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Antiphospholipid antibody: functional specificity for inhibition of prothrombin activation by the prothrombinase complex

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Summary. The antiphospholipid syndrome (APS) is associated with production of autoantibodies with lupus anticoagulant (LA) activity. These antibodies cause prolongation of *in vitro* clotting tests by inhibition of the conversion of prothrombin to thrombin in the presence of anionic phospholipid (PL). The extent to which this inhibition reflects antibody binding to, or functional inhibition of, phospholipids alone, prothrombin alone, or a prothrombin–phospholipid complex is pertinent to our understanding of the pathophysiology of this syndrome. Immunoglobulin fractions (IgG) from 18 patients with LA activity were tested for inhibitory activity against prothrombin activation by either factor Xa, in a purified prothrombinase system, or by purified fractions of snake venoms (*E. carinatus*, *E. multisquamatus*) which cleave prothrombin at the same initial site as the prothrombinase complex but do not require anionic phospholipid as a cofactor. Parallel testing of the same IgG samples for prothrombin binding by immunoassay was performed. Although all IgG samples inhibited the prothrombinase reaction, only three exhibited any inhibition of venom protease prothrombin activation in either the presence or absence of PL. Only one sample exhibited prothrombin binding by Western blot. These results suggest that lupus anticoagulant antibodies are heterogenous and that many, if not most, of the autoantibody populations responsible for LA activity impair prothrombin activation by interaction either with phospholipid alone or with a restricted range of prothrombin–phospholipid epitopes expressed by prothrombin only as part of the intact prothrombin–prothrombinase complex.

Keywords: lupus anticoagulant, antiphospholipid antibodies, snake venom proteases, antiphospholipid syndrome.

Antiphospholipid syndrome (APS) is a disorder of recurrent thrombosis, pregnancy losses and thrombocytopenia associated with positive anticardiolipin and lupus anticoagulant tests (Harris & Pierangeli, 1994). Both anticardiolipin and lupus anticoagulant tests detect antibodies which have been shown to induce pregnancy loss (Branch *et al*, 1990; Blank *et al*, 1991) and to enhance thrombosis in mice (Pierangeli *et al*, 1994a, 1995; Pierangeli & Harris, 1994). The mechanism by which these antibodies might induce thrombosis and pregnancy loss is unknown. It is generally believed that determination of the specifities of these antibodies may provide clues to their mechanism of action. There are several postulates with respect to the specificities of these antibodies. In the anticardiolipin test, binding activity is increased by the serum protein β_2-glycoprotein 1 (β_2GP1) and various investigators believe this is to be due to antibodies binding β_2GP1 (Roubey, 1994), a conformationally altered β_2GP1 molecule (Matsuura *et al*, 1994), epitopes shared by β_2GP1 and cardiolipin (McNeil *et al*, 1990, 1991), or cardiolipin that is conformationally altered by β_2GP1 (Harris & Pierangeli, 1990; Pierangeli *et al*, 1992; Sammaritano *et al*, 1992; Gharavi *et al*, 1993; Stewart *et al*, 1995).

The lupus anticoagulant test is a functional measure of antibody activity assessed by their ability to prolong *in vitro* clotting tests. This activity is the result of inhibition of conversion of prothrombin to thrombin or activation of factor X (Brandt, 1991; Galli *et al*, 1989; Goldsmith *et al*, 1994). Various groups attribute this inhibitory activity to

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antibodies specific for human prothrombin (Permpikul *et al*, 1994), a human prothrombin–phospholipid complex (Bevers *et al*, 1991; Vermylen & Arnout, 1992), a β_2GP1–phospholipid complex (McNeil *et al*, 1990; Jones *et al*, 1992), negatively charged phospholipids (Pengo *et al*, 1987; Pierangeli *et al*, 1993). Some patients with positive lupus anticoagulant tests are also markedly hypo-prothrombinaemic, and this has been attributed to antibodies directed at prothrombin (Edson *et al*, 1984; Fleck *et al*, 1988). Edson *et al* (1984) and Fleck *et al* (1988) demonstrated by crossed electrophoresis that lupus anticoagulant positive plasma had anti-prothrombin antibodies. Recent studies have also reported that pre-incubation of these antibodies with human prothrombin enhanced their ability to inhibit the prothrombin–thrombin conversion, as well as their ability to bind phosphatidylserine (Permpikul *et al*, 1994). In addition, the same group has reported direct binding of these antibodies to human prothrombin (Rao *et al*, 1995).

Recently, we have examined the effects of purified immunoglobulins and affinity purified anticardiolipin antibodies on the prothrombin–thrombin conversion reaction, in a reaction system free of plasma. Antibody preparations obtained from patients with APS inhibited the prothrombinase reaction, but it could not be determined whether this was due to binding prothrombin, to the phospholipid template, or both. To examine this question further, the present study examined the effects of 18 IgG preparations from patients with APS on phospholipid–independent prothrombin–thrombin conversion reactions catalysed by the viper venoms of *Echis carinatus* (Ecarin®) or *Echis multisquamatus* (Yukelson *et al*, 1992). In addition, binding of the IgG preparations to prothrombin by Western blot and ELISA were studied.

MATERIALS AND METHODS

Patients. Sera and plasma from 18 patients with the antiphospholipid syndrome (APS) were used to prepare IgG (IgG-APS). The patients fulfilled the criteria for APS (Harris, 1987) and were all LA positive. All except two had demonstrable IgG anticardiolipin antibodies. Sera from healthy normal controls were used to prepare normal IgG (IgG-NHS). The patients signed a consent form approved by the Human Studies Committee of the University of Louisville and the University of Utah before donating the blood.

Isolation of immunoglobulins. IgG was isolated from patients ($n = 18$) and control sera ($n = 10$) (IgG-APS and IgG-NHS) by protein G Sepharose (Pharmacia-LKB, Piscatway, N.J.) as described elsewhere (Goldsmith *et al*, 1994; Pierangeli & Harris, 1994). The protein concentration was adjusted to 5 g/l and the samples dialysed exhaustively in tris-buffered saline (TBS) solution. The purity of the preparations was determined by SDS-PAGE electrophoresis (4–12% gradient gels) and absence of β_2GP1 was determined by immunoblot utilizing a rabbit anti-human β_2GP1 as described elsewhere (Pierangeli *et al*, 1992; Kouts *et al*, 1995; Rao *et al*, 1995).

Anticardiolipin ELISA test. Anticardiolipin antibodies were determined in the sera and IgG preparations by ELISA as described elsewhere (Harris *et al*, 1994). The assay was calibrated in GPL units, using international standards (Louisville APL Diagnostics Inc., Louisville, Ky.). A result is considered negative when less than 10 GPL units).

Lupus anticoagulant activity. The lupus anticoagulant activity test in the plasma of the patients was performed as previously described (Triplett *et al*, 1983; Triplett & Brandt, 1989). In brief, the activated partial thromboplastin time (APTT), dilute Russell Viper Venom Time (dRVVT) (DVVT Test®, American Diagnostica, Greenwich, Ct.) and Kaolin Clotting time (KCT) were performed on plasma samples from the patients as screening tests using commercially available reagents. The clotting time in each case was determined in a semi-automatic fibrometer (BBL fibrosystem, Becton Dickinson, Sparks, Md.). Two commercially available plasma samples (Thromboscreen, Pacific Hemostasis, Ventura, Calif.), were run in duplicate as abnormal and normal controls on each run. Mixing studies with normal plasma were performed to exclude the presence of other inhibitors. The presence of lupus anticoagulant activity was confirmed by phospholipid neutralization using Staclot® (American Bioproducts, Parsipanny, N.J.) and DVVT confirm® (American Diagnostica). All patients studied had a lupus anticoagulant activity (data not shown).

Lupus anticoagulant activity in the IgG preparations was determined by using a modified kaolin clotting time test as described by Exner *et al* (1978). Briefly, 20 μl of each IgG preparation was added to 80 μl of normal fresh plasma and a KCT performed. The IgG sample was considered positive if the ratio of sample/control (IgG-NHS) >1.2 (Exner *et al*, 1978; Pierangeli *et al*, 1993; Goldsmith *et al*, 1994).

Effect of immunoglobulins on the prothrombinase reaction. The effect of IgG-APS preparations on the phospholipid-dependent

Table I. Anticardiolipin and lupus anticoagulant activities of the IgG-APS samples.

Sample	aCL activity (GPL units)	LA activity	
		KCT (s)	Ratio
A	311	150	2·3
B	166	123	1·89
C	69·8	108	1·66
D	631	167	2·60
E	558	143	2·2
F	76·8	103	1·58
G	792	145	2·2
H	139·8	104	1·6
J	83·6	112	1·72
K	43·9	98·0	1·72
L	692·2	145	2·23
M	43·0	89·0	1·36
N	75·9	102	1·56
P	20·4	135	2·07
Q	81·7	87	1·33
R	193·8	167·0	2·56
S	–	132·0	2·03
T	67·0	100·0	1·53

conversion of prothrombin into thrombin was determined as described elsewhere (Goldsmith *et al*, 1994; Pierangeli & Harris, 1994). Briefly, 10 μl of the IgG preparations (IgG-APS or IgG-NHS) at 5 mg/ml of protein concentration (final concentration 50 μg/ml) were incubated with 30 μl of human prothrombin (final concentration 1·0 μM; Celsus, Cincinnati, Ohio) and 50 μl of 2 μg/ml phospholipid liposomes [phosphatidylserine: phosphatodylcholine (PS:PC)] for 15 min at 37°C. A 15 μl aliquot of a mixture of factors Xa (final concentration 0·1 nM) and V (final concentration 0·2 nM) (Enzyme Res. Lab., Indianapolis, Ind.) dilutions in buffer was added to the preparations and incubated exactly for 30 min at 37°C. The formation of thrombin was evaluated by addition of a specific chromogenic substrate CBS 34·47 (0·20 μmol/ml TBS containing 3 mM EDTA) (American Bioproducts). The proteins were diluted in TBS containing 0·5 mg/ml of bovine serum albumin (TBS-HSA). Inhibition of the prothrombinase reaction by IgG-APS was calculated as described elsewhere (Goldsmith *et al*, 1994; Pierangeli *et al,* 1994b). In one series of experiments the effect of the patient IgG preparations upon prothrombin activation by the factor V/Xa mixture in the absence of phospholipid vesicles was determined. For these studies, prolonged incubation (4·5 h) was required for generation of thrombin activity at levels equivalent to those obtained with the complete prothrombinase mixture.

Effect of aPL immunoglobulins upon prothrombin activation by snake venom proteases. The effects of patient immunoglobulins upon the activation of prothrombin by *E. carinatus* and *E. multisquamatus* was determined as described above, with the exception that factor Xa was replaced by one of the two venom protease preparations Ecarin® or *Echis multisquamatus* venom. Ecarin® was purchased from Sigma Chem. Co. (St Louis, Mo.), and the venom *Echis multisquamatus* was a kind gift of Professor Barkagan (Altai Medical Institute, Russian Federation). Preliminary experiments were performed to determine rates of substrate amidolysis which correlated linearly with thrombin concentration. Ecarin® diluted in buffer (TBS-HSA) was examined in concentrations ranging from 1 U/ml to 0·001 U/ml. A concentration of 0·01 U/ml provided prothrombin activation at a rate equivalent to that observed with the concentration of Xa employed in the prothrombinase reaction. For the *Echis multisquamatus* venom, a stock solution was prepared as follows: 2 mg of the dry powder was dissolved in 10 ml of distilled water. After mixing this solution for 1 h in a water bath at 37°C, the preparation was placed for 18–24 h at 4°C. Dilutions ranging from 1:10 to 1:32 000 of this stock solution were tested in preliminary experiments. A dilution of 1:2000 was chosen to perform the experiments. The generation of meizothrombin was measured with the cromogenic substrate CBS 34.47. Prothrombin activation by the venom proteases was examined in the presence and absence of the phospholipid vesicles employed in the prothrombinase reaction.

Anti-prothrombin antibodies by ELISA. The presence of anti-prothrombin antibodies in patient sera was determined by ELISA. In brief, microtitre polystyrene ELISA plates (ICN-Flow) were coated overnight with 50 μl of 50 μg/ml human prothrombin (Celsus) in 0·5 M carbonate buffer (pH 9·8). Subsequently the wells were washed with PBS–0·05% Tween 20 buffer twice and blocked with 3% BSA-PBS solution. 100 μl aliquots of IgG-APS samples (50 μg/ml) and a positive control (rabbit anti-human prothrombin) (American Bioproducts, Parsipanny, N.J.) were each added to duplicate wells and incubated for 1 h at room temperature. Plates were subsequently washed three times then incubated with 100 μl of a 1:1000 dilution of alkaline phosphatase labelled anti-human IgG, or alkaline phosphatase labelled anti-rabbit IgG (for the positive control) for 1 h at room temperature. The plates were washed and colour reaction developed with p-nitrophenylphosphate substrate for 45 min. Net mean OD values were calculated by subtracting the mean OD value of the blank from the mean OD value of the samples. A positive value was taken as 2 standard deviations above the mean OD value of 20 normal controls.

Anti-prothrombin antibodies by immunoblot. The presence of anti-prothrombin antibodies in the IgG preparations was tested by immunoblot as described elsewhere with minor modifications (Permpikul *et al*, 1994; Rao *et al*, 1995). In brief, purified human prothrombin (Celsus Laboratories, Cincinnati, Ohio), of 50 μg/gel was subjected to SDS-PAGE electrophoresis and transblotted onto a nitrocellulose membrane at 45 V for 3 h. The membrane was then blocked with 3% bovine serum albumin in tris-buffered saline (BSA-TBS) for 1 h at room temperature. Lanes were cut and probed with IgG-APS and IgG-NHS (200 μg in 600 μl 3% BSA-TBS containing 5 mM EDTA). After 3 h, the lanes were washed five times with TBS containing 5 mM EDTA and probed with alkaline phosphatase labelled anti-human IgG for 1 h. Lanes were washed and colour developed with Immunoblot Assay Kit (BIO-RAD Laboratories, Palo Alto, Calif.).

RESULTS

Anticardiolipin and lupus anticoagulant activities of the IgG preparations

As shown in Table I, all 18 IgG-APS preparations (5 mg/ml) showed lupus anticoagulant activity (mean KCT values ± SD: 122·7 ± 25·8; mean ratios ± SD: 1·8 ± 0·5), and 16/18 also had medium-high IgG anticardiolipin levels (mean ±SD); range 43·0–692·2 GPL). One sample was low positive for aCL (patient P) and another sample was negative (patient S) for IgG aCL (Table I).

Effect of immunoglobulin preparations on phospolipid-dependent and phospholipid-independent conversion of prothrombin into thrombin

As shown in Table II, all 18 samples yielded significant inhibition (>12%) of phospholipid-dependent conversion of prothrombin to thrombin (mean per cent inhibition ±SD = 66·5 ± 23·3), but two samples (patients P and Q) yielded <30% inhibition. None of the 10 IgG-NHS significantly inhibited the phospholipid-dependent conversion of prothrombin into thrombin (all <9%). None of the samples inhibited the conversion of prothrombin into thrombin in the presence of Ecarin, but three samples (patients G, P and S) showed some degree of inhibition of the

prothrombin conversion reaction in the presence of *E. multisquamatus.*

Samples P and S showed patterns of activity different from the majority of the IgG preparations. Sample P provided moderate inhibition of the prothrombinase reaction and major inhibition of *E. multisquamatus* activation. Sample S provided weak but significant inhibition of both these reactions.

The presence of phospholipid vesicles in the venom activation mixture did not alter the observed pattern of activation of prothrombin by *E. carinatus* protease (Table II), indicating that the aPL preparations lacked inhibitory activity against activation by the venom protease of prothrombin associated with phospholipid vesicles.

Sample P was of particular interest, since it had low positive IgG anticardiolipin activity but caused only weak inhibition of phospholipid-dependent prothrombin–thrombin conversion (12·5%). However, that sample caused marked inhibition of the *Echis multisquamatus* induced prothrombin conversion reaction. Also of interest was sample S, the lupus anticoagulant positive, anti-cardiolipin negative sample. That sample weakly inhibited the prothrombin conversion reactions catalysed by both phospholipid as well as *Echis multisquamatus* and did not inhibit the *Echis carinatus* catalysed reaction (Table II). Sample G weakly inhibited the *E. multisquamatus* reaction but significantly inhibited phospholipid-dependent prothrombin-thrombin conversion reaction (Table II).

Table II. Effect of IgG-APS on conversion of prothrombin into thrombin reactions.

	Inhibition thrombin formation (%)		
		Phospholipid independent	
Sample	Phospholipid dependent	Ecarin	*E. multisquamatus**
A	95	0	0
B	50	0	0
C	57	0	0
D	72	0	0
E	82	0	0
F	92	0	0
G	82	0	20·5
H	62	0	0
J	85	0	0
K	74	0	0
L	60	0	0
M	68	0	0
N	80	0	0
P†	54·5	0	86·5
Q	23	0	0
R	99	0	0
S†	12·5	0	13
T	50	0	0

*Each value represents the mean of two determinations for each sample.

†Two patients were LA positive and aCL low positive or negative.

Effect of immunoglobulin preparations upon prothrombin activation by factor Xa in the absence of phospholipid

When affinity purified aPL IgG (two samples analysed) was incubated with the prothrombinase mixture in the presence and absence of phospholipid vesicles, the inhibitory effect was dependent upon phospholipid (Fig 1). No inhibition was observed in the absence of the vesicles.

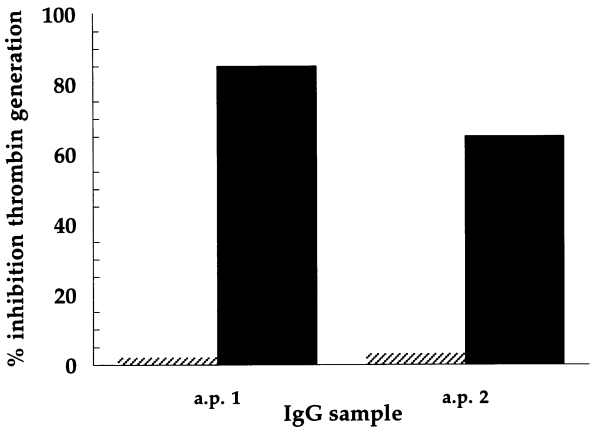

Fig 1. Inhibition of the prothrombinase reaction by two affinity purified (a.p. 1 and a.p. 2) anticardiolipin antibody preparations in the presence (■) and absence (▨) of phospholipids. The two a.p. preparations significantly inhibited the phospholipid-dependent conversion of prothrombin into thrombin (prothrombinase reaction), but did not inhibit the same reaction in the absence of phospholipids.

Determination of anti-prothrombin antibodies by ELISA

The presence of anti-prothrombin antibodies in IgG-APS was tested by ELISA. None of the IgG-APS tested sera showed significant binding to prothrombin mean OD units ± SD: 0·126 ± 0·029 (as indicated in Materials and Methods a sample was positive when it was >2 SD above the level of 20 normal controls). The positive control OD net units was 1·594, and the binding of IgG-NHS in OD units was 0·094.

Determination of anti-prothrombin antibodies by immunoblot

The presence of anti-prothrombin antibodies in IgG-APS ($n = 18$) preparations was determined. All samples, except P (which showed a mild reaction) tested negative (not shown).

DISCUSSION

Several investigators have demonstrated that antibodies with lupus anticoagulant activity inhibit the conversion of prothrombin to thrombin, both in plasma (Galli *et al*, 1989; Brandt *et al*, 1991) and plama-free reaction systems (Goldsmith *et al*, 1994; Pierangeli *et al*, 1994b). Whether inhibition is caused by antibodies specific for prothrombin (Permpikul *et al*, 1994; Rao *et al*, 1995), a complex of prothrombin with phospholipids (Bevers *et al*, 1991), or phospholipids alone (Pengo *et al*, 1987; Pierangeli *et al*, 1993), remains unresolved. Recently, Triplett *et al* (1993)

and Forastiero *et al* (1994) have reported that use of two viper venoms which differ in their requirement for phospholipids and can be used to identify LA-positive plasma. They showed that positive LA plasma samples interfered with Textarin® time which catalyses prothrombin conversion in a phospholipid-dependent manner, but not Ecarin® time, which is a phospholipid-independent reaction, suggesting that inhibition of prothrombin–thrombin conversion is phospholipid dependent. The present study employed proteases, which activate prothrombin by cleavage of the molecule at the same site as factor Xa, when functioning as part of the prothrombinase complex, but do not need anionic phospholipids for effective catalysis. Both *E. carinatus* and the prothrombinase complex initially form meizothrombin by cleavage of the Arg 320 peptide bond of prothrombin; subsequent cleavage at position 271 is catalysed by the prothrombinase complex, whereas autolytic cleavage at sites 156 and 283 yields the thrombin formed after *E. carinatus* activation. *E. multisquamatus* is thought to produce a similar sequence of cleavages (Yukelson *et al,* 1992). The inhibition of prothrombin activation by IgG-APS samples in this system was compared with that observed in a purified prothrombinase system. We correlated these functional assays, using immunoblot and ELISA, for prothrombin binding by the same IgG samples.

Patient IgG samples uniformly inhibited the prothrombinase reaction in the presence of anionic phospholipid vesicles, but no inhibition was observed in the absence of phospholipids. Prothrombin activation by *E. carinatus* was not inhibited, in the presence or absence of the same phospholipids, in a reaction system containing all elements of the prothrombinase system except factor Xa. Three samples showed some degree of inhibition of prothrombin activation by *E. multisquamatus*, despite its described similarity to *E. carinatus* in prothrombin cleavage. One of these three samples (P) also demonstrated prothrombin binding as measured by Western blot. One sample, designated S, was anti-cardiolipin negative and demonstrated only weak binding of the phospholipid-dependent coagulation reaction. It is possible that sample S may inhibit another phospholipid-dependent coagulation reaction such as activation of factor X (Shi *et al*, 1993). These findings suggest the existence of at least two populations of antibodies in these series, the larger group inhibits phospholipid-dependent prothrombin–thrombin conversion, and a small number may be directed at prothrombin alone.

Taken together, this data again supports the concept of functional heterogeneity of the family of autoantibodies identified by cardiolipin binding and lupus anticoagulant activity. In a small fraction of our patients we found evidence for inhibition resulting from direct interaction with prothrombin alone. The results obtained with the majority of our patient samples are also consistent with functional inhibition of prothrombin activation which resulted from interaction of the antibody species with certain conformers of a prothrombin–phospholipid complex. However, given the infrequency of demonstrable immunologic binding to prothrombin activation by the venom proteases, either in the presence or absence of phospholipid, it seems more likely that inhibition of the prothrombinase reaction is attributable to antibody specificity either for anionic phospholipid alone or for prothrombin–phospholipid epitopes.

The binding to prothrombin by our patient IgG samples differed from that reported by some other investigators (Permpikul *et al*, 1994; Rao *et al*, 1995). Whether this random variation reflects variation in patient selecton or differences in assay conditions is at present uncertain. In the study by Permpikul *et al* (1994) all 10 patients had significantly prolonged prothrombin times (>2 s longer than control) whereas none of the 10 patients not taking oral anticoagulant in this study had a significantly prolonged prothrombin time. It is possible that the study by Permpikul *et al* (1994) included patients with antibody heterogeneity which had greater reactivity with prothrombin itself. Despite these differences in the frequencies of prothrombin binding between the studies, the present study indicated the presence in many, if not most, patients with APS, of antibodies capable of inhibition of prothrombin activation in a purified system by interaction with either phospholipid alone or a very restricted set of prothrombin–phospholipid neoantigens.

An alternative possibility is that antibodies with more than one specificity may be responsible for LA activity. Galli *et al* (1992, 1995) found one group of antibodies with both lupus anticoagulant and anticardiolipin activities, which could both be absorbed with cardiolipin. Those antibodies exerted lupus anticoagulant activity only in the presence of β_2GP1 (Galli *et al*, 1992, 1995), a finding similar to that of Oosting *et al* (1992). They also identified another group of antibodies with lupus anticoagulant activity which were not absorbed by cardiolipin. Our samples primarily resemble the first group of antibodies reported by Galli *et al* (1992), in that both activities could be separated from the cardiolipin–antibody precipitate (Pierangeli *et al*, 1993). However, our affinity-purified anticardiolipin antibodies inhibited prothrombin–thrombin conversion without β_2GP1, and when β_2GP1 was incorporated into the reaction there was additive inhibiton of the reaction (Pierangeli *et al*, 1993). Our interpretation of the latter findings is that these antibodies bind negatively charged phospholipids or a phospholipid–prothrombin complex. We also identified one to three IgG samples which may be specific for prothrombin alone, as exemplified by inhibition of the prothrombin–thrombin conversion reaction catalysed by *E. multisquamatus* and demonstration of binding to prothrombin by Western blot in one case. These three samples may resemble those described by Rao *et al* (1995) and Permpikul *et al* (1994). It remains unclear whether these apparently separate groups of autoantibodies with lupus anticoagulant activities also have some common effect on other coagulation reactions (such as protein C activation) which result in thrombosis in patients with APS.

In summary, this study found that the majority of IgG preparations with lupus anticoagulant activity inhibit phospholipid-dependent but not phospholipid-independent prothrombin–thrombin conversion. This suggests that these antibodies may be directed at phospholipids or a phospholipid–thrombin conversion catalysed by *E. multisquamatus*.

One sample bound prothrombin, suggesting that some antibodies with lupus anticoagulant activity may be directed at prothrombin alone.

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