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ASSESSMENT OF THROMBOGENIC POTENTIAL OF LIPOSOMES

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SUMMARY

The effects of liposomes with positive, neutral and negative surface charges on platelets and the plasmatic coagulation system were investigated in several in vitro and in vivo models. Negatively charged liposomes stimulated the plasmatic contact activating system as demonstrated by significant acceleration of whole blood clotting time measured in containers with non-wettable (siliconized) surface. The same liposomes induced reversible aggregates of human platelets in vitro and circulating reversible platelet aggregates after intravenous infusion in guinea pigs. Liposomes with positive and neutral surface charges had no effect on plasmatic coagulation and platelets. The biological mechanisms and the toxicological relevance of these findings are discussed.

Key words: Liposome; Platelet aggregation; Contact activation; Thrombosis

STATEMENT OF PROBLEM

In speculating about adverse effects of intravenously (i.v.) administered liposomes, interactions with hemostatic mechanisms must be considered. Since liposomes are foreign bodies with a large and often electrically charged surface, their most likely targets are the platelets and the contact activating system of plasmatic coagulation. Introduction of liposomes into the circulation may thus lead to formation of platelet aggregates and to generation of intrinsic thromboplastin, 2 factors that are known to increase the risk of thrombosis. Experience shows that weak thrombogenic substances are

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often not detected in routine toxicological studies, and that special tests must be performed to recognize drug-induced hypercoagulability [1]. In this paper such techniques are described, using 3 liposome preparations with different electrical surface charges as test compounds.

MATERIALS AND METHODS

Liposomes

Small, unilamellar liposomes were prepared by controlled detergent dialysis procedure [2], using a Liposomat dialysis apparatus (Diachema AG, Appenzell, Switzerland). Lipids (molar ratios and amounts see, Table I) were solubilized with sodium cholate yielding mixed micelles. The detergent was removed by dialysis during 18 h at room temperature. For micelle formation as well as for dialysis phosphate buffered saline (PBS, 10 mM phosphate buffer: $Na_2HPO_4 \cdot 2H_2O$, KH_2PO_4 (pH 7.4) containing 130 mM sodium chloride) was used. Phosphatidylcholine (PC) from egg yolk and phosphatidic acid (PA) from egg lecithin, both grade I, were obtained as stock solutions in chloroform/methanol (1/1) (Lipid Products, Nutfield, U.K.). Stearylamin (SA) was from Fluka, Buchs, Switzerland. Stock solutions were prepared in chloroform. Sodium cholate and all other reagents were purchased from Merck, Darmstadt, F.R.G.

The compositions of the liposomes are listed in Table I. Liposomes were filtered with 0.2 μm pore size filter (Minisart), stored at 4 °C and checked regularly for precipitate formation. The final lipid concentration was 12 mg/ml, and the residual detergent (sodium cholate) concentration less than 1%. The liposome stocks contained between 10^{15} and 10^{16} liposomes per ml.

In vitro studies

Blood was drawn from an antecubital vein of healthy donors with plastic syringes and stainless steel disposable 21 g needles. It was anticoagulated 1:10 with 3.65% disodium citrate. Platelet-rich (PRP) and platelet-poor plasma (PPP) were prepared by conventional techniques.

For the determination of whole blood clotting time (WBCT) 3 drops of

TABLE I

COMPOSITION AND ELECTRICAL CHARGE OF LIPOSOMES

Liposome	Net charge	Lipid composition (molar ratio)	L/D[a] (molar ratio)
A	positive	PC/SA = 16/1[b]	0.6
B	neutral	PC–	0.6
C	negative	PC/PA = 8/1	0.6

[a]Lipid/detergent ratio, i.e. total lipid/Na cholate, [b]no stable micelles were obtained with a molar ratio of PC/SA = 8/1

fresh blood were added to 1 drop of the liposome stock suspensions in uncoated and silicone-coated watch-crystals. Controls contained 1 drop of PBS. Formation of fibrin thread was used as end-point. Citrated blood was incubated 10:1 with liposome stock suspensions or PBS for 30 min at 37 °C. After this, recalcification time (RT) was determined in uncoated glass tubes by mixing equal volumes (0.2 ml) of blood and 2.5×10^{-2} M $CaCl_2$, and coagulation time was determined by tilting the tubes. PPP was incubated with the same concentration of liposome suspensions or PBS for 30 min at 37 °C. One-stage prothrombin time (PT) was determined with Calcium Thromboplastin Roche and thrombin time (TT) with Thrombin Reagent Roche, using the Labor Fibrintimer (Roche) for both measurements.

One milliliter PRP, adjusted with PPP to 2×10^5 platelets per mm^3, was mixed with 0.1, 0.2 and 0.4 ml of the undiluted liposome stock suspensions or equal volumes of PBS, and was immediately transferred into a Chronolog aggrometer (Chronolog Corp. Havertown, PA) and stirred. The ability of the platelets to aggregate was controlled in the same instrument by addition of 10 µl adenosine diphosphate (ADP, Sigma Chemie GmbH BRD), 1.25×10^{-4} M).

PRP (0.1 ml) was incubated with the same volume of doubling dilutions (1:1 to 1:64) of the liposome stock suspensions or PBS (controls) for 30 min at 37 °C. Red cell pipettes were then filled to the 0.25 mark with the platelet suspensions and to the 101 mark with EDTA-formalin fixative [3] or a dilution fluid containing no formalin (1% w/v EDTA-Dilusol, Merz & Dade AG, Berne). After mixing the pipettes on an electric vibrator for 5 min, Fuchs-Rosenthal counting chambers were filled, and the percentage of platelets present as aggregates (3 or more platelets) was determined by counting a total of 200 platelets. The formalin is added to fix reversible aggregates. In EDTA diluent without formalin only irreversible aggregates are present.

In vivo studies

Male albino guinea pigs, breeder Ivanovas GmbH, Kisslegg, F.R.G., weighing at least 600 g, were anesthetized and fitted with polyethylene catheters in the right jugular vein and the left carotid artery [4]. The liposome stock suspensions were diluted with equal volumes of 0.9% NaCl and infused i.v. for 1 h at a rate of 2 ml/kg per h. Controls received equal volumes of saline. Blood samples were obtained before, 10, 30 and 60 min after the beginning of the infusion for determination of platelet counts, percentage of platelets circulating as reversible and irreversible aggregates, WBCT, Stypven-time (ST) and hemoglobin. The technique for determination of platelet aggregates was the same as that described in the previous paragraph, but fresh blood was used instead of PRP. Stypven-time (ST) was measured in citrated plasma with the Russell Viper Venom reagent of Wellcome Research Laboratories, Beckenham, GB and the labor Fibrintimer (Roche).

After the 1-h infusion, the guinea pigs were killed with an overdose of pentobarbital. Samples of lung and kidney were fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin. Samples of lung

and kidney tissue were also fixed in 2.5% glutaraldehyde, post-fixed with 1% OsO_4, and sections were prepared for transmission electron microscopy.

RESULTS

In vitro studies

Addition of liposomes A and B to fresh blood of 11 donors slightly prolonged mean WBCT measured in uncoated watch-crystals, whereas with liposomes C, a modest shortening occurred. None of the effects was statistically significant. Clotting time of blood samples containing PBS determined in silicone-coated watch-crystals was prolonged by 34 ± 16%. When the same blood samples were mixed with liposomes A and B, WBCT was, on average, slightly shorter than with PBS, but the differences were not statistically significant. However, after addition of liposomes C, WBCT was shorter than in PBS containing bloods in 10 out of the 11 samples, the difference of the means was more than 1 min and statistically significant (Table II).

The effect of liposomes on RT, PT and TT was determined in 2 or 3 blood samples of 2 donors. No changes were observed in comparison with results obtained with the same bloods with and without addition of equal volumes of PBS.

In vitro aggregation of platelets was measured with PRP of 2 donors. No aggregation was observed when the liposomes were added to PRP and stirred in the aggregometer. A prompt clumping response was induced with ADP (data not shown). Formation of platelet aggregates was also determined by microscopic observation in counting chambers. Table III contains the results obtained with PRP of one donor: with formalin-containing diluent the percentage of aggregated platelets in samples mixed with liposomes C was consistently higher than in the controls, but no clear-cut concentration-effect relationship was noted. With the EDTA diluent containing no formalin, an increase was seen at 3 dilutions. A slight increase in the percentage of aggregated platelets was also present with 2 dilutions of liposomes B when formalin-containing diluent was used. These results were confirmed with PRP of a second donor (data not shown).

TABLE II

MEAN WHOLE BLOOD CLOTTING TIME (SECONDS) (n = 11)

Test material	Glass container	
	untreated	siliconized
PBS	476 ± 102	634 ± 139
Liposomes A	550 ± 99	617 ± 155
Liposomes B	508 ± 118	602 ± 132
Liposomes C	444 ± 106	558 ± 139*

*P = 0.025, two-tailed paired t-test.

TABLE III

PERCENTAGE OF AGGREGATED PLATELETS IN VITRO[1]

Dilutions of liposome suspensions	EDTA diluent			Formalin diluent		
	Liposomes			*Liposomes*		
	A	B	C	A	B	C
Control (n = 3)	5 (range 3–10)			10.7 (range 10.5–11)		
1:1	0	3.5	12.5[a]	5	15.5[a]	18[a]
1:2	0	7	9.5	4.5	7	22[a]
1:4	0	3	11.5[a]	6	12.5[a]	18[a]
1:8	1.5	1.5	24[a]	4.5	7.5	27.5[a]
1:16	0	1.5	8	8.5	4.5	13.5[a]
1:32	0	1.5	7.5	7.5	8	23[a]
1:64	3	1.5	9.5	5	10.5	11.5[a]

[a]Values above normal range.

In vivo studies

In the blood of control guinea pigs infused with saline, a small percentage of platelets was present as reversible, circulating aggregates, and practically no irreversible aggregates were seen. The values were well within the limits of controls used in previous studies [1]. Infusion of liposomes A had no effect on the percentage of aggregated platelets. With liposomes B a modest increase of reversible aggregates was seen after 30 min. Liposomes C caused a considerable increase in the percentage of platelets present as reversible aggregates (Fig. 1). The maximum was seen after 10 min in 1, after 30 min in 3 and after 60 min in 1 animal. The highest percentage of aggregated platelets observed was 60%, and the largest aggregates consisted of 18 platelets. A slight increase of irreversible aggregates (5.5%) was seen only in 1 animal after 10 min infusion of liposomes C.

Platelet counts and WBCT did not change significantly during the infusions of all 3 types of liposomes. ST remained at or slightly above 100% of the initial values in the controls. It decreased to less than 60% in 1 guinea pig after 10, 30 and 60 min infusion with liposomes C. In 1 animal treated with liposomes B, ST reached 80% of initial values after 30 min and returned to control levels after 60 min. In all groups the repeated drawing of blood samples resulted in a modest (1.9 ± 1 g/100 ml) decrease of hemoglobin. The histopathological and electron microscopic investigations of lung and kidney did not disclose any changes attributable to the liposome administration.

DISCUSSION

The adverse effects of chemicals and particles on the hemostatic system are, to a large extent, related to the electric surface charges. For example,

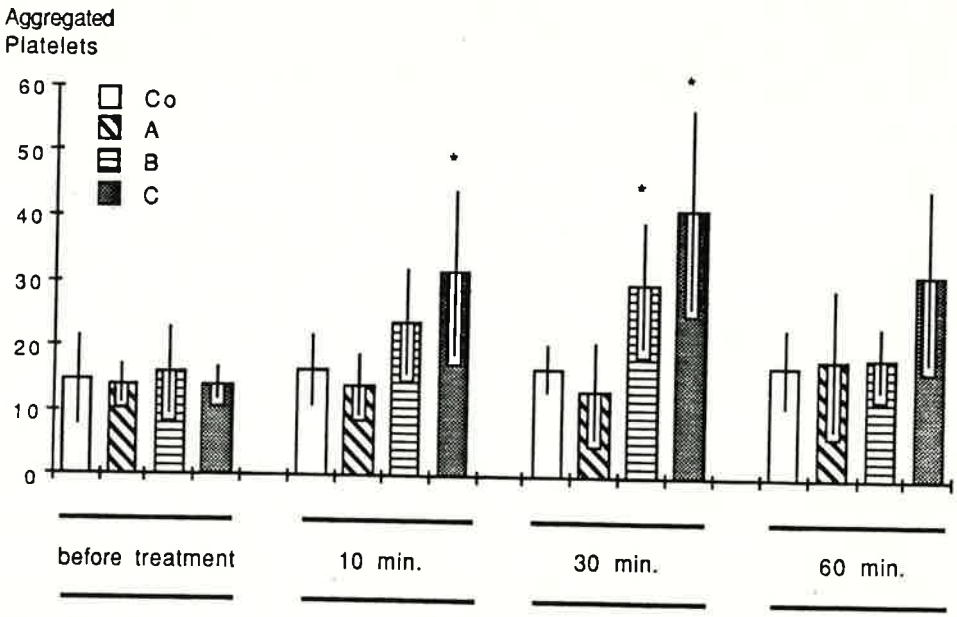

Fig. 1. Percent platelets present as reversible aggregates in guinea pigs infused with PBS (Co), and liposomes A, B, and C for 60 min. *Statistically significant difference in comparison with controls, $P < 0.05$.

activation of the contact system of plasmatic coagulation occurs with negatively charged endogenous substances such as long-chain fatty acids, uric acid and homocystin, and with many other negatively charged materials, e.g. glass, kaolin, celite etc. [5]. Thus, the negatively charged liposome preparation C was a likely candidate to activate the contact system.

A simple method to detect clot-promoting surface properties of chemicals and particles is the comparison of WBCT in wettable (glass) and non-wettable (siliconized glass) containers. With liposomes C, WBCT was shortened, particularly when measured in siliconized containers. This indicates that the contact factor XII (Hageman factor) and possibly also factor XI (plasma thromboplastin antecedent, PTA) were activated by these particles. The clinical relevance of such an effect can be judged from studies with 1-deamino-8-D-arginine vasopressin (DDAVP). In humans, this drug caused an increase in factor XII and factor VIII procoagulant activity [6], resulting in an activation of the clotting system that proved to be therapeutically useful in hemophilia and von Willebrand's disease [7]. Marked stimulation of the contact activating system leads to disseminated intravascular coagulation, as observed with the synthetic ribonucleic acid, polyinosinic-polycytidylic acid (Poly-IC) in dogs [8] and mice (unpublished personal observation).

The electric surface charges of the liposomes are also important for the interaction with the formed elements of the blood. For example, positively charged liposomes were found to agglutinate human and sheep red blood

cells (RBC) in vitro, and this effect was reduced when the cell surface potential of the RBC was made more positive by treatment with neuraminidase [9]. Since platelets have a negative net surface charge [10], it was expected that the positively charged liposomes A might cause platelet aggregation. However, no aggregates were induced by this preparation in vitro and in the circulating blood after i.v. infusion. This observation is difficult to explain. It is possible that platelets were coated with positively charged liposomes and were kept apart by electrostatic repulsion. This effect was demonstrated with RBC which failed to agglutinate after incubation with higher concentrations of positive liposomes [9].

The negatively charged liposomes C caused platelet aggregation, both after mixing with PRP in vitro and after i.v. infusion in guinea pigs. In order to understand this phenomenon, one must remember that the surface of a platelet is covered with approximatley 20.5×10^5 negatively charged groups and approximately 2.5×10^5 positive amino groups [11,12]. Negatively charged macromolecules such as heparin may combine with the amino groups of the platelets and form loose aggregates without demonstrable impairment of platelet function, possibly acting as bridging agents [13]. The same mechanism could be postulated for the aggregating effect of liposomes C. The aggregates formed in vitro and in circulating blood were small and of the reversible type. This indicates that by acting as bridging agents the liposomes kept platelets sufficiently apart to avoid fusion and platelet destruction. It also explains why no typical platelet aggregation could be measured in the aggregometer.

Reversible platelet aggregates are usually not retained in the capillary bed and thus do not lead to disseminated, microcirculatory thrombosis, as it is seen after administration of substances causing irreversible aggregates, e.g. arachidonic and other long-chain fatty acid [14]. This is confirmed by our failure to demonstrate a drop in platelet count and platelet aggregates in the blood vessels of lungs and kidneys of guinea pigs infused with the liposomes.

In conclusion, in vitro and in vivo studies showed that only negatively charged liposomes affected the hemostatic system. Activation of contact factors, suggested by accelerated clotting in non-wettable containers, probably represents a potential thrombogenic hazard, particularly if larger volumes of liposome suspensions would be infused into patients with other risk factors such as decreased antithrombin III (AT III) concentrations or depressed fibrinolytic response. The induction of reversible platelet aggregates which is also observed with various types of drugs [1], is not considered to be of clinical relevance.

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