Journal of Clinical Immunology, Vol. 12, No. 1, 1992

Phospholipid Binding of Antiphospholipid Antibodies and Placental Anticoagulant Protein

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Accepted: September 6, 1991

We evaluated the interaction of antiphospholipid antibodies (aPL) with placental anticoagulant protein I (PAP I), a calcium-dependent phospholipid binding protein which may act as a natural anticoagulant. Clotting assays showed additive prolongation of clotting times with aPL and PAP I. ELISA and vesicle phospholipid binding studies showed PAP I inhibition of aPL binding to phospholipid but no inhibition of PAP I–phospholipid binding by aPL. aPL and PAP I interact additively in anticoagulant activity in *in vitro* clotting systems and compete for phospholipid in ELISA system. These data support the hypotheses that aPL and PAP I may recognize similar phospholipid epitopes and that *in vivo* interaction may occur.

KEY WORDS: Antiphospholipid antibody; lupus anticoagulant; placental anticoagulant protein; lipocortin; phospholipid binding.

INTRODUCTION

Antiphospholipid antibodies (aPL) are strongly associated with arterial and venous thrombosis (1–5) and with fetal loss thought to be due to vascular occlusion in the placenta (6–9). The mechanism of pathogenicity is uncertain, but a procoagulant activity is likely. No definitive explanation of aPL mechanism yet exists, although many possible mechanisms have been proposed, including cross-reactivity with antiendothelial cell antibodies (10, 11), inhibition of arachidonic acid release (12, 13), and interference with thrombomodiulin (14, 15), tissue plasminogen activator activity (16), protein S (17), or antithrombin III (18). Evidence supporting each of these proposals is not definitive.

Four calcium-dependent phospholipid binding proteins [lipocortins (19)], termed placental anticoagulant proteins (PAPs), have been isolated from placenta and other endothelial tissues and have been extensively characterized (PAP I through IV) (20–23). While cDNA and protein sequences for the placental anticoagulant proteins have been determined (22, 52, 53), and PAP I has been cloned (22, 53), the precise function(s) of these proteins *in vivo* remains unclear. The four PAPs differ in isoelectric point, molecular weight (32,000 to 36,000), and relative phospholipid affinity. While all four inhibit blood clotting and phospholipase A_2 activities, potency is a reflection of their measured affinities for anionic phospholipid vesicles. PAP I has the greatest affinity; PAP II, PAP III, and PAP IV bind phospholipid vesicles 2-fold, 3-fold, and 160-fold more weakly than PAP I, respectively (20, 21).

PAPs may regulate inflammatory reactions through PLA_2 inhibition (51) and may function as vascular anticoagulants (21–23). PAP I, a 36-kd single-chain protein, has been demonstrated in erythrocytes, leukocytes, platelets, and extracellular fluids including plasma, amniotic fluid, and culture medium of endothelial cells (24, 25). PAP I is present in placenta in greatest quantity (20). By binding to anionic phospholipid, PAP I prevents binding of activated factor X and prothrombin, thus decreasing the rate of thrombin formation *in vitro* (20–23, 25, 26). PAP I also inhibits the phospholipid-dependent factor VII-tissue factor activation of factors IX and X *in vitro* (27).

The placental anticoagulant proteins are of interest because aPL also bind to anionic phospholipids.

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0271-9142/92/0100-0027$06.50/0

The lupus anticoagulant (LAC), which by definition prolongs coagulation assays when mixed with normal plasma, is generally viewed as one part of the spectrum of antiphospholipid antibodies (28–31). While some controversy exists as to whether LAC and anticardiolipin antibodies are identical or different antibody populations, and as to which is a more sensitive or specific predictor for clinical complications, both are clearly associated with thrombosis and fetal loss (32–35). LAC prolongs phospholipid-dependent coagulation assays by binding to phospholipid and reducing the amount of phospholipid available to clotting factors. This results in an inhibition of the prothrombinase complex activation of prothrombin which is reversible with addition of excess phospholipid (36–38). Because of the similarity in *in vitro* anticoagulant mechanisms, and because a correction of the PAP I-induced aPTT prolongation by addition of aPL has been suggested (39), we investigated the interaction of aPL with PAP I. We address the question of whether aPL and PAP I recognize the same phospholipid epitope and whether phospholipid binding with possible restructuring by aPL or PAP I removes available phospholipid from the system.

MATERIALS AND METHODS

Sera

IgG fractions from sera of 12 patients with high-titer IgG aPL (>35 GPL U/ml) and 8 normal controls were evaluated. IgG was isolated using a protein G–Sepharose 4B column (Zymen, San Francisco, CA). Antiphospholipid activity was measured by ELISA.

Coagulation Assays

Kaolin Clotting Time (KCT). KCT was performed using pooled platelet-free plasma (PFP) as described by Exner (40). PFP was prepared by cellulose acetate filtering (Nalgene, Rochester, NY) of platelet poor plasma. KCT was performed by incubation of PFP (100 μl) with 50 μl acid-washed kaolin [20 mg/ml (Fisher Scientific, Fair Lawn, NJ)] for 3 min at 37°C, followed by the addition of 100 μl 0.025 *M* $CaCl_2$, with the time until clot formation measured. All KCTs were done in duplicate. Anticoagulant activity was defined as KCT greater than the mean normals plus 3 standard deviations.

To test anticoagulant activity of column fractions, 50 μl of Tris buffer A (50 m*M* Tris-HCl, pH 7.9, with 50 m*M* NaCl) or 50 μl of column fraction was added to the PFP before testing. To test anticoagulant activity of IgG, 100 μl of either normal or aPL-positive IgG (5–12 mg/ml) or 100 μl of Tris buffer A (as a control for dilutional effect) was added to the PFP. To test for the combined effect of IgG and PAP I, 240 ng of PAP I (final concentration, 600 ng/ml) or Tris buffer A was added before the addition of kaolin.

Dilute Activated Partial Thromboplastin Time (aPTT). aPTT was performed using Actin FSL reagent (American Dade, Aguada, Puerto Rico) at a 1:4 dilution. Twenty-five microliters of Actin FSL reagent (incubated at 37°C for 1 min) was added to 100 μl of PFP and incubated for 3 min at 37°C. One hundred microliters of 0.025 *M* $CaCl_2$ was added and the time until clot formation measured. To test anticoagulant activity of IgG with and without PAP I, 100 μl of normal or aPL-positive IgG and 50 μl of Tris buffer A or PAP I (final concentration, 5 μg/ml) were added to PFP before incubation. To demonstrate reversibility of anticoagulant effect with phospholipid excess, aPL IgG, PAP I, and both in combination were also tested with addition of cardiolipin (CL) vesicles. CL vesicles were prepared by evaporation of CL in ethanol under nitrogen, addition of Tris buffer A, and sonication. Sixty-five microliters of a 1 mg/ml solution of CL vesicles, or 65 μl of Tris buffer A as control, was added.

Placental Anticoagulant Protein I: Isolation and Characterization

Human PAP I was isolated and purified using the method of Funakoshi *et al.* (23) with some modification. One hundred twenty-five grams of fresh human placenta was chopped, washed in Tris buffer A with EDTA and benzamidine, and homogenized. After filtering, ammonium sulfate was added to the filtrates to 40% saturation, followed by a second precipitation at 80% saturation. The precipitate was dissolved in Tris buffer A, dialyzed, added to DEAE-Sepharose CL-6B (Pharmacia LKB Biotechnology, Piscataway, NJ), and eluted with a linear gradient formed by Tris buffer A and Tris buffer A plus 0.45 *M* NaCl. Fractions were assayed for protein concentration (absorbance at 280 nm with spectrophotometer) and anticoagulant activity (KCT). Active fractions were pooled and precipitated with 80% saturated ammonium sulfate, dis-

solved in Tris buffer B (50 m*M* Tris, pH 7.9, with 0.2 *M* NaCl), and applied to a Sephadex G-75 column (Pharmacia LKB Biotechnology, Piscataway, NJ). Trace albumin was removed with an albumin affinity filter (Affinity Technology Inc., Leonia, NJ). The concentration of purified PAP I was determined by the Bradford method (41). PAP I was characterized on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions (42) and by western blot technique (43).

Anti-PAP I Antibody

Anti-PAP I antibody was raised in NIH/Swiss mice by immunization with 100 μg of purified human PAP I in Freund's adjuvant at 2-week intervals for a total of three injections. To ensure specificity, antisera were immunoabsorbed against cross-linked human plasma proteins (44). Specificity was confirmed by immunoblotting technique and ELISA (including inhibition studies with preincubation with PAP I which showed complete inhibition of anti-PAP I activity).

Antiphospholipid ELISA

APL ELISA was performed using the method of Gharavi *et al.* (45). IgGs were tested at 50 μg/ml in 10% adult bovine serum (ABS) in phosphate-buffered saline, pH 7.4 (PBS). When PAP I effect on aPL activity was evaluated, the assay was performed in the presence of 1.0, 2.5, and 7.0 m*M* $CaCl_2$.

PAP I ELISA

Phospholipid binding of PAP I to cardiolipin (CL) in solid phase was assayed by ELISA. Microtiter plates coated with cardiolipin in ethanol (50 μg/ml) were blocked with 2% bovine serum albumin (BSA) in Tris buffer C (50 m*M* Tris-HCl, pH 7.9, 50 m*M* NaCl, and 10 m*M* $CaCl_2$). All washes and dilutions were with Tris buffer C unless otherwise noted. Serial dilutions of PAP I in 10% ABS–Tris buffer C (15 to 250 μg/ml) were incubated for 2.5 hr (final calcium concentration, 2.5 m*M*). Mouse anti-human PAP I antibody in 2% human serum albumin (HSA)–Tris buffer C (1/100) was applied, followed by goat anti-mouse IgG alkaline-phosphatase conjugated antibody (Sigma Chemical Company, St. Louis, MO) in 10% ABS–Tris buffer C (1/1000). Color development with *p*-nitrophenyl phosphatase, 1 mg/ml, in diethanolamine buffer was read at 405 nm.

The effect of normal and aPL IgG on PAP I binding to CL on ELISA plate was evaluated using 350 and 575 μg/ml IgG with a constant concentration of PAP I (100 μg/ml) in 10% ABS–Tris buffer C (final $CaCl_2$, 2.5 m*M*) in the ELISA system described above. Eleven aPL and eight normal IgGs were tested at each concentration.

Absorption and Inhibition Studies

PAP I binding to phospholipid (PL) vesicles was tested by absorption studies. PL vesicles of phosphatidylserine (PS) and phosphatidylcholine (PC), 10 mg/ml, were prepared in the presence of either $CaCl_2$ or EDTA at PS/PC molar ratios of 0/100 and 20/80. [PAP I does not bind to pure PC, but does bind to vesicles at a 20/80 PC/PS ratio (23, 25, 26). Pure PS vesicles could not be generated in the presence of calcium: PC of at least 20% is needed to stabilize a lamellar configuration (46)].

PL in chloroform was evaporated under nitrogen, buffer added, and the sample sonicated. Vesicles (50 to 350 μl) were incubated with PAP I (40 μg/ml) and centrifuged at 14,000 rpm, and the resulting aqueous layer was separated and applied to a microtiter plate. After overnight incubation at 4°C, the plate was blocked with 2% BSA–Tris buffer C, followed by application of anti-human PAP I antibody in 2% HSA–Tris buffer C (1/100) and anti-mouse IgG alkaline phosphatase-conjugated antibody in 10% ABS–Tris buffer C (1/1000). Color development was read as above. Percentage of PAP I absorbed to PL vesicles was calculated using ELISA optical density (OD):

$$\% \text{ binding} = \frac{\text{OD PAP I control} - \text{OD PAP I with PL}}{\text{OD PAP I control}} \times 100$$

PAP I bound to PS/PC vesicles (20/80 molar ratio) in the presence of calcium, but not EDTA. No significant binding (1%) was seen with 0/100 PS/PC vesicles.

Inhibition studies with normal and aPL IgG were performed as detailed above, with addition of 200 μg/ml of IgG (eight aPL and five normal). PAP I and PS/PC concentrations were chosen from the linear part of the PAP I binding curve to give binding of approximately 50%.

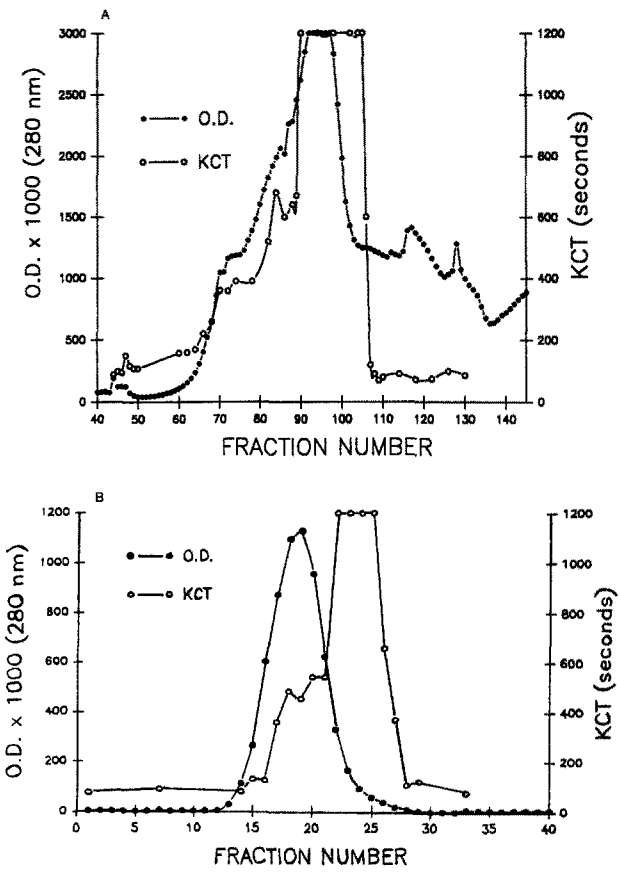

Fig. 1. Isolation of PAP I from human placenta. (A) Purification of PAP I on DEAE-Sepharose CL-6B. (B) Final purification of PAP I on Sephadex G75. OD, optical density at 280 nm. KCT, kaolin clotting time in seconds.

Statistical Analysis

Clotting times and aPL and normal IgG effect on PAP I–phospholipid binding were evaluated using Student's *t* test for unpaired sample means. The PAP I effect on aPL–phospholipid binding was analyzed using Student's *t* test for paired samples.

RESULTS

PAP I Isolation and Characterization

In both column separations, anticoagulant activity followed the major protein peak (Fig. 1) as previously described (23). SDS-PAGE (10%) revealed two bands at 35 and 65 kd under nonreducing conditions (Fig. 2A), representing monomeric protein and dimer (31). Coomassie blue staining determined purity of the final PAP I to be greater than 95%, and immunoblot using anti-PAP I antibody confirmed the protein identity (Fig. 2B).

Fig. 2. Characterization of PAP I. (A) SDS–polyacrylamide gel electrophoresis (10%) showing PAP I as monomer and dimer with approximate MW 35 and 65 kd, respectively. Standards: 70, 50, 36, 25, and 18 kd. (B) Immunoblot using anti-PAP I antibody showing specificity of isolated protein.

Normal and aPL IgG

The mean optical density (OD) at 405 nm for normal IgGs was 0.138 ± 0.021 (range, 0.133–0.172) and that for aPL IgGs was 1.259 ± 0.682 (range, 0.413–2.242). Normal and aPL IgG means differed significantly ($P < 0.001$). Binding of a panel of five aPL IgGs and one normal IgG did not differ significantly when phosphatidylserine (PS), 50 μg/ml, was used as antigen in aPL ELISA instead of cardiolipin (CL).

Coagulation Assays

All 12 aPL IgGs demonstrated anticoagulant activity. The KCT means of the aPL and normal IgGs differed significantly. PAP I markedly prolonged the clotting time of PFP, and addition of aPL IgG but not normal IgG significantly enhanced prolongation of the clotting time (Table I). When increasing quantities of aPL IgG (0 to 200 μg/ml) were added to PFP with a constant amount of PAP I (240 ng), aPL IgG alone showed a plateau in KCT at approximately 200 sec at concentrations ≥100 μg/ml, and addition of PAP I at each concentration of aPL IgG caused further prolongation of KCT, up to 420 sec.

Table I. Effects of Normal and aPL IgG on KCT in the Presence and Absence of PAP I

PFP[a]	KCT mean (sec above control)	KCT range (sec above control)
+ PAP I	368 ± 3	n/a[b]
+ Nl IgG (n = 8)	7 ± 7	−10–12
+ aPL IgG (n = 12)	126 ± 48*	55–191
+ Nl IgG + PAP I (n = 8)	286 ± 56	299–376
+ aPL IgG + PAP I (n = 12)	593 ± 166**	290–850

[a]Control, PFP alone: 110 ± 6 sec.
[b]Not applicable.
*aPL IgG significantly prolongs the KCT compared with normal IgG, $P < 0.001$.
**aPL IgG plus PAP I significantly prolongs the KCT compared with normal IgG plus PAP I, $P < 0.001$.

The dilute aPTT assay confirmed the KCT results. APL IgGs again produced additive prolongation of the aPTT when incubated with PAP I (Table II). Addition of excess CL (65 μg CL vesicles) partially reversed the PAP I-induced aPTT prolongation (161 sec with PAP I plus PL, 195 sec with PAP I alone), as well as the PAP I- and aPL IgG-induced aPTT prolongation (180 sec with PAP I and aPL IgG plus CL, 250 sec with PAP I and aPL IgG alone). Addition of aPL IgG to PFP in the presence of CL did not prolong the aPTT.

Addition of normal IgG with PAP I decreased the PAP I prolongation in both the KCT and, especially, the aPTT assays. This effect was also noted with the addition of a nonimmunoglobulin protein, bovine serum albumin (10 mg/ml), and likely reflects a nonspecific steric hindrance.

PAP I/aPL Interaction

PAP I bound to CL-coated ELISA plates in the presence of 2.5 m*M* calcium in a dose-dependent fashion, with binding demonstrated from 15 to 250 μg/ml.

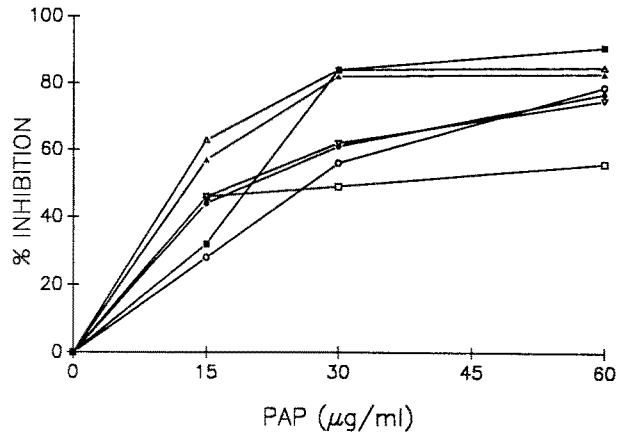

Fig. 3. Inhibition of aPL IgG binding by PAP I. PAP I-inhibited binding of seven aPL IgGs (50 μg/ml) to CL in ELISA at a calcium concentration of 2.5 m*M*. PAP I significantly inhibited aPL binding at 15, 30, and 60 μg/ml: $P < 0.02$, $P = 0.01$, and $P < 0.01$, respectively. (Symbols represent seven individual aPL IgGs.)

When incubated with aPL IgGs, PAP I inhibited aPL binding to CL in the presence of calcium in a dose-dependent manner in all seven aPL IgGs tested (Fig. 3). Inhibition by PAP I was calculated using calcium-containing controls since calcium affects aPL binding to CL (47): decreased aPL IgG binding occurred in the presence of 1, 2.5, and 7 m*M* $CaCl_2$ in the absence of PAP I. PAP I at a low concentration (10 μg/ml) inhibited aPL binding in the presence of calcium in a dose-related manner, whereas at 80 μg/ml PAP I inhibition was equal for each calcium concentration (Table III). In contrast, 11 aPL IgGs did not inhibit the binding of 100 μg/ml PAP I to CL-coated ELISA plates, even at a high IgG concentration.

Table II. Effects of Normal and aPL IgG on Dilute aPTT in the Presence and Absence of PAP I

PFP[a]	aPTT mean (sec above control)	aPTT range (sec above control)
+ PAP I	203 ± 36	n/a[b]
+ Nl IgG (n = 4)	−7 ± 14	−27–5
+ aPL IgG (n = 4)	48 ± 35*	0–84
+ Nl IgG + PAP I (n = 4)	154 ± 17	134–165
+ aPL IgG + PAP I (n = 4)	226 ± 37**	188–277

[a]Control, PFP alone: 75 ± 7 sec.
[b]Not applicable.
*aPL IgG significantly prolongs the aPTT of PFP compared with normal IgG, $P < 0.05$.
**aPL IgG plus PAP I significantly prolongs the aPTT of PFP compared with normal IgG plus PAP I, $P = 0.01$.

Table III. PAP I Inhibition of aPL IgG Binding Varies with $CaCl_2$ Concentration

aPL IgG	Percentage decrease in OD in aPL ELISA[a]		
	1 m*M* $CaCl_2$	2.5 m*M* $CaCl_2$	7.5 m*M* $CaCl_2$
Alone[b]	13	22	38
+ PAP I 10, μg/ml[c]	34	49	58
+ PAP I 80, μg/ml[c]	91	93	91

[a]OD, optical density at 405 nm. aPL IgG concentration: 50 μg/ml.
[b]Percentage decrease in OD compared to calcium-free control: calcium alone inhibits aPL binding in a dose-dependent manner.
[c]Percentage decrease in OD in presence of PAP I compared to aPL IgG binding at the same calcium concentration.

aPL IgG did not significantly modify the binding of PAP I to PS/PC vesicles at concentrations tested. PAP I binding to PS/PC vesicles in the absence of IgG was 50.5%. PAP I binding in the presence of aPL and normal IgG was 50.2 ± 4.4 and 55.3 ± 7.5%, respectively (the difference was not statistically significant).

DISCUSSION

Six distinct lipocortins have now been identified (20), with characteristics including reversible calcium-dependent binding to anionic phospholipids (19, 48) and phospholipase A_2 (PLA_2) inhibition (49, 50). Although PAP I, like all lipocortins, is predominantly intracellular, it has been demonstrated in extracellular fluids at low levels (24) and shown to bind membranes of platelets and activated macrophages (54). PAP I may be released as a local anticoagulant, preventing intravascular coagulation and possibly representing an important factor in aPL associated thrombosis.

PAP I and aPL both bind anionic phospholipid necessary for activation of coagulation factors (36, 37). Like PAP I, lupus anticoagulants (LAC) *in vitro* cause prolongation of coagulation which is enhanced in tests utilizing a minimum of phospholipid and may be abolished with the addition of excess phospholipid (38, 54, 55).

Because clinical studies suggest that aPL functions as a procoagulant *in vivo*, we hypothesized that it might interfere with the anticoagulant action of PAP I and might, thus, act as an antianticoagulant. Realizing that the paradoxical *in vitro* LAC effect might preclude demonstration of *in vivo* interaction in coagulation assays, we also evaluated more specific solid-phase phospholipid binding interactions. There are reports that lupus anticoagulant and anticardiolipin activities of antiphospholipid antibodies can be separated (56, 57), although not all authors agree (30, 58). We did not address this issue, having chosen to use aPL-positive IgG fractions rather than aPL affinity purified on phospholipid vesicles to avoid the possibility of phospholipid contamination. We do not assume that our results reflect purified antiphospholipid antibody populations and, so, cannot ascribe results to one or the other antibody population. All IgG preparations we evaluated demonstrated both LAC and anticardiolipin activities after purification.

Although both aPL and PAP I are known to bind phospholipid, it is not clear that they would necessarily interact, since they may each recognize very different epitopes, or phases, of anionic phospholipids. If binding is to the same phospholipid epitope, aPL and PAP I could either effectively deplete the system of phospholipid or, alternatively, restructure the phospholipid and enhance recognition by the other. Postulating such an interaction is not intuitive, since precise aspects of phospholipid binding for both proteins are incompletely defined. APL binding may be phase dependent (59) and will decrease with increasing calcium molarity (47), while PAP I binding to phospholipid is calcium dependent (53).

Our data support an aPL–PAP I interaction in which competition for phospholipid does occur, since aPL and PAP I compete for phospholipid in both clotting assays and ELISA system. It is likely that phase preferences do not differ, if one assumes a uniform lipid phase distribution. In the coagulation assays we found a prolongation of clotting induced by all aPL IgGs, marked prolongation by PAP I, and further prolongation when aPL and PAP I were used together. Normal IgG and bovine albumin decreased the PAP I prolongation somewhat, probably due to a nonspecific steric hindrance effect. Our results contrast with a report suggesting that affinity-purified aPL partially corrected a PAP I-induced aPTT prolongation (39). Since aPL affinity purified with cardiolipin vesicles may contain contaminating phospholipid, we studied the effect of adding a small amount of cardiolipin vesicles to concentrated aPL IgG and demonstrated correction of aPL prolongation of the aPTT. The discrepancy between these data likely reflects addition of contaminating cardiolipin with affinity-purified aPL IgG.

The meaning of the further prolongation in KCT with PAP I when LAC effect of aPL IgG has plateaued is not self-evident but would be consistent with either differing avidities (with PAP I avidity greater), differences in the phospholipid or phospholipid complex recognized, or a combination of both. A difference in avidity is supported by the solid-phase phospholipid binding data. We found that aPL does not inhibit PAP I binding to phospholipid in vesicle form or on ELISA plates. This is consistent with several calculated avidities of PAP I for phospholipid (25, 53). K_d estimates of <0.1 nM have been made, and PAP I is estimated to have a 1000-fold greater avidity for phospholipid than do coagulation factors. Pengo *et al.* (37) found that >100 nM LAC IgG was required for prolongation of

the dilute Russell viper venom time; in contrast, Tait *et al.* (25) and others (54) found prolongation of the less sensitive aPTT with PAP I in the nanomolar range, and we demonstrate here prolongation of the more sensitive KCT with picomolar amounts of PAP I.

The precise nature of aPL IgG and PAP I phospholipid binding and their competition may be even more complex. A serum cofactor which enhances IgG aPL binding has been identified as $beta_2$-glycoprotein I (60–62), a constituent of normal serum. This cofactor may restructure phospholipid to make it more attractive to aPL. By experimental design, cofactor was present in our clotting assays and ELISA experiments. PAP I clearly does not function as a cofactor for aPL binding and may act by inhibiting $beta_2$-glycoprotein I binding to phospholipid and reducing aPL binding through interference with the PL–cofactor complex rather than through direct competition with the antibody. Unlike PAP I, binding of $beta_2$-glycoprotein I to anionic phospholipid is not calcium dependent. PAP I itself does not seem to require a cofactor for binding (23, 25, 53).

It is not clear that PAP I inhibits binding of aPL to phospholipid *in vivo*. Physiologic interactions may be difficult to predict, given the possible effects of phospholipid phase, calcium molarity, aPL cofactor, and shift in local concentrations of PAP I and aPL. We do demonstrate an *in vitro* interaction in both coagulation assays and ELISA. The apparent competition for phospholipid suggests recognition of similar phospholipid epitopes: that PAP I inhibits aPL binding to phospholipid but aPL does not block PAP I binding more likely reflects differing avidities than different binding sites. Further work will better define precise mechanisms of phospholipid binding underlying the aPL–PAP I interaction described here and may yield insight into the basis of aPL pathogenicity.

ACKNOWLEDGMENTS

This work was supported by NIH Grant AR-32929. Dr. Sammaritano was supported in part by a grant from the New York Chapter of the Arthritis Foundation. Dr. Levy was supported by the Charles and Irene B. Jacobs Foundation.

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