Impaired Catalytic Function of Activated Protein C: A New In Vitro Manifestation of Lupus Anticoagulant

By Ewa Marciniak and Edward H. Romond

Lupus anticoagulant (LA), an antibody against anionic phospholipid with anticoagulant laboratory manifestations, is paradoxically associated with a high incidence of thrombosis. In the present study we analyzed the phospholipid- and platelet-dependent degradation of factor Va following clotting in plasma from 15 consecutive patients with LA to provide evidence for a distinct procoagulant effect of the antibody. After clotting with 25 μg phospholipid/mL, all samples containing LA showed markedly decreased rates of factor Va degradation (k = 0.01 to 0.14 min⁻¹ v 0.27 to 0.35 min⁻¹ in controls). Also with higher phospholipid concentrations (up to 100 μg/mL), as well as in the presence of platelets (5 to 33 × 10⁷/mL), significantly less of the procoagulant activity disappeared per unit of time in samples with LA than in controls. Plasma with LA was to a variable extent capable of decreasing or abolishing factor Va inhibition in normal plasma. Most importantly, exogenous activated protein C failed to correct the ineffective factor Va destruction despite adequate protein S levels. These data suggest that LA prevents the formation of the complex essential for rapid proteolysis of factor Va both on phospholipid and on the platelet membrane, thereby compromising the catalytic function of activated protein C. Our findings offer a new opportunity for a more comprehensive evaluation of patients with antiphospholipid antibody in defining the pathogenesis of thrombosis in this clinical condition.

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UPUS ANTICOAGULANT (LA), which appears in a variety of clinical conditions, has been defined as an antibody with affinity for negatively charged phospholipid micelles. The presence of this abnormal protein in plasma is usually detected in routine coagulation tests because it disturbs binding of vitamin K-dependent coagulation factors to phospholipid reagents preventing efficient formation of thrombin and delaying clotting in vitro. Paradoxically, this laboratory abnormality is clinically associated with a high incidence of thrombosis, both venous and arterial, while bleeding occurs only in rare cases complicated by hypoprothrombinemia or thrombocytopenia.

The relationship between LA and thrombosis remains unclear. It has been generally assumed that anticardiolipin activity of LA or related antibodies occurring simultaneously may affect adversely the endothelial cell membrane, thereby disturbing its antithrombotic properties such as production of prostacyclin or activation of protein C. However, the apparent role of LA in preventing binding of vitamin K-dependent proteins to anionic phospholipid suggests yet another possibility, namely, that of a direct interference of the antibody with factor Va degradation catalyzed by activated protein C.

Activated by thrombin, factor Va plays a key role in blood coagulation functioning as a nonenzymic cofactor for the activation of prothrombin by factor Xa. Degradation of factor Va is the fundamental end step in the regulatory function of protein C. However, rapid proteolysis of factor Va by activated protein C takes place only when both enzyme and substrate are bound to phospholipid, platelet, or endothelial cell surface. If this binding is diminished, protein C may no longer effectively control clot formation. It has been well established that the activation of protein C by thrombin complexed with thrombomodulin on the vascular surface of the endothelium plays a major role in the prevention of fibrin deposition in vivo. Whether and to what extent activation of protein C by free thrombin controls in vitro clotting is much less apparent. Studies on molecular factor V transformations implied, however, that the loss of factor V procoagulant activity normally occurring in clotted blood or plasma results solely from a partial in vitro conversion of protein C to the enzyme form. Consistent with this supposition is the finding that no significant factor Va usage occurs in serum from a child with homozygous protein C deficiency.

In this study we used the plasma system to investigate the possibility that LA interferes with the expression of anticoagulant properties of activated protein C, thereby limiting factor Va inactivation.

MATERIALS AND METHODS

Human protein C was purified as described by Suzuki et al except that the activation process was terminated in the absence of heparin using 30 μg of antithrombin III per 1 μg of thrombin. The concentration of activated protein C was based on the amount of protein C used for activation accepting E(1%, 1 cm, 280 nm) of 14.5. Phospholipid reagent was prepared according to the method of Bell and Alton. The desiccated material suspended in saline and ultrasonicated was stored at –30°C at 1.5 mg/mL concentration. Further dilutions of this stock suspension were made in 0.15 mol/L NaCl, 0.02 mol/L Tris, pH 7.5.

Blood was collected in one-tenth volume of 3.8% sodium citrate. Platelet-poor plasma was obtained by centrifugation at 4,000 x g for 10 minutes at room temperature. For platelet-rich plasma, blood was centrifuged at 150 x g for 15 minutes at room temperature. To isolate platelets, the platelet-rich plasma was centrifuged at 1,500 x g for 15 minutes and the platelets were washed twice with 0.15 mol/L NaCl, 0.02 mol/L Tris, 0.001 mol/L EDTA. The same buffer but without EDTA was used for the third wash and for the final suspension of platelets. Plasmas selectively deficient in factors XII, XI, and VII were obtained from George King Biomedical Inc. (Overland Park, KS). Plasmas selectively deficient in factors VIII and IX or protein C were obtained, respectively, from the regional blood bank.
population of hemophiliacs and from a child with homozygous protein C deficiency (cord blood) recently born in a previously described family.18

Amidase activity of activated protein C was estimated as published with Spectrozyme PCa, (American Diagnostica Inc, Greenwich, CT). Anticoagulant activity of activated protein C was measured in factor Xa clotting time prolongation test.15 Human antithrombin III19 and human α-thrombin20 were purified in the laboratory. The prothrombin time, activated partial thromboplastin time, and thrombin time were performed using, respectively, Thromboplastin C and Actin from Dade (Agudada, Puerto Rico) and thrombin from Armour Pharmaceutical Co (Kankakee, IL). Our normal ranges for these tests are 11 to 13 seconds, 26 to 31 seconds, and 10 to 14 seconds, respectively. Factor VIII coagulant activity was measured routinely by a one-stage assay. Protein C levels in plasma were determined by measuring the amidolytic activity generated during incubation of plasma with Southern Copperhead snake venom using Coatest protein C kit (Kabivitrum Diagnostica, Sweden). Total and free protein S levels were determined by Laurell rocket immunoelectrophoresis according to the method of Comp et al22 with reagents from American Diagnostica (Greenwich, CT). The tissue thromboplastin inhibition test (TTI) was performed according to the method of Schleider et al using a 1:500 dilution of thromboplastin. The presence of LA was diagnosed when the ratio of patient/control clotting time (TTI ratio) in the test was 1.3 or greater and the abnormal clotting time was not corrected by addition of normal plasma in equal volume. The factor V activity was determined by the clotting method in the Fibrometer coagulation timer. Factor V-deficient plasma was prepared according to Bloom et al.24 The sample to be tested was diluted in saline containing 1 U/mL of purified antithrombin III and incubated at room temperature for 5 minutes before the assay. Thereafter, a 50 μL aliquot of the diluted sample was mixed with 50 μL of the deficient plasma substrate and incubated at 37°C for 60 seconds. Clotting was initiated by the addition of 100 μL of thromboplastin C reagent (Dade). Standard curves were prepared with pooled normal human plasma. One unit of factor V was defined as the activity present in 1 mL of this pool. Intraassay and interassay coefficients of variation were 5.2% and 7.3%, respectively.

For evaluation of factor V activity during clotting the test plasma, 0.5 mL, was mixed with 0.5 mL of buffer (0.15 mol/L NaCl, 0.02 mol/L Tris, pH 7.5), or phospholipid reagent, or platelet preparation, and incubated at 37°C for 2 minutes. Subsequently, 0.5 mL of 0.025 mol/L CaCl2 was added. While the incubation continued at 37°C, aliquots of 100 μL were taken from the reaction mixture and diluted for evaluation of factor V activity. The clot that formed during incubation of the plasma mixture was removed mechanically with a small plastic rod. The first-order inhibition rate constant, k, for the decay of factor Va generated during clotting was calculated from the straight-linear portion of the semilogarithmic plot of activity versus time using the equation: \( \frac{d[VA]}{dt} = -(VA)k \).

Investigated plasma samples were from 15 consecutive patients (seven women, eight men) who between August 1988 and January 1989 were diagnosed positively for the presence of LA in our laboratory. These samples were referred to the laboratory for evaluation of a prolonged activated partial thromboplastin time. In all of them the thrombin time was normal, which excluded the presence of heparin. The concentration of factor VIII:C ranged from 55% to 139% of the control level. None of the patients at the time of diagnosis was receiving immunosuppressive medications or anticoagulant therapy. Their laboratory values are presented in Table 1.

Approval was obtained from the Institutional Review Board for these studies. All subjects who were approached to donate a blood sample were informed that the donation was for research purposes and that their privacy would be protected.

RESULTS

Factor Va inactivation in clotted plasma. The time course of changes in factor V activity associated with clotting of normal plasma and a representative plasma containing LA is illustrated in Fig 1. In both plasma samples clotting induced about a fivefold increase in factor V activity indicating formation of factor Va, which subsequently decayed in

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<th>Case No.</th>
<th>PT SEC</th>
<th>APTT SEC</th>
<th>TTI Ratio</th>
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<th>PS Total (%)</th>
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Normal range 11-13 26-31 1 67-140 66-135 57-120 0.27-0.33 0.31 0.28

Abbreviations: PC, protein C; PS, protein S; LAP, LA plasma; NP, normal plasma; PHL, phospholipid; PLT, platelets; PT, prothrombin time; APTT, activated partial thromboplastin time.

*Values of first-order factor Va inhibition rates (k) calculated as described in Materials and Methods after clotting of LAP or LAP/NP mixtures in the presence of PHL or PLT suspension.
apparent first order fashion. After clotting with calcium alone, the factor Va inactivation rate in platelet-poor plasma was very slow, and more than 1 U of activity still remained after 50 minutes. Addition of platelets or a small amount of phospholipid to normal plasma before recalcification accelerated factor V activation and markedly enhanced the rate of factor Va destruction. In contrast, the inactivation rate of factor Va in plasma with LA was not significantly altered by identical amounts of phospholipid or platelets. Under similar experimental conditions all of the remaining plasma samples diagnosed positively for the presence of LA showed more or less ineffective rates of factor Va inactivation (Table 1). However, no strong correlation between the suppression of factor Va inhibition and the prolongation of clotting time in the TTI test was found (Fig 2).

Effect of exogenous activated protein C on residual factor Va activity. A question to be answered was whether LA as an antibody with reactivity toward anionic phospholipid interferes directly with the proteolysis of factor Va by activated protein C or whether, by delaying formation of thrombin, it simply prevents activation of protein C. Since the amount of protein C activated during clotting cannot be directly measured, we investigated the effect of exogenous activated protein C added to samples of clotted plasma at the time when factor Va had achieved its peak activity. In the case of normal plasma clotted in the absence of phospholipid, exogenous activated protein C mixed with phospholipid induced an equally rapid inactivation of factor Va as that seen with phospholipid alone, suggesting that endogenous enzyme had been generated in sufficient quantity (Fig 3). As expected, purified activated protein C without phospholipid had no apparent effect on factor Va inactivation under these conditions. In the case of LA plasma, significant reduction of factor Va was not observed after addition of either phospholipid or exogenous activated protein C-phospholipid mixture.

To demonstrate that the 1.5 mmol/L concentration of activated protein C, clearly ineffective in the presence of LA, can otherwise provide for complete destruction of factor Va generated during clotting, we used plasma from a newborn child with homozygous protein C deficiency. As illustrated in Fig 4, factor Va remained stable at a high activity level following clotting of this plasma with calcium and phospholipid. A normal decay of the procoagulant activity occurred on addition of activated protein C to serum at 0.75 mmol/L and 1.5 mmol/L concentrations. Even smaller quantities of the enzyme distinguishably enhanced factor Va consumption although a normal rate of inhibition was not achieved. These observations indicate that activation of about 50 to 100 ng (1% to 2%) of protein C per milliliter of plasma will assure a complete and rapid inactivation of factor Va after clotting initiated in the presence of phospholipid.

Effect of LA plasma on phospholipid- and platelet-enhanced factor Va inactivation in normal plasma. It was our initial prediction that when LA is present in plasma, the rate of inactivation of factor Va will depend both on the activity of the antibody and on the quantity of phospholipid or platelets available. This prediction was first confirmed by studying factor Va destruction in mixtures of normal plasma and LA plasma at a constant phospholipid concentration. As shown in Fig 5, the normal k value for factor Va inhibition decreased by more than one-half when the investigated LA plasma constituted only 5% of the mixture. When the abnormal plasma constituted 50% of the mixture, the phospholipid-enhanced inhibition of factor Va was abolished. All 15 samples with LA were clearly inhibitory, suppressing the phospholipid-enhanced rate of factor Va decay when mixed...
with normal plasma in equal volumes (Table 1). With eight of these samples (cases 1 through 8) the value of k in the mixture was equal to, or even less than, that in LA plasma alone, while with the other seven, factor Va inactivation was less severely impaired. These differences between samples apparently reflect individual variations in LA activity.

In five patients with LA we determined the rate of platelet-dependent factor Va decay using 1:1 mixtures of their platelet-poor plasma and normal plasma with a platelet count of $6.6 \times 10^9$/mL. In all these cases factor Va decayed much less rapidly than in the control consisting of the platelet-rich and platelet-poor normal plasma mixture (Table 1).

**Quantitative requirements for phospholipid and platelets.** We next studied the dependence of factor Va inhibition on the concentration of phospholipid and platelets facilitating the reaction (Fig 6). In normal plasma a steady increase in k values was observed with increasing concentrations of phospholipid, from 0 to 100 µg/mL. In contrast, low concentrations of the reagent (up to 25 µg/mL) failed to enhance the inhibition in the LA sample while at higher concentrations only a modest acceleration occurred. In parallel experiments with platelets, similar results were obtained.

A high concentration of phospholipid (100 µg/mL) failed to enhance the rate of factor Va inactivation to a considerable degree in six of the 15 LA samples tested (Fig 7). These were the samples (cases 1 through 6 in Table 1) that also more strongly inhibited factor Va consumption in normal plasma. In the other nine cases, phospholipid at high concentration had a marked stimulatory effect although the k values for factor Va inhibition still remained below the normal range. As we predicted, the rate of factor Va decay in LA plasma falls as the activity of the antibody rises and increases concomitant with the availability of the supportive surface provided by phospholipid or platelets.

**Factor Va inactivation in coagulation factor deficiencies.** To further document that our findings were specific for LA activity, we studied plasma with coagulation factor deficiencies in which abnormal inactivation of factor Va might have been expected. In selective deficiency of factors XII and XI, the decay of factor Va occurred at a normal rate despite a markedly delayed clotting enhanced by phospholipid (25 µg/mL). Normal factor Va inactivation was also recorded in factor VII deficiency and in patients with mild
hemophilia A and B (factor VIII or IX from 0.12 to 0.22 U/mL). Abnormally slow degradation was seen after clotting in severe hemophilia and factor X deficiency, but normalized promptly with exogenous activated protein C. In the case of patients treated with coumadin, normal plasma corrected both impaired clotting and subsequent loss of factor Va. Exogenous activated protein C in this situation was less effective in normalizing factor Va inhibition, presumably due to associated deficiency of protein S.

DISCUSSION

Our study demonstrates under relatively simple and well-defined conditions a new and specific activity expressed to a variable degree in every plasma diagnosed positively for the presence of LA. The activity prevents inhibition of factor Va on a membrane-like surface and may therefore support a state of increased clottability of blood.

The fact that the procoagulant activity of factor V disappears rapidly from serum has been known for many years. As shown more recently, this phenomenon results from the cleavage of the 110 Kd factor Va polypeptide by a small amount of activated protein C generated during clotting in vitro. The amount of activated protein C formed in clotted blood and, as shown in this study, necessary for rapid factor Va use in clotted plasma, corresponds to the activation of only 2% or less of protein C normally available. In such small quantities the enzyme cannot be detected and evaluated by

![Graph 1](image1)

**Fig 6.** Dependence of the first-order inhibition rates of factor Va on phospholipid and platelet concentrations during clotting. ■, normal plasma; □, LA plasma from case no. 5.

![Graph 2](image2)

**Fig 7.** The effect of phospholipid concentration on factor Va inhibition rates in 15 plasma samples with LA. Shaded area is the range obtained with seven normal plasma samples.
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direct assay methods. Limited in vitro protein C activation is most likely caused by ionized calcium which inhibits substrate recognition by free thrombin. One can speculate that, if a defect in the clotting system occurs, including the one induced by LA, the activation of protein C might decrease further and the degradation of factor Va in serum will then be impaired. On the other hand, incomplete activation of factor V due to a scarcity of thrombin could also contribute to increased factor V stability in serum. Our findings from analyses of coagulation factor deficiencies suggest, however, that impaired factor Va use after clotting takes place only in severe disorders associated with a virtual absence of factors VIII, IX, or X in blood. Other defects, such as factor XI or XII deficiency, despite remarkably prolonged clotting times, do not restrict factor Va inhibition. Regardless of the enzyme-substrate concentration, activated protein C as a vitamin K-dependent protein must interact with a membrane surface to accomplish factor Va proteolysis. An additional vitamin K-dependent protein, protein S, serves as a cofactor for the high-affinity interaction of activated protein C with these surfaces.

The above considerations form a context for interpreting the results of our study, which demonstrated an adverse effect of LA on the inactivation of factor Va during clotting supported by phospholipid or platelets. Similar to the well-known anticoagulant manifestations of LA, this newly described activity of the antibody is most clearly seen when phospholipid or platelets are used at a minimal concentration required for stimulatory effects. Although under such conditions LA strongly inhibits binding of factor X and prothrombin to the phospholipid micelles, there are some indications from our study that insufficient formation of prothrombinase does not necessarily alter protein C activation. We have shown that normally the absence of phospholipid and platelets during clotting will not obviate generation of the activity capable of destroying factor Va once a proper surface is provided. The activation of protein C as a process independent of a catalytic surface may thus remain unaffected by LA.

Most importantly, however, we provided evidence that exogenous activated protein C does not catalyze degradation of factor Va in samples containing LA. This occurs with adequate levels of protein S in these samples. Evidently LA compromises the surface-dependent function of activated protein C. Thus the antibody termed LA has not only anticoagulant properties but is also capable of inducing a strong procoagulant effect by supporting and stabilizing in blood an activated clotting factor otherwise marked for destruction. Such in vitro behavior is consistent with the definition of LA as an antibody that blocks the catalytic surface for calcium-mediated binding of vitamin K-dependent proteins including coagulation enzymes as well as activated protein C. However, only further studies on molecular changes occurring in factor Va and protein C during their activation and subsequent interaction can definitively resolve the issue of increased stability of factor Va in the presence of LA in blood.

We have further shown that LA, believed to react specifically with anionic phospholipid, strongly prevents factor Va inhibition supported not only by the phospholipid vesicle but also by the platelet surface. Whether immunoglobulins with LA activity do interact directly with the platelet membrane remains undetermined. The substitution of platelets for phospholipid reagent usually leads to normalization of clotting tests in plasma containing LA. Our data, however, provide evidence that in this type of plasma native or isolated platelets are in most cases as ineffective as phospholipid in carrying out factor Va inhibition. A discrepancy between the effects of the antibody on catalytic phenomena occurring on the platelet membrane as opposed to those taking place on phospholipid vesicle perhaps results from platelet-induced structural alterations of factor Va. These changes tend to stabilize factor Va and may offset the effect of LA on prothrombinase formation but will only further deter the destruction of factor Va procoagulant activity by activated protein C.

Our in vitro finding that LA interacts with platelets in preventing factor Va inhibition raises an interesting possibility, namely, that a similar situation may occur in vivo involving cell surfaces that support the catalytic function of activated protein C. In addition to platelets, vascular endothelium has been found to promote cleavage of factor Va by the activated protein C-protein S system. It would be of interest to demonstrate whether or not LA may interfere with this endothelial support. The persistence of factor Va in blood despite adequate levels of activated protein C would create a rather unique hypercoagulable condition. Such a condition may, in part, account for an increased risk of thrombosis in patients with LA.

The data in this report offer a new opportunity for a more comprehensive laboratory evaluation of patients with antiphospholipid antibodies. Future studies correlating these laboratory findings with the clinical status of the patient may provide a new insight into the pathogenesis of thrombosis associated with the presence of LA in blood.

ACKNOWLEDGMENT

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Impaired catalytic function of activated protein C: a new in vitro manifestation of lupus anticoagulant

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