Autoantibodies to Phospholipid-Binding Plasma Proteins: A New View of Lupus Anticoagulants and Other "Antiphospholipid" Autoantibodies

By Robert A.S. Roubey

Lupus Anticoagulants, and "antiphospholipid" autoantibodies in general, are of considerable clinical importance because of their strong association with thrombosis, recurrent fetal loss, and thrombocytopenia, i.e., the "antiphospholipid" antibody syndrome. This review focuses on recent evidence that "antiphospholipid" autoantibodies are not directed against anionic phospholipids, as has previously been thought, but are part of a larger group of autoantibodies against certain phospholipid-binding plasma proteins. At present, the most common and best characterized antigenic targets are β2-glycoprotein I (β2GPI) and prothrombin.

Other phospholipid-binding proteins, particularly protein C and protein S, may be important targets as well. Autoantibodies with these specificities differ from acquired coagulation factor inhibitors in that they preferentially bind antibodies that are immobilized on anionic phospholipid membranes or certain synthetic surfaces. In most instances, antibody binding to the antigens in the fluid-phase is weak or nondetectable. Thus, such autoantibodies usually do not decrease plasma antigen levels. Although direct evidence for a physiologic role of "antiphospholipid" autoantibodies is lacking, it is hypothesized that autoantibodies to phospholipid-binding proteins contribute directly to a thrombotic diathesis by interfering with hemostatic reactions that occur on anionic phospholipid membranes in vivo. Interestingly, the effect of autoantibodies on target antigen function may vary. For example, certain antibodies to β2GPI enhance its activity, whereas antibodies to phospholipid-bound activated protein C are inhibitory. Taken together, these new observations explain much of the confusion regarding the laboratory tests used to detect "antiphospholipid" antibodies and are providing key insights into the pathophysiology of the "antiphospholipid" antibody syndrome.

TERMINOLOGY

The nomenclature of "antiphospholipid" antibodies has always been confusing. For example, lupus anticoagulants occur in many individuals who do not have systemic lupus erythematosus, and are associated with thrombosis, not bleeding. Recently this problem has been exacerbated because changes in terminology have not kept pace with research developments, e.g., "antiphospholipid" autoantibodies that are not directed against phospholipids. For the purposes of this review, the following definitions will apply:

Lupus Anticoagulants

Antibodies that inhibit certain in vitro phospholipid-dependent coagulation reactions, typically the conversion of prothrombin to thrombin, are lupus anticoagulants. This inhibitory activity is thought to be directly related to the antigenic specificity of these antibodies, rather than to some other property. The anticoagulant activity of these antibodies is accentuated by lowering the phospholipid concentration, eg, in the dilute Russell viper venom time assay, and inhibited by raising the phospholipid concentration or by preincubation with excess phospholipid, eg, the platelet neutralization procedure. Mixing patient plasma with normal plasma does not correct the prolongation of the coagulation reaction caused by lupus anticoagulants, distinguishing them from factor deficiencies. The various coagulation assays used for the diagnosis of lupus anticoagulants have been recently reviewed elsewhere. Lupus anticoagulant assays have been thought to detect antibodies against anionic phospholipids. However, these tests use whole plasma and recent data from prothrombinase assays using purified components indicate that certain lupus anticoagulants are specific for either phospholipid-bound prothrombin or β2GPI. Thus, lupus anticoagulants are heterogeneous, and include autoantibodies to at least two phospholipid-bound plasma proteins.

Anticardiolipin Antibodies

Anticardiolipin antibodies are antibodies detected in solid-phase immunoassays, typically enzyme-linked immunosorbent assays (ELISAs), in which the putative antigen is cardiolipin dried onto a microtiter plate. However, in addition to detecting antibodies to cardiolipin this assay also detects antibodies to serum or plasma proteins that bind to the cardiolipin coated on the plate, in particular, antibodies to β2GPI. These proteins may be present in the serum or plasma samples and/or in bovine serum, which is often a component of the blocking buffer and sample diluent. Because of the conditions of the assay, the relevant proteins are those that bind to anionic phospholipids independently of calcium. Thus, like lupus anticoagulants, the antibodies detectable in the anticardiolipin assay are heterogeneous, and include both antibodies to cardiolipin and antibodies to cardiolipin-bound proteins.

Antiphospholipid Antibodies

Antiphospholipid antibodies are antibodies detected in lupus anticoagulant assays, anticardiolipin assays, and certain
**Table 1. Reactivity of Autoantibodies to Phospholipid-Binding Plasma Proteins in “Antiphospholipid” Antibody Assays**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>“Anticardiolipin”</th>
<th>Lupus Anticoagulant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-β2GPI</td>
<td>Yes</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Anti-prothrombin</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Anti-factor X</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Anti-protein C</td>
<td>No</td>
<td>No</td>
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<tr>
<td>Anti-protein S</td>
<td>No</td>
<td>No</td>
</tr>
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</table>

**Antibodies to Phospholipid-Binding Proteins**

This is a broader term encompassing “antiphospholipid” autoantibodies as well as antibodies to other phospholipid-binding proteins that are not detectable in lupus anticoagulant or anticardiolipin assays, eg, antibodies to protein C and protein S. These latter antibodies appear to be associated with “antiphospholipid” antibodies and may have pathologic significance. This phrase more accurately describes the group of autoantibodies associated with the “antiphospholipid” antibody syndrome. Table 1 lists the phospholipid-binding proteins known to be antigenic targets and the reactivity of autoantibodies to these proteins in “anticardiolipin” and lupus anticoagulant assays.

**Antiphospholipid Antibody Syndrome**

The association of moderate- to high-titer “antiphospholipid” autoantibodies with arterial thrombosis, venous thrombosis, recurrent fetal loss, or thrombocytopenia is termed antiphospholipid antibody syndrome. The syndrome often occurs in patients with systemic lupus erythematosus or related autoimmune diseases. When it occurs in the absence of an associated autoimmune disease, it has been termed the primary antiphospholipid antibody syndrome. Antiphospholipid antibodies in patients with syphilis, detectable in serologic tests for syphilis and anticardiolipin assays, are not associated with the antiphospholipid syndrome.

**Inadequacy of the Phospholipid Model**

Until recently it was held that “antiphospholipid autoantibodies” were directed against anionic phospholipids and, by virtue of this specificity, inhibited phospholipid-dependent reactions. However, this model does not adequately explain the subsets of “antiphospholipid” autoantibodies observed in current laboratory testing or the clinical heterogeneity of the antiphospholipid antibody syndrome. In the laboratory, sera from patients with syphilis are often reactive in the anticardiolipin assay, but do not have lupus anticoagulant activity. In contrast, “anticardiolipin” antibodies and lupus anticoagulants are closely associated in patients with autoimmune diseases, although some patients clearly have one specificity but not the other. In some patients, “anticardiolipin” antibodies and lupus anticoagulants appear to be a single antibody population, whereas in other patients the two are distinct and separable. Heterogeneity also exists among lupus anticoagulants. For example, Brandt tested the ability of lupus anticoagulant IgG fractions to inhibit the phospholipid-dependent formation of factor Xa and thrombin, using the same phospholipid reagent. Most lupus anticoagulants inhibited thrombin generation to a significantly greater degree than Xa generation, whereas one exhibited significantly more inhibitory activity in the Xa assay.

More importantly, the basis of the heterogeneity of the clinical features of the antiphospholipid syndrome is not well understood. If one hypothesizes that these antibodies are pathogenic on the basis of their antigenic specificity, it is not clear why certain patients have venous thrombosis versus arterial thrombosis versus thrombocytopenia versus recurrent fetal loss, or some combination of these manifestations. It is also not clear why only about 30% of individuals with “antiphospholipid” antibodies will develop the clinical syndrome, although greater risk is associated with higher antibody titers.

**Specificities of Autoantibodies to Phospholipid-Binding Plasma Proteins**

This section will review recent data regarding the antigenic specificities of “antiphospholipid” and related autoantibodies. It should be remembered that most of these studies are limited to relatively small numbers of patients with moderate- to high-titer IgG autoantibodies. In retrospect, several early reports clearly suggest a role for plasma proteins in the activity of lupus anticoagulants. In 1959, Loeiger was among the first to describe the so-called lupus anticoagulant “cofactor” phenomenon, ie, the addition of normal plasma to patient plasma increased the inhibition of coagulation. He concluded that the cofactor was most likely prothrombin itself. In 1965, in an elegant and often overlooked report, Yin and Gaston purified a lupus anticoagulant, showed it to be a 7S immunoglobulin (IgG), and clearly demonstrated that its anticoagulant activity required a protein cofactor. The cofactor, although not fully characterized, was present in both normal and patient plasma and was not prothrombin.

**β2-Glycoprotein I (β2GPI)**

In 1990 two independent reports challenged the conventional wisdom that antiphospholipid autoantibodies were directed against anionic phospholipids. Both groups of investigators prepared affinity-purified IgG “anticardiolipin” antibodies from patients sera and showed that these antibodies did not bind to solid-phase cardiolipin or cardiolipin liposomes in serum-free assay systems. Addition of normal serum restored antibody binding. The serum component required for the binding of “anticardiolipin” antibodies to solid-phase cardiolipin was found to be β2GPI, a phospholipid-binding plasma protein.
P2GPI is a 50-kD protein present at ~200 µg/mL in normal plasma. Although its physiologic role is not known, in vitro data suggest that β2GPI may play a role in coagulation. β2GPI binds to anionic phospholipids and inhibits the contact phase of intrinsic blood coagulation, adenine-dependent platelet aggregation, and the prothrombinase activity of platelets. Recently preliminary data suggest that β2GPI may play a regulatory role in the protein C pathway by inhibiting the interaction between protein S and C4b-binding protein. Although these data suggest an anticoagulant role for β2GPI, deficiency of this protein is not a clear risk factor for thrombosis. Population and family studies indicate that the plasma concentration of β2GPI is controlled by codominant alleles. A recent study of patients with familial thrombophilia indicated that heterozygous partial β2GPI deficiency (plasma levels of ~60 to 140 µg/mL) was not associated with thrombosis. Two brothers with homozygous β2GPI deficiency were identified; one had a history of recurrent venous thrombosis whereas the other was asymptomatic at age 35. Patients with "antiphospholipid" antibodies have normal or somewhat elevated levels of β2GPI.

Structurally, β2GPI is a noncomplement member of the complement control protein family. β2GPI has five of the consensus repeats, or so-called "sushi domains," characteristic of such proteins. The fifth domain may contain a phospholipid-binding region. The amino acid sequence of P2GPI was originally determined by Lozier et al. and several other groups previously found no reactivity to P2GPI alone. Conversely, Avi et al. and Keeling et al. reported antibody binding to β2GPI in the absence of phospholipid. Our laboratory and several other groups previously found no reactivity to P2GPI alone. Two possible explanations are (1) the antibodies require a relatively high density of immobilized antigen which cannot be achieved on untreated polystyrene, or (2) that the antibodies are specific for a conformational epitope of β2GPI formed when the protein is bound to an anionic surface, such as irradiated polystyrene or cardiolipin. Our data support the first explanation, suggesting that anti-β2GPI autoantibodies are of intrinsically low affinity and bind to β2GPI only when the density of immobilized antigen is sufficient to allow bivalent attachment. The low affinity of a single IgG antigen binding site for β2GPI does not mean that these antibodies are trivial or irrelevant with regard to pathophysiology. The marked enhancement of the antibody-antigen interaction provided by antibody bivalency means that these are high-affinity antibodies when β2GPI is immobilized at a sufficiently high density. In vivo such antibodies may bind to β2GPI clustered on an anionic phospholipid surface, eg, an activated platelet or endothelial cell, but would not bind to β2GPI in the circulation. In this way antibodies to β2GPI are analogous to another autoantibody to a plasma protein, rheumatoid factor. Rheumatoid factors have low affinity for monomeric IgG in solution, but bind avidly to IgG that is aggregated or bound to particles, because of the enhancement provided by multivalency. Others have observed antibody binding to β2GPI on irradiated polystyrene and support the alternative explanation, that the antibodies recognize conformational epitopes formed when β2GPI binds to an anionic surface. These two possibilities are not mutually exclusive.
Fig 1. Binding of autoimmune "anticardiolipin" antibodies to cardiolipin requires the presence of β2GPI. The serum-free anticardiolipin ELISA was performed in the absence (A) or presence (B) of 25 µg/ml human β2GPI. Purified IgG fractions from representative patients with the antiphospholipid antibody syndrome (A) and syphilis (B) were assayed. See text for references.

Some, but not all, autoantibodies to β2GPI have lupus anticoagulant activity. This is interesting in light of the fact that β2GPI itself inhibits prothrombinase activity in vitro. β2GPI-dependent lupus anticoagulants appear to enhance the anticoagulant effect of β2GPI. Several monoclonal and polyclonal antibodies to β2GPI have similar lupus anticoagulant-like activity. Enhancement of β2GPI's anticoagulant effect could be caused by the steric effect of antibodies bound to β2GPI on a phospholipid surface. Another possibility is that these antibodies could cross-link surface-bound β2GPI, increasing the affinity of the β2GPI-phospholipid interaction. Indeed, "antiphospholipid" autoantibodies enhance the binding of β2GPI to cardiolipin-coated microtiter plates. As will be discussed below, the effect of autoantibodies to β2GPI on the interaction of β2GPI with phospholipid surfaces may contribute to the pathogenesis of the antiphospholipid antibody syndrome. The reason why some anti-β2GPI antibodies have lupus anticoagulant activity whereas others do not is unclear, but may be related to avidity and/or epitope specificity.

Prothrombin

The occurrence of acquired hypoprothrombinemia in a subset of patients with lupus anticoagulants has been recognized for some time. Loe-liger suggested that prothrombin was the lupus anticoagulant cofactor in one such case. Bajaj et al. were the first to demonstrate the presence of autoantibodies to prothrombin in two patients with the lupus anticoagulant-hypoprothrombinemia syndrome. These antibodies were of relatively high affinity and it is likely that hypoprothrombinemia was caused by the clearance of prothrombin antigen-antibody complexes from the circulation. Subsequently, circulating prothrombin-antibody complexes were also observed in 38 of 55 patients with lupus anticoagulants and normal prothrombin levels. Fleck et al. showed that three affinity-purified antiprothrombin autoantibodies had lupus anticoagulant activity. These investigators concluded that, in some patients, the autoantibodies to prothrombin were of relatively low affinity. This was based on the observation that, although these antibodies bound to immobilized prothrombin, the antibodies and prothrombin were not bound to each other in patient plasma.

More recently, others have demonstrated that some lupus anticoagulants are directed against phospholipid-bound prothrombin. Bevers et al. studied 16 patients with both antiphospholipid antibodies and lupus anticoagulants. Plasma samples were incubated with cardiolipin-containing liposomes fol-
lowed by centrifugation. In 11 of 16 patients, the lupus anti-coagulant activity remained in the supernatant while the anticardiolipin antibody activity pelleted with the liposomes. In a purified prothrombinase assay, these non–phospholipid-binding lupus anti-coagulants were specific for human prothrombin bound to phospholipid. In the remaining 5 patients, lupus anti-coagulant activity co-sedimented with the anticardiolipin antibodies; these lupus anti-coagulants were antibodies to β2GPI. Oosting et al showed that 4 of 22 lupus anti-coagulants inhibited endothelial cell-mediated prothrombinase activity. This inhibitory activity could be adsorbed by prothrombin-coated cardiolipin vesicles, but not by vesicles alone, or vesicles coated with β2GPI, factor Va, or factor Xa.

Taken together these data strongly suggest that some lupus anti-coagulants are directed against immobilized or phospholipid-bound prothrombin. Analogous to the discussion of antibodies to β2GPI, these antibodies may be directed against cryptic or neo-antigens formed when prothrombin binds to anionic phospholipid, and/or they may be low-affinity antibodies binding bivalently to immobilized prothrombin. The observation that the antibodies could be adsorbed by prothrombin bound to agarose beads favors the latter explanation. The occurrence of hypoprothrombinemia in a small subset of patients with the lupus anti-coagulant suggests that this group of patients has higher affinity antibodies, or antibodies that recognize native prothrombin. These autoantibodies bind to prothrombin in the circulation leading to the formation and clearance of prothrombin-containing immune complexes. It is not known whether some antibodies to prothrombin also bind to thrombin.

**Protein C and Protein S**

Limited data suggest that autoantibodies may be directed against components of the protein C pathway, ie, protein C and protein S. Although such antibodies are not detectable in standard anticardiolipin or lupus anti-coagulant assays, they are included here as part of a broader view of “antiphospholipid” autoantibodies as antibodies to phospholipid-binding plasma proteins. Oosting et al demonstrated inhibition of the protein C-mediated inactivation of factor Va by IgG fractions from 7 of 30 patients with antiphospholipid antibodies and/or a history of thrombosis. This inhibitory activity could be adsorbed by protein C-coated cardiolipin vesicles in 3 patients, and by protein S-coated vesicles in the remaining 4 patients. No inhibition was observed by β2GPI-coated vesicles or by vesicles alone. These investigators concluded that antibodies were specific for phospholipid-bound protein C or protein S. In another recent report antibodies to protein C were detected in 12 of 108 SLE serum samples by an ELISA using high-binding microtiter plates. These antibodies were not associated with decreased protein C levels or functional protein C deficiency. In contrast, antibodies to protein C or protein S were not detected by immunoblotting in 11 patients with the antiphospholipid antibody syndrome, 7 of whom had low levels of free protein S. As is the case with antibodies to β2GPI, antibodies to protein C and protein S may be detectable under certain experimental conditions but not others.

**Other Potential Targets**

Inhibition of endothelial cell prostacyclin production has been cited as a potential mechanism by which antiphospholipid autoantibodies may predispose to thrombosis. In view of recent data indicating that this may be caused by inhibition of phospholipase A₂ activity, Vermyleen and Arnout suggested that these antibodies might be directed against a phospholipase A₂-phospholipid complex. A difficulty with this hypothesis is that the fraction of phospholipase A₂ thought to mediate arachidonic acid release is intracellular, ie, in the cytosol or associated with the inner leaflet of the plasma membrane. However, nonpancreatic secretory phospholipase A₂ has also been reported to contribute to the synthesis of arachidonic acid metabolites. At the present time there is no direct evidence of autoantibodies to any type of phospholipase A₂.

Annexins are a family of calcium-dependent phospholipid-binding proteins thought to play important roles in membrane processes such as exocytosis. The potential interaction of antiphospholipid autoantibodies with annexin V (human placental anticoagulant protein b) has recently been studied. Primarily an intracellular protein, very low levels of annexin V are detectable in human plasma, amniotic fluid, and conditioned medium of cultured endothelial cells. There are no data suggesting that extracellular annexin V plays a physiologic role in coagulation. Because of its high affinity for anionic phospholipid surfaces, annexin V can block the binding of “antiphospholipid” antibodies to anionic phospholipids in vitro. “Antiphospholipid” antibodies do not recognize annexin V.

Anecdotally, an antibody to factor X with lupus anti-coagulant activity has been identified in a patient with high titer “anticardiolipin” antibodies and a hemorrhagic diathesis (Dr D. Triplett, personal communication, January 1994). Autoantibodies with apparent specificity for phosphatidylethanolamine have been reported in a few patients. Sugi et al showed that the binding of these antibodies to phosphatidylethanolamine in ELISA requires a 140-kD serum glycoprotein, the precise identity of which is not yet known.

**Antibodies to Endothelial Cell Surface Proteins**

Although not phospholipid-binding plasma proteins, thrombomodulin and vascular heparan sulfate proteoglycan are included as potential antigenic targets because of their respective roles in the control of thrombosis. Autoantibodies to thrombomodulin have been identified in few patients. However, in a larger study of antithrombomodulin antibodies, Gibson et al observed no significant differences among patients with lupus anti-coagulants, patients referred for lupus anti-coagulant testing but found to be negative, and normal individuals. Fillit et al have demonstrated autoantibodies to both heparan sulfate and the protein core of vascular heparan sulfate proteoglycan in the sera of certain patients with sys-
temic lupus erythematosus (SLE). Given that sera were used in these assays and that β2GPI binds to heparin, it is possible that the apparent specificity of autoantibodies for heparan sulfate is, in fact, caused by antibodies to β2GPI.

**Phospholipids**

Are some “antiphospholipid” autoantibodies specific for anionic phospholipids? Previous studies indicating such a specificity are difficult to interpret because of the fact that most experiments were performed in the presence of serum or plasma. Therefore, the possible role of plasma proteins and/or protein-phospholipid complexes may not have been appreciated. Additionally, contamination of purified antibodies and other reagents with plasma proteins now known to be important, eg, β2GPI, was not directly evaluated. A look at the key report by Pengo et al highlights these difficulties. These investigators studied IgG lupus anticoagulants from normal plasma, and the anticardiolipin ELISA was performed with 10% fetal calf serum as the blocking agent and sample diluent, as is often the case for this immunoassay.

In the absence of plasma or serum, these lupus anticoagulants inhibited the binding of prothrombin and factor X to solid-phase phospholipids. As discussed above, prothrombin, and possibly factor X, are the targets of some “antiphospholipid” autoantibodies. Further, this experiment was performed in the presence of bovine serum albumin, some commercial preparations of which may contain significant amounts of bovine β2GPI (unpublished observation, December 1992). More recent evidence that some lupus anticoagulants may be directed against anionic phospholipid alone is limited.

As discussed above, some investigators have suggested that β2GPI is not the target of “anticardiolipin” autoantibodies, but rather enhances antibody binding to cardiolipin by modifying the phospholipid conformation. However, at present there are no direct data to support this hypothesis. Anionic phospholipids do appear to be the target of anticardiolipin antibodies associated with syphilis and other infectious diseases, as well as those detected in many normal sera after heat inactivation.

Lupus anticoagulants have been reported to recognize non-bilayer, hexagonal (II) phase phospholipid structures; however, this specificity has not been shown in the absence of serum or plasma. Binding of β2GPI to hexagonal (II) phase phospholipid may explain some of these data.

Although not antigenic targets, anionic phospholipids may play an important role in vivo in the binding of autoantibodies to phospholipid-bound plasma proteins. As discussed, these antibodies may be specific for protein conformations induced when the proteins bind to phospholipids. Further, antibody binding may depend on the phospholipid surface to provide a high local concentration of antigen, thereby promoting high-avidity bivalent binding of intrinsically low-affinity antibodies.

A New Model: Autoantibodies to Phospholipid-Binding Plasma Proteins

Taken together, recent data strongly suggest that phospholipid-binding proteins and/or protein-phospholipid complexes are the physiologically relevant targets of “antiphospholipid” autoantibodies. Some, but not all, of these autoantibodies are detectable in the “antiphospholipid” antibody assays currently in clinical use (see Table 1). Autoantibodies to β2GPI are detected in the “ anticardiolipin” ELISA, β2GPI being present in the bovine sera used in the blocking buffer and in the patient’s serum sample. Also detected in this assay are “true” anticardiolipin antibodies seen in association with syphilis and other infections. A lupus anticoagulant assay, such as the dilute Russell viper venom time, detects certain antibodies to β2GPI, as well as antibodies to prothrombin and factor X. Antiprothrombin and anti-factor X antibodies are probably not detectable in “anticardiolipin” ELISAs because of several factors including the use of serum rather than plasma and the low concentration of Ca²⁺ in the assay. Autoantibodies to protein C and protein S are not detected in either assay. Thus, the detection of different antigenic specificities in the currently used “antiphospholipid” assays appears to explain much of the heterogeneity and inconsistent overlap of “anticardiolipin” antibodies and lupus anticoagulants. For example, it clarifies why, in some patients, “anticardiolipin” antibodies and lupus anticoagulants are the same antibodies, whereas in others they are clearly distinct antibodies.

Immunologically, it is of interest that autoantibodies to different phospholipid-binding proteins occur together in various combinations. As recently demonstrated by Oosting et al, some patients may have autoantibodies to β2GPI, prothrombin, protein C, and protein S, whereas others may have antibodies against one, two, or three of these proteins in different combinations. Speculatively, this is similar to certain linked sets of antinuclear antibodies that occur in patients with systemic lupus erythematosus, eg, antibodies to constituents of small nuclear ribonucleoprotein particles. Antibodies may be directed against different protein components of such particles, such as Sm and U1-RNP proteins, suggesting that the immune response is antigen-driven. By analogy, the co-occurrence of antibodies to different phospholipid-binding proteins suggests that the relevant antigenic particle in this instance may consist of proteins bound in close proximity to one another on an anionic phospholipid surface, perhaps a damaged vascular endothelial cell or activated platelet.

**PATHOGENETIC MECHANISMS**

If “antiphospholipid” autoantibodies play a role in the pathogenesis of the antiphospholipid syndrome, the spectrum of antigenic specificities discussed may offer an explanation for the confusing variety of proposed pathophysiologic mechanisms as well as the heterogeneity of clinical manifestations of the syndrome. Although no association between a particular antigenic specificity, a particular mechanism of action, and a particular clinical manifestation of the antiphospholipid syndrome could be demonstrated for any of the autoantibodies, the following may be consistent with the definition of an autoimmune reaction:
phospholipid syndrome has yet been established, it is reasonable to speculate that antibodies to different proteins would have different effects and may be associated with different types of events. For example, it has been suggested that antibodies which alter the prostacyclin/thromboxane balance may be associated with arterial thrombosis, whereas antibodies that inhibit the protein C pathway may be associated with venous thrombosis. Some specificities may not be related to any disease manifestation, thus explaining why many patients are asymptomatic. In this section, the numerous mechanisms associated with arterial thrombosis, whereas antibodies that inhibit the protein C pathway may be associated with venous thrombosis, whereas others inhibited equally well with and without protein S. As previously discussed, similar results were obtained by Oosting et al, who further demonstrated that the antibodies responsible for inhibiting Va degradation were directed against phospholipid-bound protein C or protein S. Interestingly, one patient in this study had a history of thrombosis and autoantibodies to phospholipid-bound protein S, in the absence of "anticardiolipin" antibodies or lupus anticoagulants inhibited the activation of protein C both in solution and on endothelial cells. Similar data have been reported by others. Some of these reports have suggested that the antibodies may be directed against thrombomodulin or protein C. Given that β2GPI-dependent lupus anticoagulants appear to enhance the binding of β2GPI to anionic phospholipid in the prothrombinase reaction, it is possible that such antibodies could similarly inhibit the activation of protein C. Although β2GPI has been shown to inhibit protein C activation by thrombomodulin incorporated in cardiolipin vesicles, there was little or no effect on the endothelial cell-mediated activation of protein C, in the presence or absence of anti-β2GPI autoantibodies. This discrepancy may be explained by the fact that protein C activation, while enhanced by anionic phospholipids, can occur on neutral phospholipid membranes. The kinetics of protein C activation on cultured endothelial cells are comparable to those observed with neutral synthetic membranes. Theoretically, antibodies to thrombin might also inhibit protein C activation.

Several laboratories have observed inhibition of the anticoagulant activity of activated protein C by "antiphospholipid" antibodies. Marciniak and Romond reported a decreased rate of factor Va degradation in the plasma of 15 patients with lupus anticoagulants. Exogenous protein C did not correct this deficiency, leading these investigators to conclude that the antibodies inhibited the formation of the anionic phospholipid/protein C/protein S complex. However, Malia et al found that IgG fractions from certain patients prevented Va degradation only in the presence of protein S, whereas others inhibited equally well with and without protein S. As previously discussed, similar results were obtained by Oosting et al, who further demonstrated that the antibodies responsible for inhibiting Va degradation were directed against phospholipid-bound protein C or protein S. In some of these differences. Cameras et al, in a recent review of this field, have suggested several methodologic reasons for this discrepancy including the heterogeneity of the vascular systems studied, eg, rat aortic rings, bovine aortic endothelial cells, or human umbilical vein endothelial cells, different cell culture conditions, differences in agonists, and different methods of antibody of serum/plasma

### Table 2. Potential Mechanisms of Autoantibody-Mediated Thrombosis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Potential Mechanisms</th>
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<tr>
<td>Anti-β2GPI</td>
<td>Inhibition of protein C activation</td>
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<tr>
<td></td>
<td>Inhibition of protein C/protein S-mediated inactivation of factors Va and Vlla</td>
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<tr>
<td></td>
<td>Inhibition of factor XII/prekallikrein-mediated fibrinolytic activity</td>
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<td>Inhibition of heparan sulfate-mediated activation of antithrombin III</td>
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<td>Enhanced platelet activation</td>
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<td>Enhanced factor Xa generation by platelets</td>
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<td>Decreased free protein S levels via inhibition of the interaction of β2GPI and C4b-binding protein</td>
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<td>Anti-prothrombin/ Anti-thrombin</td>
<td>Binding to thrombin-activated platelets</td>
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<td>Inhibition of thrombin-mediated endothelial cell prostacyclin release</td>
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<tr>
<td>Anti-vascular heparan sulfate proteoglycan</td>
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**Endothelial Cell Interactions**

**Inhibition of the protein C pathway.** "Antiphospholipid" antibodies may inhibit phospholipid-dependent reactions of the protein C pathway: (1) the thrombin/thrombomodulin activation of protein C, and/or (2) the activated protein C/protein S degradation of factors Va and Vlla. Comp et al reported that IgG from two of seven patients with lupus anticoagulants inhibited the activation of protein C both in solution and on endothelial cells. Similar data have been reported by others. Some of these reports have suggested that the antibodies may be directed against thrombomodulin or protein C. Given that β2GPI-dependent lupus anticoagulants appear to enhance the binding of β2GPI to anionic phospholipid in the prothrombinase reaction, it is possible that such antibodies could similarly inhibit the activation of protein C. Although β2GPI has been shown to inhibit protein C activation by thrombomodulin incorporated in cardiolipin vesicles, there was little or no effect on the endothelial cell-mediated activation of protein C, in the presence or absence of anti-β2GPI autoantibodies. This discrepancy may be explained by the fact that protein C activation, while enhanced by anionic phospholipids, can occur on neutral phospholipid membranes. The kinetics of protein C activation on cultured endothelial cells are comparable to those observed with neutral synthetic membranes. Theoretically, antibodies to thrombin might also inhibit protein C activation.

Several laboratories have observed inhibition of the anticoagulant activity of activated protein C by "antiphospholipid" antibodies. Marciniak and Romond reported a decreased rate of factor Va degradation in the plasma of 15 patients with lupus anticoagulants. Exogenous protein C did not correct this deficiency, leading these investigators to conclude that the antibodies inhibited the formation of the anionic phospholipid/protein C/protein S complex. However, Malia et al found that IgG fractions from certain patients prevented Va degradation only in the presence of protein S, whereas others inhibited equally well with and without protein S. As previously discussed, similar results were obtained by Oosting et al, who further demonstrated that the antibodies responsible for inhibiting Va degradation were directed against phospholipid-bound protein C or protein S. Interestingly, one patient in this study had a history of thrombosis and autoantibodies to phospholipid-bound protein S, in the absence of "anticardiolipin" antibodies or lupus anticoagulant. Decreased levels of protein S have been reported in a small number of patients with the antiphospholipid antibody syndrome. Although this could be caused by antibodies to protein S itself, autoantibodies to β2GPI might alter the level of free protein S by interfering with the proposed interaction of β2GPI with C4b-binding protein.

**Inhibition of prostacyclin production.** Although some investigators have demonstrated inhibition of endothelial cell prostacyclin production by plasma or purified IgG from patients with "antiphospholipid" antibodies, others have reported no effect, enhanced prostacyclin production, or mixed results. True differences among patients and small numbers of patients in these studies may explain some of these differences. Carreras et al, in a recent review of this field, have suggested several methodologic reasons for this discrepancy including the heterogeneity of the vascular systems studied, eg, rat aortic rings, bovine aortic endothelial cells, or human umbilical vein endothelial cells, different cell culture conditions, differences in agonists, and different methods of antibody of serum/plasma
Preparation. Another important factor in these studies is the relative state of activation of the endothelial cells. Activation of endothelial cells by cytokines and inflammatory mediators significantly alters the expression of cell surface proteins. For example, "antiphospholipid" antibodies potentiate tumor necrosis factor-induced endothelial cell procoagulant activity. Inhibition of endothelial cell procoagulant production could be explained by inhibition of phospholipase A2 activity, and it has been suggested that autoantibodies may be directed against phospholipase A2 or a phospholipase A2-phospholipid complex. Antibodies to thrombin could potentially interfere with thrombin-induced procoagulant production. At the present time, there are no data regarding other antigens, either phospholipid-binding proteins or endothelial cell-surface molecules, which might explain antibody-mediated alteration of arachidonic acid metabolism.

Increased tissue factor expression. As mentioned above, it has been reported that six patient sera containing "antiphospholipid" antibodies acted synergistically with tumor necrosis factor to enhance endothelial cell procoagulant activity. The likely mechanism for this activity is upregulation of tissue factor expression. In support of this hypothesis, sera or IgG from 14 of 16 lupus patients have been shown to induce tissue factor production in cultured endothelial cells. Whether this effect is caused by serum or by the engagement of specific endothelial cell antigens is not known.

Inhibition of antithrombin III activity. Autoantibodies to vascular heparan sulfate proteoglycan could contribute to a thrombotic diathesis by blocking the heparan sulfate-mediated activation of antithrombin III. Monoclonal antibodies to heparan sulfate have been shown to have such an activity. Compatible with this mechanism, Cosgriff and Martin described a patient with the lupus anticoagulant and recurrent thrombosis who had high antigenic, but low functional, levels of antithrombin III. More recently, Chamley et al showed that IgM purified from the serum of a patient with "anticardiolipin" autoantibodies inhibited the heparin-dependent activation of antithrombin III. In view of the fact that β2GPI binds to heparin, this effect might be caused by anti-β2GPI autoantibodies.

Impaired fibrinolysis. Studies of tissue plasminogen activator levels, before and after venous occlusion, have generally been inconclusive. The most consistent finding is an elevated level of plasminogen activator inhibitor-1 (PAI-1) in patients with SLE. However, there is no strong correlation between increased PAI-1 levels, or presence or level of "antiphospholipid" autoantibodies, and a history of thrombosis.

The contact system may play an important role in fibrinolysis. Two groups have observed inhibition of the factor XII-dependent fibrinolytic pathway in a total of 14 of 22 patients, and suggested that this is a mechanism of lupus anticoagulant-associated thrombosis. In view of the fact that β2GPI inhibits factor XII and prekallikrein activation on anionic phospholipid surfaces, anti-β2GPI autoantibodies may amplify this inhibitory activity by enhancing the binding of β2GPI to the phospholipid surface, as previously discussed. Decreased activation of factor XII and kallikrein generation would lead to decreased cleavage of prekallikrein, thus impairing fibrinolysis.

Enhanced platelet-activating factor production. Silver et al reported that "anticardiolipin" autoantibodies enhance the endothelial cell production of platelet-activating factor. However, platelet-activating factor, like prostacyclin, is released by phospholipase A2-mediated hydrolysis. Accordingly, these data conflict with those of Schorer et al, who observed decreased platelet-activating factor, in accordance with their findings that some antiphospholipid antibodies inhibit phospholipase A2 activity.

Platelet interactions.

*Thrombocytopenia.* Autoimmune thrombocytopenia is part of the antiphospholipid antibody syndrome and thought to be caused by the antiplatelet activity of a subset of these antibodies. "Antiphospholipid" antibodies have been shown to bind to freeze-thawed platelets and activated platelets; however, there is little evidence for binding to fresh, unstimulated platelets.

Recently Shi et al studied the platelet-binding activity of "anticardiolipin" antibodies and lupus anticoagulants separated by antiphospholipid affinity chromatography and other physicochemical techniques from the plasma of two patients with both activities. The lupus anticoagulant fractions bound to thrombin-activated but not resting platelets. Together with the observation that lupus anticoagulants lacking "anticardiolipin" activity are directed against phospholipid-bound prothrombin, it is intriguing to speculate that the antiplatelet activity of these lupus anticoagulants is caused by antibodies binding to thrombin. "Anticardiolipin" antibody fractions bound to activated platelets as well, but required the presence of β2GPI. This suggests that platelet-bound β2GPI may be a physiologic target of these autoantibodies.

Effects on platelet activation. A number of studies suggest that "antiphospholipid" autoantibodies may alter platelet activity themselves or in association with other agonists. Antiphospholipid antibodies have been reported to increase thromboxane A2 production. Measuring urinary metabolites of prostacyclin and thromboxane A2, two groups have observed increased average thromboxane:prostacyclin ratios in a total of 56 patients with the antiphospholipid syndrome. This imbalance was primarily caused by increased platelet thromboxane A2. Further, IgG F(ab')2 fragments from six patients with elevated ratios were shown to enhance platelet generation of thromboxane in vitro. The mechanism by which this occurs and the relevant platelet antigen are not known. There are conflicting data regarding the effect of "antiphospholipid" on platelet aggregation. Some investigators have reported that "antiphospholipid" autoantibodies may induce platelet aggregation, whereas others have found that lupus anticoagulants block platelet aggregation in response to some agonists but not others. In the study by Shi et al discussed above, neither lupus anticoagulant nor "anticardiolipin" antibody fractions affected platelet degranulation or aggregation. Murine monoclonal antibodies to β2GPI have been shown to...
bind to platelets in the presence of β2GPI and lead to platelet activation in the presence of subthreshold concentrations of weak agonists. Both Fab and Fc portions of the antibodies were required for platelet activation, suggesting a role for the platelet IgG Fc receptor, FcγRII.

It has been reported that β2GPI inhibits the factor Xa generating activity of platelets and that "anticardiolipin" autoantibodies block this inhibitory activity. This would result in unopposed Xa generation, which may contribute to a thrombotic tendency. However, these data are in contrast to those observed in the prothrombinase assay in which "anticardiolipin" antibodies enhance the inhibitory activity of β2GPI.

CONCLUSIONS AND FUTURE DIRECTIONS

Recent data support the paradigm that "antiphospholipid" autoantibodies are part of a linked set of autoantibodies directed against various phospholipid-binding plasma proteins. This group includes autoantibodies that are detected in "anticardiolipin" and/or lupus anticoagulant assays, eg, anti-β2GPI and anti-prothrombin antibodies, as well as antibodies not detected by currently used clinical laboratory tests, eg, anti-protein C and anti-protein S antibodies. Additional specificities may well be discovered as research in this field progresses. In contrast, antibodies specific for anionic phospholipids occur in association with syphilis and other infectious diseases, and do not appear to be associated with autoimmune diseases or the "antiphospholipid" antibody syndrome. Although evidence that "antiphospholipid" autoantibodies are directed against a number of proteins involved in the control of thrombosis and hemostasis should stimulate further investigation regarding the potential pathologic role of these antibodies, it should be kept in mind that direct evidence for such a role is not yet available. These antibodies may represent an epiphenomenon rather than a causative factor in the development of the "antiphospholipid" antibody syndrome.

This new model explains much of the heterogeneity of "antiphospholipid" autoantibodies observed in laboratory testing. More importantly, it offers great promise in explaining the clinical heterogeneity and pathophysiology of the "antiphospholipid" antibody syndrome. Future studies are needed to determine if different antigenic specificities, alone or in combination, are associated with particular clinical manifestations, or carry greater or less risk for the development of the syndrome. Important areas for investigation include (1) the effect of different antibodies on the physiologic functions of their target antigens; (2) the location, accessibility, and density of the antigens on phospholipid membranes in vivo; (3) the role of titer, avidity, and epitope specificity of the antibodies; and (4) characterization of the immune response that leads to the development of these autoantibodies. The use of purified antibodies and assay systems utilizing purified plasma components, rather than serum or plasma, will be of critical importance.

If autoantibodies to particular phospholipid-binding proteins are shown to be associated with different clinical presentations or to confer different risks, then clinical testing will need to be revised. Although current "anticardiolipin" ELISAs and lupus anticoagulant assays are clinically useful, these tests do not clearly differentiate antibodies with different specificities nor do they detect potentially important antibodies, such as those directed against protein C and protein S. For example, "anticardiolipin" assays detect both antibodies to β2GPI, associated with the "antiphospholipid" antibody syndrome, and antibodies to cardiolipin, which are not associated with this syndrome. Thus, an assay that detects only antibodies against β2GPI would be expected to have greater specificity for the "antiphospholipid" antibody syndrome. Studies are needed to determine if it is clinically important to differentiate between lupus anticoagulants that are antibodies to β2GPI and those that are antibodies to prothrombin. Antigenic and functional assays for the detection of autoantibodies to protein C and protein S may be shown to have clinical utility.

Finally, with regard to nomenclature, recent data highlight the inadequacy of the current classification of antiphospholipid antibodies as anticoagulant antibodies and lupus anticoagulants. Vercymen and Arnoult have suggested that antiphospholipid antibodies be renamed antiphospholipid-protein antibodies. Because antibody binding to β2GPI and prothrombin has been observed in the absence of phospholipid, the term autoantibodies to phospholipid-binding plasma proteins has been used in this review. If warranted by future studies, a classification based on antigenic specificity may prove to be useful. For example, designations such as anti-β2GPI antibodies or anti-prothrombin antibodies may confer more relevant information than the term lupus anticoagulant.

NOTE ADDED IN PROOF

The protein required for the binding of autoantibodies to phosphatidyethanolamine, initially thought to be a 140-kD serum glycoprotein, has recently been identified as low molecular-weight kininogen and/or nonfragmented high molecular-weight kininogen.

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