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The Binding of Phosphatidylglycerol Liposomes to Rat Platelets Is Mediated by Complement

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Summary

Previous work has shown that intravenous administration of phosphatidylglycercol (PG) containing liposomes to rats results in a rapid transient decline in platelet count (1). Here the interactions of PG liposomes with rat platelets in vitro have been examined with the aim of characterizing factors associated with the decline. It is shown that PG liposomes induce formation of rat (but not human) platelet-liposome microaggregates in vitro. The PC liposome dependent thrombocytopenia observed in vivo can therefore be attributed to sequestration of PG liposome-platelet aggregates. Further, the aggregation of platelets with PG liposomes, which can be monitored as a reduction in platelet count using a coulter counter, is shown to be mediated by a serum complement factor, likely C3b. This is indicated by a requirement of plasma for the in vitro reduction in platelet count induced by PG liposomes, and the inhibition of this effect by heat treatment of plasma, by incubation of plasma with purified cobra venom factor, or by removal of C3 from plasma.

Intrbduction

Recent work from this laboratory has shown that intravenous injection of negatively charged liposomes induces a transient thrombocytopenia in rodents (1,2). This effect is most striking for liposome preparations containing the negatively charged lipid phosphatidylglycerol (PG). The transient reduction in platelet counts is associated with sequestration of platelets and liposomes in the liver and lung (2). This indicates a potential role of platelets in the removal of liposomes from the circulation following intravenous injection,

Preliminary investigations suggest that the transient thrombocytopenia results from a transient liposome-platelet interaction (1). The nature of this interaction is clearly of interest. In this regard, it has been well documented that liposomes bind a variety of serum components (3). Specifically, negatively charged PG containing liposomes bind significantly more serum proteins than neutral liposomes. These serum components may play a role in binding of liposomes to platelets.

In the present study, we show that PG containing liposomes associate with rat platelets in platelet rich plasma. This interaction, which results in the formation of platelet microaggregates, is shown to be dependent on the presence of plasma. The sensitivity of this adhesion process to treatment of plasma with heat, purified cobra venom factor and removal of the complement factor C3, are consistent with a requirement for complement in the binding of PG liposomes to platelets.

Materials and Methods

Materials

Apyrase (grade l), Na heparin (porcine intestinal mucosa, grade l), 4-chloro-naphthol and cholesterol were obtained from Sigma. Egg phosphatidylcholine (PC) and egg phosphatidylglycerol (PG; a synthetic derivative prepared by transphosphatidylation of PC in the presence of glycerol) were purchased from Avanti Polar Lipids. Purified cobra venom factor ***(Naja naja kaouthia)*** was obtained from Diamedix. Goat anti-rat C3 was obtained from Organon Teknika and peroxidase labelled anti-goat IgG (H and L) was purchased from Jackson Immunoresearch Laboratories Inc. Cyanogen bromide activated Sepharose CL-4B was obtained from Pharmacia. All other chemicals were of standard analytical grade. Female rats of albino Wistar strain were obtained from the University of British Columbia Animal Care Center or from Charles River Laboratories. The animals weighed between 225-250 g at the time of use.

Preparation of Platelets and Plasma

Rats were injected with heparin (10 units/kg) prior to collection of blood by heart puncture. Blood (7 ml) was collected in tubes containing 100 units of heparin. Platelet rich plasma (PRP) and platelet poor plasma (PPP) were prepared by standard haematological techniques (4). Briefly, whole blood was centrifuged at 400 x g for 20 min at 22" C. The resulting supernatant was spun at 800 x g for 30 min at 22" C to generate PPP. Plasma was heat inactivated by heatjng plasma at 54" C for 30 min. Plasma was treated with cobra venom factor (160 μg/ml, ref. 5) for 30 min at 37" C. The hemolysis of antibody-sensitized erythrocytes (6) in the presence of Ca^{2+} and Mg^{2+} was negligible for the plasma preparations either after heat treatment or cobra venom treatment. EDTA plasma (45%) containing 20 mM EDTA was titrated to pH 7.5 with NaOH. For generation of plasma deficient in C3, goat anti-rat C3 was coupled to cyanogen bromide activated Sepharose CL-4B by standard procedures (1.5 mg antibody bound/ml of packed sepharose, ref.7). Plasma (1 ml) was incubated with goat anti-rat C3 sepharose (1 ml packed sepharose in 1 ml HBS) for 1 h at 4" C. The resulting preparation showed no detectable C3 as determined by Western blot analysis. Serum was obtained from coagulated blood by centrifuging at 800 x g for 20 min at room temperature. Washed platelets were isolated from PRP (9 volumes diluted with 1 volume of acid citrate dextrose) by centrifuging at 800 x g for 20 min at room temperature and resuspended in Tyrodes-HEPES buffer (8) containing apyrase (0.5 mg/ml) and heparin (50 units/ml). The concentration of platelets was adjusted to 400,000/ml and the final concentrations of HEPES, Ca^{2+} and Mg^{2+} were 20 mM, 2 mM and 1 mM respectively.

Abbreviations

EDTA, ethylenediaminetetracetic acid; EGTA, ethyleneglycol-bis-(b-aminoethyl ether) N,N-tetraacetic acid; HEPES, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; HBS, 25 mM HEPES 125 mM NaCl; PRP, platelet rich plasma; PPP, platelet poor plasma; PBS, phosphate buffered saline; PC, egg phosphatidylcholine; PG, egg phosphatidylglycerol and CHOL, cholesterol.

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Preparation of Liposomes

Frozen and thawed multilamellar vesicles (FATMLVs, ref. 9) were prepared by first dissolving the lipids in chloroform and then evaporating the solvent under nitrogen, followed by drying under high vacuum for 1 h. The dried lipid film was hydrated in 25 mM HEPES, 150 mM NaCl pH 7.5 (HBS), and then freeze-thawed 5 times employing liquid nitrogen for the freezing cycles.

Assay of Platelet-Liposome Interaction

Initially, the interaction of liposomes with platelets was visualized by light microscopy. Briefly, liposomes (20 μl of 5 mM total lipid in HBS) were incubated with PRP (400,000 platelets/ml, 80% plasma) at room temperature. Phase contrast photomicrographs of samples were taken after a 15 min incubation period with a 35 mm camera mounted on a Leitz Laborlux-D light microscope. The association of liposomes with platelets was also indicated by a reduction of platelet count as determined using an automated blood counter (Coulter Counter Model T660) after gentle mixing of liposomes with platelets. The basis of the assay is that PG liposome induced aggregation of platelets results in the exclusion of the aggregate from the platelet window, resulting in a decline in the platelet count recorded by the counter. Since FATMLVs can be of a similar size to platelets, lipid samples (PC/cholesterol and PG/PC/cholesterol; 50 μM) contributed to the platelet count (approx. 1 and 2% respectively, see results). In this study, the platelet count at time t is expressed as a percentage of the platelet count at time zero (which also includes the small contribution from liposomes).

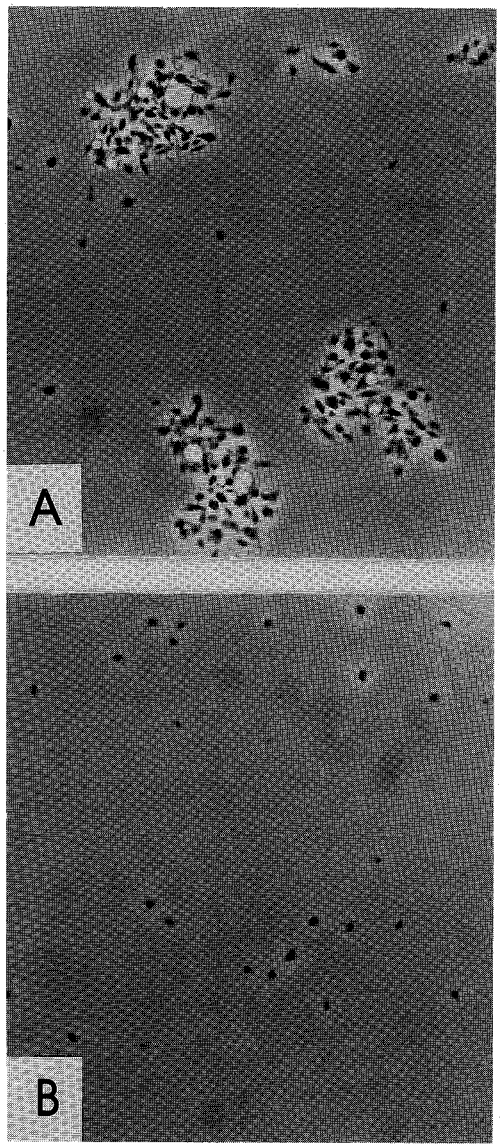

Fig. 1 Formation of platelet-liposome microaggregates: requirement of PG. Liposomes [100 nmole total lipid of (A) 10 mole % PG, 45 mole % PC, 45 mole % CHOL or (B) 55 mole % PC, 45 mole % CHOL] were incubated with PRP (400,000 platelets/ml, 80% plasma) for 15 min at room temperature and subsequently examined by light microscopy (final magnification of photomicrographs is 480 x)

Gel *Electrophoresis and Western Blots*

Liposomes with associated plasma proteins were prepared by incubating liposomes (5 μmoles total lipid in 0.1 ml) with normal or EDTA (20 mM final concentration) treated plasma (4.5 ml) diluted with 100 mM HEPES, 150 mM NaCl pH 7.5 (total volume 6 ml) for 15 min at room-temperature and were washed extensively at 4" C with 25 mM HEPES buffered saline in the presence of Ca^{2+} (2 mM) and Mg^{2+} (1 mM) for normal plasma liposomes or in the absence of divalent cations for EDTA plasma liposome samples. Liposomes with associated plasma proteins (1 μmole lipid equivalent) were prepared for gel electrophoresis by delipidation according to the method of Wessel and Flugge (10), and solubilized in reducing sample buffer (100 μl) for 1 h at 37" C. Plasma (diluted 50 x with reducing sample buffer) was prepared for gel electrophoresis as described for the liposome samples. Aliquots (1 μl) were examined by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10-15% gradient resolving gel using the PhastGel system (Pharmacia). Gels were washed in transfer buffer [20 mM Tris-acetate, 2 mM EDTA and 0.01% SDS (w/v) at pH 7.41 for 20 min and electrophoretically transferred for 45 min onto nitrocellulose (Nitroplus 2000, Micron Separations) in a mini electrophoretic transfer apparatus (Pharmacia) at 50 mA at 4" C. After gel transfer, nitrocellulose was quenched with 5% powdered milk (w/v), 2% BSA (w/v) in phosphate buffered saline (PBS) overnight, washed and incubated with goat anti-rat C3 (75 μg/ml in PBS) for 3 h. After extensive washing, rabbit anti-goat antibody [1,000 x diluted in 2% BSA (w/v), 5% skim powder milk (w/v) in PBS] was incubated for 90 min, washed and labelled proteins were visualized with the peroxide immuno-stain using the chromagen 4-chloro-naphthol.

Results

In previous studies we have observed a transient thrombocytopenia induced by negatively charged liposome preparations in rodents (1, 2). This in vitro study was initiated in order to determine what factors are involved in this event. Initially the interactions of liposomes with platelets were examined by light microscopy. In Fig. 1, it is shown that incubation of PG liposomes with platelet rich plasma (PRP) for 15 min resulted in the formation of platelet microaggregates, in which the negatively charged liposomes form an integral part (Fig. 1A). The requirement of PG in this process was shown by the absence of aggregates on incubation of PC/cholesterol liposomes with platelet rich plasma (Fig. 1B). No platelet aggregates were observed in PRP at 15 min (data not shown).

This association of PG liposomes with platelets was also shown to result in a time dependent reduction in platelet counts as recorded by a coulter counter (Fig. 2 and Table 1). The effect of various concentrations of plasma and PG liposomes on the PG induced decline in platelet counts was examined in order to maximize this response. As shown in Fig. 2, a rapid decline in platelet count was observed over 15 min on addition of PG liposomes to PRP The extent of the decline in platelet count induced by PG liposomes (50 nmoles lipid) was minimally affected by the levels of plasma (25-65%). Thus, in all subsequent plasma reconstitution experiments, the final concentration of plasma was 45%. In Table 1, a significant decrease in platelet counts was observed at 15 min for a range of PG liposome concentrations (50-500 μM lipid). As liposomes at higher lipid concentrations interfere with platelet count estimates (Table 1), lipid concentrations of 50 μM lipid were employed subsequently.

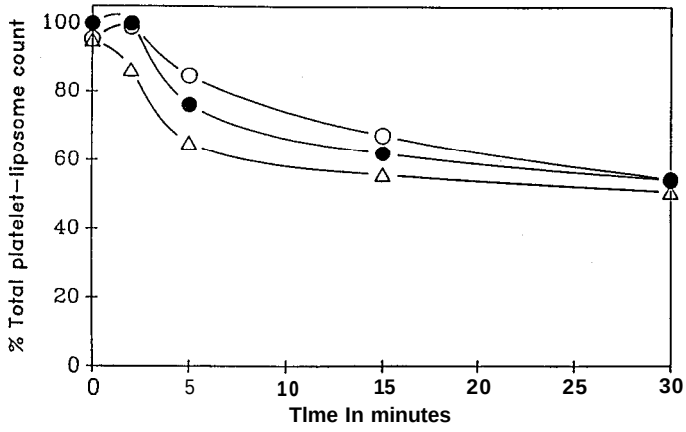

Fig. 2 Effect of plasma concentration on the interaction of PG liposomes with platelets. PG liposomes (10 μl of 5 mM) were added to washed platelets reconstituted with plasma (25-65%) and platelet number was determined over time as in Materials and Methods. (0) 25% plasma, (●) 45% plasma, and (△) 65% plasma

Table I Effect of PG liposome concentration on platelet count[a]

Lipid concentration (μM) to platelet count[b]	Lipid contribution at 15 min[c] (%)	Platelet count (%)
0	0	95
50	2	64
100	4	58
250	10	48
500	20	45

[a] PG liposomes (10 μl of 5-50 mM lipid) were added to washed platelets reconstituted with plasma (45%) and platelet count was determined as described in Materials and Methods.

[b] This is expressed as the percentage of the total count in the platelet window (at zero time) which arises from the liposomes.

[c] This is expressed as the counts in the platelet window at 15 min as a percentage of the counts at time zero.

In Fig. 3 the requirement for plasma is illustrated by the absence of a reduction in the platelet count for washed platelet preparations in the presence of PG liposomes. Addition of plasma resulted in a rapid decline in platelet count which was equivalent to that obtained for PRP. The lipid dependence of this interaction indicated by light microscopy studies, is also illustrated by the slight, much slower platelet count decline observed on incubation of PC/cholesterol liposomes with PRP.

To identify the plasma factor(s) required for the liposome dependent reduction in platelet count, PG liposomes were incubated with washed platelets to which serum or plasma derivatives had been added (Fig. 4 and 5). The substitution of serum by plasma did not affect the rate or extent of PG liposome binding to platelets, indicating that blood coagulation activity was not involved in the binding process. However, the addition of heat inactivated plasma (54" C for 30 min) did not result in any decline in platelet counts induced by PG liposomes, demonstrating that the plasma factor is heat labile. Treatment of plasma with purified cobra venom factor also resulted in loss of the capacity of PG liposomes to induce aggregation as indicated by a decline in platelet count. Finally, a requirement for divalent cations in the process was shown by the lack of any PG liposome induced decline in platelet count in the presence of EDTA treated plasma. These results suggest that the PG liposome induced reduction in platelet counts is dependent on a complement factor.

To conclusively demonstrate the requirement of complement for platelet-liposome interactions, plasma deficient in C3 was prepared by immunoaffinity chromatography on goat anti-rat-sepharose. Removal of C3, as confirmed by Western blot analysis (data not shown), totally inhibited the reduction in platelet counts (Fig. 5).

To further demonstrate the possible involvement of C3 and/or C3 cleavage products in the binding process, a Western blot of protein associated with PG liposomes after incubation with plasma was probed with goat anti-rat C3. The third component of complement C3 is composed of two disulphide linked polypeptide chains of molecular weight (MW) 123,000 (α chain) and 76,000 (β chain, 11). As shown in Fig. 6, two major bands were detected by blot analysis of plasma (lanes 1 and 2) with goat anti-rat C3

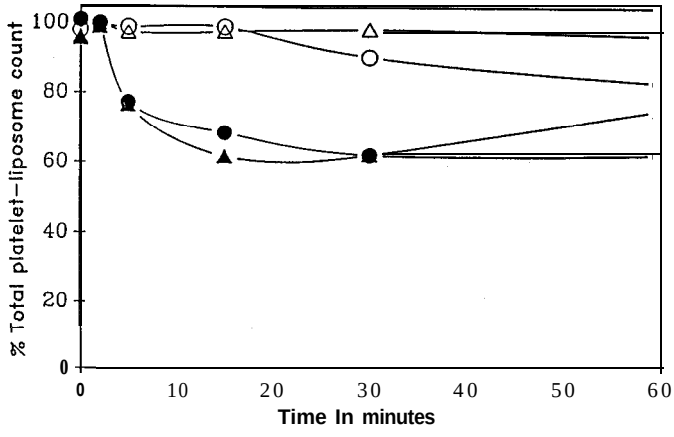

Fig. 3 Binding of PG liposomes to platelets: requirement of plasma. Liposomes (50 nmoles of 10 mole % PG, 45 mole % CHOL, 45 mole % PC or 55 mole % PC, 45 mole % CHOL) were added to PRP or washed platelets in the presence or absence of plasma, gently mixed and sampled at various times for platelet count as outlined in Materials and Methods. (A) PRP and PG liposomes, (●) washed platelets, plasma and PG liposomes, (△) washed platelets and PG liposomes and (0) washed platelets, plasma and PC liposomes

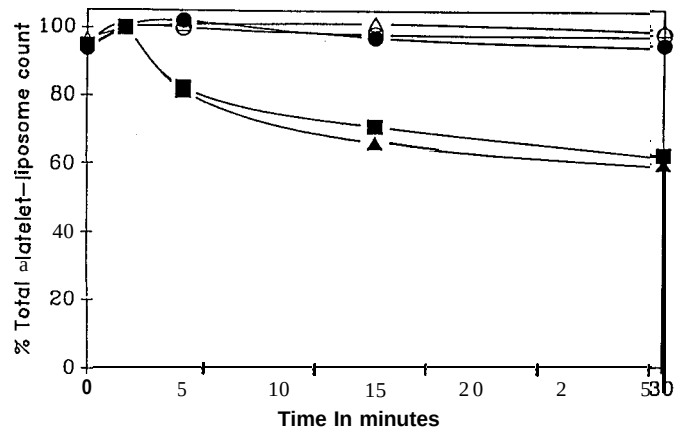

Fig. 4 Determination of the plasma component(s) involved in the binding of PG liposomes to platelets: Liposomes (50 nmoles PG) were added to washed platelet preparations reconstituted with various types of plasma or serum (45%) and assayed for platelet count. Characterization of plasma component: (A) plasma; (■) serum; (△) EDTA plasma; (●) heat inactivated plasma and (0) cobra venom treated plasma

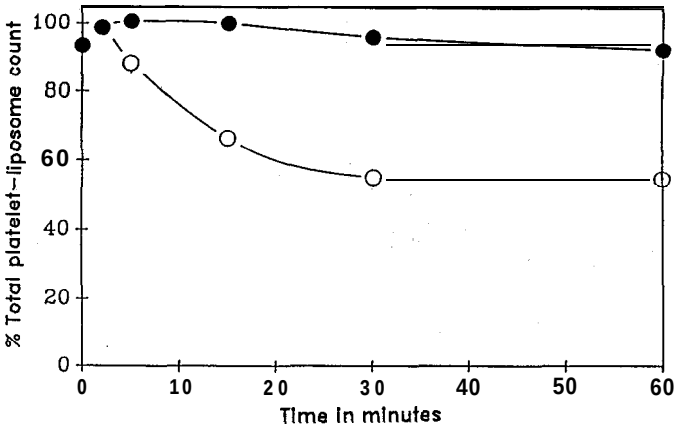

Fig. 5 Effect of removal of C3 from plasma on binding of PG liposomes to platelets; PG liposomes (50 nmoles) were added to washed platelets reconstituted with normal plasma (45%, 0) or plasma that had been treated with goat anti-rat C3 coupled sepharose (●)

IgG on a 10-15% reducing SDS-PAGE gel. Some minor labelling of the band of MW 65-70 kDa with control non-Immune goat serum was observed with all samples (data not shown). To confirm the identity of the higher MW band as the a chain of C3, plasma was treated with purified cobra venom factor (lane 3). This results in the disappearance of the high molecular weight band with the concomitant appearance of a low molecular weight band (MW: approximately 40 kDa), a pattern which is consistent with the formation of the rat equivalent of the C3 cleavage fragment, iC3b. Analysis of protein associated with PG liposomes after 15 min incubation with plasma (lane 4), indicates a similar protein profile to that obtained for cobra venom treated plasma, with the additional appearance of an unidentified high molecular protein band (approximate 140-150 kDa).

In this investigation, the interaction of liposomes with platelets is shown to require the presence of negatively charged lipids, cations and intact complement factors. To demonstrate that levels of liposomally associated C3 (and C3 cleavage fragments) reflect these requirements, protein associated with PC liposomes incubated with plasma (lane 5), and PG liposomes incubated with EDTA plasma (lane 6) or cobra venom treated plasma were screened with anti-rat C3. The results indicate that negligible levels of the complement factor C3 or its cleavage products associate with liposomes under these conditions.

Discussion

Here it is shown that platelet-liposome microaggregates readily form in vitro on incubation of negatively charged PG liposomes with platelets in the presence of plasma. This investigation was initiated in order to determine the nature of the factors involved in this adhesion process. The extent of liposome-platelet binding was followed by platelet count measurements using a coulter counter assay. The participation of complement in platelet-liposome interactions was initially suggested by the heat labile nature of the mediator. This was further confirmed by experiments in which treatment of plasma with cobra venom factor, which specifically degrades the complement factor C3 (5), or removal of C3 by treatment of plasma with goat anti-rat C3 coupled to sepharose, abolished the PG liposome-platelet interaction.

Previous work from this laboratory has shown that the rapid clearance of PG liposomes from blood is associated with a transient drop in circulating platelets (1, 2). Also biodistribution studies indicate that PG liposomes and platelets are sequestered together within the lung as well as the liver (2). These observations suggest that platelets are involved in the clearance of PG liposomes from the circulation. As it is well established that particular matter is cleared in vivo by the reticuloendothelial system in a size dependent manner (19, 20), the observation that PG liposome-platelet microaggregates of >20 μm in diameter readily form in vivo would suggest that the size of the platelet-liposome aggregate leads to an enhanced clearance rate of PC

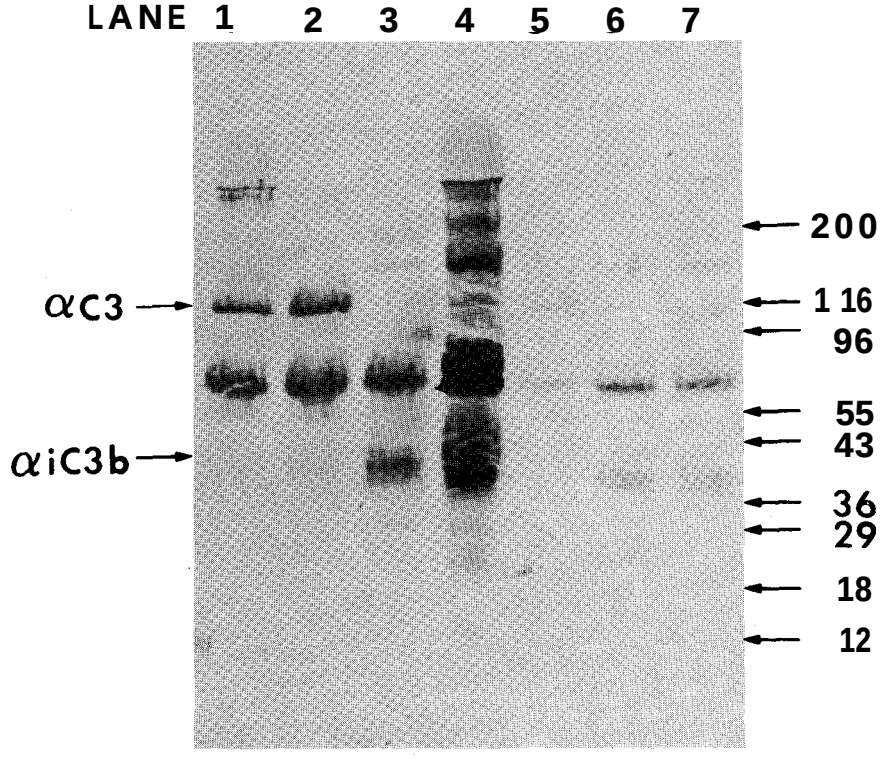

Fig. 6 Western blot of protein bound to PC and PG liposomes using goat anti-rat C3 IgG. Samples were prepared as in Materials and Methods and run on a 10-15% reducing SDS-PAGE gel. C3 and iC3b cleavage products are identified. Lane 1, plasma treated as described for liposome samples; lane 2, plasma; lane 3, cobra venom treated plasma; lane 4, C3 and C3 cleavage fragments associated with PG liposomes after 15 min incubation with plasma; lane 5, PC liposomes incubated with plasma; lane 6, PG liposomes incubated with EDTA plasma; lane 7, PG liposomes incubated with cobra venom treated plasma

liposomes from the circulation. Furthermore. the in vivo transient reduction in platelet count may arise from trapping of these microaggregates within the microvasculature of the lung and the liver.

In this report we show that proteolytic fragments of C3, corresponding to the rat equivalent of human iC3b (12), are associated with PG liposomes under conditions which result in maximum aggregation of PG liposomes and platelets. It is well established that platelets from rodents have CR1 type receptors that bind C3b and iC3b avidly (16, 17). Our findings support the possibility that the deposition of C3b and/or iC3b on PG liposomes results in the adhesion of PG liposomes to rat platelets via the CR1 receptor. This interpretation is consistent with the observations that negatively charged liposomes consume complement (14, 15) and can bind purified C3b in vitro (21). Furthermore, human platelets which lack CR1 receptors (16), do not interact with PG liposomes in the presence of human plasma (unpublished observations), even though incubation of PG liposomes with human plasma results in consumption of C3 (15). Muramatsu et al. (13) have proposed a similar role of complement in the adhesion of rabbit platelets to negatively charged microcapsules. However, other components such as C4b (which associate with negatively charged liposomes, ref:2l) as well as adhesive proteins such as fibronectin (which bind to negatively charged liposomes and C3 cleavage fragments, refs. 3,22) may play a role in mediating this interaction.

In summary, the results of this investigation show that in order for liposomes to bind to platelets in vitro, a negatively charged lipid such as PG and the complement component C3 must be present. The formation of platelet-liposome microaggregates of significant size offers a feasible explanation for the rapid removal of negatively charged liposome from the blood circulation. Furthermore, these results emphasize the pivotal role that liposomally bound serum proteins such as complement play in the in vivo clearance behavior of liposomes. Eurther studies are required to address the mechanism of complement mediated binding of PG liposomes to platelets.

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