelium.

**Processing of vessel segments for morphometric evaluation**

At the end of the perfusion, the rod with the vessel segment was rinsed with phosphate buffered saline (PBS), fixed with PBS containing 2.5% glutaraldehyde and finally removed from the annular chamber. The fixed segments were embedded in JB-4 plastic compound (Polyscience Warrington, PA,
Fibrin deposition onto perfused subendothelium was morphometrically evaluated using a light microscope equipped with a split prism. Fibrin deposits on subendothelium were expressed as a percentage of the total length of the vessel screened after evaluation of 20 different microscopic fields. Details of this method have been provided elsewhere.10,12,17

Fragment 1+2 determinations
Aliquots of blood were systematically collected to test plasma levels of prothrombin fragment 1+2 (F1+2). Samples before and after filtration were taken to confirm that the filtration procedure had not activated the coagulation system. Blood aliquots were also obtained in this way after addition of liposome preparations and immediately after the perfusion had been stopped. Blood samples were always immediately mixed with a solution of citrated phosphate dextrose (CPD) to achieve 19 mmol/L citrate to prevent further activation of the coagulation system. Plasma, obtained from blood in the absence or in the presence of liposomes, was separated by centrifugation (1,000 × g, 20 min), frozen at -40°C and stored for further determination of F1+2. Plasma F1+2 levels were determined by enzymoimmunoassays (Enzygnost F1+2, Behring, Germany), as previously reported by Bruhn et al.19

Particle size analysis
The vesicle size distribution was determined by photon correlation spectroscopy (PCS) with an Autosizer II spectrometer (Malvern Instruments, Malvern, UK) at 25°C. Samples (liposomes alone or plasma with liposomes) contained in 5 mL glass cuvettes were placed in a thermally packed sample holder at 25°C.

Basically, the size of the particles present in the samples was determined by their ability to scatter the light produced by a helium-neon laser (632.8 nm and 5 mW) focused on the sample, and detected at 90° to the incident beam by a photomultiplier tube, which was connected to a quantum photometer.

Fluctuations in the scattered light intensity generated by the diffusion of vesicles in solution were analyzed, and the autocorrelation function was obtained via a Malvern 7032-N, 72-channel multibit correlator.

The method of cumulant analysis was applied20 for sizing vesicles obtained by extrusion, which afforded a unimodal vesicle distribution, while for sizing MLV vesicles (a polydispersed system) model-independent analysis, which does not assume any particular form of distribution, was used.

Determination of aminophospholipids
The aminophospholipids PE and DPPS were determined spectrophotometrically using fluorescamine as a labeling reagent.21 It was confirmed in advance that fluorescamine had no disruptive effect on the liposome structure.

Determination of total aminophospholipid in the vesicle
Aliquots (100-200 µL) of liposomes were disrupted with 2.0 mL of borate buffer. Fluorescamine solution (0.2 mL of a solution of 3 mg in 100 mL of acetone) was added, and the vesicle sample was shaken vigorously for 30 s. After 1 min, 2 mL of 1.6% Triton X-100 in borate buffer was added to the sample, and then mixed. The resulting fluorescence was read within 2 h of the reaction by exciting the sample at 381 nm and measuring the radiation emitted at 471 nm.

Data analysis
Results are expressed as mean ± standard error of the mean (S.E.M.). The number of experiments for each preparation was at least n=6. Statistical analyses were performed by one-way ANOVA test for independent experiments. Differences were considered statistically significant if p<0.05.

Results
Characterization of liposomes
IUVs of any composition (DPPC alone or combined with PE or DPPS) presented a unimodal distribution, the average size of which was 137±16 nm, measured by PCS. After three days, no significant change in this parameter was observed. As a general rule, liposome preparations are heterogeneous in size, so that polydispersity is one of their main characteristics. Polydispersity is a parameter that can vary from 0 for a totally monodispersed sample to 1 for a polydispersed one. A polydispersity value ≤ 0.1 was obtained at any case, and did not change during this short period of time.
The distribution of DPPS or PE between the outer and the inner layers of the vesicles afforded values higher than 50% for all compositions tested. Individual values remained unaltered during the three days of the assay.

Measurements of plasma by PCS yielded average diameters ranging from 40 to 150 nm. This wide variation was due to the nutritional state of the donors; the low value corresponded mainly to very low density lipoproteins (VLDL), whereas the high value corresponded to chylomicrons. When liposomes were mixed with plasma, the average size of the mixture, in the case of DPPC and DPPC:DPPS populations, was always lower than that obtained for the liposomes alone (Figure 1). However, for PE-containing liposomes, the average size was higher.

With respect to MLV liposomes, the average size ranged from 1 to 4 mm, and their polydispersity was close to 0.9. The large size of this kind of vesicles makes them quite unstable and, after a short period, liposomes flocculated although, when shaken gently, the system returned to its original state. Three days later, size and polydispersity remained the same. Mixing liposomes with plasma gave average sizes lower than that obtained with vesicles, irrespective of the lipid composition. In this case, the distribution of aminophospholipids between the outer layer and the inner layer was always lower than 50%. No changes in these values were observed.

Morphologic differences between MLV and IUV liposomes are shown in Figure 2.

Hemostatic potential of liposome preparations

The deposition of fibrin on the subendothelium observed in control perfusions performed under conditions of severe thrombocytopenia (<6000 plts/µL) covered 11.5±1.2% of the subendothelial surface.
IUV liposomes (IUV)
Addition of IUV liposomes containing DPPC produced the most relevant statistically significant increase (46.61±11.0% *p<0.05). Liposomes containing different proportions of DPPS and DPPC (1DPPS:9DPPC and 1DPPS:3DPPC) in the thrombocytopenic blood resulted in an increase in fibrin deposition vs. control experiments (39.76±6.75% and 40.69±10.54; p<0.01). A similar effect was observed with IUV liposomes containing 1PE:1DPPC (32.22±7.35; p<0.01), whereas the composition formed by 1DPPS:9PE had no significant effect (11.13±3.13%). These results are summarized in Figure 3.

Three days after obtaining the IUV we performed experiments with some of the most representative liposomes. Thus, liposomes containing 1DPPS:3DPPC resulted in a decrease in the percentage covered by fibrin (30.50±5.27% vs 40.69±10.54 on day 0). However, experiments performed with liposomes containing 1PE:1DPPC showed an increase vs. day 0 (42.57±4.86% vs. 32.22±7.35%) (Figure 4).

MLV liposomes
Following data published in the literature and our own results, we produced MLV liposomes with different compositions: DPPC, 1DPPS:3DPPC, 3DPPS:1DPPC, 1PE:1DPPC. As with previous results, liposomes containing DPPC alone were the most effective, allowing fibrin to cover 85.50±5.95% of the surface. Addition of 1DPPS:3DPPC and 1PE:1DPPC into the thrombocytopenic blood also resulted in an increase in fibrin formation (59.86±11.55% and 43.73±7.84%, p<0.05 vs. control). No effect was shown when we used MLV liposomes containing 3DPPS:1DPPC. All these results are summarized in Figure 5. After 3 days, fibrin formation on the subendothelium for 1PE:1DPPC liposomes was similar (46.67±7.51%). However, the effect of DPPC alone and 1DPPS:3DPPC MLV liposomes was less (50.62±10.65%, 36.55±3.85%, respectively) (Figure 4).

Selected micrographs illustrating the most remarkable features observed in the perfusion studies are shown in Figure 6.

Detection of prothrombin fragment 1+2
After perfusion experiments, F1+2 levels in plasma were increased. Plasma levels of F1+2 in severe thrombocytopenic blood were 0.65±0.08 nM. After
10 min of perfusion experiments F1+2 levels increased significantly (1.71±0.21 nM/L; p<0.05 vs. pre-perfusion values).

Addition of liposomes into the blood produced levels of activation of coagulation similar to those of control experiments. However, experiments performed in the presence of MLV liposomes containing DPPC resulted in the most significant increase of fibrin formation vs. control experiments, levels for F1+2 increasing into the range allowed for other blood products. Activation of coagulation levels in plasma for each preparation at 0 day are summarized in Table 1.

Preparations tested after 3 days showed a mild decrease in F1+2 levels. Thus, IUV liposomes containing 1PE:1DPPC decreased to 1.56±0.1 nM on day 3 after their preparation vs. 2.43±0.24 nM on day 0. Similar tendencies were observed when experiments were performed with 1DPPS:3DPPC (1.49±0.26 nM vs. 2.19±0.64 nM). Mild decreases were also observed in those experiments performed with MLV liposomes.

Discussion

In recent years, efforts to improve platelet storage and to develop new platelet substitutes have been made.22,23 This study has taken a step forward in the search for synthetic phospholipid preparations that could be used as a platelet substitute. Our results indicate that liposomes of adequate chemical and physical characteristics could promote fibrin deposition on damaged vascular surfaces. Furthermore, moderate increases in the overall coagulation system were observed in plasma.

Studies from several laboratories have demonstrated that platelets, besides their adhesive and cohesive functions, have the ability to promote a coagulant action.12,24-26 The relevance of platelet membrane phospholipids in blood coagulation mechanisms has been demonstrated by different laboratories.27-29 Over the last few years, our group

Figure 5. Fibrin deposition onto perfused subendothelium after addition of MLV liposomes to thrombocytopenic blood (TPB). (DPPC: dipalmitoyl-DL-α-phosphatidylcholine, PE: dipalmitoylphosphatidylethanolamine, DPPS: dipalmitoyl-DL-α-phosphatidylserine, Mean ± S.E.M.; *p<0.05 vs. TPB).

Figure 6. Light micrographs illustrating the most remarkable features observed in cross-sections of the perfused vascular segments (800× magnification). A) Perfusion with thrombocytopenic blood (TPB). Fibrin deposition on the subendothelium is only occasionally observed. B) Perfusion with TPB plus DPPC MLV liposomes. The procoagulant effect is evident, as can be seen from the abundant bunches of fibrin strands on the subendothelium.
has generated experimental and clinical evidence confirming that transfused platelets can promote procoagulant activity and eventually exert a favorable effect on hemorrhage. The accepted mechanism for this procoagulant activity is that platelets, or platelet fragments, could provide the adequate phospholipid composition to support the activation of coagulation mechanisms. In vivo, this catalytically active surface is provided by exposure of aminophospholipids in platelet membrane during platelet activation. This exposure is also related to the shedding of microvesicles from the platelet plasma membrane and is related to local fibrin formation.

Previous studies by our group using the same experimental system have demonstrated that fragments of platelets and microvesicles from plasma promote procoagulant activity onto the subendothelium. Not only platelet fragments but even raw preparations of synthetic phospholipids were capable of activating the coagulation system and promoting fibrin formation on damaged vascular surfaces. However, those preparations produced an increase in F1+2 levels that could result in a high risk of intravascular coagulation. Following these previous results, this study has focused on obtaining more elaborated liposomes with specific characteristics in terms of size and composition. In the present study two types of liposomes, MLV and IUV, were tested and the results indicate that some of the preparations induced a localized procoagulant effect only at sites of vascular injury and have a minimal impact on systemic coagulation.

Table 1. Post-perfusion levels of F1+2 expressed as nM after addition of liposomes to TPB (day 0).

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>IUV Liposomes</th>
<th>MLV Liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPB</td>
<td>1.71±0.21</td>
<td>1.71±0.21</td>
</tr>
<tr>
<td>TPB+DPPC</td>
<td>2.8±0.30</td>
<td>2.73±0.17</td>
</tr>
<tr>
<td>TPB + 3DPPS:1DPPC</td>
<td>1.93±0.35</td>
<td>1.72±0.25</td>
</tr>
<tr>
<td>TPB + 1DPPS:3DPPC</td>
<td>2.19±0.64</td>
<td>1.72±0.22</td>
</tr>
<tr>
<td>TPB + 1PE:1DPPC</td>
<td>2.43±0.24</td>
<td>2.87±0.36</td>
</tr>
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Other factors, such as shelf life, should be considered in order to obtain a potential platelet substitute. In our study, in the experiments performed three days after obtaining the liposomes, the presence of DPPS in liposomes decreased fibrin formation on the subendothelium. However, three-day old liposomes containing neutral phospholipids produced a mild increase of fibrin formation. These results indicate the relevance of phospholipid charges in the effectiveness of liposomes.

Despite the favorable results obtained with various prepared liposome preparations, further clinical studies will be necessary to discover whether or not the actions observed in blood experimentally depleted of platelets are reproduced in blood from real patients with thrombocytopenia. We believe that synthetic liposome preparations promote procoagulant activity at sites of vascular damage, and this combination of DPPS and DPPC promotes the effect attributed to platelet membranes in intravascular coagulation tests. However, in the present study liposomes containing DPPC alone provided the most effective preparation to produce fibrin formation on the subendothelium. This finding highlights the relevance of phospholipid composition and reinforces the differences between static and flow systems.

Mixing liposomes with plasma usually produces an apparent size reduction in IUV and MLV liposomes containing DPPS and/or DPPC (Figure 4). In such conditions, the size is obtained by averaging the sizes of all the components present. The much larger number of small-sized plasma components thus accounts for the apparent size reduction in the liposomes. The increase in the average size observed when IUV liposomes containing PE were mixed with plasma could be explained by the adsorption of some components of the blood on liposomes due to the physicochemical properties of PE. In the same conditions the MLV PE liposomes suffered a decrease in average size.

MLV liposomes seem to have much more effect than IUV on fibrin formation on a damaged vessel. However, they are less stable in solution and in plasma. IUV liposomes of small size, on the other hand, are more effective on fibrin formation, but more stable. An ideal platelet substitute would retain a localized effect on a damaged vessel but carry a low risk of overall activation of the coagulation system in plasma. Our results for F1+2 could be explained by the fact that certain liposomes induce a localized procoagulant effect only at sites of vascular injury and have a minimal impact on systemic coagulation.

Despite the favorable results obtained with various liposome preparations, further clinical studies will be necessary to discover whether or not the actions observed in blood experimentally depleted of platelets are reproduced in blood from real patients with thrombocytopenia. We believe that synthetic liposome preparations promote procoagulant activity at sites of vascular damage, and this
action could have an effect in controlling hemorrhagic episodes. The development of synthetic platelet substitutes with a low risk of biological contamination, long shelf-life and hemostatic effectiveness is a challenge to be met.

Contributions and Acknowledgments

AMG and EC were the principal investigators; they carried out the experimental part of the study and the statistical analysis. JE and GE were responsible for direct supervision, analysis and interpretation of results. AMG, EC, JE and GE wrote the paper. MRH, JB and RM contributed with their technical expertise to reproduce experimental conditions established in previous series of studies. JCR contributed with his expertise to evaluation of coagulation system. RM also provided his expertise in blood banks for the conception and design of the studies. AO participated in drafting and revising the intellectual content of the paper and contributed to the discussion. GE participated in the design of study and contributed to the conception and discussion of the present manuscript.

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Disclosures

Conflict of interest: none.

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**Hemostatic activity of liposomes**

**Peer Review Outcomes**

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Carlo Balduini, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor Balduini and the Editors. Manuscript received March 1, 2002; accepted April 10, 2002.

What is already known on this topic

In spite of all the improvements in the production of liquid stored platelets, recipients continue to be at risk of febrile transfusion reactions, infections and alloimmunization. Efforts to overcome these shortcomings resulted in an array of novel platelet products and platelet substitutes, but at present the only accepted alternatives are frozen platelets.

What this study adds

Some of the platelet synthetic substitutes described in this paper were able to promote in vitro fibrin formation on the damaged subendothelium without significant activation of the coagulation system. These platelets substitutes are therefore suitable for pre-clinical testing in thrombocytopenic animal models.

Potential implications for clinical practice

With advances in basic science, there is reason to believe that effective and safe platelet substitutes will become available for clinical use in thrombocytopenic patients.

Carlo Balduini, Associate Editor