JCI The Journal of Clinical Investigation

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J Clin Invest. 1980;66(3):397-405. https://doi.org/10.1172/JCI109869.

Research Article

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Monoclonal Immunoglobulin MλCoagulation Inhibitor with Phospholipid Specificity

MECHANISM OF A LUPUS ANTICOAGULANT

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ABSTRACT Prolongation of all phospholipid-dependent coagulation tests was found in a patient with macroglobulinemia, despite absence of bleeding manifestations. The purified monoclonal IgMλ protein and its Fab_μ tryptic fragment induced similar changes in normal plasma. Patient IgM and Fab_μ completely inhibited Ca^{++}-dependent binding of radiolabeled prothrombin and Factor X to mixed phospholipid micelles. The patient's IgMλ paraprotein reacted with phosphatidylserine and, to a lesser extent, with phosphatidylinositol and phosphatidic acid, but not with phosphatidylcholine or phosphatidylethanolamine. Prior incubation of phospholipid with patient Fab_μ blocked the positive reactions. Substitution of washed platelets for phospholipid led to normalization of patient coagulation tests and corrected all abnormalities produced in normal plasma by patient IgM. Furthermore, binding of ^{125}I-Factor Xa to thrombin-treated platelets was entirely normal in the presence of patient IgM. These studies support the concept that platelets, rather than phospholipid micelles, are the primary locus of prothrombin and Factor X activation in normal hemostasis.

INTRODUCTION

In 1952, Conley and Hartmann (1) called attention to a coagulation inhibitor in patients with systemic lupus erythematosus (SLE).[1] Similar inhibitors have been described in a variety of diseases (2–5). The presence of such an inhibitor, to which the term "lupus anticoagulant" has been applied, is characterized by the impaired conversion of prothrombin to thrombin, most frequently reflected in the prolongation of prothrombin and partial thromboplastin times. The immunoglobulin nature of these anticoagulants has been demonstrated but their precise mechanism of action has not been delineated (2–5). Individuals with lupus anticoagulants rarely have deficiency of only a single coagulation factor, although hypoprothrombinemia has been reported occasionally (3), and no specific coagulation factor inhibition has been demonstrated for such anticoagulants.

We have studied a patient with macroglobulinemia and a lupus-type anticoagulant whose purified monoclonal IgMλ paraprotein possessed the anticoagulant activity. We have demonstrated that the mechanism of coagulation inhibition is the result of a specific immunologic reaction with acidic phospholipids.

METHODS

Purification of IgM. 7 ml of serum from the patient were applied to a 2.5 × 90-cm column of Sephadex G-200 equilibrated with a buffer consisting of 0.15 M NaCl and 0.02 M Tris, pH 7.4. The ascending limb of the void volume peak was pooled and tested by Ouchterlony double diffusion against antisera to IgM, IgG, IgA, fibrinogen, β-lipoprotein, and α_2-macroglobulin. The only impurity detected, α_2-macroglobulin, was removed by passage through a 2 × 15-cm column of Sepharose 4B-coupled anti-α_2-macroglobulin (goat antiserum obtained from Atlantic Antibodies, Westbrook, Maine). The effluent was concentrated by Amicon ultrafiltration using a PM10 membrane (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). Similarly, an IgMλ paraprotein was purified from the serum of another patient with macroglobulinemia whose coagulation tests were normal. Normal polyclonal IgM was prepared from 200 ml of serum by thorough dialysis against deionized water at 4°C for 48 h, by centrifugation of the precipitated protein, and by resus-

A preliminary report of this research was presented at the 22nd meeting of the American Society of Hematology, Phoenix, Arizona, and published in abstract form in 1979. *Blood.* **54**: 306A.

Received for publication 17 March 1980 and in revised form 20 May 1980.

[1] *Abbreviations used in this paper*: PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; RVV, Russell's viper venom; SLE, systemic lupus erythematosus.

pension in the column buffer followed by Sephadex G-200 gel filtration and ultrafiltration. This material was used only in some coagulation systems and was not passed through the anti-α_2-macroglobulin immunoadsorbent column.

Purification of prothrombin and Factor X. Human prothrombin and Factor X were purified from cryosupernate according to the method of Miletich et al. (6) with the following modifications. After barium citrate adsorption, ammonium sulfate elution, and quaternary aminoethyl-Sephadex chromatography, the eluate was dialyzed overnight at 4°C against a buffer consisting of 0.05 M imidazole, 0.001 M benzamadine, pH 7.0, and applied to a 2 × 30-cm column of dextran sulfate-Sepharose 4B (7) equilibrated with the same buffer. A 1-liter linear gradient of NaCl (0.0–1.0 M) was used to develop the column. Prothrombin elutes first (0.05 M NaCl), followed by Factor X (0.3 M NaCl) and then Factor IX (0.55 M NaCl).

The Factor X peak was pooled and dialyzed against 0.2 M potassium phosphate, pH 6.8, and applied to a 0.7 × 56-cm column of hydroxyapatite (Bio-Rad Laboratories, Richmond, Calif.). Factor X was eluted with 20 ml of 0.27 M potassium phosphate, pH 6.8, as described by Rosenberg et al. (8). The fractions containing Factor X activity were pooled, ultrafiltered to a concentration of 1–2 mg/ml, quick-frozen with acetone dry ice, and stored at −70°C. The preparation contained ~200 Factor X U/mg and was at least 95% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Factor Xa was prepared by incubation of Factor X for 15 min at 37°C with 1% (wt/wt) purified Factor X-converting enzyme from Russell's viper venom (RVV) in 0.15 M NaCl, 0.02 M Tris, and 0.007 M $CaCl_2$, pH 7.4. The purified Factor X-converting enzyme was a gift from Dr. B. Furie, Tufts University School of Medicine, Boston, Mass.

Tryptic hydrolysis of IgM. Tryptic cleavage of IgM was carried out at 65°C according to the method of Plaut and Tomasi (9). Tryptic cleavage at this temperature produces intact Fab_μ and $Fc_{5\mu}$ fragments. In a typical experiment, 10 mg of IgM in 6.6 ml of a pH 8.1 buffer consisting of 0.15 M NaCl, 0.05 M Tris, and 0.0115 M $CaCl_2$ was preincubated in a 65°C water bath until the temperature of the solution reached 65°C, at which time 0.5 mg of twice-crystallized *N*-α-*p*-tosyl-L-lysine chloromethyl ketone trypsin (Worthington Biochemical Corp., Freehold, N. J.) in 100 μl of water was added. At the end of exactly 7.5 min, the incubation mixture was cooled in an ice-water bath. The cleaved fragments were separated on a 2 × 90-cm Sephadex G-200 column using a buffer containing 0.05 M Tris and 0.15 M NaCl, pH 8.1. The Fab_μ and $Fc_{5\mu}$ peaks were pooled and concentrated against 20% polyethylene glycol (PEG 20,000; Fisher Scientific Co., Pittsburgh, Pa.). The concentrated peaks were immunologically pure when tested by Ouchterlony double diffusion against antibodies to Fc_μ and Fab (Atlantic Antibodies).

Phospholipid binding by prothrombin and Factor X. Ca^{++}-dependent binding of prothrombin and Factor X to phospholipid micelles was studied by the method of Gitel et al. (10), with some modifications: 12.5 mg of phospholipid (Centrolex P; Central Soya Co., Inc., Fort Wayne, Ind.) was suspended in 5 ml buffer (0.1 M NaCl, 0.04 M Tris, pH 7.4) in a glass ampule. The suspension was deaerated by bubbling with dry nitrogen for 40 min. The ampule was sealed, placed in a water bath, and the phospholipid suspension emulsified by sonication (Heat Systems Ultrasonics, Inc., Plainview, N. Y.) for 1 h, the temperature being maintained at 20–25°C by addition of ice to the bath. The resulting sonicate was centrifuged at 10,000 *g* for 20 min at 22°C and the supernate drawn off with a Pasteur pipette and used for binding experiments. A typical incubation mixture contained 300 μl phospholipid, 0.27 mg of cold prothrombin or Factor X in 300 μl of the same buffer, 20 μl of ^{125}I-prothrombin or ^{125}I-Factor X, 5 μg (in 5 μl) of purified antithrombin III, 5 μl (5 U) of heparin, and 400 μg (in 400 μl) of patient or control whole IgM or Fab_μ fragment. Presence of antithrombin III and heparin was necessary to prevent activation during the experiment. 10 μl of 0.25 M calcium chloride was added and the mixture incubated at 37°C for 15 min. A sample of this incubation mixture, usually 250 μl, was applied to a 1 × 30-cm column of Bio-Gel A 0.5 M, 200–400 mesh (Bio-Rad Laboratories) equilibrated with a buffer containing 0.1 M NaCl, 0.04 M Tris, and 0.005 M $CaCl_2$, pH 7.4. In each series of experiments, positive and negative binding controls were run, omitting any IgM, in buffers containing 5 mM $CaCl_2$ or 5 mM EDTA, respectively.

Immunodiffusion of IgM against phospholipids. Chromatographically pure phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidic acid (PA) dissolved in chloroform-methanol were obtained from Supelco, Inc. (Bellefonte, Pa.). 1 mg of each phospholipid was dried in a stream of dry nitrogen in a small glass ampule, suspended in 1 ml of buffer (0.15 M NaCl, 0.02 M Tris, pH 7.4), and bubbled with dry nitrogen for 40 min. The ampules were sealed and the phospholipids sonicated and centrifuged as described above. The supernates were diluted in the same buffer to a final absorbance at 320 nm of 0.066. These preparations were used for immunodiffusion studies in 1% agarose gel in a buffer containing 0.15 M NaCl and 0.08 M Tris, pH 7.4 (11). Patient and control IgM were used at a concentration of 1 mg/ml. In some experiments, patient Fab_μ was mixed with specific phospholipids before immunodiffusion against patient IgM. Precipitin lines were stained for protein with Coomassie Blue for phospholipid with Sudan black B.

Platelet-Factor Xa binding studies. Factor Xa binding to thrombin-treated platelets was studied according to the method of Miletich et al. (6), except that some incubation mixtures contained 1 mg/ml of patient or control IgM. Correction for nonspecific binding was done according to the methods of these authors. Purified human thrombin used in these experiments was a gift from Dr. John Fenton, New York State Department of Health Laboratories, Albany, N. Y.

Protein iodination. Prothrombin was iodinated by the iodine monochloride method, as described (12), to a specific activity of 1.3×10^6 cpm/μg. Factor X was iodinated as described by Miletich et al. (6) using the Bolton and Hunter reagent (Amersham Corp., Arlington Heights, Ill.). 12 μg of Factor X in 20 μl of pH 8.5 buffer containing 0.1 M borate were mixed with 1 mCi of nitrogen-dried reagent (1,500 Ci/mM) for 15 min in an ice bath. Unbound reagent was removed by gel filtration on a 0.5 × 5-cm column of Sephadex G-25 equilibrated with a pH 7.4 buffer containing 0.15 M NaCl and 0.02 M Tris, and further dialyzed against the same buffer. The specific activity of Factor X was 4×10^6 cpm/U, corresponding to ~1,000 cpm/ng.

Protein concentration was determined from absorbance at 280 nm and corrected for scattering as described (13), with the following $A_{280}^{1\%}$ values: prothrombin 13.6 (13), Factor X 11.6 (6), and IgM 11.8 (9).

Coagulation studies. Venous blood was collected into 0.1 vol of 3.8% trisodium citrate; plasma was obtained by centrifugation at 2,300 *g* at 4°C for 15 min. Routine coagulation studies and coagulation factor assays were done by conventional methods (14, 15). Taipan venom times were performed as described by Pirkle et al. (16), and *Echis carinatus* times were performed as described by Franza et al. (17). The pro-

TABLE I
Screening Coagulation Tests

Test	Clotting time Patient/control
	s
Prothrombin time	
Simplastin (rabbit brain + lung)	18.4/12.4
Permaplastin (rabbit brain)	19.4/14.2
Human brain	20.3/16.4
Partial thromboplastin time	
Thrombofax	96.0/61.4
+ kaolin	76.4/47.8
+ ellagic acid	85.3/40.1
Human brain extract	96.4/78.4
+ kaolin	48.4/42.6
+ ellagic acid	56.1/43.3
RVV time	12.8/5.4
Taipan snake venom time	27.9/15.9
Echis carinatus time	15.8/16.2
Thrombin time	17.8/18.2

thrombin-activating enzyme from *Echis carinatus* venom was purified as described by Franza et al. (18). Human brain extract was prepared according to the method of Bell and Alton (19). The following commercial reagents were used in coagulation assays: Thrombofax and activated Thrombofax (Ortho Diagnostics Inc., Raritan, N. J.); actin and Cephaloplastin (Dade Diagnostics, Inc., Miami, Fla.); Simplastin (General Diagnostics, Morris Plains, N. J.); RVV (Burroughs Wellcome & Co., Raleigh, N. C.); Taipan snake venom and *Echis carinatus* venom (Miami Serpentarium, Miami, Fla.).

Platelet-rich plasma was obtained by centrifugation of citrated venous blood at 180 *g* for 15 min at 22°C. The platelets were sedimented by further centrifugation at 22°C, 1,500 *g*, for 15 min. The supernate plasma was removed and the platelet pellet suspended in a buffer consisting of 0.15 M NaCl, 0.02 M Tris, and 1 mM EDTA, pH 7.4. The platelets were washed three times in the same buffer by sedimentation for 15 min at 1,200 *g* and finally suspended at a concentration of 200,000 platelets/μl in a buffer consisting of 0.15 M NaCl and 0.02 M Tris, pH 7.4, containing 1 mg/ml of glucose.

All coagulation factor assays, using either phospholipid or washed platelets, were performed using 1:5, 1:10, and 1:20 dilutions of patient or standard normal plasma. When phospholipid was used, patient plasma dilution curves were not parallel to the standard curve; assay results are based on the 1:20 dilution only.

Clinical summary. The patient was a 72-yr-old man in good health until March 1978 when he began to have episodes of epigastric distress and noted a 10-lb wt loss. He had no past history of spontaneous bleeding or excessive bleeding with surgery, and did not have a bleeding tendency at the time of diagnosis or throughout the course of his illness. On physical examination he had splenomegaly and hepatomegaly but no lymphadenopathy. His blood count showed: hemoglobin 11.8 gm/dl, platelets 235,000/μl, leukocytes 5,700/μl with 34% neutrophils, 8% bands, 47% lymphocytes, 8% atypical lymphocytes, and 3% monocytes. The bone marrow was hyperplastic and infiltrated with lymphocytes. There was an IgMλ paraprotein present in his serum, with a concentration ranging between two and three gm/dl during the course of the illness. He died 1.5 yr after the initial diagnosis. At autopsy he had disseminated lymphoma but no evidence of internal or external hemorrhage.

RESULTS

Coagulation tests. Results of the screening coagulation tests are shown in Table I. The prothrombin, partial thromboplastin, RVV, and Taipan snake venom times were prolonged, whereas the *Echis carinatus* and thrombin times were normal. All but the latter two tests have a requirement for phospholipid. The whole blood coagulation time was also normal. Prolongation of the prothrombin time was independent of the tissue or species source of tissue factor. Prolongation of the partial thromboplastin time was present both in activated and in nonactivated systems; this effect was less obvious with human brain extract. The bleeding time, platelet count, and platelet aggregation studies were entirely normal.

Addition of the patient's purified monoclonal IgM paraprotein to normal plasma reproduced all the coagulation abnormalities seen in the patient's plasma (Table II). Addition of control monoclonal IgM had no effect. Inhibition of coagulation tests could be achieved by addition to normal plasma of patient Fab_{μ} but not $Fc_{5\mu}$ (Table II). Neither the Fab_{μ} nor the $Fc_{5\mu}$ from the control monoclonal IgM had any effect. Approximately

TABLE II
*Effect of Addition of Patient and Control IgM and their Tryptic Fragments on Coagulation Tests of Normal Plasma**

Test	Buffer	IgM‡	Fab_{μ}‡	$Fc_{5\mu}$‡
		s		
Prothrombin time§	20.3	32.1/17.8	27.8/18.1	18.8/17.9
Activated partial thromboplastin time	33.2	81.4/30.1	63.1/27.8	29.8/29.3
Nonactivated partial thromboplastin time	72.4	116.4/74.2	98.4/73.7	68.4/76.4

* IgM, Fab_{μ}, and $Fc_{5\mu}$ added at concentration of 0.35 absorbance U/ml in the normal plasma.
‡ Patient purified protein/control purified protein.
§ Prothrombin time was performed with permaplastin diluted 1:3 with buffer.

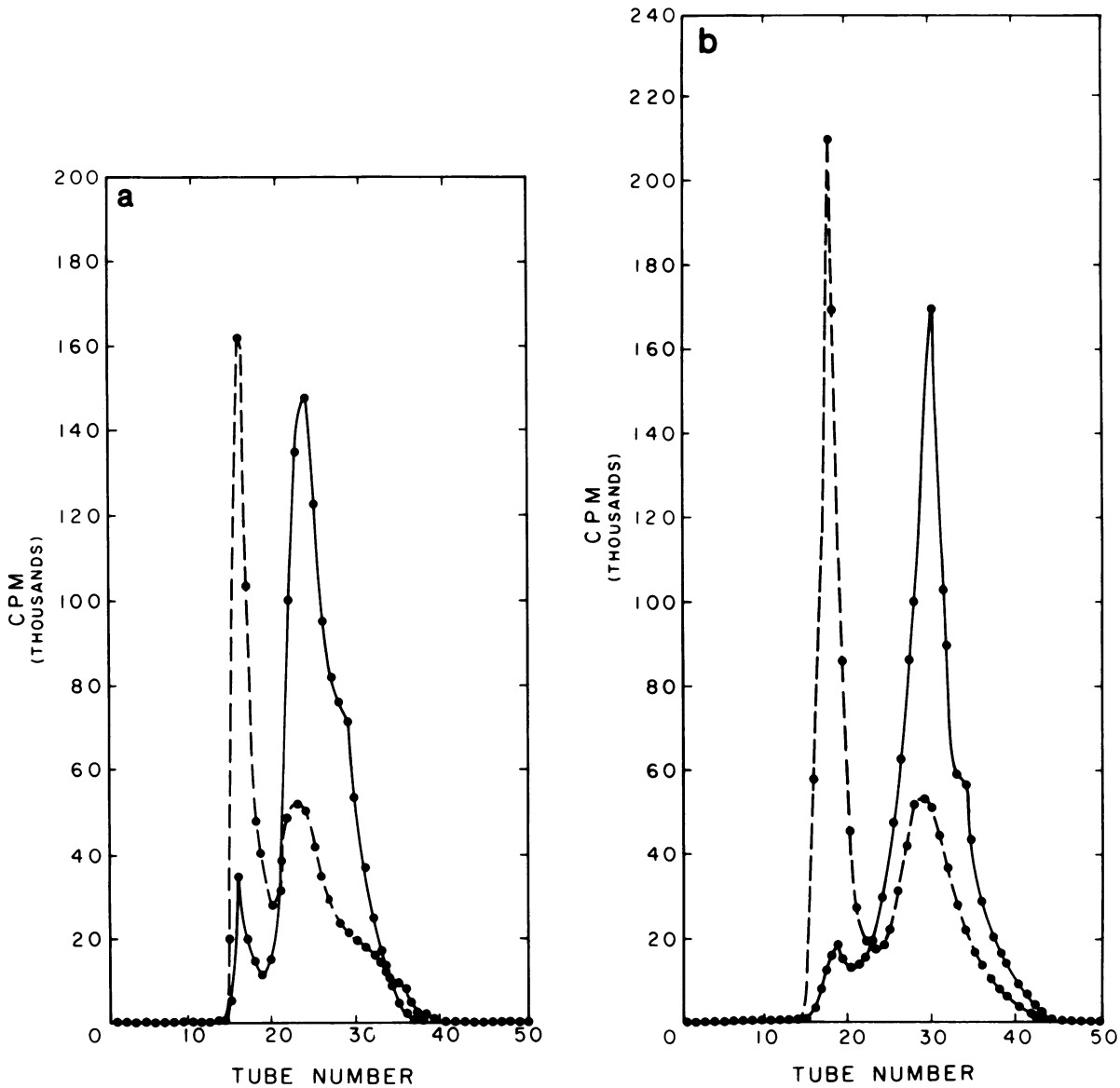

FIGURE 1 Effect of the Fab_μ fragment of patient and control IgM on the Ca^{++}-dependent binding of prothrombin and Factor X to phospholipid micelles. Cold prothrombin or Factor X, iodinated prothrombin or Factor X, Fab_μ and $CaCl_2$ were incubated with phospholipid at 37°C for 15 min in the presence of heparin and antithrombin III. The mixture was gel-filtered on Bio-Gel A 0.5 M. a, Prothrombin; b, Factor X; ——, patient Fab_μ; – – –, control Fab_μ.

equal concentrations of IgM and Fab_μ produced equivalent prolongations of coagulation tests. Because each pentameric IgM molecule contains 10 Fab_μ fragments, representing roughly half the total molecular weight, these data indicate that the isolated Fab_μ fragments are roughly half as potent as the intact IgM in coagulation tests.

Phospholipid binding of prothrombin and Factor X. In view of the inhibition of all phospholipid-dependent coagulation tests, we studied the effect of patient IgM and Fab_μ on calcium ion-dependent binding of prothrombin and Factor X to phospholipid micelles. Results for Fab_μ are illustrated in Fig. 1. In the presence of calcium ions, prothrombin and Factor X bind to phospholipid micelles and appear in the void volume of Bio-Gel A 0.5-M gel-filtration columns; in the presence of EDTA, the proteins do not interact with phospholipid micelles and appear instead in the included volume of the column. Patient IgM completely blocked the binding of radioactive prothrombin and Factor X to phospholipid micelles, whereas control monoclonal IgM had no effect. Similarly, in

the presence of patient Fab_μ (Fig. 1), binding of prothrombin and Factor X to phospholipid was almost completely blocked.

Immunologic specificity of patient IgM towards phospholipid. The immunologic specificity of patient IgM toward individual phospholipids is shown in Fig. 2A. A strong precipitin line is present against PS, PI, and PA, but no reaction is seen against PC or PE. Neither control monoclonal nor normal polyclonal IgM showed any reaction in this system. The positive precipitin reactions could be completely blocked by prior addition of patient Fab_μ to the phospholipids (Fig. 2B), indicating the immunologic nature of the interaction of patient IgM with the phospholipids. Patient Fab_μ itself does not produce a precipitin line with any phospholipids. Patient IgM showed no reaction against human prothrombin or Factor X when tested in immunodiffusion. When patient IgM and human prothrombin were incubated, no evidence of complex formation was detected by gel-filtration on Bio-Gel A 0.5 M.

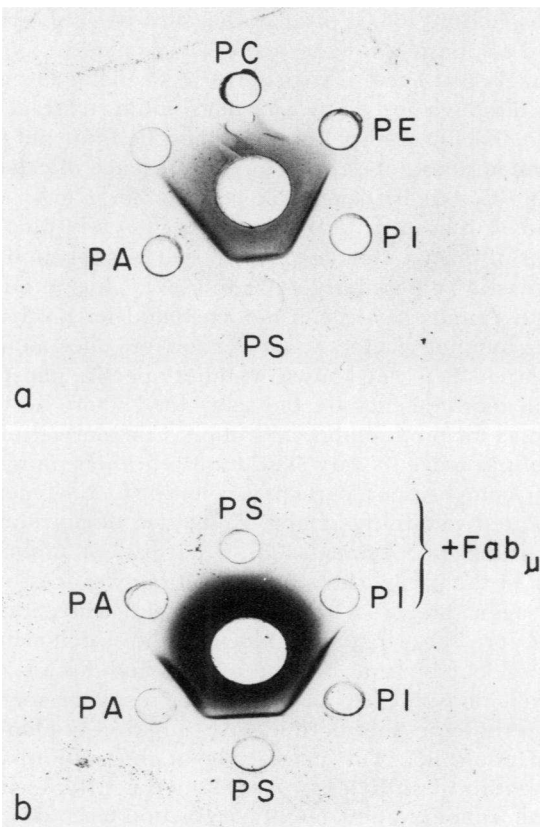

FIGURE 2 Immunodiffusion of IgM against phospholipids. The precipitin lines were developed for 24 h. Patient IgM is in the center well. a, PC, PE, PI, PS, and PA are in the peripheral wells. b, top, PS, PA, and PI were each mixed with patient Fab_μ before being added to the peripheral wells.

TABLE III

Comparison of Platelets and Phospholipid in Phospholipid-dependent Coagulation Tests

Test	Patient plasma	Control plasma
	s	
Partial thromboplastin time		
Phospholipid (thrombofax)	85.3	40.3
Normal platelets	48.2	46.0
Patient platelets	44.2	49.0
RVV time		
Phospholipid (thrombofax)	12.8	5.4
Normal platelets	7.9	7.2
Patient platelets	6.8	7.4
Taipan snake venom time		
Phospholipid (thrombofax)	27.9	15.9
Normal platelets	16.4	14.3
Patient platelets	—	—

Effect of platelets in coagulation systems. The patient's whole blood coagulation time and platelet-rich plasma recalcification time were normal. In addition, substitution of washed platelets for phospholipid resulted in normalization of all abnormal plasma coagulation tests (Table III). Similarly, apparent deficiencies of several coagulation factors disappeared when one-stage assays were performed using washed platelets instead of phospholipids (Table IV). Control congenitally deficient plasmas gave the same results when assayed either with phospholipid or platelets. Both normal and patient platelets were effective, as were also platelet preparations frozen and thawed five times.

Effect of patient IgM on Factor Xa-platelet interaction. Patient IgM had no effect on the binding of Factor Xa to platelets, the binding curve being superimposable with that found in the presence of control

TABLE IV

Specific Coagulation Assays on Patient Plasma Performed with Phospholipid or Platelets

	Phospholipid	Platelets
	U/ml	
Assay		
Factor VIII	0.38	2.16
Factor IX	<0.01	0.66
Factor XI	0.20	1.55
Factor X	0.06	0.70
Factor V	<0.01	1.00
Controls		
Factor VIII (hemophilia A)	<0.01	<0.01
Factor IX (hemophilia B)	<0.01	0.01
Factor X (congenital deficiency)	0.02	0.03
Factor XI (congenital deficiency)	<0.01	0.03

monoclonal IgM or buffer alone (Fig. 3). Consistent with these observations, high specific radioactivity patient Fab_μ did not bind to human platelets before or after thrombin treatment. Radioactive Fab_μ also did not react with sonicated platelet membrane preparations, even though chloroform-methanol extracts of such preparations did react with patient IgM in immunodiffusion systems.

DISCUSSION

Lupus anticoagulants have been reported in a variety of disease settings. Despite the abnormalities in coagulation tests, sometimes of a marked degree, the presence of a lupus anticoagulant, per se, is seldom associated with a bleeding tendency (1–5). In fact, thrombosis has been reported in at least 13 patients with lupus anticoagulants (20–22), one of whom has even been anticoagulated with warfarin sodium without any bleeding complications (20). The nature of the immunologic inhibition of coagulation tests is not known. It has been suggested by several investigators that the inhibitory activity might be directed toward the phospholipid component involved in prothrombin activation (1–5, 23), although no direct evidence for this concept has been adduced.

Antibodies to phospholipids and related substances are surprisingly common, although their precise immunochemistry is poorly understood (24). Antibodies to purified phospholipids have been raised in experimental animals; in many cases these antibodies cross-react with other phospholipids besides the immunizing species. In addition, many myeloma proteins derived from mineral oil-induced plasma cell tumors in BALB/c mice have phosphorylcholine specificity (25). Monoclonal IgM antibodies to phospholipids in patients with macroglobulinemia have been reported on at least three prior occasions (26–28), and a single case of an IgM paraprotein with specificity against phosphorylcholine has also been described (29). No coagulation studies were given in any of these cases. Polyclonal antibodies to cardiolipin occur during normal pregnancy (30), after vaccination (31), and in a variety of disease states (30). It is of great interest that perhaps as many as 20% of the patients with SLE have a biologically false positive serology and that a correlation between this finding and the presence of a lupus anticoagulant has been noted (2–5).

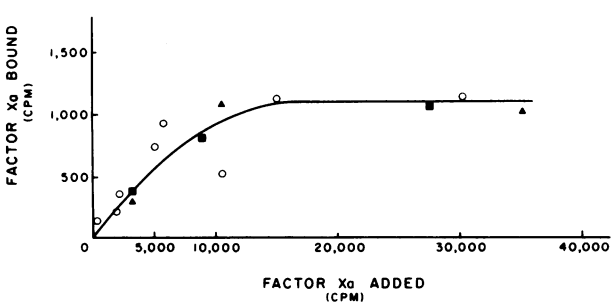

FIGURE 3 Effect of IgM on Factor Xa binding to platelets. Reaction mixtures contained 0.5 U/ml thrombin, 75 μg/ml prothrombin, 10^8 platelets/ml, varying concentrations 0.5–35 ng of ^{125}I-Factor Xa, and 1 mg/ml patient or control IgM in a Tris-NaCl buffer, pH 7.4, containing 5 mg/ml bovine serum albumin, 1 mg/ml glucose, and 2.5 mM $CaCl_2$. Binding was determined after 30 min incubation. ▲, patient IgM; ■, control monoclonal IgM; ○, buffer (no IgM).

The nature of the phospholipid involvement in coagulation has been the subject of controversy (32, 33). Our current concept is that negatively charged phospholipid micelles function as a surface upon which both the activation of Factor X by Factor IXa in the presence of Factor VIII and the activation of prothrombin by Factor Xa in the presence of Factor V can occur. Factor X and prothrombin each bind to negatively charged phospholipid micelles in a process with a complex requirement for calcium ions; γ-carboxyglutamic acid residues, whose presence in these proteins is dependent on vitamin K action, are required for Ca^{++}-binding and subsequent binding to phospholipid (34). Factor Xa has also been shown to bind to phospholipid by a similar mechanism (35). It is likely that Factor IXa behaves in an analogous manner, although no evidence for such binding exists at this time. Factor V also binds to phospholipid, but this reaction does not depend on the presence of calcium ions (35, 36). It is not known whether Factor VIII binds to phospholipid, although some early preparations of this clotting factor were rich in phospholipid, suggesting a possible interaction (37). Thus, minimal requirements have been demonstrated for prothrombin, but not Factor X, conversion on phospholipid surfaces. It is not known whether specific phospholipid requirements are the same for Factor X activation as for prothrombin activation. Commercial phospholipid reagents vary significantly in their phospholipid compositions, perhaps accounting for the recently reported variability among reagents in demonstrating lupuslike anticoagulants (38). In our patient, prolongation of the partial thromboplastin time was least with a reagent prepared from human brain. Our patient's IgM or Fab_μ fragment blocked the calcium ion-dependent binding of prothrombin and Factor X to mixed phospholipid micelles as a result of specific immunologic interaction with negatively-charged phospholipids. The magnitude of this abnormality is certainly sufficient to explain the prolongation of phospholipid-dependent coagulation tests. Binding of calcium ions, as a mechanism of the inhibition, seems unlikely in view of the inability of patient IgM to block the interaction of Factor Xa and platelets (see below), a reaction also requiring calcium ions.

Despite grossly abnormal in vitro phospholipid-

dependent coagulation tests, our patient did not have a bleeding tendency. A likely explanation for this apparent paradox is supplied by the observations that platelets, when substituted for phospholipids in these tests, supported normal coagulation in the presence of the patient's IgM inhibitor. Moreover, the patient's whole blood clotting time and platelet-rich plasma recalcification time were entirely normal.

Because phospholipids can substitute for platelets in prothrombin conversion and in various in vitro coagulation assays, it has been assumed that platelets promote prothrombin conversion by providing phospholipid at their surface. This activity has been referred to as platelet Factor 3 (39). Several lines of argument lead to questioning of this hypothesis. Marcus et al. (40) suggested that more than phospholipid was involved in platelet Factor 3 activity because 20 times more extracted platelet phospholipid was required to achieve the same clotpromoting activity as that produced by intact platelet membranes. Moreover, PS, the most active phospholipid in vitro, is apparently present largely or exclusively on the inner aspect of the platelet membrane (41). There is little evidence, in general, that "flip-flop" translocation of membrane phospholipids occurs at a significant rate (42, 43), and conflicting evidence suggests that platelet membrane PS becomes exteriorized in response to platelet aggregating agents (41, 44). Finally, Factor X and prothrombin, though they bind to negatively charged phospholipid micelles in vitro, have not been found to bind to platelet membranes (45, 46).

Nevertheless, the concept that the platelet membrane may serve as a surface upon which prothrombin activation occurs finds strong support in the work of Miletich et al., who have demonstrated the formation of a high affinity Factor Xa binding site on the surface of platelets (46). The receptor has the properties of activated Factor V and has a higher affinity for Factor Xa than the affinity of phospholipid micelles for Factor X (6). Receptor-bound Factor Xa can generate thrombin from prothrombin in the absence of added phospholipids. The in vivo significance of this mechanism is indicated by the finding by the same authors of a better correlation of the bleeding tendency in Factor V deficiency with platelet Factor V than with plasma Factor V (47), and also by the recent report of a patient with a hemorrhagic diathesis who has normal plasma and platelet Factor V but a deficiency of platelet Factor Xa binding sites (48). It appears that the sole requirement for formulation of the Factor Xa binding site is the activation of Factor V because Factor Xa is able to bind to intact (unstimulated) platelets in the presence of Factor Va (49). It is known that Factor Va can bind with high affinity to platelets (50) and prothrombin (51), and binding of Factor Va to Factor Xa on a mole-for-mole basis can be inferred from kinetic data. (52).

The nature of the phospholipid or phospholipid analog, if any, in the platelet membrane participating in the platelet-Factor Va-Factor Xa interaction is not clear. We have been unable to demonstrate any binding of highly radioactive patient Fab_{μ} to resting or thrombin-treated platelets or to platelet membrane preparations, even though chloroform-methanol extracts of such preparations do react with patient IgM in immunodiffusion. The Fab_{μ} was of high enough specific radioactivity to detect as few as 200 PS residues/platelet. Thus, the head group of PS may be relatively inaccessible in cell membranes even when the inner, PS-rich, membrane surface is exposed, a possibility also suggested by the inability of chemical probes such as 2,4,6-trinitrobenzenesulfonate to bind >50–60% of the PS present in erythrocyte membranes (53). The inaccessibility of phospholipid head groups in cell membranes is further suggested by the observations that neither animals nor humans with circulating antibodies to phospholipid polar head groups show any evidence of general cellular destruction or dysfunction, even when the antibody is directed against the head group of PC, a phospholipid known to be on the external surface of cell membranes (53).

The present case defines a new mechanism of action for a coagulation inhibitor. The anticoagulant mechanism delineated here is not restricted to this patient. Preliminary results in our laboratory on the plasmas from three patients with SLE or SLE-like illnesses and lupus-type anticoagulants have revealed the presence of anti-PS and anti-PA reactivity in their immunoglobulin fractions and correction of the plasma coagulation defect by platelets. Whether this mechanism is generally applicable to all lupus anticoagulants remains to be determined. Our observations provide an explanation for the dissociation of abnormal tests and normal in vivo hemostasis in our patient and perhaps in others, and give strong support to the central role of platelets in the coagulation mechanism. Nevertheless, they raise several fundamental questions: What, if any, is the role of phospholipid in coagulation in vivo? How do the γ-carboxyglutamic residues of the vitamin K-dependent coagulation factors, whose function is based entirely on the phospholipid micelle model but whose absence is associated with a bleeding tendency, operate in vivo? Why are phospholipid head groups partially or completely unavailable to some chemical and immunologic probes, despite the fact that the classical membrane model predicts that they would be available? We anticipate that the study of lupus anticoagulants may be useful for approaching some of these problems.

ACKNOWLEDGMENTS

We wish to thank Dr. Scott Murphy for bringing the patient to our attention, Miss Susannah McCord for expert technical

assistance, and Mrs. Jeanne Romanic for typing the manuscript.

This work was supported in part by grant HL 09163 and training grant HL 07371 (P.T.) from the National Institutes of Health, and by a grant from the Delaware Valley Chapter of the National Hemophilia Foundation.

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