Case report

Antibody to phosphatidylethanolamine in a patient with lupus anticoagulant and thrombosis*

HENRIQUE LUIZ STAUB, E NIGEL HARRIS, MUNTHER A KHAMASHTA, GEOFF SAVIDGE, WILLIAM HABIB CHAHADE, AND GRAHAM R V HUGHES

From the Lupus Research Unit, The Rayne Institute, St Thomas's Hospital, London, UK

SUMMARY Most patients with lupus anticoagulant (LA) activity have coincident antibodies to a group of negatively charged phospholipids, and it is suggested that LA and anticardiolipin tests detect antibodies with overlapping specificities. Some discordance between the two assays has been described, however. One patient presenting with severe thrombotic disease (recurrent deep vein thrombosis, pulmonary embolism, inferior venocaval obstruction, myocardial infarction, and digital gangrene) showed strong LA activity in February 1987. An enzyme linked immunosorbent assay (ELISA) showed no binding to the negatively charged phospholipids cardiolipin, phosphatidylserine, and phosphatidic acid, but binding to zwitterionic phosphatidylethanolamine (PE) was demonstrated. Inhibition studies and affinity purification confirmed this finding. Interestingly, the serum did not bind to the kaolin cephalin clotting time reagent when used as antigen in an ELISA. The pathogenic significance of anti-PE antibodies and their relation to LA remains to be clarified. Further studies of the occurrence of anti-PE antibodies in patients with LA activity who have negative anticardiolipin tests are suggested.

Key words: antiphospholipid antibody.

There are convincing data to suggest a relation between the lupus anticoagulant (LA) and thrombosis, fetal loss, and thrombocytopenia.^{1 2} The term 'antiphospholipid syndrome' has been used to describe this particular group of patients,³ and the predictive value of high concentrations of anticardiolipin for these features has been demonstrated.⁴

Anticardiolipin test by radioimmunoassay has been performed since 1983,⁵ and approximately 90% of plasmas with LA activity bind to cardiolipin in an enzyme linked immunosorbent assay (ELISA). The LA and anticardiolipin antibodies need not bind to identical antigens, however. We describe

here a patient whose serum bound to phosphatidylethanolamine (PE) but not to negatively charged phospholipids, and whose LA activity was demonstrated by coagulation assays but not by an ELISA.

Case report

In February 1987 a 52 year old man was admitted to St Thomas's Hospital presenting with arterial insufficiency in the fifth toe. Previous medical history included several episodes of deep vein thrombosis in both legs in the last 20 years. In 1975, after stopping warfarin treatment, he suffered a pulmonary embolus; at this time an inferior venocavogram showed thrombotic changes in the common iliac system and inferior cava. In 1977 a myocardial infarction was diagnosed. He had been using warfarin regularly until that admission. Significant smoking and alcohol consumption were denied. Family history was unremarkable. Clinical examination showed col-

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Correspondence to Dr Munther A Khamashta, Lupus Research Unit, The Rayne Institute, St Thomas's Hospital, Lambeth Palace Road, London SE1 7EH, UK.

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lateral veins in the lower abdomen and signs of necrosis in the fifth toe. Cardiac, pulmonary, and neurological examinations were normal.

Investigations at this time included a negative antinuclear antibody, extractable nuclear antigen, and DNA binding test, normal complement, normal platelet count, and negative Venereal Disease Research Laboratory test. Antithrombin III concentration was normal. Protein C concentration was decreased (37 mg/l, normal range 82–146 mg/l). Antiphospholipid studies were carried out in our unit (see below).

Materials and methods

COAGULATION STUDIES

The lupus anticoagulant test was performed by the Exner test with modifications by Rosner *et al.*⁶ A kaolin cephalin clotting time (KCCT)⁷ and prothrombin time⁸ were also determined.

ELISA FOR ANTIPHOSPHOLIPID ANTIBODIES

Cardiolipin, phosphatidic acid, phosphatidylserine, and PE (egg and bovine brain) were purchased from Sigma Chemical Company (UK). Thrombafax (activated form) was purchased from Ortho Diagnostics (UK). Cardiolipin was dissolved in ethanol, and the other phospholipids were dissolved in methanol: chloroform (3:1) at final concentration of 50 µg/ml. Thrombafax was dissolved in phosphate buffered saline (1:8). Cardiolipin (30 µl) and 50 µl of other phospholipids were applied to each well. Thrombafax preparation (75 µl) was applied to wells coated with poly-L-lysine (concentration 100 µg/ml). IgG and IgM anticardiolipin standards were used as positive controls. An ELISA was performed as described by Gharavi *et al.*⁹

INHIBITION STUDIES AND AFFINITY PURIFICATION

Inhibition studies and affinity purification were performed as described by Harris *et al.*¹⁰ with minor modifications. In brief, 5 ml of patient serum was mixed with 5 mg/ml (PE) liposomes prepared as described previously¹⁰ and incubated at 37°C for one hour and at 4°C overnight. On the following day the mixture was centrifuged at 15 000 g for one hour and the precipitate separated. The precipitate was washed with phosphate buffered saline and the suspension centrifuged at 15 000 g for one hour. This process was repeated twice. After the third wash the precipitate was suspended in 1.5 M NaI, vortex mixed, and left to stand for 15 minutes. Then an equal volume of chloroform was added, vortex mixed, and allowed to stand. The aqueous layer was separated and dialysed overnight against phosphate

buffered saline. The final preparation was characterised by double immunodiffusion (Ouchterlony technique) using antisera to human IgG, IgM, IgA, and human serum albumin (Sigma Chemical Company, UK).

Results

COAGULATION ASSAYS

Table 1 lists the means (patients and controls) for prothrombin time, KCCT, kaolin clotting time (KCT), and the index of circulating anticoagulant. The presence of LA was detected by very prolonged KCCT and KCT and confirmed by a high index of circulating anticoagulant (meaning no correction of patient KCT by normal plasma) A raised prothrombin time resulted from warfarin treatment (8 mg daily).

BINDING TO DIFFERENT PHOSPHOLIPIDS IN AN ELISA

Figure 1 shows the IgM binding activity to PE, phosphatidic acid, phosphatidylserine, cardiolipin, and Thrombafax. Significant binding to PE was observed, whereas no significant binding to other phospholipids was detected. No IgG binding activity was demonstrated using these phospholipids as antigens.

INHIBITION OF IgM ANTI-PE ACTIVITY BY PE AND CARDIOLIPIN

Figure 2 shows the percentage inhibition of IgM anti-PE activity when serum was mixed with decreasing concentrations of PE and cardiolipin. PE caused more marked inhibition of IgM anti-PE activity than did cardiolipin.

AFFINITY PURIFIED IgM ANTI-PE ACTIVITY

Incubation of the patient's serum with PE liposomes, then precipitation of these liposomes by

Table 1 Coagulation results

	<i>Time (s)</i>	<i>Further details</i>
PT*		
Control	12	Therapeutic range for warfarin use: 23–41 s
Patient	26	
KCCT*		
Control	37	1:1 mixture: 71 s
Patient	82	
KCT*		
Control	70	1:1 mixture: 237 s
Patient	246	
Index of circulating anticoagulant 67.8 (normal range 0–15)		

*PT=prothrombin time; KCCT=kaolin cephalin clotting time; KCT=kaolin clotting time.

ultracentrifugation, washing, and elution of antibody bound to the liposomes, as described above, yielded an immunoglobulin preparation of the IgM isotype. Binding of this preparation to ELISA plates coated with PE was significantly higher than equivalent concentrations of human monoclonal IgM antibody preparation (Fig. 3). This preparation did not bind plates coated with cardiolipin or any other negatively charged phospholipids.

Fig. 1 IgM optical densities achieved when serial dilutions of the serum were tested to phosphatidylethanolamine (●), phosphatidic acid (▲), phosphatidylserine (△), cardiolipin (○), and Thrombofax (□).

Fig. 2 Inhibition of IgM anti-phosphatidylethanolamine activity by varying concentrations of phosphatidylethanolamine (●) and cardiolipin (▲).

Fig. 3 Optical densities achieved by affinity purified IgM anti-phosphatidylethanolamine antibody (○) and human IgM control (●).

Discussion

There is good evidence that LA is an antiphospholipid antibody.¹¹ Whether or not the antigenic determinants measured on LA and anticardiolipin test are the same, is still a matter of controversy. The great majority of LA positive patients have anticardiolipin activity by ELISA.¹¹ Both antibodies with LA activity and anticardiolipin antibodies cross react with negatively charged phospholipids.^{9 10 12 13} Antibodies that have been affinity purified using cardiolipin liposomes have been shown to have LA activity.^{10 13} On the other hand, our own and other groups have identified patients with LA activity who have negative anticardiolipin tests. In this report we describe a patient who had a strongly positive LA test but negative anticardiolipin test. Further study showed that this serum did not bind other negatively charged phospholipids but bound PE. That this binding was specific was shown by inhibition studies and by demonstration that anti-PE antibodies could be affinity purified with PE liposomes. Previous studies of plasma with LA activity¹¹⁻¹³ and of sera with cardiolipin binding activity¹⁰ have shown little or no binding to PE. Hence preferential binding of this patient's serum to PE compared with negatively charged phospholipids is unusual in our experience. Decreased protein C level may be related either to warfarin treatment or to congenital deficiency. The latter is a known cause of recurrent thrombosis,¹⁴ and its role in this case cannot be

ruled out. To date, no association between protein C deficiency and anti-PE antibodies has been described.

In summary, we describe a patient with recurrent thrombosis and unusual binding to PE; LA activity was detected in the absence of antibody binding activity to negatively charged phospholipids. We suggest that in those patients in whom recurrent thrombotic disease is thought to have an antibody mediated mechanism, but in whom anticardiolipin antibodies are absent, a search for antibodies to PE may be warranted.

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References

- 1 Harris E N, Gharavi A E, Hughes G R V. Antiphospholipid antibodies. *Clin Rheum Dis* 1985; **11**: 591–609.
- 2 Hughes G R V. Thrombosis, abortion, cerebral disease and the lupus anticoagulant. *Br Med J* 1983; **287**: 1088–9.
- 3 Hughes G R V. The anticardiolipin syndrome. *Clin Exp Rheumatol* 1985; **3**: 285–6.
- 4 Harris E N, Chan J K H, Asherson R A, *et al*. Thrombosis, recurrent fetal loss and thrombocytopenia: predictive value of the anticardiolipin antibody test. *Arch Intern Med* 1986; **146**: 2153–6.
- 5 Harris E N, Gharavi A E, Patel S P, Hughes G R V. Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in SLE. *Lancet* 1983; **ii**: 1211–4.
- 6 Rosner E, Pautzner R, Lusky A, Modam M, Many A. Detection and quantitative evaluation of circulating LAC activity. *Thromb Haemost* 1987; **57**: 144–7.
- 7 Proctor R R, Rapaport S I. The partial thromboplastin time with kaolin. *Am J Clin Pathol* 1961; **36**: 212–9.
- 8 Bowie E J W, Thompson J H, Didisheim P, Owen C A. *Mayo Clinic laboratory manual of hemostasis*. Philadelphia: Saunders, 1971: 61.
- 9 Gharavi A E, Harris E N, Asherson R A, Hughes G R V. Anticardiolipin antibodies: isotype distribution and phospholipid specificity. *Ann Rheum Dis* 1987; **46**: 1–6.
- 10 Harris E N, Gharavi A E, Tincani A, *et al*. Affinity purified anticardiolipin and anti-DNA antibodies. *J Clin Lab Immunol* 1985; **17**: 155–62.
- 11 Shapiro S S, Thiagarajan P. Lupus anticoagulant. *Prog Hemost Thromb* 1982; **6**: 263–8.
- 12 Thiagarajan P, Shapiro S S, de Marco L. Monoclonal immunoglobulin M lambda coagulation inhibitor with phospholipid specificity. Mechanism of a lupus anticoagulant. *J Clin Invest* 1980; **66**: 397–405.
- 13 Pengo V, Thiagarajan P, Shapiro S S, Heine M J. Immunological specificity and mechanism of action of IgG lupus anticoagulants. *Blood* 1987; **70**: 69–76.
- 14 Griffin J H, Evatt B, Zimmerman T S, Kleiss A J, Wideman C. Deficiency of protein C in congenital thrombotic disease. *J Clin Invest* 1981; **68**: 1370–3.