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INTERACTION OF FACTOR VIII WITH PHOSPHOLIPIDS: ROLE OF COMPOSITION AND NEGATIVE CHARGE

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ABSTRACT

Radiolabelled human anti-FVIII:C antibody was affinity-purified according to its ability to bind to factor VIII-phospholipid (FVIII-PL) complexes, yielding a fraction directed against the phospholipid binding-site (PL-site antibody). This antibody was used as a specific probe for FVIII binding to PL vesicles containing a variety of natural and synthetic PLs.
Of purified PLs tested for FVIII binding, phosphatidyl serine (PS) and phosphatidic acid (PA) were highly active, phosphatidyl inositol (PI) much less so, and both phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) inactive: the apparent dissociation constant (K_d app) for FVIII binding to PS:PC vesicles showed a strong dependence on PS content.
Free-flow electrophoresis of vesicles confirmed FVIII binding to PS:PC required both net negative charge and specific head-group: neither PS vesicles given a positive charge with stearylamine nor PC vesicles made negative with dicetyl phosphate bound FVIII.
It is concluded that the negative charge required for FVIII binding must be presented on the phospholipid surface in the correct orientation: phosphatidyl serine supplies this charge in coagulant-active PL preparations.

INTRODUCTION

The binding of FVIII* to PL surfaces has been shown to be required for expression of its cofactor activity in the proteolysis of factor X (FX) by activated factor IX (FIXa) (1, 2). In addition, numerous studies have demonstrated that a negatively-charged PL surface is necessary for FVIII binding (3-8). Such negatively charged PLs include PS and PA.

Key Words: Factor VIII, phospholipids, blood coagulation

A negative surface charge on PL vesicles is also required for the generation of 'thromboplastic activity' (essentially the ability to generate activated factor X (FXa) from FVIII, FIXa, PL and calcium ions) (9). *In vitro*, PL vesicle surfaces are understood to mimic the membranes of activated platelets *in vivo*. Following platelet activation by thrombin and collagen, the inner PS-rich leaflet of the platelet surface membrane 'flip-flops' with the outer PS-depleted leaflet to expose a negatively-charged surface for the binding and interaction of coagulation factors (10). The PS content of these membranes has been stated to be responsible for the procoagulant activity of both platelets (10) and PL extracts used in *in vitro* coagulation studies (11).

Binding of FVIII to PL surfaces or to activated platelets takes place via its 80 kDa light chain (LC) (12-14): the binding area has been localised to residues 2302-2332 in the C2 domain (15). That binding of FVIII to PL surfaces is essential to FVIII function *in vivo* has been recently underscored by studies in which homologous inhibitory anti-FVIII antibodies binding to the C2 region have been shown to prevent binding of FVIII to PS (16). Also using homologous anti-FVIII antibodies, we developed an immunoradiometric assay (IRMA) for FVIII binding to PL vesicles (17): we have now studied in more detail the charge, head-group and acyl chain saturation requirements for FVIII binding to PL vesicles, with special emphasis on phosphatidyl serine content.

MATERIALS AND METHODS

Materials

Factor VIII concentrates used for phospholipid-binding studies were intermediate purity, ampouled and freeze-dried at NIBSC. Mixed PL extracts (bovine brain) were prepared by the method of Barrowcliffe et al (18). Their PS content was approximately 25% as estimated by thin layer chromatography. Purified phospholipids: bovine spinal cord PS Grade 1 and soybean PI Grade 1 were obtained from Lipid Products, South Nutfield, Surrey, UK. Synthetic dipalmitoyl L-α-PC (product P-0763), synthetic dioleoyl L-α-PA (product P-2767) and synthetic dilauroyl L-α-PE (product P-6270) were purchased from Sigma, Poole, Dorset, UK. P-L Biochemicals (Northampton, UK) supplied hydrogenated bovine PS (PSH_2, product 6638), dicetyl phosphate (DiCP) (product 6518) and stearylamine (SA) (product 6516).

^{125}I-labelled PL-site anti-FVIII Fab' was prepared from inhibitor haemophilic plasma CC8000 as previously described (17). Briefly, Fab' fragments were prepared from inhibitor IgG and iodinated, then incubated with a mixture of FVIII/vWf complex and an excess of PS:PC (1:1) vesicles. ^{125}I-Fab' not bound to FVIII-PS:PC was separated by gel filtration and further incubated with excess FVIII/vWf alone: bound ^{125}I-Fab' was recovered from immune complexes by acid treatment and gel filtration to provide a pool of anti-FVIII Fab' able to bind the FVIII in the absence of excess PS:PC but not in its presence. Buffer used for all vesicle preparations, incubations of PL vesicles with factor VIII and IRM assay was TBS-azide (50 mM Tris, 150 mM NaCl, 3 mM NaN_3, pH 7.4).

Preparation of homogeneous PL vesicles

Small unilamellar vesicles were prepared as follows. Purified PLs in chloroform solution were mixed and dried down in a glass tube with a stream of oxygen-free nitrogen: TBS was added and the PL whirlimixed vigorously until all the PL was suspended. The liquid was transferred to a shallow glass vial and sonicated using an MSE Soniprep 150 with 3 mm probe for 5 periods of one minute each, with one minute of cooling in ice water in between. Where purified PLs with saturated acyl chains were used (eg dipalmitoyl PC), the vesicles were annealed by incubation at 45°C for 30 minutes.

Transmission electron micrography

This was performed on vesicle suspensions to confirm that small unilamellar vesicles had been successfully prepared: preparations were dried by evaporation onto carbon film, then stained with 4% (w/v) sodium silicotungstate negative stain before visualisation. The instrument used was a Philips TEM 201. In all cases vesicles with a size range of 25-75 nm were obtained.

Estimation of FVIII antigen (FVIII:Ag) binding to PL vesicles

FVIII concentrate was mixed with test preparations of PL vesicles for 20 minutes at 37°C before addition of ^{125}I-Fab' and subsequent IRMA. FVIII: Ag binding was estimated by fluid phase IRMA (7) in the presence of varying concentrations of different PL preparations (17): using the PL-site ^{125}I anti-FVIII Fab', reductions in precipitated label following incubation of PL with FVIII indicated binding of FVIII:Ag to PL, and were expressed as a percentage of FVIII:Ag measured in the absence of PL. Two replicates of each PL dilution were assayed in triplicate. Available PL concentration (in uM) of vesicles was obtained by assuming an average molecular weight of 800 for PLs, then dividing by two to account for the fact that approximately half the weight of PL in suspension constitutes the inner leaflet of the bilayer. By analogy with the binding study of Factor V (FV) to PS:PC vesicles by Pusey and Nelsestuen (19), where it was shown that most or all of the FV molecule was external to the vesicle surface, the inner PL leaflet most probably does not interact with the factor VIII protein.

Apparent dissociation constants (apparent K_d app)

In order to derive a quantitative measure of the strength of interaction between FVIII and a given PL sample, apparent dissociation constants (K_d app) were calculated: at equilibrium between free and PL-bound FVIII:Ag,

$$K_d = [PL/n]_{free} . [FVIII:Ag]_{free} / [nPL\text{-}FVIII:Ag]_{complex}$$

where n is the stoichiometry of the interaction (20).

Since PL vesicles are in large excess, at 50% bound FVIII:Ag

$$K_d = [PL/n]_{total}$$

and since values for n are not derived by these studies, we use

$$K_d \text{ app} = [PL]_{50}$$

ie the PL concentration at which 50% of FVIII:Ag is bound to PL.

Measurement of PL vesicle surface charge

The net surface charge in millivolts, or zeta potential, ζ, of PL vesicles was assessed by measurement of their electrophoretic mobility as previously described (21). A minimum of five vesicles were measured in each PL preparation.

The microelectrophoresis equipment used for vesicle surface charge measurements was built in the laboratory of A D Bangham, MD FRS, used by his kind permission and with the assistance of Nigel Miller, Department of Immunology, Institute of Animal Physiology, Babraham, Cambridge, UK.

Preparation of PL vesicles for electrophoretic mobility measurements

Since sonication would result in vesicles too small to be observed, PL dispersions were made by prolonged vortex-mixing after drying down from chloroform solution. Suspensions of 1 mg/ml PL were made, to ensure a large number of vesicles would be visible. After these measurements, however, the suspensions were sonicated for 5 x 60 seconds before testing in the IRMA for ability to bind FVIII:Ag.

RESULTS

FVIII:Ag Binding to Purified PLs Studied by PL-Site IRMA

Comparison of PS, PA, PI and PC. PS:PC, PA:PC and PI:PC vesicles (all 1:1) were prepared and incubated with FVIII (0.9 iu/ml FVIII:C) at various PL concentrations, then the mixtures were assayed for residual FVIII:Ag using the PL-site labelled antibody. The results are shown in Fig 1.

Both PS:PC and PA:PC vesicles showed strong FVIII:Ag binding, with virtually complete loss of measurable antigen above 10 μM PL. PI:PC also had an effect, but only at higher concentrations, binding to FVIII between one-tenth and one-hundredth as strongly as PS or PA. When vesicles composed of PC alone were tested, no FVIII:Ag reduction was observed.

Effect of Saturation of PL Fatty Acid Chains. FVIII was incubated with bovine spinal cord PS (largely unsaturated) or hydrogenated bovine PS (both 100% and 1:1 with PC), then assayed for residual FVIII:Ag using the PL-site antibody (Fig 2).

There was no significant difference between hydrogenated PS and the unmodified PS, whether tested alone or mixed with PC.

Also shown is that binding of FVIII was much stronger to 100% PS vesicles than to 1:1 PS:PC vesicles, regardless of extent of acyl chain saturation.

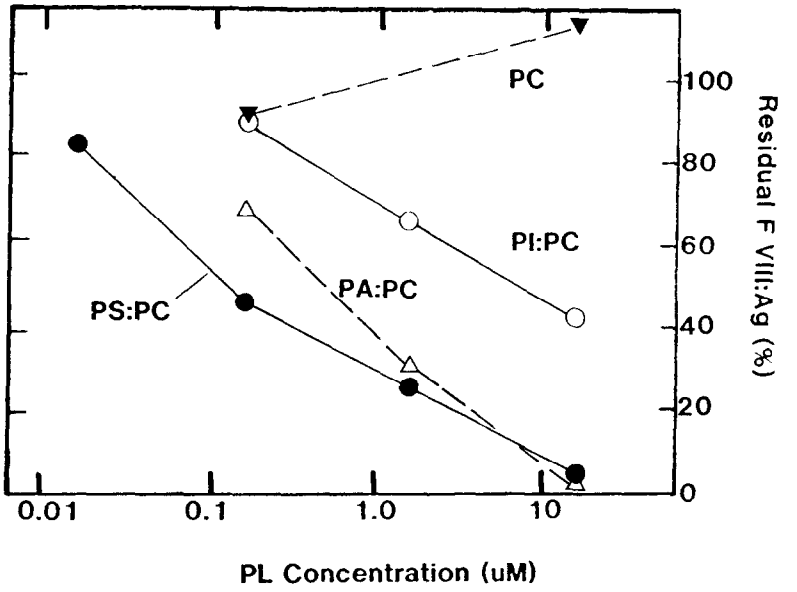

Fig 1.
Effect of different purified PL vesicles on IRMA for FVIII:Ag. Factor VIII concentrate was incubated with PS:PC (1:1), PA:PC (1:1), PI:PC (1:1) and PC alone at the concentrations indicated, and the IRMA for FVIII:Ag performed using PL-site antibody as described in Materials and Methods.

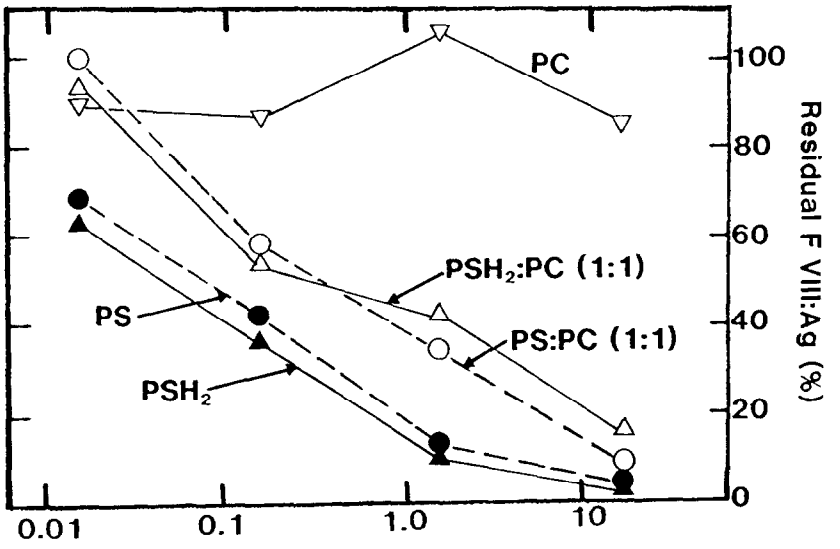

Fig 2.
Binding of FVIII:Ag to PS:PC vesicles: effect of PL fatty acid chain saturation.
FVIII concentrate was incubated with either PS, PSH_2, PS:PC (1:1), PSH_2:PC (1:1) or PC alone at the concentrations indicated, and FVIII:Ag measured using PL-site antibody. Abbreviation: PSH_2, hydrogenated bovine PS.

Dependence of FVIII:Ag Binding on Vesicle PS Content. Dependence of FVIII binding on the percentage of PS in PS:PC vesicles was studied by mixing FVIII with different vesicle compositions: Fig 3 shows the results obtained.

As the PS composition of the vesicles was increased from 0% to 50%, there was an increase in FVIII:Ag binding, as seen by the shift of binding curves towards lower PL concentrations. Apparent K_d values for FVIII binding to each PS:PC vesicle preparation are shown in Fig 4: there is a strong dependence of K_d app on the PS content of vesicles, in this case up to 50% PS. As shown in Fig 2 above, binding of FVIII is even stronger with 100% PS vesicles.

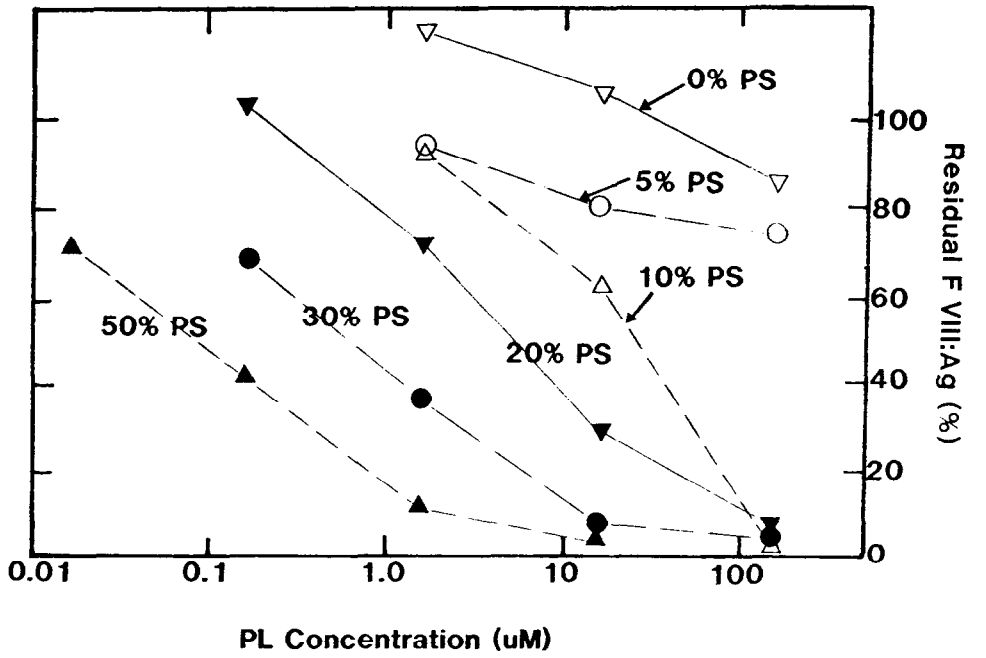

Fig 3.
Dependence of FVIII:Ag binding on vesicle PS content.
PL vesicles with a range of PS content between 0% PS and 50% PS were prepared and tested for FVIII binding as assessed by residual FVIII:Ag measured on the IRMA using PL-site antibody.

As stated earlier, to give true dissociation constants apparent K_d values must be divided by the stoichiometry of the interaction: however, the apparent values allow for a comparison of strength of binding to be made.

Correlation of FVIII:Ag Binding with PL Surface Charge

Results presented above indicate a close association between the PS content of a vesicle and its ability to bind FVIII:Ag: in addition, the fact that PA-containing vesicles were able to interact similarly with the protein suggested a correlation between binding and negatively-charged head-groups of PLs. To investigate this correlation experiments were performed using a

technique designed to measure the net surface charge (zeta potential) of PL vesicles, and then compare this with FVIII:Ag/PL binding as assessed by the IRMA using PL-site antibody.

This aim was pursued in two ways: firstly, by preparing vesicles using various negatively-charged PLs; and secondly, by introducing the charged non-PL compounds dicetyl phosphate and stearylamine (negatively and positively charged respectively) into PL membranes.

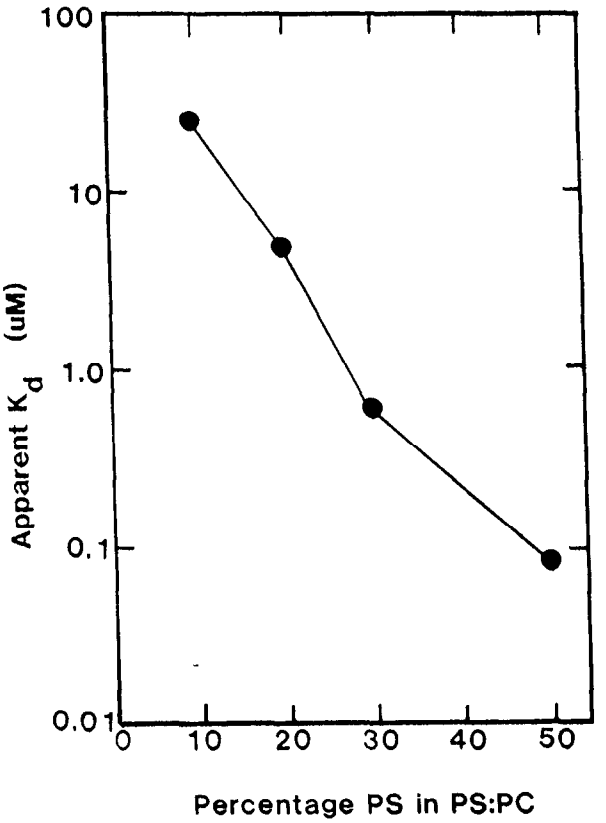

Fig 4.
Apparent dissociation constant (K_d app) for the FVIII-PL interaction: influence of PS content of vesicles.
Apparent K_d values were calculated from FVIII:Ag binding curves to vesicles of different compositions as described in Materials and Methods.

Measurement of Zeta Potentials. The following preparations were made from purified PLs: PC alone, PE:PC (50 and 100% PE), PA:PC (25, 50 and 100% PA), PSH_2:PC (10, 20, 30, 50 and 100% PSH_2) and PS:PC (5, 10, 15, 20, 30, 50 and 100% PS). In addition, negatively-charged DiCP:PC vesicles were also made by addition of different proportions (10% to 50%) or DiCP to PC before drying down and vortexing. Two bovine brain mixed-PL extracts (NIBSC 83/555 and 86/516) were also tested.

Mobilities of at least five separate vesicles from each suspension were measured, averaged and zeta potentials for each vesicle type calculated. All of the preparations tested appeared homogeneous with respect to charge on individual vesicles. Fig 5 shows the relationship of net surface charge (in mV) to the vesicle composition, expressed in the Figure as the percentage of PC by weight in the preparation.

Both PC and PE, and PC:PE (1:1) were uncharged, as previously reported (9). PS and PSH_2 vesicles showed steeply-increasing negative zeta potential as the PS content was increased to 50%; a further small increase in charge was seen when vesicles of pure PS or PSH_2 were tested. PA:PC preparations also showed a strong negative charge, increasing with PA content; however the magnitude was less than for PS vesicles of corresponding composition.

DiCP:PC vesicles showed a similar curve of surface charge to PS up to 30% DiCP by weight; thus these preparations were used to test whether the surface charge of PL vesicles is the sole requirement for FVIII:Ag binding, by comparison with PS:PC vesicles of similar charge.

Vesicles from the two bovine brain mixed-PL extracts gave zeta potentials intermediate between those of 20% PS and 30% PS vesicles.

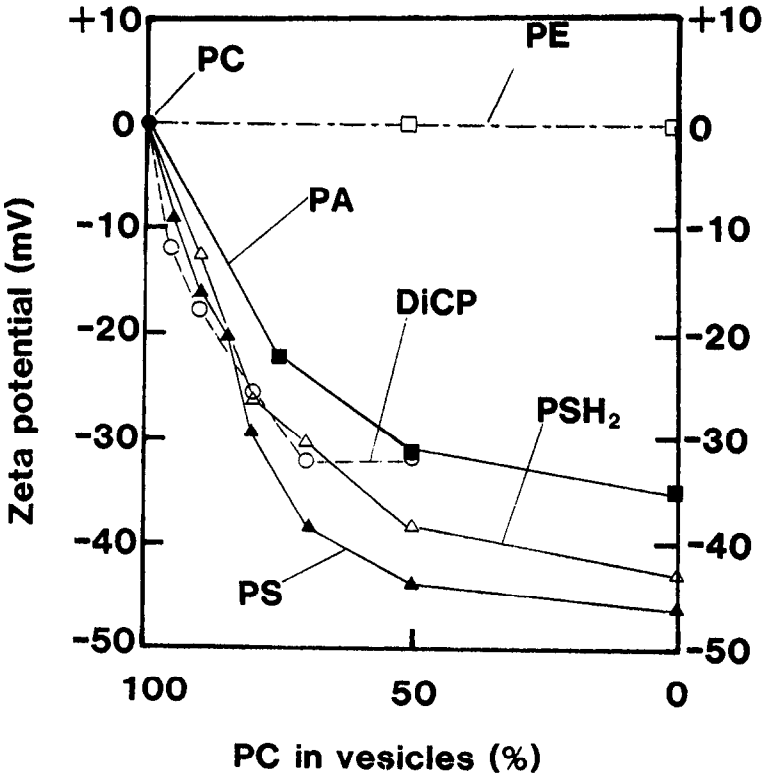

Fig 5.
Effect of PL vesicle composition on surface charge.
PL vesicles of different compositions were prepared as described in Materials and Methods, except that sonication was omitted. Mobilities of vesicles in an electric field were measured and zeta potentials for each vesicle composition derived as described in Materials and Methods.

Correlation of Vesicle Surface Charge with FVIII:Ag Binding. PL suspensions used for measurement of zeta potentials above were sonicated to yield small unilamellar vesicles, then tested for FVIII:Ag binding as above. A range of dilutions of each preparation were tested in order to estimate the concentration of each (the apparent K_d) necessary to bind 50% of the antigen (see above). In addition, the two bovine brain mixed-PL preparations (NIBSC 83/555 and 86/516) were sonicated and tested.

A comparison between zeta potentials (net surface charge) of all these PL vesicles and their apparent K_ds for binding FVIII:Ag is shown in Fig 6.

Firstly, PS- and PSH_2-containing vesicles show similar behaviour, both displaying a strong relationship between zeta potential and apparent K_d; this relation is not affected by the degree of saturation of the PL fatty acid chains. Secondly, PC and PE vesicles are uncharged, and show no significant binding of FVIII:Ag (apparent K_d >300 uM). Thirdly, PA vesicles bind FVIII:Ag increasingly as their negative charge rises, but the

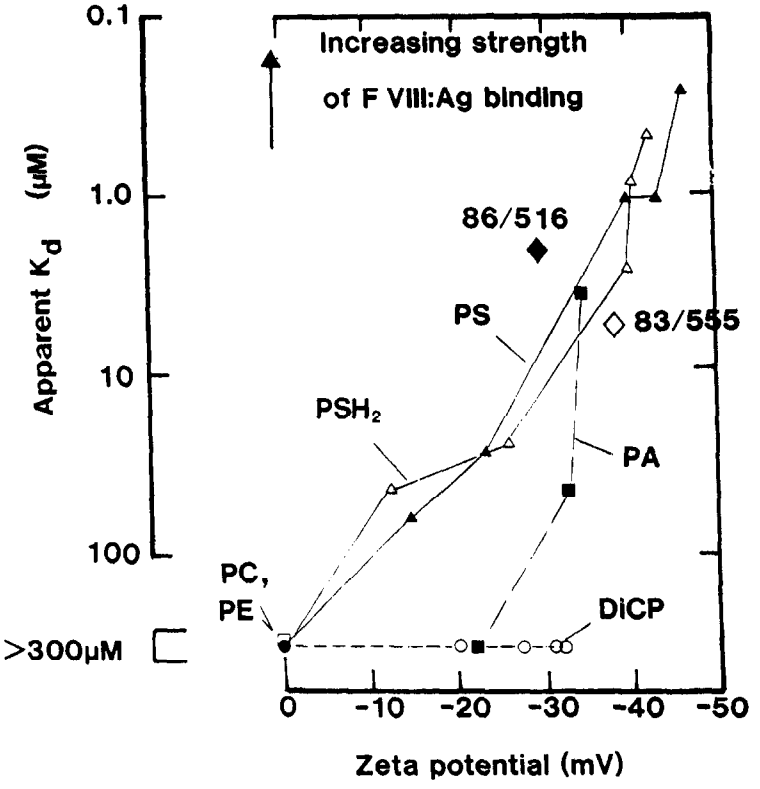

Fig 6.
Correlation of FVIII:Ag binding to PL vesicles with vesicle surface charge (zeta potential).
Zeta potentials were derived from mobility measurements of unsonicated vesicles: the vesicles were then sonicated and used for estimation of K_d app in FVIII:Ag IRMA for PL binding as described in Materials and Methods.

relationship between charge and binding differs from that of PS-containing vesicles, indicating a qualitative difference in FVIII:Ag interaction between these two PLs. Fourthly, DiCP-containing vesicles, despite carrying the same net negative charge as PS vesicles of the same percentage up to 30% DiCP (Figure 5), show no interaction with FVIII:Ag. Finally, the data obtained from the two coagulant-active mixed-PL extracts (83/555 and 86/516) shows that their FVIII:Ag binding is related to their surface charge in a similar way to that of PS-containing vesicles.

Effect of Stearylamine on PS-Containing Vesicles. Stearylamine, a positively-charged amphiphile, was introduced in various proportions (10%, 30% and 50%) into 30% PS:PC vesicles, replacing PC. These were then tested for zeta potential and FVIII:Ag binding by IRMA: the results are shown in Table I.

The inclusion of SA in increasing amounts led to abolition of the net negative charge of the vesicles, and at higher SA levels net positive charge was observed. Coincident with this charge-reversal was raising of the K_d of these vesicles above 300 uM (the practical upper limit of measurement). In the case of 10% SA/30% PS vesicles, the charge distribution among the vesicles observed was noticeably heterogeneous, so that a zeta potential could not be unambiguously assigned (this was the only preparation which showed this behaviour).

TABLE 1

Binding of FVIII:Ag to PS:PC Vesicles Containing Added Stearylamine

Positively-charged stearylamine was incorporated at the percentage shown into PS:PC vesicles by sonication as detailed in Methods: the proportion of PS was kept constant at 30% of the total, while PC was reduced. Apparent K_d and zeta potential were measured as stated in Methods.

Vesicle preparation	K_d app, uM	ζ, mV	Charge distribution
30% PS	1.1	-40	Homogeneous
30% PS, 10% SA	-	-	Heterogeneous
30% PS, 30% SA	>300	+18	Homogeneous
30% PS, 50% SA	>300	+27	Homogeneous

DISCUSSION

The fractionation of antibodies from a human anti-FVIII inhibitor plasma by their ability to bind to FVIII in the

presence or absence of coagulant-active PL vesicles produced a pool of labelled PL-site Fab' which could be used in an IRMA measuring FVIII:Ag binding to PL with sensitivity and specificity (17). Preliminary results of studies of FVIII:Ag binding to different PL classes (22) have now been extended; the influence of PL head-group, vesicle surface charge and acyl chain saturation have been studied.

Papahadjopoulos and Watkins (23) found that of the naturally-occurring phosphatides, only PS and PA showed a large net negative charge on their head-groups at neutral pH. Our studies of FVIII:Ag binding to preparations of various purified PLs confirmed the pronounced binding to PS and PA, previously noted (22, 24): of these two, only PS is present in any quantity in blood cell membranes (25). PC and PE were inactive, while PI showed a marginal effect in FVIII binding. In the case of PS, the phosphatide responsible for the 'procoagulant' qualities of activated platelets (10) or PL extracts (11), in our studies there was a strong dependence of strength of binding on the PS content of the vesicle, with the strongest binding found at 100% PS. In addition, we found that the net negative charge of PS-containing vesicles was critical for FVIII:Ag binding, since removal of the negative charge by inclusion of the positively-charged amphiphile stearylamine abolished the interaction.

However, vesicle charge is not the sole determinant of binding: PC vesicles made negatively charged by inclusion of a non-phospholipid component (dicetyl phosphate) were unable to bind FVIII:Ag. There is therefore a stereochemical requirement to the presentation of the charged group(s) on the surface of the vesicle, which PS and PA can satisfy, but DiCP-containing vesicles cannot. The difference between PA and PS when their curves of apparent K_d with surface charge are compared may reflect the different ways in which their negative charges are presented on the vesicle surface to the light chain binding area of FVIII: alternatively PS molecules may group together more easily to form the local PS-rich domains forming FVIII binding sites.

In a study of the influence of PL surface charge on FVIII cofactor activity Papahadjopoulos et al (9) demonstrated that maximum FXa generation took place when added PL had a particular negative surface charge: PS:PC vesicles of around 30% PS possessed such a charge and were maximally active, whereas vesicles given greater charge (either by increasing PS content or altering the pH of the PL buffer) had less clot-promoting activity. A similar correlation with surface charge was found for the prothrombinase system (26), although the optimal PS requirements for the two systems are not identical (27). The results presented here demonstrate that this reduction in procoagulant activity above approximately 30% PS is not due to a decrease in binding of FVIII but to some other aspect of the PL surface's involvement in generation of FXa or thrombin, for example the reduced binding of FX and possibly other prothrombin complex enzymes to vesicles of high PS content (28).

Tans et al (29), using synthetic disaturated or unsaturated PS and PC, showed that below the transition temperature of the

PLs (in the gel phase) prothrombinase activity of vesicles was much less than above it (in the liquid crystal state), presumably due to the great difference in membrane fluidity between the two states affecting lateral diffusion of bound clotting factors. Sterzing and Barton (30), using natural PS and PC, also found a decrease in procoagulant activity on hydrogenation (saturation of double bonds) of PLs. These reports indicate that the extent of fatty acid saturation affects the overall clot-promoting character of PS:PC vesicles. However, studies reported here demonstrate that when hydrogenated (saturated) PS was compared with unmodified (mainly unsaturated) PS the degree of saturation of PS fatty acid chains was not found to affect FVIII:Ag binding: we conclude that binding of FVIII:Ag is a surface process essentially independent of membrane fluidity, although expression of its cofactor activity is not.

Bevers et al (10) reported that, following activation of platelets by thrombin and collagen, the PS-rich (approximately 25% PS) inner leaflet of the platelet membrane bilayer 'flip-flops' with the outer PS-depleted leaflet (around 0% PS), thus abruptly presenting a PS-enriched PL surface for the binding of plasma coagulation factors: this is probably a transient event, although platelet microparticles are shed which show sustained recombinant FVIII binding (31). Other studies by Nesheim et al (13) demonstrated that recombinant FVIII showed enhanced binding to thrombin-activated over resting platelets, with a calculated K_d of 2.9 nM: since bound FVIII was not displaced by excess FV they suggested that a specific receptor, possibly a membrane-bound protein, was responsible for the binding of FVIII. However Gilbert et al (20), in fluorescence studies of FVIII binding to purified PS:PC vesicles, also found a K_d of about 2-4 nM for the interaction over a range of PS mole fractions between 2.5% and 30%: using the stoichiometry data derived from those studies the apparent K_ds derived in our studies may be converted to true K_ds: thus at 30% PS we obtain a value of 6-7 nM (stoichiometry n=89). Further, Gilbert et al (31) obtained a figure of 5 nM for binding of recombinant FVIII to platelet microparticles, in addition to demonstrating competition between FVIII and activated FV to such particles at high FVa levels. The similarity of these four estimates for the dissociation constant may therefore indicate that ultimately the interaction of FVIII with activated platelets is indeed directly with PS in the membrane bilayer: this would not however exclude other more subtle interactions between FVIII or FV and the other components of coagulation enzyme complexes.

From a clinical perspective, management of haemophilic patients with inhibitors remains one of the most serious concerns. We have previously demonstrated that the inhibition of FVIII by certain haemophilic anti-FVIII antibodies can be prevented by binding of FVIII to PS-containing vesicles (7). The PL binding-site on the FVIII molecule has been shown to be on the light chain polypeptide in studies using both purified phospholipids (12, 14) and thrombin-activated platelets (13), and a probable major site has been further localised to residues 2303-2332 in the C2 domain (15). Additional evidence that some inhibitors' interference in PL binding via the C2 region is responsible for their effect *in vivo* has been obtained by studies in which purified C2-binding antibodies from haemophiliacs with

anti-FVIII inhibitors have been shown to prevent the binding of FVIII to PS (16). It is possible that such patients could be treated successfully with a preformed complex of FVIII with appropriately-formulated PL vesicles (22, 32): the studies presented here confirm that PS would be the essential active constituent of such vesicles.

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