Biochimica et Biophysica Acta 900 (1987) 1–9
Elsevier

BBA 73569

Interactions of polymerizable phosphatidylcholine vesicles with blood components: relevance to biocompatibility

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(Received 18 November 1986)
(Revised manuscript received 9 March 1987)

Key words: Polymerizable lipid; Phospholipid bilayer membrane; Blood protein; Platelet aggregation; Fibrin coagulation cascade; Biocompatible surface

We have studied the biocompatibility properties of polymerizable phosphatidylcholine bilayer membranes, in the form of liposomes, with a view toward the eventual utilization of such polymerized lipid assemblies in drug carrier systems or as surface coatings for biomaterials. The SH-based polymerizable lipid 1,2-bis[1,2-(lipoyl)dodecanoyl]-*sn*-glycero-3-phosphocholine (dilipoyl lipid, DLL) and the methacryl-based lipid 1,2-bis[(methacryloyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (dipolymerizable lipid, DPL) were studied in comparison to 'conventional' zwitterionic or charged phospholipids. We examined binding of serum proteins to liposomes and effects of liposomes on fibrin clot formation and on platelet aggregation. All types of liposomes tested bound complex mixtures of serum proteins with IgG being the most abundant bound component. DPL vesicles and anionic vesicles bound substantially more protein than other vesicle types. Polymerized DPL vesicles uniquely bound a protein of about 53 kDa which was not bound to other types of phosphatidylcholine liposomes. Likewise polymerized DPL vesicles, but not other types of phosphatidylcholine vesicles, caused a marked alteration in coagulation as measured by activated partial thromboplastin time (APTT) and prothrombin time (PT) tests; this effect was shown to be due to binding and depletion of clothing factor V by the DPL polymerized vesicles. Polymerized DPL liposomes and DLL liposomes in polymerized or nonpolymerized form, were without substantial effect on platelet aggregation. However, DPL nonpolymerized vesicles, while not causing aggregation, did impair ADP-induced aggregation of platelets. These studies suggest that SH based polymerizable lipids of the DLL type may be very suitable for in vivo use in the contexts of drug delivery systems or biomaterials development. Methacryloyl-based lipids of the DPL type seem to display interactions with the hemostatic process which militate against their in vivo utilization.

Abbreviations: DLL, 1,2-bis[1,2-(lipoyl)dodecanoyl]-*sn*-glycero-3-phosphocholine; DPL, 1,2-bis[(methacryloyloxy)-dodecanoyl]-*sn*-glycero-3-phosphocholine; PV, polymerized vesicle; NPV, nonpolymerized vesicle; PT, prothrombin time; APTT, activated partial thromboplastin time.

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Introduction

Polymerizable lipids represent a novel approach for the development of drug carrier systems and of biocompatible surfaces [1–4]. The physical properties and characteristics of polymerizable lipids have been extensively investigated, but there are not many data on their biological behavior. Despite their similarities to natural lipids, polymerizable phospholipids have shown some unique aspects in terms of physical stability, permeability properties and interactions with cells [5,7]. Thus Juliano et al. [7] have found that photopolymerized liposomes of a phosphatidylcholine analog (DPL) are more rapidly taken up by reticuloendothelial cells (macrophages) as compared to conventional liposomes. This behavior is paralleled by a rapid clearance of the polymerized DPL vesicles from the circulation into reticuloendothelial cell rich organs such as liver and spleen [8]. Since polymerized lipids may be used to coat surfaces [3] thus altering the physical properties of the surface, they may be of substantial value in the development of novel biomaterials. In order to pursue such development, however, more information is needed on the interaction of polymerized lipid surfaces with cellular and macromolecular components of blood. As a simple initial approach to this problem, we report here on the interactions of platelets and of blood proteins with polymerized lipid surfaces in the form of liposomes.

Methods

Materials. Phosphatidylcholine derivatives 1,2-bis[(methacryloyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (designated dipolymerizable lipid or DPL) and 1,2-bis-[1,2-(lipoyl)dodecanoyl]-*sn*-glycero-3-phosphocholine (designated dilipoyl lipid or DLL) were synthesized as described previously [5,9]. Other lipids, including dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), phosphatidylserine (PS) and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Avanti Polar Lipids (Birmingham, AL). All materials were stored at $-20\,^{\circ}C$ under N_2 in the dark until needed and the purity checked by thin-layer chromatography (TLC) prior to use. Human serum albumin, γ-globulin, haptoglobin, antitrypsin and apolipoprotein A-I were purchased from Sigma Chemical Co. Human α_2-macroglobulin was purchased from Calbiochem and human transferrin from Miles Laboratories. Apolipoprotein E was provided by Dr. W. Bradley (Methodist Hospital, Houston).

Preparation of liposomes. Multilamellar liposomes (MLVs) of DPL were formed and photopolymerized for 1 h using ultraviolet light in a Rayonet photochemical reactor as described previously [6]. Multilamellar liposomes of DLL were prepared by dissolving the lipid in methylene chloride, drying the lipid extensively onto the wall of a glass tube with a nitrogen stream, adding 10 mM Tris buffer, 0.15 M NaCl (pH 8.5) and then allowing liposome formation during vortexing at room temperature. Polymerization was accomplished by adding 10 mol% of dithiothreitol, and incubation for 3 h at 65 °C followed by overnight incubation at room temperature. Polymerized multilamellar vesicles were washed with isotonic phosphate-buffered saline or with 10 mM Tris buffer, 0.15 M NaCl at pH 7.4 and sedimented at $20000 \times g$ for 20 min. DPL small unilamellar vesicles were formed by sonication at 50 °C of multilamellar vesicles until a clear suspension was obtained. The use of sonication with DLL leads to the polymerization of the lipids. Thus, for controlling DLL vesicle size we used vesicles formed by extrusion under pressure of DLL MLVs through a 0.1 μm polycarbonate filter [10]. For DPL and DLL liposomes polymerization was checked by TLC with chloroform/methanol/water (65 : 25 : 4, v/v) and development in iodine vapor. All polymerized lipids remain as a single spot at the origin of the TLC plate. MLVs from conventional lipids were prepared by vortex dispersion of a dried lipid film in buffer as largely described previously. Phospholipid content was determined using an organic phosphorus assay [11].

Protein binding to liposomes. Multilamellar liposomes (10 mg lipid) were incubated with 1 ml human pooled serum for 1 h at 37 °C. To minimize the variability of individual serum proteins, we used a single batch of pooled serum. The MLVs were then diluted in 10 ml of buffer, sedimented at $20000 \times g$ using a Beckman centrifuge, the pellet was resuspended in buffer and washed three times. The bound proteins were extracted with sodium dodecyl sulfate (SDS) 1%, lipid content was determined using the organic phosphorus assay and protein content using a B.C.A. kit (Pierce Chemical Co.). The proteins bound were analyzed by SDS-polyacrylamide gel electrophoresis (7.5 or 12% running gel) using reducing (with mercaptoethanol) or nonreducing (without mercaptoethanol) conditions [12]. The gels were stained with Coomassie brilliant blue. Molecular weight standards were myosin 200 000, phosphorylase *b* 97 000, transferrin 80 000, bovine serum albumin 68 000, ovalbumin 45 000, and trypsin inhibitor 21 000. A number of partially purified, commercially available serum proteins were used as standards for the identification of the bands on the gel. γ-Globulin amounts were estimated by scanning gel bands (under nonreducing conditions) using a scanning densitometer (L&B instrument, courtesy of P. Davies) and comparing to known amounts of γ-globulin run on the same gel.

Effects of liposomes on platelets. Effects of liposomes on platelet aggregation at 37°C were studied using a Chronlog's aggregometer of the type normally used for clinical laboratory assay of platelet function as previously described [13]. Liposomes were added to the aggregometer cuvette as small aliquots (50 to 150 μl) into 0.5 ml platelet-rich plasma. In all experiments platelet count was adjusted to 200 000/μl and ADP was used to stimulate aggregation at a final concentration of 8 μM. Phospholipid content was determined using the phosphorus assay [11]. Stimulated platelet aggregation versus dose of liposomes added was recorded. The liposomes used in these studies were sonicated or filtered vesicles: this was necessary so as to reduce the light scatter contribution of the liposomes themselves.

Effects of liposomes on the clotting cascade. Activated partial thromboplastin time (APTT) and prothrombin time (PT) assays were used to evaluate the influence of the polymerizable lipids on the intrinsic system and the extrinsic system of the clotting cascade, respectively [14,15]. Citrated plasma was collected from fresh human blood and incubated with multilamellar vesicles of DPL or DLL (either polymerized or not) at concentrations up to 20 mg of lipid/ml plasma. Liposomes were pelleted at 16 000 × *g*, and the plasma supernatant was collected carefully and measured by APTT, PT or coagulation factor assays.

For APTT and PT tests, the basic techniques are similar [16]. The plasma to be studied is added to either a thromboplastin calcium mixture (PT) or incubated with activated partial thromboplastin and then calcium (APTT) and the time which is required to form fibrin is measured using an automated instrument with photo-optical clot detection system (Coag-A-mate model X-2, Organon Technica, Morris Plains, NJ). All factor assays were based on the correction of the clotting time of factor deficient plasma when mixed with the test plasma. The factor activity was calculated by comparison of the sample clotting time with a curve prepared from dilutions of normal plasma. Thromboplastin reagent containing calcium chloride (Pacific Hemostasis, Ventura, CA) was used to initiate clotting for PT, factor V and factor VII assays. APTT reagent (General Diagnostic Organon Technica, Morris Plains, NJ) was used for APTT and factor VIII activity. Factor V deficient plasma was provided by Pacific Hemostasis, Ventura, CA and Factor VII, VIII deficient plasma by George King Biomedical, Overland Park, KA. Fibrinogen was assayed by measurement of clotting time after addition of Thrombin (General Diagnostic Organon Technica, Morris Plains, NJ) to a dilution of the test plasma.

Results

Binding of serum proteins to liposomes

We examined the binding of human serum proteins to different types of liposomes during

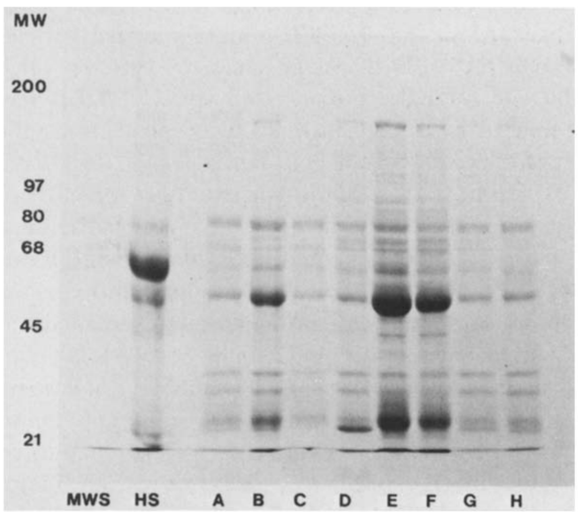

Fig. 1. Binding of human serum proteins on neutral, negatively charged and polymerizable liposomes. 7.5% SDS-polyacrylamide gel, reducing conditions. MWS, molecular weight standard; HS, human serum; A, DPL NPV; B, DPL PV; C, DPPC; D, DPPE; E, DPPG; F, PS; G, DLL NPV; H, DLL PV.

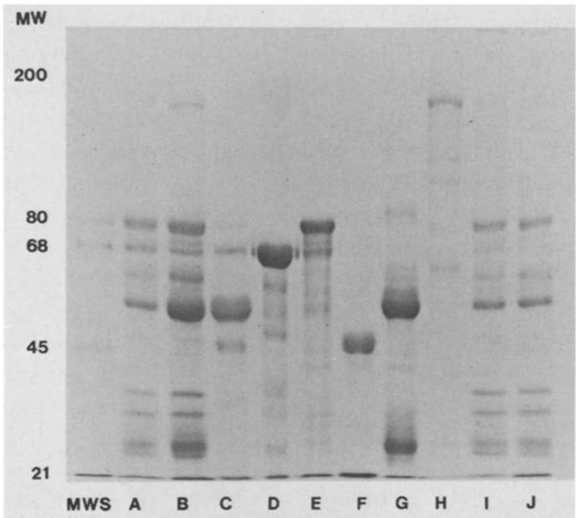

Fig. 2. Identification of proteins bound to polymerizable liposomes. 7.5% SDS-polyacrylamide gel, reducing conditions. MWS, molecular weight standard; A, DPL NPV; B, DPL PV; C, antitrypsin; D, human serum albumin; E, transferrin; F, haptoglobin; G, γ-globulin; H, macroglobulin; I, DLL NPV; J, DLL PV.

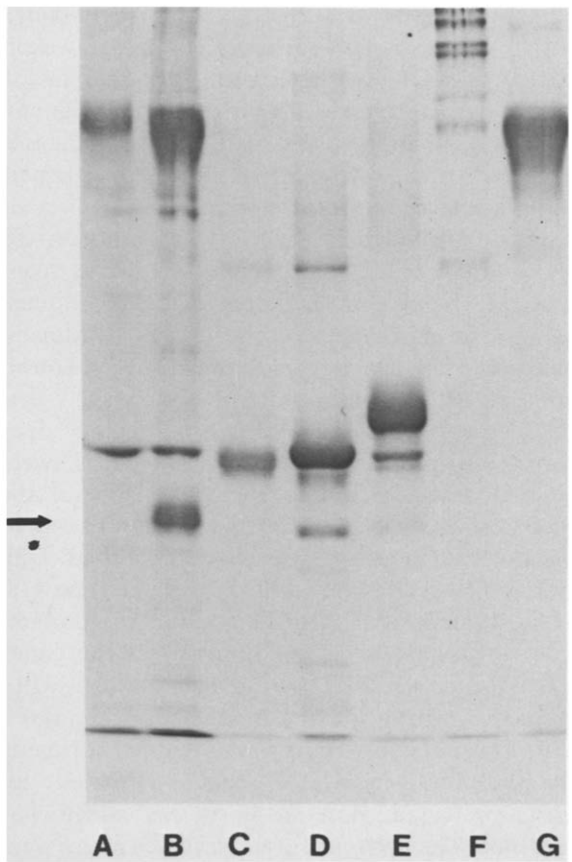

Fig. 3. Identification of γ-globulin binding onto polymerizable liposomes. 7.5% SDS-polyacrylamide gel, nonreducing conditions. A, DPL NPV; B, DPL PV (arrow indicates unknown protein); C, antitrypsin; D, human serum albumin; E, transferrin; F, haptoglobin; G, γ-globulin.

incubation for 1 h at 37 °C, since previous experience [17] indicated the protein binding was complete during this time. The binding of proteins to conventional and polymerizable liposomes is illustrated in Fig. 1. DPL nonpolymerized vesicles (DPL NPV), DLL nonpolymerized vesicles (DLL NPV) and DLL polymerized vesicles (DLL PV) seem to bind the same array of proteins as the conventional zwitterionic lipids DPPC or DPPE. By contrast, the binding patterns for DPL polymerized vesicles (DPL PV) were almost the same as for negatively charged lipids such as DPPG or PS. We tried to identify the bound proteins by comparison to co-migrating standard purified human proteins, under reducing or nonreducing conditions. As shown in Fig. 2 (reducing conditions) and Fig. 3 (nonreducing conditions) the major protein bound to all liposome types is likely to be γ-globulin (IgG), which co-migrates with the major band at 53–54 kDa (reduced) or 150 kDa (not reduced). Surprisingly, under nonreducing conditions we separated a protein (X) which on reducing gels had co-migrated with IgG. As shown in Fig. 3, this band X (nonreduced) appears principally associated with DPL polymerized vesicles. It also appeared, but at a lower level, in gels of proteins bound to charged lipids, but never with DPL NPV or DLL PV or DLL NPV. Table I summarizes the identification of the major serum proteins bound to the different kinds of polymerizable liposomes studied.

We were also interested in examining low molecular weight proteins bound to the various liposome types. As shown in Fig. 4, we demonstrated with a 12% running gel, that apolipoprotein A-I (M_r 27000), the major subunit of high density lipoprotein, is bound to all polymerizable lipids studied. We have also quantified the amount of serum proteins bound to polymerizable lipids, and more specifically the IgG bound

TABLE I

BINDING BEHAVIOR OF MAJOR HUMAN SERUM PROTEINS TO POLYMERIZABLE LIPIDS AS DETERMINED BY SDS GEL ELECTROPHORESIS

	DPL	DPL PV	DLL NPV	DLL PV
Apolipoprotein A-I	+	+	+	+
Apolipoprotein E	−	−	−	−
Antitrypsin	−	−	−	−
Serum albumin	+	+	+	+
Transferrin	−	−	−	−
Haptoglobin	−	−	−	−
γ-Globulin	+	+	+	+
Macroglobulin	+	+	+	+
Protein (X)	−	+	−	−

to each kind of polymerizable liposome (Table II). DPL vesicles whether polymerized or not, bind more protein than DLL vesicles. The amount of IgG per mg of lipid doesn't noticeably differ

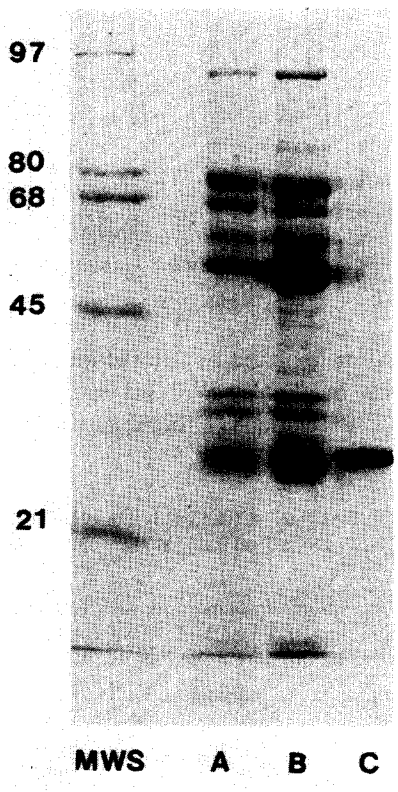

Fig. 4. Identification of apolipoprotein A-I binding onto polymerizable liposomes. 12% SDS-polyacrylamide gel, reducing conditions. MWS, molecular weight standard; A, DPL NPV; B, DPL PV; C, apolipoprotein A-I.

TABLE II

QUANTITY OF TOTAL PROTEINS AND GAMMA GLOBULIN BOUND TO POLYMERIZABLE LIPIDS

These values are mean values ± S.D. for four experiments.

Vesicles	μg protein per mg lipid	μg IgG per mg lipid
DPL NPV	237 ± 5	66 ± 6
PV	152 ± 12	48 ± 5
DLL NPV	56 ± 6	10 ± 4
PV	69 ± 11	11 ± 3

between the polymerized or non polymerized form of each lipid. Compared to its average percentage in serum (10–15% total protein), IgG represents respectively for DPL and DLL, almost 30% and 18% of the total proteins bound. However, each mg of DPL binds almost 5- or 6-times more γ-globulin than DLL, in rough proportion to the increased total protein binding to DPL.

Effects on platelet aggregation

No direct effects on platelet aggregation were observed with any of the liposome preparations studied. Nevertheless, as shown in Fig. 5A, a marked decrease of ADP-induced aggregation was observed with DPL nonpolymerized vesicles. DPL nonpolymerized vesicles at 1 mg/ml plasma markedly inhibited ADP-induced platelet aggregation. This inhibitory effect of DPL NPV is dose dependent and appears at relatively low doses (380 μg/ml plasma) as compared to DPL po-

TABLE III

EFFECT OF LIPOSOMES ON THE ACTIVATED PARTIAL THROMBOPLASTIN TIME (APTT) AND PROTHROMBIN TIME (PT)

Samples were incubated with liposomes (10 mg/ml plasma) for 1 h at 37 °C prior to testing PT or APTT. Clotting time was expressed as % of control plasma. Values are the average of triplicate determinations differing by less than 10%.

Liposomes	APPT (% of control)	PT (% of control)
DPL NPV	83	97
PV	160	143
DLL NPV	90	101
PV	93	108

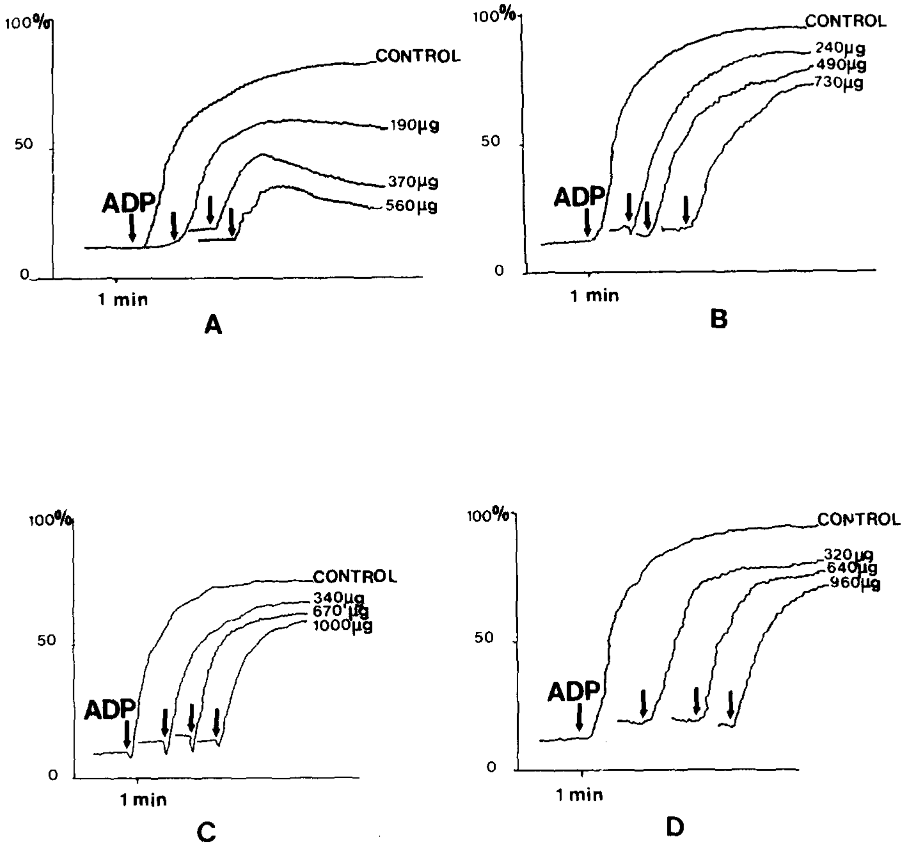

Fig. 5. Effect of polymerizable lipids in platelet aggregation. Different doses (μg of lipid) of liposomes were added to platelet-rich plasma (0.5 ml at 200000/μg) and after 2 min of incubation, aggregation was induced by addition of 8 μM of ADP. Percentage of aggregation versus time is recorded with an aggregometer. Light scattering arbitrary scale 100% indicates maximum aggregation. A, DPL NPV; B, DLL NPV; C, DPL PV; D, DLL PV.

lymerized liposomes or DLL liposomes (Figs. 5B, C, D).

Effects on the clotting cascade

The prothrombin time (PT) is a measure of the extrinsic system of coagulation while the activated partial thromboplastin time (APTT) evaluates the intrinsic system of coagulation [14,15]. After 1 h incubation of plasma with liposomes at 10 mg lipid per ml of plasma, both parameters were found to have normal values except for the case of DPL polymerized vesicles (Table III). In that case a lipid concentration dependent increase of the clotting times (both PT and APTT) was observed in the concentration range studied (0 to 20 mg lipid/ml plasma). A prolonged PT and APTT indicates a depletion of one or more of the coagulation factors. We studied factors which most likely would be involved or depleted by interaction with lipids. As shown in Table IV, the levels of fibrinogen, Factor VIII and VII found in plasma

TABLE IV

LEVEL OF COAGULATION FACTORS IN PLASMA WITH OR WITHOUT INCUBATION WITH DPL POLYMERIZED VESICLES

The data given as % refer to a comparison with untreated normal pooled plasma as an 100% standard. Average values of two or three determinations differing by less than 10%.

Factors studied	Normal	Mock incubated plasma	Plasma after incubation with liposomes
Fibrinogen	200–400 mg/dl	249 mg/dl	239 mg/dl
Factor VIII	50–150%	61%	66%
Factor VII	50–150%	142%	122%
Factor V	50–150%	66%	29%

after incubation with DPL PV were normal, but the level of factor V was reduced. Control plasmas were used to check that the values obtained were not due to the incubation temperature and time or to the dilution process.

Discussion

Polymerizable phosphatidylcholines may provide novel constituents in the development of liposomal drug carrier systems or of surface coatings for biomaterials. However, the use of polymerizable lipids in these context is predicated upon an understanding of the in vivo behavior of these lipids, particularly in terms of interactions with cellular and macromolecular elements of blood. In this report we describe the binding of blood proteins to polymerizable lipid vesicles, as well as effects of such vesicles on platelet aggregation and the fibrin coagulation cascade.

As reported previously, lipid vesicles bind a complex variety of serum protein components [17,18], but the bound components are not simply a random sample of the total serum proteins. The major protein bound to all types of vesicles tested here is IgG; this is true of polymerizable liposomes, liposomes composed of 'conventional' phosphatidylcholines as well as liposomes containing anionic phospholipids. Albumin, which is by far the most abundant serum protein, is bound to a lesser degree than IgG or another, as yet unidentified, protein running at a mol. wt. of 80 000. While the overall pattern of protein binding to the various types of lipid vesicles tested was similar, each vesicle type did display unique quantitative and qualitative aspects of protein binding. We attempted to identify some of the components in the complex mixtures of bound proteins by comparison of migration patterns to those of commercially available purified serum proteins under both reducing and nonreducing conditions. This was only partially successful, resulting in the identification of IgG, albumin, α_2-macroglobulin and apolipoprotein A-I; the remaining bound components cannot be identified with any certainty at this point. There were also marked quantitative differences in total protein or total IgG bound, with polymerized DPL vesicles binding substantially more protein than DLL vesicles or 'conventional' DPPC vesicles; previous results have shown that anionic vesicles also display relative high protein binding [17,19].

The binding of IgG and apolipoprotein A-I to vesicles may have important functional consequences. Thus both HDL and its major subunit apolipoprotein A-I, are known to destabilize liposomes [20,21]. The binding of IgG may also be important in terms of the interaction of liposomes with the phagocytic cells of the reticuloendothelial system, many of which bear receptors for the Fc domain of IgG [22,23]. DPL vesicles bind particularly large amounts of IgG, especially when they are polymerized. We have observed previously that DPL vesicles are taken up by macrophages in vitro much more rapidly than 'conventional' PC vesicles [7]. We have also observed that polymerized DPL vesicles are cleared very rapidly from the circulation [8]. At this time it is not clear that the high binding of IgG to DPL vesicles accounts for these biological behaviors, but it certainly seems a possibility.

One of our most interesting findings is the existence of a serum component (protein X) which can apparently discriminate between polymerized and nonpolymerized DPL vesicles, binding strongly to the former; this component also bind to a lesser degree to anionic PS and PG vesicles. Since polymerization of the methacryloyl groups of DPL takes place within the hydrophobic region of the bilayer, while protein binding takes place at the liposome surface, this suggests that the polymerization reaction leads to a modification of vesicle surface topology which can be recognized by certain proteins. At this point we have not yet identified protein X with any well known serum component.

In evaluating a new chemical moiety for in vivo use it is important to determine whether the moiety either provokes or inhibits hemostasis. The platelet plug and the fibrin clot are the final products of the hemostatic process. Thus we evaluated the effects of polymerizable liposomes on spontaneous or ADP induced platelet aggregation and on fibrinogenesis. None of the liposomes tested here initiated aggregation of platelets in platelet rich plasma, even when lipid doses in excess of 1 mg/ml were tested. Further most of the conventional or polymerizable liposomes tested did not significantly impede ADP-induced platelet aggregation, with the single exception of non-polymerized DPL. The basis for the marked inhibitory effect of nonpolymerized DPL vesicles on platelet aggregation is unclear at this time; one might speculate that it is due to the previously reported cellular toxicity of methacryloyl moieties [7], but this has not been definitely tested. In any case it seems that polymerized DPL or DLL vesicles are relatively innocuous in terms of thrombogenic interactions with platelets, as has been previously established for 'conventional' PC vesicles [13].

The interaction between plasma coagulation factors and lipids is known to play a key role in blood clotting particularly in accelerating some of the reaction steps leading to the clot formation [18,24]. According to Hunt [25], the majority of the in vivo studies with liposomes are carried out using lipid concentrations of 6–9 μmol/ml plasma (5–10 mg of lipid/ml plasma). Thus it was interesting to study the influence of DPL and DLL on the clotting process in similar concentration ranges. We used simple hematology tests such as PT and APTT, to have a measure of the overall coagulant activity. Among the different lipids tested, only DPL polymerized vesicles caused a marked modification of the clotting time, indicating binding and depletion of one or more essential factors by DPL polymerized vesicles. Using factor deficient plasmas we were able to investigate different coagulation factors which might be involved in this effect. On the factors tested, only factor V was found to be depleted by DPL polymerized vesicles (Table IV). Factor V is a single chain glycoprotein which participates in the activation of prothrombin to thrombin leading to the formation of fibrin; a negative lipid surface charge is supposed to be necessary to its binding to phospholipids [26,27]. Factor V appears as a single band of 330 kDa on SDS-polyacrylamide gel electrophoresis before and after reduction of disulfide bridges [28,29]. Therefore, it cannot be identical to protein X, the component which is abundantly bound to DPL polymerized vesicles which has a molecular weight of approx. 53 000 under reducing evaluations.

In this study we have examined the interactions of a photopolymerizable lipid (DPL) and a chemically polymerizable lipid (DLL), both in the form of liposomes, with soluble and cellular components of blood, with a view to assessing their suitability for in vivo use. DPL seems to present some problems in this regard since (a) in polymerized form it strongly binds a number of serum proteins including clotting factor V and thus may affect fibrin clot formation; (b) in non polymerized form it has a toxic or inhibitory effect on platelet aggregation and could potentially impair hemostasis in this manner. By contrast DLL, in either polymerized or nonpolymerized forms, seems to effect neither fibrin clot formation not platelet aggregation and thus is unlikely to perturb hemostasis. In this respect DLL vesicles seems similar to 'conventional' phosphatidylcholine vesicles, which are innocuous in terms of hemostasis, while DPL vesicles seem more like charged vesicles which can affect both soluble and cellular aspects of coagulation [24] (although there is no evidence for a surface charge on DPL vesicles [7]). This is not to say that DLL vesicles will be devoid of biological activity, since they clearly bind IgG and a number of other serum proteins which may promote a variety of interactions with cells and tissues. To a first approximation, however, the SH based polymerizable lipids of the DLL type seem a more promising approach than the methacryloyl based moieties of the DPL type for future development of biocompatible drug carrier systems and biomaterials.

Acknowledgements

We wish to thank The Institute de Recherches Internationales Servier (France) for their support of F.B.. Supported by a contract from the Office of Naval Research to R.L.J. and by NIH grant CA 42056 to S.L.R.

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