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Interactions of Liposomes and Platelets

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Key words

Liposomes – Platelets – Biodistribution – Transient thrombocytopenia

Summary

Rats were injected intravenously with liposomes of various compositions and sizes and blood platelet count measured. It was found that negatively-charged liposomal systems produced a transient reduction in platelet count in the first 5 minutes after injection which recovered by 60 minutes post-injection. This effect was most striking for multilamellar vesicles (MLV's) containing phosphatidylglycerol (PG). Dose levels of 25 mg/kg of MLV's containing 10 mole% PG caused the platelet count to drop from a control value of 1,086 ± 21 × 10^9/l to 193 ± 14 × 10^9/l by 2 minutes post-injection, an 82% decline. This thrombocytopenic effect was observed to diminish as vesicle size or vesicle dose was decreased. Positively-charged liposomes produced a less pronounced transient reduction in platelet count while neutral liposomes caused only a mild, transient platelet decline. This transient thrombocytopenic effect was not blocked by common anticoagulants and fibrinolytic agents but was prevented by liposomal pretreatment. Radiolabeled platelet studies revealed that transient sequestration of platelets occurs in the liver and spleen 2 minutes after PG:EPC:CHOL MLV injection with a normalization of platelet distribution by 60 minutes post-injection. In vitro studies, using an automated blood counter, suggest a transient association of liposomes and platelets occurring following injection. Liposomally-induced transient thrombocytopenia suggests a role for platelets in the biodistribution of liposomes.

Introduction

Liposomes are receiving considerable attention as drug delivery systems (for review see 1, 2). They have potential as carriers for anticancer drugs (3, 4), antifungal agents (5), antibacterials (6). As noted by Juliano et al. (7), however, limited information exists on the interaction of liposomes with the cellular elements of the blood.

Donald and Tennent (8), studying the role of platelets and macrophages in clearing carbon particles from the blood of the Sprague-Dawley rat, showed that in the early phases (less than 10 minutes) following injection of carbon particles several factors contribute to clearance. These include aggregation of the particles with platelets in the pulmonary circulation, with a resulting decline in circulating platelet count. Donald also observed that for low carbon doses the number of platelet-particle aggregates in the lung tended to diminish with time and demonstrated a dose-dependent relationship between platelet count depression, carbon particle clearance and carbon particle dose. The latter relationship between particle dose and particle clearance bears certain similarities to behaviour observed for liposomal systems. As a particulate system, it could be expected that liposomes might interact with blood cellular components. The purpose of this study is to examine the effect of the injection of liposomes on circulating platelet counts in the Wistar rat. The understanding of these interactions is important for the rational development of liposomal drug preparations as such interactions might be expected to affect circulation lifetimes and biodistributions. These results indicate that liposomal composition, size, and dose are important variables when the effects of liposomal administration on platelets are examined.

Materials and Methods

Materials

Egg phosphatidylcholine (EPC), egg phosphatidylglycerol (PG), bovine brain phosphatidylserine (PS), and bovine cardiolipin (CL) were obtained from Avanti Polar Lipids, Inc., Birmingham, AL, U.S.A. Stearylamine (SA) and cholesterol (CHOL), as well as epsilon-amino caproic acid (EACA), Heparin, Streptokinase, Acetylsalicylic acid (ASA), and Ancrod were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were of reagent grade. I^{125}-labeled dipalmitoylphosphatidylethanolamine (I^{125}-DPPE) was synthesized according to the method of Schroit (9) and purified by thin-layer chromatography. The iodinated lipid was then checked for lysolipid by measuring fatty acid exchange to bovine serum albumin. All I^{125}-DPPE used for experiments contained less than 2% fatty acid.

Female rats of the albino Wistar strain were obtained from the University of British Columbia Animal Care Centre or from Charles River Laboratories of St. Constant, Quebec. The animals weighed between 200–250 g at the time of use.

Methods

Multilamellar vesicles (MLV's) were prepared by first dissolving the lipids in chloroform and then evaporating the solvent under high vacuum for 1 hour. The dried lipid was hydrated in 0.9% NaCl, 25 mM HEPES buffer (HBS) at pH 7.5 and the solution freeze-thawed five times in liquid nitrogen to ensure uniform solute distribution (10). Large unilamellar vesicles were produced employing the Extruder (Lipex Biomembranes, Vancouver, B.C.) by passing the MLV's under pressure through double-stacked polycarbonate filters (Nuclepore Corp., Pleasanton, CA, U.S.A.) ten times as described previously (11, 12). Vesicles which had been sized through filters 0.4 μm or smaller were measured using a Nicomp Model 20 Submicron Particle Sizer. The total phospholipid content of the vesicles was always 55 mole%, with the remaining 45% composed of cholesterol. I^{125}-labeling of vesicles was accomplished by adding I^{125}-DPPE to the initial lipid-chloroform solution. For the dose of 25 mg total lipid (t.l.)/kg, 0.02 μCi I^{125}-DPPE was added per μmol of total lipid. For experiments which employed Cr^{51}-labeled platelets, platelets were first isolated from 10 ml of rat blood that was collected into polypropylene tubes containing 3 ml acid-citrate-dextrose (ACD). The blood was then centrifuged at 400 × g for 20 minutes and the platelet rich plasma (PRP) collected. The PRP was centrifuged at 800 × g for 10 minutes and the plasma removed. The platelets were resuspended in buffer [8.1 m*M* $Na_2HPO_4 \cdot 7H_2O$, 1.5 m*M* KH_2PO_4, 27 m*M* KCl, 138 m*M* NaCl, 55 m*M* glucose (pH 7.5)] with 10% rat plasma and transferred to a clean tube. The platelets were resuspended in 1.5 ml buffer-10% plasma and labeled with 130 μCi Cr^{51} for 60 minutes at 37° C. Buffer was added to a total volume of 10 ml and

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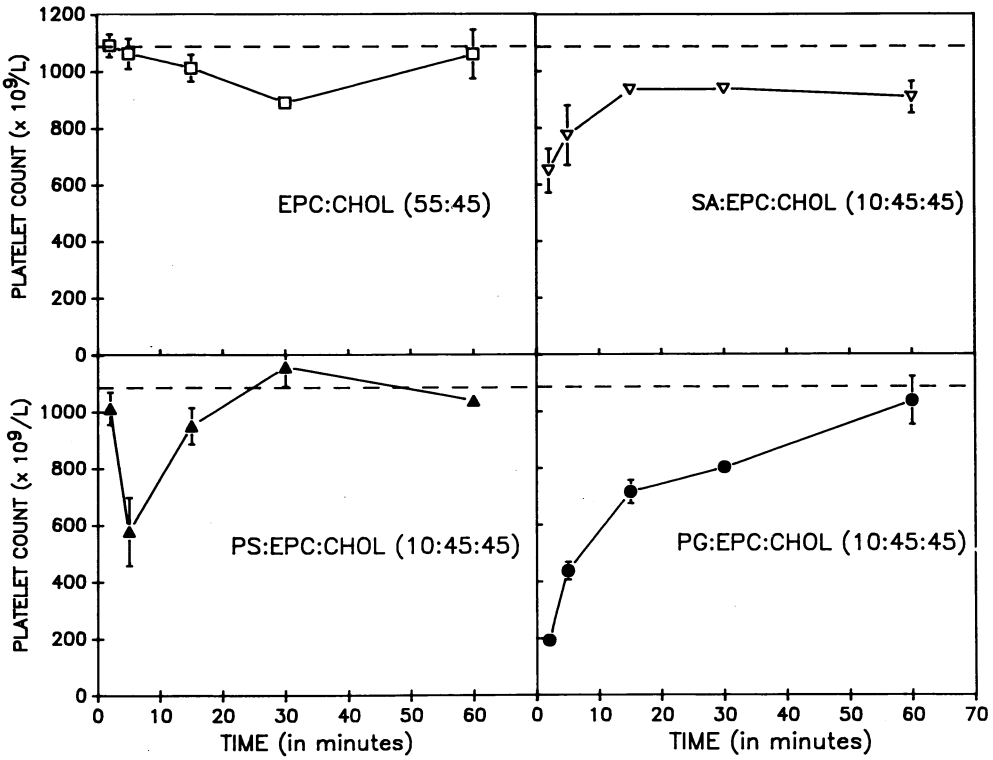

Fig. 1 Effects of injection of 25 mg t.l./kg of various lipid compositions on platelet count vs. time post-injection for: (a) EPC:CHOL (55:45 mole%) MLV's, (b) SA:EPC:CHOL (10:45:45 mole%) MLV's, (c) PS:EPC:CHOL (10:45:45 mole%) MLV's, (d) PG:EPC:CHOL (10:45:45 mole%) MLV's

the platelets centrifuged at 800 × g for 10 minutes. The Cr^{51}-labeled platelets (Cr^{51}-platelets) were resuspended in 3 ml buffer-10% plasma. To prepare platelet poor plasma (PPP), the whole blood used in the preparation of PRP was recentrifuged for 20 minutes at 800 × g and the PPP removed. For those experiments which used Cr^{51}-labeled RBC's, 4 ml of rat blood anticoagulated with 10 m*M* EDTA were isolated by washing cells three times with 150 m*M* NaCl, 20 m*M* Pi at pH 7.5 (phosphate-buffered saline or PBS) at 400 × g for 10 minutes. The RBC's were incubated with 200 μCi Cr^{51} for 60 minutes at 37° C. The cells were then washed four times with PBS at 400 × g for 10 minutes and adjusted to a hematocrit of 55% with PBS to give 300,000 DPM/200 μl.

In Vivo Experiments

Groups of 3–7 animals were administered liposomes of various lipid compositions and sizes in doses which ranged from 0.5–100 mg t.l./kg animal (0.8–160 μmol t.l./kg). Animals were anaesthetised with ether and injected via a lateral tail vein. The total volume per injection was 250 μl per 250 g animal weight. The time taken for injection was 15 seconds. Blood was collected from free-flowing tail blood immediately prior to sacrifice. When blood was to be sampled within 5 minutes of injection the animals were maintained under ether with spontaneous respiration. For time points beyond this, the animals were re-anaesthetized prior to blood collection. Platelet (PLT), Red Blood Cell (RBC), and White Blood Cell (WBC) counts were determined from a 300 μl sample of EDTA-treated blood using an automated blood counter (Coulter Counter Model T660). Blood smear slides were made from the undiluted blood samples and viewed with a light microscope under an oil-immersion lens for the presence of platelet aggregates. Aggregates, if present, could produce a falsely low platelet count by the automated particle counter technique. Changes in PLT counts are expressed as a percentage of control counts: $(Count_1 - Count_0)/Count_0 \times 100$. Prothrombin time (PT) was determined by standard hematological methods. Plasma fibrinogen was determined by the Dade Data-Fi® procedure, which is based on the quantitative fibrinogen assay developed by Clauss (13). For those animals which received I^{125}-labeled vesicles, Cr^{51}-labeled RBC's or Cr^{51}-labeled platelets, blood and organs were counted for radioactivity using a gamma counter (Beckman Instruments, Model Gamma 8000). Total per cent recovery was determined by summing the amount of radioactivity in the tissues, corrected for circulating blood counts, and dividing the sum by the total amount of radioactivity injected. Organ and blood I^{125}-labeled liposome, Cr^{51}-labeled RBC and Cr^{51}-labeled platelet recoveries are expressed as a percentage of the total radioactivity injected. For liposome and platelet distribution experiments, estimation of total and organ blood volumes of the 225–250 g female Wistar rat were determined by Cr^{51}-labeled RBC technique in a separate series of experiments.

All groups are compared using either the paired or unpaired one- or two-sided Student's t-test (14). Differences between groups are considered significant for $p < 0.05$. Values are expressed as the mean ± standard error of the mean (SEM).

In Vitro Experiments

Whole blood from rats was collected in lithium heparin Vacutainer™ tubes (Becton and Dickenson Vacutainer™ Systems, Rutherford, NJ, USA). Platelet rich plasma (PRP) was prepared as described earlier and diluted to a volume of 1 ml with 500 μl of PPP and 200 μl of HBS to give a platelet count of approximately 400,000 platelets per 1 μl. At 0 time, lipid (5 or 10 μl of 50 μmoles/ml) was added to 1 ml of the diluted PRP, gently shaken and at subsequent time points, the platelet count was determined using the automated blood counter. Lipid backgrounds were measured as an equivalent volume of lipid (5 or 10 μl) diluted in HBS at pH 7.5 (1 ml).

Results

In order to determine normal blood values and to examine the effects of tail blood sampling and injection on these indices, six rats were anaesthetized and tail blood obtained prior to saline injection. Five minutes after saline injection, a second blood sample was taken. The WBC and PLT counts did not differ significantly between pre- and post-injection values. RBC count did decline by 3% ($p < 0.01$ vs. pre-injection values), which is consistent with blood loss. In the following studies, all PLT counts were taken post-injection and compared using Student's t-test with a group of 40 animals from which control (pre-injection) samples were taken. Examination of the peripheral blood smears for all samples did not reveal significant numbers or sizes of platelet aggregates.

The effect of liposomes of neutral, positive or negative charge on platelet count was first examined. Fig. 1 shows the effects of

25 mg t.l./kg MLV's containing (a) EPC:CHOL (55:45 mole%), (b) SA:EPC:CHOL (10:45:45 mole%), (c) PS:EPC:CHOL (10:45:45 mole%), and (d) PG:EPC:CHOL (10:45:45 mole%). For the neutral EPC:CHOL vesicles the platelet depression observed was 18% vs. controls ($p < 0.005$) and occurred at 30 minutes post-injection. The maximal effect of the charged systems was more pronounced, with a decline of 47% vs.

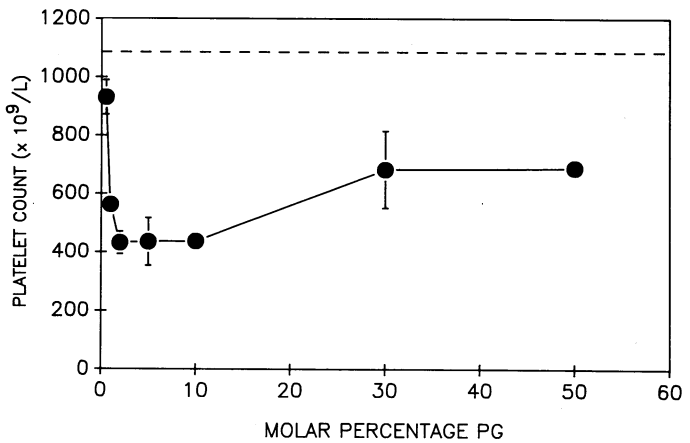

Fig. 2 The influence of molar percentage of PG in PG:EPC:CHOL MLV's on platelet count depression at 5 minutes post-injection of 25 mg t.l./kg

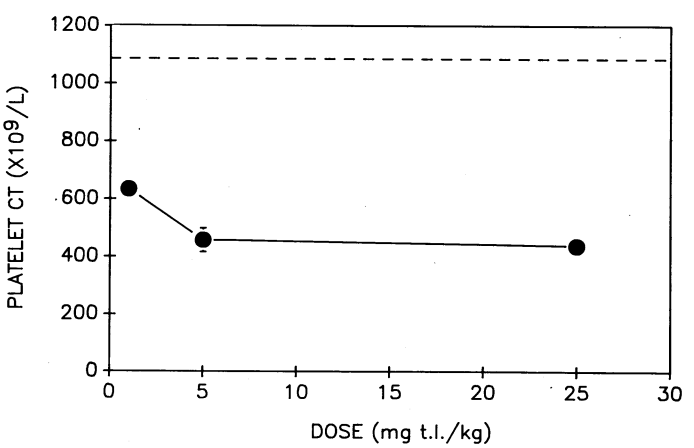

Fig. 3 Effect of injected dose of PG:EPC:CHOL (10:45:45 mole%) MLV's on platelet count at 5 minutes post-injection

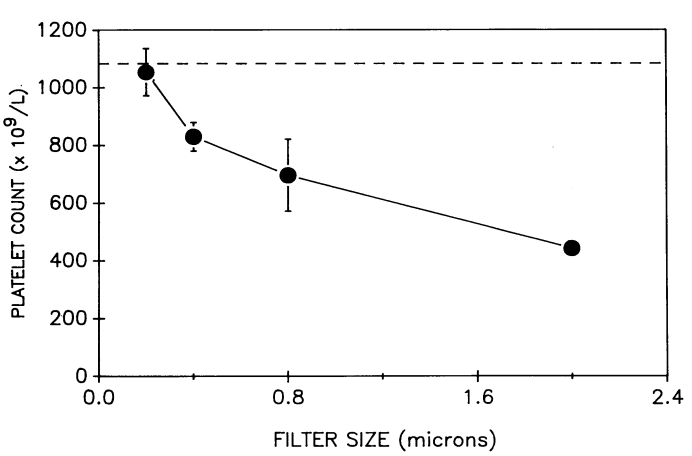

Fig. 4 Effect of filter size on the platelet count depression produced by extruded PG:EPC:CHOL (10:45:45 mole%) vesicles at 5 minutes post-injection of 25 mg t.l./kg

Table 1 Effect of 25 mg t.l./kg of EPC:Chol (55:45 mole %) or PG:EPC:Chol (10:45:45 mole %) MLV injection on circulating platelet counts, expressed as a percentage of control (pre-injection) counts. Platelet counts were determined by radiolabeled platelet counting or by automated blood counting techniques

Lipid type	Labeled platelets		Unlabeled platelets	
	2 min (% control)	60 min (% control)	2 min (% control)	60 min (% control)
EPC:CHOL	101.5	96.1	111.5	97.8
PG:EPC:CHOL	27.5	94.5	34.7	89.3

controls ($p < 0.0005$) occurring at 5 minutes post-injection for the PS:EPC:CHOL vesicles and a decline of 82% vs. controls ($p < 0.0005$) at 2 minutes post-injection for the PG:EPC:CHOL vesicles. The peak decline in platelets following SA:EPC:CHOL liposomes was 40% ($p < 0.05$) at 2 minutes post-injection vs controls. Because of the platelet count depression seen following administration of PG:EPC:CHOL MLV's was the largest, this system was the major focus of subsequent experiments.

In order to confirm that the decline in circulating platelet counts as measured by the automated blood counter does not represent an artifact through interference of liposomes with the counting process, Cr^{51}-labeled platelets were infused into a group of rats prior to liposome injection. Five minutes after the administration of the radiolabeled platelets, a blood sample was taken for isotope counting and for automated blood counting. Two minutes later, 25 mg t.l./kg of either PG:EPC:CHOL (10:45:45 mole%) or EPC:CHOL (55:45 mole%) MLV's were injected. A second blood sample was taken at either 2 or 60 min following liposome injection. As shown in Table 1, at both 2 and 60 min post-EPC:CHOL injection the labeled and unlabeled platelet counts remain at or above control values. In contrast, at 2 min following PG:EPC:CHOL MLV injection both the labeled and unlabeled platelet counts decline by approximately 70% from control values ($p < 0.05$, $p < 0.02$ respectively). By 60 min post-injection of PG:EPC:CHOL MLV's both labeled and unlabeled platelet counts have returned to control levels. Thus in these systems, where there is a good correlation between circulating platelet counts determined by radiolabeled and automated blood counting techniques, automated blood counting provides an accurate means to measure the transient thrombocytopenia which can be induced by liposomes.

The sensitivity of the initial drop in platelet count to the mole percentage of PG is clearly of interest. The molar percentage of PG in the liposomal preparation was therefore varied from 0.5% to 50%. These systems were administered at a dose of 25 mg t.l./kg and blood samples taken 5 minutes post-injection. As shown in Fig. 2, there appears to be a very specific liposome-platelet interaction with PG-containing vesicles such that an MLV system containing only 1 mole% PG is able to elicit a nearly 50% reduction in circulating platelets at 5 minutes post-injection. The maximal effect at this time and dose appears to be with MLV system containing 2–10 mole% PG.

The effect of liposome dose on platelet count at 5 minutes post-injection was examined for the PG:EPC:CHOL (10:45:45 mole%) MLV system (Fig. 3). As shown, lipid dose is important for determining the magnitude of the platelet count depression, with a lipid dose of 1 mg t.l./kg producing a reduced depression of platelet count, although all doses given caused a significant ($p < 0.0005$) platelet decline.

In addition to liposome composition and dose, liposome size can also be varied appreciably. In order to examine the effect of liposome size on platelet count, 25 mg t.l./kg PG:EPC:CHOL (10:45:45 mole%) of liposomes of various sizes were administered. As shown in Fig. 4, as filter size is decreased, and thus

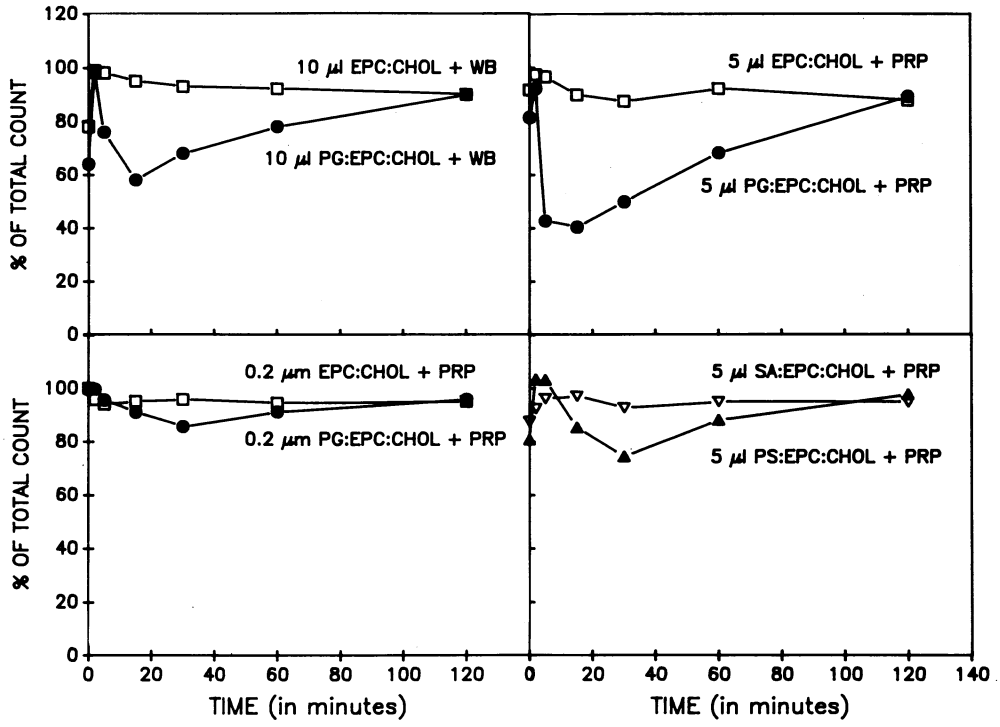

Fig. 5 Effect of the presence of vesicles of various lipid compositions and sizes in whole blood (WB) or platelet rich plasma (PRP) on the number of particles measured in the platelet channel of an automated blood counter vs. time. Values are expressed as a percentage of the total number of particles added (the sum of lipid only + WB or PRP only)

liposomal size is decreased, the magnitude of the platelet count depression diminishes until it becomes non-significant for MLV's sized through 0.2 µm filters (measured vesicle diameter: 148 ± 48 nm). Thus vesicle size can dramatically affected the extent of platelet count depression.

In order to better understand the nature of the probable liposome-platelet interaction suggested by in vivo studies, several in vitro experiments were carried out using either rat whole blood (WB) or platelet rich plasma (PRP) prepared as described in Methods. As shown in Fig. 5, the addition of 500 nmoles of EPC:CHOL (55:45 mole%) or PG:EPC:CHOL (10:45:45 mole%) MLV's to (a) 1 ml of WB produces a transient decline in the number of particles counted in the platelet "window" of the automated blood counter at about 15 minutes after addition of PG:EPC:CHOL MLV's, with a gradual return to baseline occurring after that time. In (b), addition of 250 nmoles EPC:CHOL (55:45 mole%) or PG:EPC:CHOL (10:45:45 mole%) MLV's to 1 ml of PRP also produces a transient drop in particle counts for the PG-containing vesicles. The zero time point represents platelet counts only. The two minute time point contains both platelet and liposome counts. Addition of 250 nmoles of MLV's alone to 1 ml of HBS produces approximately 40–90 × 10^9/l counts in the platelet channel and 5–10^9/l in the WBC channel. In (c), addition of 250 nmoles of EPC:CHOL or PG:EPC:CHOL 0.2 µm LUVET's does not produce a rise in counts at the 2 minute time point. A much smaller transient decline in particle counts is seen upon addition of the 0.2 µm PG:EPC:CHOL LUVET's to PRP than was seen in (b) for PG:EPC:CHOL MLV's. As shown in (d), addition of 250 nmoles of PS:EPC:CHOL (10:45:45 mole%) MLV's produces a modest decline in counts, however addition of 250 nmoles of SA:EPC:CHOL (10:45:45 mole%) MLV's does not affect the counts in the platelet window throughout the time course of this experiment.

Macroaggregation of platelets in whole blood (50% diluted with HBS) as measured by impedance in a lumiaggregometer was not detectable on addition of PG:EPC:CHOL (10:45:45 mole%) or EPC:CHOL (55:45 mole%) MLV's (data not shown).

It thus appears that the addition of negatively charged MLV's to rat WB or PRP produces a temporary association between the liposomes and platelets which may then, in vivo, be transiently sequestered from the circulation. As was shown in Table 1, the transient thrombocytopenia observed in vivo following liposome injection represents an actual decline in circulating platelets and not an artifact due to a "shift" of liposome-platelet complexes out of the platelet counting window of the automated blood counter. The absence of an in vitro response to the addition of SA-containing vesicles may indicate a different mechanism for the SA-induced thrombocytopenia seen in vivo. Such an alternate mechanism may account for the long-lasting SA-induced thrombocytopenia observed in vivo (Fig. 1).

The possibility of preventing the observed platelet count decline produced by PG-containing MLV's by pretreatment with vesicles was addressed in a series of experiments in an attempt to better understand the mechanism of action of the liposomally-induced thrombocytopenia. Five animals were challenged with 25 mg t.l./kg PG:EPC:CHOL (10:45:45 mole% MLV's) and 2 minutes later blood samples were taken. Two of the animals had not received liposomal pretreatment, the other three had been pretreated with the identical lipid composition and dose 1 hour prior to challenge. Platelet count decline to 335 ± 77 × 10^9/l or 31% of control value in the two animals which had not received pretreatment prior to challenge. In the pretreated animals, the platelet count 2 minutes following the second dose of PG-containing liposomes was 1,006 ± 60 × 10^9/l or 93% of control value (n.s. vs. controls). The absence of a platelet decline in this second group of animals suggests a mechanism of action for the platelet count depression which may involve the following factors: (1) saturation of endothelial receptors by the first lipid dose thus preventing either liposome-platelet complexes from transiently binding, (2) the initial liposome-platelet interaction may release platelet factors or change platelet receptors making the platelets refractory to a second liposomal challenge, (3) vesicles from the pretreatment dose may be associated with the circulating platelets, preventing interaction with the second dose of vesicles which would be required for transient platelet sequestration.

Table 2a Effect of injection of 25 mg t.l./kg EPC:CHOL (55:45 mole %) MLV's or PG:EPC:CHOL (10:45:45 mole%) MLV's on the biodistribution of Cr^{51}-labeled platelets

Tissue type	2 Minutes EPC:CHOL (% inj)	PG:EPC:CHOL (% inj)	60 Minutes EPC:CHOL (% inj)	PG:EPC:CHOL (% inj)
Blood	63.8	18.2*	64.2	57.0*
Heart	0.0	0.3	0.3	0.1
Lung	6.4	26.6**	5.0	3.5
Liver	10.9	30.0**	11.6	11.8
Spleen	6.4	7.6	13.1	14.6
Kidney	1.0	1.1	0.3	0.3
Total recovery	88.5%	83.8%	94.5%	87.3%

* : $p < 0.05$, ** : $p < 0.02$

Table 2b The biodistribution of I^{125}-labeled EPC:CHOL (55:45 mole %) MLV's or PG:EPC:CHOL (10:45:45 mole%) MLV's injected at 25 mg t.l./kg at 2 or 60 minutes post-injection

Tissue type	2 Minutes EPC:CHOL (% inj)	PG:EPC:CHOL (% inj)	60 Minutes EPC:CHOL (% inj)	PG:EPC:CHOL (% inj)
Blood	55.6	45.9**	3.8	13.4
Heart	1.2	0.6	0.3	0.6
Lung	21.4	10.2***	4.2	0.6***
Liver	10.1	31.6****	36.6	26.8*
Spleen	2.0	1.5	32.8	18.3
Kidney	1.9	1.3***	0.8	1.1****
Total recovery	92.2%	91.1%	78.5%	60.8%

* : $p < 0.05$, ** : $p < 0.02$, *** : $p < 0.01$, **** : $p < 0.001$

In an attempt to investigate a possible role for fibrinogen in liposome-mediated platelet depression, a group of three rats were pretreated with Ancrod, an agent which cleaves fibrinogen without producing platelet aggregation or the release of ADP, ATP, calcium or serotonin from platelets (15). One hour following pretreatment with 25 U/kg Ancrod, at which time fibrinogen was below detectable levels, injection of 25 mg t.l./kg PG:EPC:CHOL (10:45:45 mole%) MLV's produced a decline in platelet counts at 2 minutes post-injection ($282 \pm 10 \times 10^9$/l) comparable to that seen in animals which were not pretreated ($191 \pm 14 \times 10^9$/l). Thus, there does not appear to be a dependence on plasma fibrinogen for the platelet decline observed, although platelets are known to contain an endogenous pool of fibrinogen (16). In a separate attempt to inhibit the platelet decline seen with the PG-liposomes, three rats were pretreated over 24 hrs with 25 mg/kg aspirin, a drug which is known to prolong the bleeding time through interference with platelet aggregation. Aspirin pretreatment did not however block the liposomally-induced transient thrombocytopenia, with the platelet count dropping to $357 \pm 34 \times 10^9$/l following PG:EPC:CHOL (10:45:45 mole%) MLV injection. In another trial, pretreatment with 1,000 U/kg heparin one hour prior to PG:EPC:CHOL (10:45:45 mole%) MLV injection did not prevent the platelet count from declining to $407 \pm 53 \times 10^9$/l. Ancrod, aspirin, or heparin given alone did not affect platelet counts. Thus it appears as though the transient thrombocytopenia observed following liposomal injection does not represent activation of several of the more common pathways of blood coagulation nor appear to involve the aggregation of platelets but may represent an association of platelets with liposomes.

As it appears that the PG-containing liposomes associate in some way with platelets and that these platelets are transiently removed from the circulation, it might be expected that the biodistribution of liposomes or platelets would be different for PG:EPC:CHOL systems than for EPC:CHOL systems (Tables 2a and 2b). For the platelet distribution studies, an injection of Cr^{51}-labeled platelets was followed 5 minutes later by unlabeled PG:EPC:CHOL or EPC:CHOL liposomes (Table 2a). For the liposome biodistribution studies, animals were injected with 25 mg t.l./kg PG:EPC:CHOL (10:45:45 mole%) MLV's or EPC:CHOL (55:45 mole%) MLV's containing 0.02 μCi I^{125}-DPPE/μmol t.l. (Table 2b). Tissue distribution was determined at either 2 or 60 minutes post-injection. Two minutes after liposome injection, animals which received PG:EPC:CHOL (10:45:45 mole%) MLV's have significantly less of the injected liposome dose recovered in the lung than those which received EPC:CHOL (55:45 mole%) MLV's (10.2% vs. 21.4%, $p < 0.001$) yet lung recovery of radiolabeled platelets is four-fold higher in the PG:EPC:CHOL-treated animals than EPC:CHOL-treated (26.6% vs. 6.4%, $p < 0.01$). At 2 minutes the PG:EPC:CHOL-treated animals also have significantly greater recoveries of labeled platelets and liposomes in the liver than the EPC:CHOL-treated animals. The lower blood recovery of platelets at 2 and 60 minutes in the PG:EPC:CHOL-treated animals correlates with the transient thrombocytopenia measured by automated blood counting techniques. Note that by 60 minutes post-injection, organ recoveries of radiolabeled platelets in the PG:EPC:CHOL treated animals are not significantly different than in those animals which received EPC:CHOL MLV's. Thus the interaction of PG:EPC:CHOL MLV's with platelets alters the biodistribution of platelets in a transient manner such that the normalization of the peripheral platelet count by 60 minutes post-injection reflects a redistribution of those platelets which had been temporarily sequestered in the lung and liver and does not appear to represent a mobilization of noncirculating platelets from splenic or bone marrow stores.

Discussion

We have examined the effects of various liposomal compositions and sizes on blood platelets in the rat and found that negatively-charged systems produce a transient reduction in platelet count in the first 5 minutes post-injection. This effect is most pronounced for liposomes containing PG; MLV's containing 10 mole% PG caused the platelet count to decline by approximately 75% vs. controls at 2 minutes post-injection as measured

by both radiolabeled platelet and automated blood counting methods. PG is able to elicit this response when comprising only 0.5 mole% of liposomal lipid and at low lipid doses. The transient effect seen with the negatively-charged systems as well as the absence of a decline with neutral systems or small negatively-charged systems agrees with in vitro studies. The inability to prevent the transient platelet depression with ASA, heparin and Ancrod and the absence of a detectable macroaggregation in vitro suggests a transient liposome-platelet interaction and not an activation of platelet aggregation. These observations may implicate platelets as a mediator in the clearance of liposomes, particularly those with a negative charge. Neutral and positively-charged systems produce a less striking decline in platelet count. A number of substances have long been known to cause an acute, significant and reversible thrombocytopenia. Heparin was shown by Gollub and Ulin (17) to produce such a change in man approximately 30 minutes after injection. He observed that heparin-induced thrombocytopenia could be immediately reinduced after platelet count recovery in the same subject, suggesting a transient sequestration of platelets, rather than their destruction with subsequent mobilization of stored platelets. Colloidal carbon is also known to transiently decrease the platelet count in experimental animals (8, 18). Villalobos examined hypothermia-induced decreases in circulating platelets and WBC's and showed that some of these cells are sequestered in the liver and probably also the spleen but suggested that since hepatectomy and splenectomy did not completely abolish hypothermia-induced platelet and WBC drops, other sinusoidal organs may play a role in their sequestration (19).

Although liposomes are also particulate systems, there is a very limited literature which examines in vivo interactions between liposomes and platelets. Weissmann et al. (20) reported an in-vivo liposome-leukocyte interaction when he examined the effects of intraperitoneal liposomes on circulating polymorphonuclear leukocytes (PMN's). He observed a 50–70% decline in total circulating leukocytes in the New Zealand rabbit at 5 hours post-injection of charged and uncharged multilamellar vesicles. At 24 hours post-injection he observed a peripheral leukocyte count at 130–202% of pre-injection values. While we also observed a time-dependent relationship between liposomal administration and platelet count depression, the time course was completed over a 1 hour period with blood platelet counts returning to, but not above, control values. WBC counts from in vivo experiments determined by the automated blood counter were not reported in our study due to the interference of liposomes with the WBC counting window of the instrument which tends to produce falsely high measured WBC values, as seen in the in vitro results section.

In summary, liposomes have been shown to induce a transient reduction in platelet count in vivo. It has been shown that liposomal charge, size and dose are important determinants of the extent of platelet depression. A better understanding of this interaction between liposomes and platelets is likely to be important for understanding liposome behaviour in the circulation, and could offer ways to enhance the therapeutic potential of liposomes.

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