



Advances in Basic, Laboratory and Clinical Aspects of Thromboembolic Diseases*

LUPUS ANTICOAGULANTS AND THROMBOSIS: THE ROLE OF PHOSPHOLIPIDS

NAOMI L. ESMON,*^o MIKHAIL D. SMIRNOV,* CHARLES T. ESMON*^{o#@}

*Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation; Departments of ^oPathology, [#]Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, and [@]Howard Hughes Medical Institute, Oklahoma City, Oklahoma, USA

ABSTRACT

Background and Objective. Lupus anticoagulants (LAs) are loosely defined as immunoglobulins that inhibit phospholipid dependent coagulation assays. Antiphospholipid antibodies (APAs) are those immunoglobulins that are observed to bind to phospholipids, usually cardiolipin, in ELISA type assays. Interest in these antibody populations derives from the observation that rather than being associated with bleeding disorders as would be expected, they correlate with an increased risk of thrombosis. Many mechanisms have been proposed to account for the prothrombotic activity of some LAs and APAs. These mechanisms are as diverse as inhibition of the production of endothelial prostacyclin synthesis or impaired fibrinolysis to interaction with β 2-glycoprotein I or prothrombin bound to phospholipids. For the purposes of this review, we would like to focus on a potential mechanism that has been proposed by several labs in addition to our own, namely inhibition of the protein C anticoagulant pathway.

Information sources. The authors have been working in this field and contributing original papers. In addition, the material examined in the present paper includes articles published in journals covered by the Science Citation Index[®] and Medline[®].

State of art and Perspectives. In general, correla-

tion of phospholipid specificity and thrombosis has not been performed on a large scale. We were therefore led to ask two questions. Are the membrane requirements of the protein C anticoagulant pathway really the same as those for the procoagulant complexes? Secondly, if they are not, do the membrane requirements of the anticoagulant complexes mimic those of the thrombotic LAs? The membrane requirements for the activated protein C anticoagulant complex differ from those of the prothrombinase complex. These requirements, i.e. the need for phosphatidylethanolamine for optimal activity, mimic the lipid requirements for at least a population of lupus anticoagulants associated with thrombosis. These observations may provide both the specificity and the link between the activated protein C pathway, lupus anticoagulants and thrombosis. Of course, no conclusion is ever that black and white. Only future studies into the fine specificity of lupus anticoagulants and anti-phospholipid antibodies associated with thrombosis will bear out the hypothesis that those directed towards the activated protein C pathway will be predictive of thrombotic risk.

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Key words: phospholipids, lupus anticoagulant, thrombosis

Lupus anticoagulants (LAs) are loosely defined as immunoglobulins that inhibit phospholipid dependent coagulation assays. Anti-phospholipid antibodies (APAs) are those immunoglobulins that are observed to bind to phospholipids, usually cardiolipin, in ELISA type assays. Depending on the laboratory, protein cofactors such as β 2-glycoprotein 1 or prothrombin, are also included in these binding assays. Interest in these antibody populations derives from the observation that rather than being associated with bleeding disorders as would be expected, they correlate with an increased risk of thrombosis. Exactly how they are associated has been difficult to determine for several reasons. First, there is not a direct correlation between LA

activity and phospholipid binding activity, there being approximately a 60% concordance between the presence of LA and APA in a single plasma.¹ Second, several different assays can be used to identify and confirm the presence of LAs in a plasma.¹ In general, a particular plasma does not result positive for LA activity in all the assays employed.² This may be due to the different lipids or concentration of lipid used in the assays, or the factors to which the assays are most sensitive. In other cases, the reasons for discrepancy are not always clear.

Many mechanisms have been proposed to account for the prothrombotic activity of some LAs and APAs. These mechanisms are as diverse as inhibition of the production of endothelial prostacyclin

Correspondence: Naomi L. Esmon, Ph.D., Oklahoma Medical Research Foundation, Cardiovascular Biology Research, 825 N.E. 13th Street, Oklahoma City, Oklahoma 73104, USA. Tel. international +1.405.2717399, Fax. international +1.405.2713137.

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synthesis³ or impaired fibrinolysis⁴ to interaction with β 2-glycoprotein I⁵⁻⁷ or prothrombin^{7,8} bound to phospholipids. For the purposes of this review, we would like to focus on a potential mechanism that has been proposed by several labs in addition to our own, namely inhibition of the protein C anticoagulant pathway. There have been several reports in the literature in which antibodies have been found in thrombotic patients that react with various proteins involved in the protein C pathway.⁹⁻¹⁵ It is not difficult to understand how antibodies to the proteins which interfere with this activity might be prothrombotic. These antibodies could be directed to neoepitopes on the proteins induced by interaction with phospholipid. However, this would not explain how immunoglobulins that appear to interact with phospholipid alone, or are defined by an activity in clotting assays in which the protein C pathway is not active, could lead to hypercoagulability. In looking for clues, it is useful to consider the background on phospholipid involvement in coagulation and what is known about the interaction of LAs, APAs and phospholipids.

Coagulation complexes are known to assemble on negatively charged phospholipids.¹⁶ It has long been held that phosphatidylserine (PS) is the best lipid for the binding of these proteins. However, this is based mainly on functional studies and binding measurements of components of the prothrombinase complex as a model for all coagulation complexes. In addition, some phospholipids, such as phosphatidylethanolamine (PE), have not been studied to any great extent because of their non-ideal properties. The reported reactivities of LAs and APAs are not in complete agreement. The standard test for APAs has been reactivity with cardiolipin \pm β 2-glycoprotein I.¹⁷ LAs have been reported to react with naturally derived PS, phosphatidylinositol or phosphatidic acid, but not phosphatidylcholine (PC) or PE by Thiagarajan *et al.*¹⁸ Alternatively, they have been reported to react best with PE,¹⁹ with only PE²⁰ or only with PE in the hexagonal II phase.²¹ Normally, PE is not present on the outside leaflet of natural membranes; however, it can be found on thrombin stimulated endothelial cells²² and up to almost 40% on activated platelets.²³ Hexagonal II phase lipid can be formed when microparticles vesiculate off cell membranes.

In general, correlation of phospholipid specificity and thrombosis has not been performed on a large scale. We were therefore led to ask two questions. Are the membrane requirements of the protein C anticoagulant pathway really the same as those for the procoagulant complexes? Secondly, if they are not, do the membrane requirements of the anticoagulant complexes mimic those of the thrombotic LAs?

Membrane requirements of the coagulation complexes

When various phospholipids were added to liposomes already containing 20% PS, the composition considered to be optimal for binding and function of the coagulation proteins, it was found that the ability of activated protein C to inactivate factor Va was considerably enhanced (Figure 1).²⁴ Of those that enhanced this activity, PE was found to be the best. Further studies indicated several features (Figure 2). First, there was a dose dependent effect of PE on APC function. Second, it was clear that compared to the activity observed when 40% PE is included in the vesicles, the standard 20%PS:80%PC vesicles are essentially inactive. In contrast, the effect of adding 40% PE to prothrombinase reaction mixtures was slight. The anticoagulant activity in plasma was also dramatically affected by the presence of PE in the membrane (Figure 3), i.e. vesicles devoid of PE showed minimal activity when compared to those containing PE.

Membrane preferences of lupus anticoagulants and the protein C system

It would thus appear that the anticoagulant complexes do indeed have different membrane requirements for optimal activity from those of the procoagulant complexes. The question then becomes, are these differences reflected in LA plasma (Figure 4)?

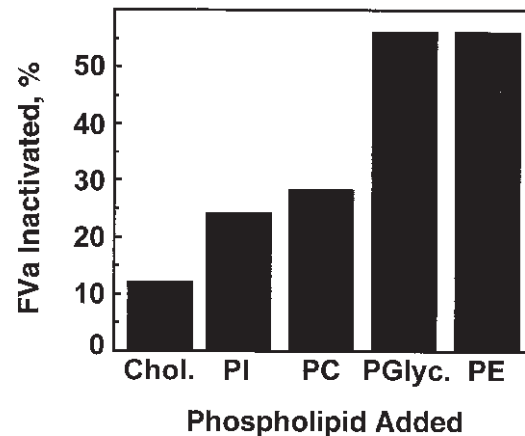


Figure 1. The effect of phospholipid vesicle composition on activated protein C activity. The ability of activated protein C to inactivate factor Va was compared on phospholipid vesicles of varying composition. Inactivation proceeded for 30 min at 25°C and the mixtures contained 7 pM factor Va, 117 pM APC, 10 μ g/mL protein S and 1 μ g/mL phospholipid. Vesicles contained 60% PC, 20% PS and 20% of the additional lipid indicated. Chol., cholesterol; PI, phosphatidylinositol; PGlyc., phosphatidylglycerol. Residual factor Va activity was monitored by its activity in a prothrombinase assay as described.²⁴ A similar PE effect was observed when the factor Va concentration was increased as much as 1000-fold. Bovine proteins were used in the experiment shown.

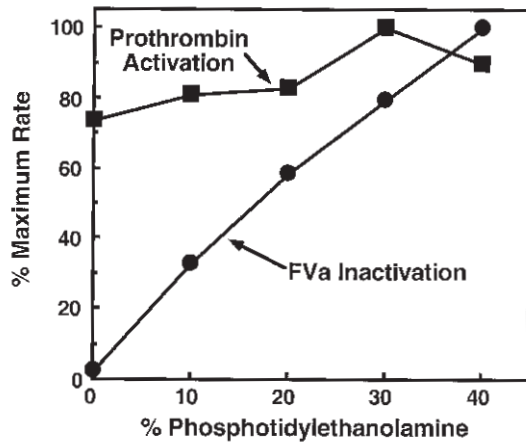


Figure 2. The influence of liposome PE content on prothrombin activation and factor Va inactivation by APC. All vesicles contained 20% PS, the % PE indicated on the abscissa and the remainder made up with PC. Factor Va inactivation was measured as described in Figure 1. Prothrombinase reactions contained 1.4 μM prothrombin, 20 pM factor Xa, 7 pM factor Va and 4 $\mu\text{g}/\text{mL}$ phospholipid and proceeded for 5 minutes at 25°C. Bovine proteins were used in the experiment shown.

Although the clotting time under normal conditions was somewhat prolonged (because it is lupus anticoagulant plasma), there was a much more dramatic effect when APC was present in the clotting assay. This was only observed when PE was included in the phospholipid vesicles. Additional studies²⁵ of a panel of LA plasma from thrombotic patients indicated that the degree of inhibition observed on the anticoagulant pathway, i.e. when APC was included in the assays, was dependent on the presence of PE in all cases and independent of the apparent LA titer. This can be directly traced to the fact that in normal plasma APC abolishes prothrombin activation, whereas in LA plasma the lag until significant thrombin is produced is somewhat delayed but by no means stopped.²⁵ In studies using purified reagents, although the production of thrombin could be inhibited as much as 50% by immunoglobulin purified from this plasma when PE was present in the membrane vesicles, APC activity was inhibited > 90% (Figure 5).²⁶ Clinically, when patients are reduced to 50% prothrombin with oral anticoagulants they are not yet therapeutically anticoagulated.²⁷ However, patients with 50% or less protein C are at significant risk for thrombosis.²⁸

Conclusions

In conclusion, the membrane requirements for the activated protein C anticoagulant complex differ from those of the prothrombinase complex. These requirements, i.e. the need for phos-

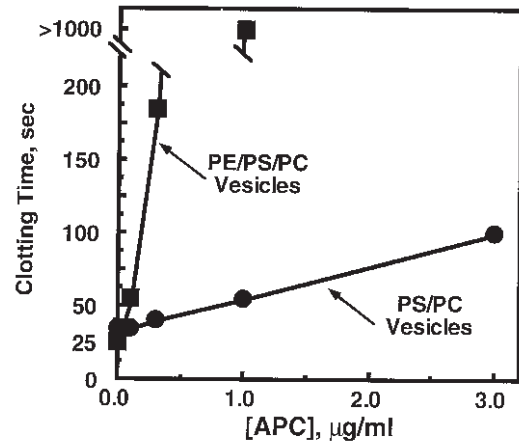


Figure 3. The effect of PE in liposomes on plasma clotting assays. Vesicles containing 20% PS, 80% PC (30 $\mu\text{g}/\text{mL}$) or 40% PE, 20% PS, 40% PC (8 $\mu\text{g}/\text{mL}$) were used in the one-stage clotting assay in the presence or absence of varying concentrations of human APC. Different phospholipid concentrations were necessary for the starting clotting times in the absence of APC to be similar. Mixtures contained 6 ng/mL factor X-activating enzyme from Russell's viper venom and a standard human plasma pool. Clotting was initiated by the addition of CaCl_2 .

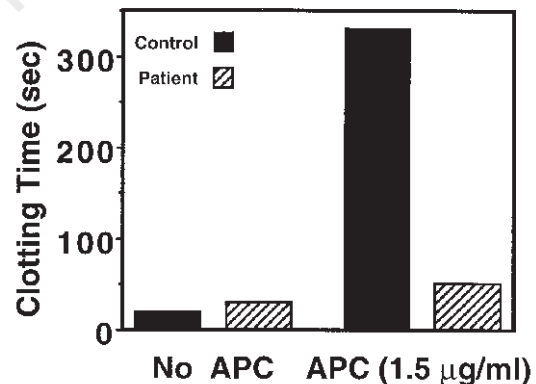


Figure 4. Influence of LAs on APC anticoagulant activity. Normal (solid bars) or lupus anticoagulant (hatched bars) plasma was clotted in the presence or absence of APC. Vesicles containing 40% PE were used in all assays.

phatidylethanolamine for optimal activity, mimic the lipid requirements for at least a population of lupus anticoagulants associated with thrombosis. These observations may provide both the specificity and the link between the activated protein C pathway, lupus anticoagulants and thrombosis. Interestingly, Berard *et al.*²⁹ recently reported a high incidence of thrombosis or recurrent fetal loss in patients in whom anti-PE is the sole anti-phospholipid detected.

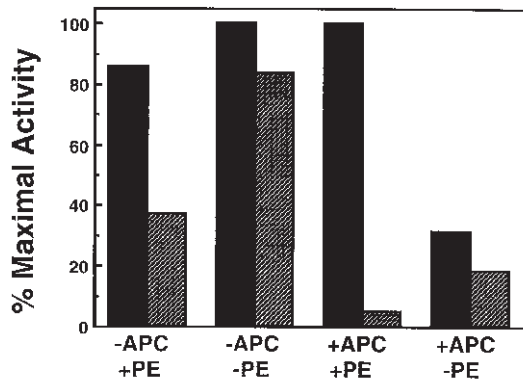


Figure 5. The effect of a purified lupus anticoagulant immunoglobulin on the activation of prothrombin and inactivation of factor Va on vesicles of different phospholipid composition. Phospholipid vesicles either did or did not contain 40% PE in addition to 20% PS as indicated. Prothrombin activation reactions (-APC) consisted of 1.4 μ M prothrombin, 0.2 nM Va, 2 nM Xa and 40 μ g/mL phospholipid. Factor Va inactivation reactions (+APC) contained 0.2 nM factor Va, 32 pM APC, 70 nM protein S and 40 μ g/mL phospholipid; 20% PS:80% PC vesicles were added to 60 μ g/mL total phospholipid concentration to optimize prothrombin activation and overcome LA activity in the second stage. When present, the LA immunoglobulin was at 10 mg/mL. Solid bars, normal immunoglobulin; hatched bars, immunoglobulin derived from a lupus anticoagulant plasma. For the -APC reactions, the activity in the absence of PE and LA was set at 100%. For the +APC reactions, the activity in the presence of PE and absence of LA was set at 100%.

Of course, no conclusion is ever that black and white. Recently, it has been reported that at suboptimal phosphatidylserine concentrations PE can affect the activation of prothrombin.^{26,30} PE has also been reported to have effects on the tissue factor-factor VIIa³¹ and factor VIII-factor IXa³² activation complexes. It is possible that antibody populations directed towards these complexes (in addition to the APC complex?) might actually be protective. Only future studies into the fine specificity of lupus anticoagulants and anti-phospholipid antibodies associated with thrombosis will bear out the hypothesis that those directed towards the activated protein C pathway will be predictive of thrombotic risk.

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