

## Differences in Functional Activity of Anticardiolipin Antibodies from Patients with Syphilis and Those with Antiphospholipid Syndrome

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**Anticardiolipin antibodies are produced both in patients with the antiphospholipid syndrome (APS) and in patients with syphilis, but lupus anticoagulant activity has been reported only for the former group. To understand these differences, affinity-purified immunoglobulin G anticardiolipin antibodies from APS ( $n = 11$ ) and syphilis ( $n = 5$ ) patients were compared. Only the antibodies from the APS group inhibited prothrombin conversion to thrombin and cross-reacted with phosphatidylserine. These findings may enable better definition of the phospholipid epitopes involved in the hemostatic abnormalities of APS.**

Anticardiolipin (aCL) antibodies are induced both in patients with syphilis and in patients with the antiphospholipid syndrome (APS), but thrombosis, pregnancy loss, and thrombocytopenia occur only in the latter disorder (14). If the presence of aCL antibodies is associated with clinical complications seen in APS, then investigators must determine why patients with syphilis do not get such complications. Several groups have demonstrated differences between the binding specificities of antibodies associated with APS and those associated with syphilis, variously concluding that these differences are attributable to differences in specificity for phospholipids (5, 6, 17, 22, 23, 28) or for the  $\beta_2$  glycoprotein 1 ( $\beta_2$ GP1)-cardiolipin complex (26). However, it is likely that the different pathogenetic effects of APS and syphilis antibodies may be better ascertained by studying their functional effects. One particular effect shared by most antibodies with lupus anticoagulant (LA) activity is inhibition of phospholipid-dependent hemostatic reactions, such as the conversion of prothrombin to thrombin (3, 8, 11) and the activation of protein C (4, 24, 25). Inhibition of the conversion of prothrombin into thrombin is believed to account for prolonged clotting times of LA-positive plasma *in vitro* (1, 31, 32), and inhibition of protein C-dependent reactions might account for enhanced thrombosis *in vivo* (4, 24, 25). On the basis of a study showing that syphilis plasma did not have LA activity (20), we speculated that aCL antibodies of syphilis patients might differ from aCL antibodies of APS patients in their effects on phospholipid-dependent hemostatic reactions. Such a difference in functional effect might account for reported differences in clinical complications.

Immunoglobulin G (IgG) aCL antibodies were affinity purified from 11 patients with APS (IgG-APS) and from 5 patients with syphilis (IgG-syph). Previously published criteria were used to define the APS (12). All APS patients had moderately to highly (15) positive IgG aCL and positive LA tests (33). Sera from 19 patients with syphilis were collected. Five sera contained quantities of IgG aCL antibodies sufficient for affinity purification (exceeding 40 GPL units), and these were used for the studies described here.

Affinity purification was performed with cardiolipin (CL) liposomes as described elsewhere (11, 16). Further purification of the IgG preparations was accomplished by utilizing protein G-Sepharose. Purity of the IgG preparations was demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the absence of  $\beta_2$ GP1 was assessed by immunoblotting (19, 30).

The CL binding activities of the affinity-purified samples were determined by the standard aCL enzyme-linked immunosorbent assay (ELISA) method (10, 13). Samples were diluted in 10% adult bovine serum in phosphate-buffered saline for these determinations. CL binding activity was expressed in GPL units per microgram of sample, where 1 GPL unit is defined as the CL binding activity of a 1- $\mu$ g/ml concentration of a standard sample termed Rey (15).

The LA activities of the affinity-purified IgG samples were determined by a modified kaolin clotting time (KCT) method as described by Exner et al. (7). In brief, 20  $\mu$ l of each affinity-purified preparation (IgG-APS, IgG-syph, or IgG from normal human serum [IgG-NHS]) was mixed with 80  $\mu$ l of normal plasma (20:80) and then incubated with 50  $\mu$ l of a 2% kaolin suspension (Sigma Chemical Co., St. Louis, Mo.) for 3 min at 37°C. The clotting reaction was started by adding 100  $\mu$ l of 0.03 M CaCl<sub>2</sub> to the mixture, and clotting time was determined in a semiautomatic BBL fibrometer (Becton Dickinson). LA activity was defined as a ratio of KCT of an affinity-purified sample to control KCT (IgG-NHS) in excess of 1.2.

Inhibition of prothrombin-to-thrombin conversion by the two groups of affinity-purified preparations was measured by utilizing a chromogenic substrate for thrombin in a plasma-free system (Tris-buffered saline containing 0.5 mg of human serum albumin per ml was used as the diluent for the reaction) (30). Thrombin generated by a mixture of purified human prothrombin (Diagnostics Stago), human factor Xa and factor V/Va (Enzyme Research Laboratory), and phospholipid vesicles (Sigma Chemical Co.) was quantitated by measurement of thrombin-dependent amidolysis of the synthetic thrombin substrate CBS 34.47 (H-D-CHG-But-Arg-pNA). All protein reagents and buffers lacked contaminating  $\beta_2$ GP1 as measured by immunoblot analysis sensitive to 0.2  $\mu$ g of  $\beta_2$ GP1 per ml. Details of the assay are described elsewhere (11). Phospholipid vesicles used to support the reaction were either phosphatidyl-

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TABLE 1. Inhibitory activity of affinity-purified IgG-APS samples in PS-PC and CL-PC thrombin generation reaction systems

Sample	Concn ( $\mu\text{g/ml}$ ) <sup>a</sup>	% Inhibition of thrombin generation with:	
		PS-PC	CL-PC
1	85	80	48
2	39	61	44
3	89	28	19
4	42	33	56
5	92	79	55
6	29	23	17
7	45	49	60
8	49	38	46
9	56	43	60
10	67	96.5	100
11	78	71	76
Mean $\pm$ SD	61.0 $\pm$ 22.2	54.7 $\pm$ 24.3	52.8 $\pm$ 23.3

<sup>a</sup> Final concentration of affinity-purified sample in the prothrombinase reaction.

serine (PS)-phosphatidyl choline (PC) (1:3, 4  $\mu\text{g/ml}$ ) or CL-PC (1:3, 40  $\mu\text{g/ml}$ ).

Inhibition of prothrombin-to-thrombin conversion by test samples (IgG-APS or IgG-syph) was compared with that of IgG isolated from NHS. Inhibitory activity was expressed as follows: inhibition of prothrombin activation by test samples =  $[1 - (\text{thrombin activity in the presence of affinity-purified IgG-APS}) / (\text{thrombin activity in the presence of IgG-NHS})] \times 100$ . Inhibition of the prothrombinase reaction was considered to be significant when it was  $\geq 15\%$ . This value was determined by replicate testing eight different IgG-NHS preparations on different days.

Statistical analysis utilized Student's *t* test to compare means when appropriate. Differences exhibiting  $P \leq 0.05$  were considered significant.

The mean ( $\pm$  standard deviation) CL binding activity of the 11 IgG-APS samples was  $2.95 \pm 1.78$  GPL units per  $\mu\text{g}$ . The mean ( $\pm$  standard deviation) CL binding activity of the five IgG-syph samples was  $2.40 \pm 0.90$  GPL units per  $\mu\text{g}$ . There was no significant difference between the mean CL binding activities of the two groups of antibodies studied.

LA activity was present in all 11 IgG-APS samples when they were added to normal plasma, as demonstrated by prolongation of the KCT (the KCT ranged from 86.5 to 158.4 s; the control value, obtained with IgG-NHS, was 60.9 s). None of the five IgG-syph samples had LA activity.

Inhibition of the conversion of prothrombin to thrombin was significant ( $>15\%$ ) in all 11 IgG-APS samples tested. Inhibition occurred when either PS-PC or CL-PC was used to support the reaction. The mean ( $\pm$  standard deviation) inhibition by IgG-APS samples was  $54.7 \pm 24.3\%$  (range, 28 to 96.5%) when PS-PC was used as the catalyst and  $52.8 \pm 23.3\%$  (range, 17 to 100%) when CL-PC was used as the catalyst (Table 1). There was good correlation of antibody concentration with percent inhibition of prothrombin conversion into thrombin in two IgG-APS samples examined (Fig. 1).

Only one of the five IgG-syph samples exhibited significant inhibition of prothrombin-to-thrombin conversion (inhibition of 32% with PS-PC as the catalyst and 36% with CL-PC as the catalyst).

Because PS is thought to be the main phospholipid which promotes the conversion of prothrombin into thrombin, we postulated that differences between the functional activities of

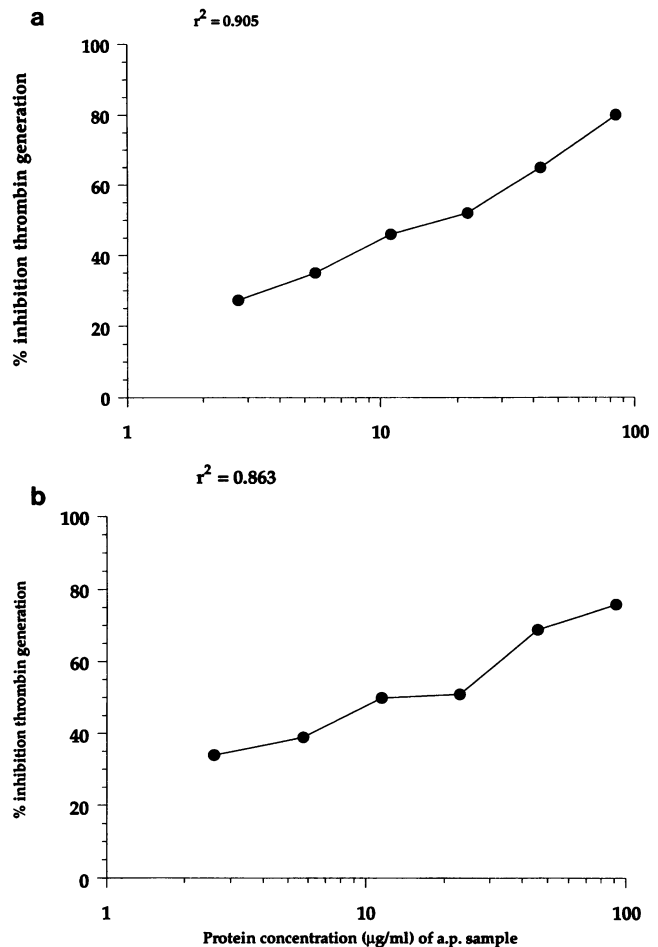


FIG. 1. Prothrombinase-inhibitory activity of serial dilutions of two affinity-purified IgG-APS samples. Serial dilutions of affinity-purified (a.p.) IgG-APS samples 1 (85  $\mu\text{g/ml}$ ) (a) and 5 (92  $\mu\text{g/ml}$ ) (b) were tested for inhibitory activity in the prothrombinase reaction system with 2  $\mu\text{g}$  of PS-PC liposomes per ml to support the reaction, as described in the text.

the CL-binding IgG-APS and IgG-syph preparations, as demonstrated above, might be attributable to differing cross-reactivities with an epitope on PS. Thus, we compared binding of the affinity-purified preparations to PS alone, PS-PC, CL alone, CL-PC, and PC alone. The IgG-APS samples bound all phospholipid antigens except PC (Fig. 2), while the IgG-syph samples bound only CL and CL-PC (Fig. 3). Within the IgG-APS group, the CL binding activity of individual IgG-APS preparations correlated better with inhibition of prothrombin activation with the PS-PC template ( $r^2 = 0.536$ ) than with inhibition with the CL-PC template ( $r^2 = 0.283$ ).

These results suggest that in APS patient sera, CL binding yields an antibody population that is cross-reactive with an epitope(s) on PS. This target epitope also appears to be critical for proper function of phospholipids in support of the prothrombinase reaction (Fig. 4). If the same epitope(s) is important in supporting other phospholipid-dependent hemostatic reactions, such as protein C activation, this would explain how these antibodies might cause a hypercoagulable state in vivo (4, 24, 25). In contrast, IgG aCL antibodies from syphilis sera exhibit limited inhibitory activity and bind an epitope(s) specific to CL (Fig. 4). The CL-specific epitope appears to be less

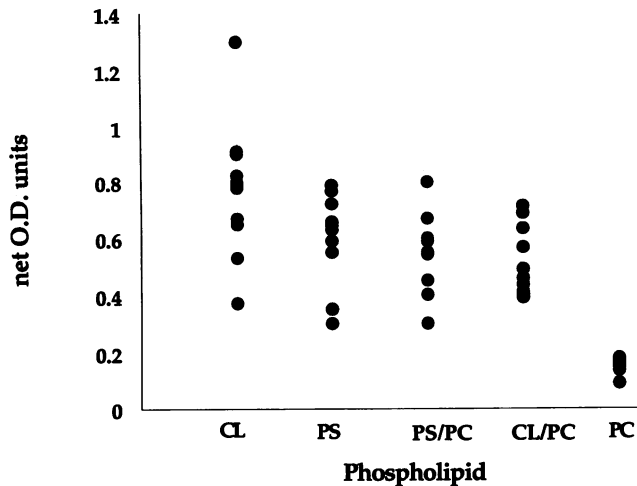


FIG. 2. Binding of affinity-purified IgG-APS to different phospholipids as determined by ELISA. The binding of 11 IgG-APS samples to CL, PS, PS-PC, CL-PC, and PC was determined by coating parallel rows of microtiter plates with the different phospholipids or phospholipid mixtures and performing an ELISA, as explained in Materials and Methods. Samples ranged in concentration from 29 to 89  $\mu\text{g/ml}$ . Binding was calculated by subtracting the optical density (O.D.) of an IgG-NHS sample from that of the affinity-purified IgG-APS sample.

critical in supporting prothrombin-to-thrombin conversion. If this epitope is also not important in phospholipid-dependent hemostatic reactions (e.g., protein C activation), one might predict that aCL antibodies from syphilis patients will also have no effect on this reaction. This might explain why patients with syphilis do not have a hypercoagulable state. (The effects of these affinity-purified antibodies on protein C activation are currently being tested in our laboratory.)

These data do not preclude the possible interaction of antiphospholipid antibodies with molecular species other than phospholipids during inhibition of phospholipid-dependent coagulation reactions (2, 9, 29). In a previous study, we found

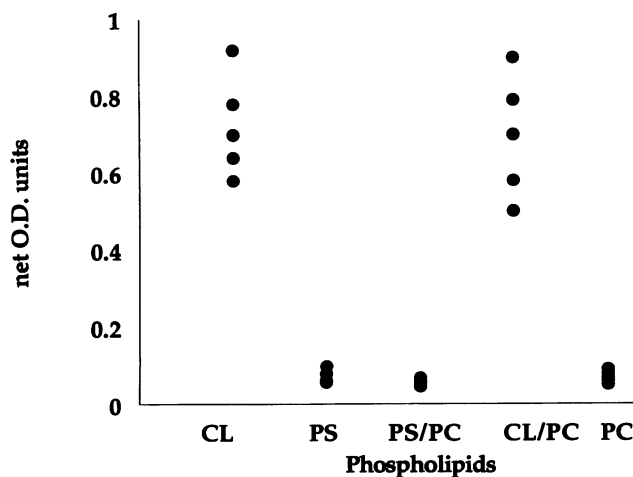


FIG. 3. Binding of affinity-purified IgG-syph to different phospholipids as determined by ELISA. The binding of five affinity-purified IgG-APS samples to CL, PS, PS-PC, CL-PC, and PC was determined as described in the legend to Fig. 2. O.D., optical density. Sample concentrations ranged from 26 to 85  $\mu\text{g/ml}$ .

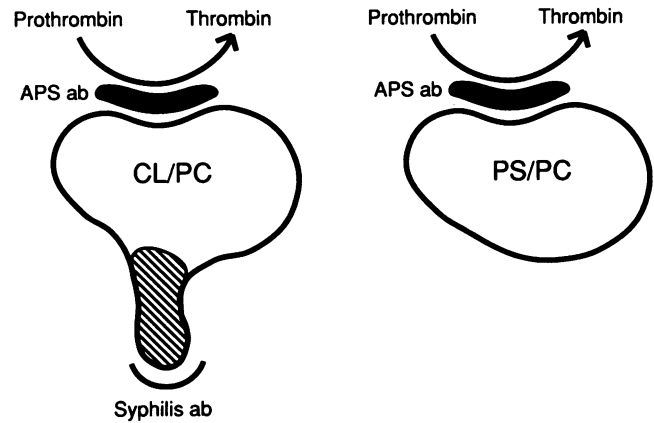


FIG. 4. Hypothetical interpretation of results obtained in this study. We propose that antibodies from patients with APS (APS ab) bind sites on CL-PC liposomes that are different from those bound by antibodies from patients with syphilis (syphilis ab). The sites recognized by IgG-APS antibodies are present on CL-PC and PS-PC liposomes and are the same sites required for support of the prothrombin-to-thrombin conversion reaction. The site recognized by IgG-syph is specific to CL-PC and does not play a role in catalyzing prothrombin-to-thrombin conversion.

that IgG-APS preparations were capable of direct inhibition of prothrombin activation in the absence of  $\beta_2\text{GP1}$  (11), and the experiments in the present study were also carried out in the absence of this protein. It is also possible that CL-binding antibodies from patients with APS may also react with neoantigens resulting from phospholipid-protein complex formation (2, 18, 21, 27).

The present study extends our understanding of the relationship between phospholipid binding specificity and functional interactions with phospholipid-dependent coagulation reactions. Further clarification of the basis for functional differences between CL-binding antibody populations present in syphilis sera and in patients with APS will require more precise identification of the differing epitopes that are the targets of these antibodies.

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