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Research Article

It has been reported that antiphospholipid autoantibodies do not recognize phospholipid alone, but rather the plasma protein beta 2-glycoprotein I (beta 2GPI), or a beta 2GPI-phospholipid complex. In vitro beta 2GPI binds to anionic phospholipids and inhibits the prothrombinase activity of procoagulant membranes. In light of the fact that lupus anticoagulants, a type of antiphospholipid antibody, have similar anticoagulant properties, the relationship of beta 2GPI to lupus anticoagulant activity was investigated. IgG from patients with autoimmune diseases or syphilis were tested for anticardiolipin reactivity and lupus anticoagulant activity in the presence and absence of beta 2GPI. As expected, anti-cardiolipin reactivity associated with autoimmune disease was beta 2GPI dependent. In contrast, IgG from a patient with syphilis recognized cardiolipin alone and binding was inhibited by beta 2GPI. Autoimmune antiphospholipid antibodies prolonged the dilute Russell viper venom time of normal plasma, but had no effect on beta 2GPI-depleted plasma. Antiphospholipid antibodies associated with syphilis had no anticoagulant effect. RP-1, an anti-beta 2GPI mAb, had anticoagulant effects similar to those of autoimmune antiphospholipid antibodies. These data demonstrate that antiphospholipid autoantibodies exert lupus anticoagulant activity via an interaction with beta 2GPI. These antibodies and RP-1 appear to amplify the anticoagulant effect of beta 2GPI itself.

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Lupus Anticoagulant Activity of Autoimmune Antiphospholipid Antibodies Is Dependent upon β_2 -Glycoprotein I

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Abstract

It has been reported that antiphospholipid autoantibodies do not recognize phospholipid alone, but rather the plasma protein β_2 -glycoprotein I (β_2 GPI), or a β_2 GPI-phospholipid complex. In vitro β_2 GPI binds to anionic phospholipids and inhibits the prothrombinase activity of procoagulant membranes. In light of the fact that lupus anticoagulants, a type of antiphospholipid antibody, have similar anticoagulant properties, the relationship of β_2 GPI to lupus anticoagulant activity was investigated. IgG from patients with autoimmune diseases or syphilis were tested for anticardiolipin reactivity and lupus anticoagulant activity in the presence and absence of β_2 GPI. As expected, anticardiolipin reactivity associated with autoimmune disease was β_2 GPI dependent. In contrast, IgG from a patient with syphilis recognized cardiolipin alone and binding was inhibited by β_2 GPI. Autoimmune antiphospholipid antibodies prolonged the dilute Russell viper venom time of normal plasma, but had no effect on β_2 GPI-depleted plasma. Antiphospholipid antibodies associated with syphilis had no anticoagulant effect. RP-1, an anti- β_2 GPI mAb, had anticoagulant effects similar to those of autoimmune antiphospholipid antibodies. These data demonstrate that antiphospholipid autoantibodies exert lupus anticoagulant activity via an interaction with β_2 GPI. These antibodies and RP-1 appear to amplify the anticoagulant effect of β_2 GPI itself. (*J. Clin. Invest.* 1992. 90:1100–1104.) **Key words:** apolipoprotein H • cardiolipin • phospholipid • systemic lupus erythematosus • thrombosis

Introduction

Antiphospholipid antibodies (aPL)¹ occur in certain autoimmune diseases, particularly systemic lupus erythematosus

(SLE) and the primary aPL syndrome, as well as in syphilis and other infectious diseases. Autoimmune aPL include the lupus anticoagulant and are associated with thrombosis, recurrent fetal loss, and thrombocytopenia (1). New insights into the pathophysiology of autoimmune aPL have been provided by the recent observation that these antibodies do not react with phospholipid alone, but rather with the plasma protein β_2 -glycoprotein I (β_2 GPI, also designated apolipoprotein H) or with a complex antigen comprised of both phospholipid and β_2 GPI (2, 3).

While its physiological role is not known, β_2 GPI binds to anionic phospholipids (4) and exhibits anticoagulant properties in vitro. For example, β_2 GPI has been shown to inhibit the contact phase of intrinsic blood coagulation (5), ADP-dependent platelet aggregation (6), and the prothrombinase activity of platelets (7). β_2 GPI is thought to inhibit the prothrombinase activity of platelets or phospholipid vesicles via binding to anionic phospholipids expressed on these membranes (7). β_2 GPI does not competitively inhibit binding of constituents of the prothrombin activator complex, i.e., Factors X_a or V_a, but may decrease the number of functional prothrombinase sites via a structural alteration of the phospholipid membrane (7). Interestingly, lupus anticoagulants are also thought to inhibit prothrombinase activity by binding to the phospholipid portion of the prothrombin activator complex (8).

Population and family studies demonstrate that the plasma level of β_2 GPI, $\sim 200 \mu\text{g/ml}$ in normal individuals, is under genetic control (9–12). Although the in vitro data cited above imply an in vivo anticoagulant role for β_2 GPI, no signs or symptoms of disease have been reported in individuals with inherited low or absent plasma levels (9). A small number of patients with disseminated intravascular coagulopathy have been reported to have very low β_2 GPI levels, although this appeared to be an acquired phenomenon associated with the coagulopathy (5). Slightly increased to normal levels of β_2 GPI have been observed in a small group of patients with SLE (13).

The finding that β_2 GPI constitutes at least part of the antigen targeted by antiphospholipid antibodies suggests that this protein may play a role in the pathogenesis of lupus anticoagulant activity and the thrombotic diathesis associated with these antibodies. In the current study aPL from patients with autoimmune disease or syphilis were characterized with regard to their requirement for β_2 GPI in a modified anticardiolipin ELISA and in a lupus anticoagulant assay, the dilute Russell viper venom time (dRVVT). Additionally, the anticoagulant properties of β_2 GPI itself and of a murine mAb to β_2 GPI, RP-1, were investigated.

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1. Abbreviations used in this paper: aPL, antiphospholipid antibodies; β_2 GPI, β_2 -glycoprotein I; dRVVT, dilute Russell viper venom time.

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Methods

Patients and sera. Sera were obtained from two patients with the primary antiphospholipid antibody syndrome (14, 15), one patient with SLE (16), and one patient with primary syphilis, all previously identified as having high titer anticardiolipin antibodies in a standard assay (17), and from a normal individual. Sera were stored at 4°C after the addition of 0.02% sodium azide. IgG was purified from sera by protein A-Sepharose 4B (Zymed Laboratories, South San Francisco, CA) column chromatography under low salt conditions as described (18). IgG preparations contained < 100 ng/ml β 2GPI by ELISA.

Purification of β 2GPI. β 2GPI was purified from normal human plasma as a by-product of the protein C inhibitor preparation (19). Plasma was made 0.2 M in barium chloride, centrifuged at 4,000 g, and the supernatant collected. Material precipitated by ammonium sulfate (50–70% saturation) was collected by centrifugation, resuspended in 50 mM Tris, 100 mM NaCl, pH 7.0, and applied to a dextran sulfate-agarose column. Proteins were eluted with a linear salt gradient (100–600 mM NaCl). Fractions containing putative β 2GPI were identified by SDS-PAGE, pooled, dialyzed against 50 mM Tris, pH 9.0, applied to DEAE-Sepharcel (Pharmacia-LKB, Piscataway, NJ) and eluted with 50 mM Tris-phosphoric acid, pH 6.0. β 2GPI was dialyzed against HNP buffer (20 mM HEPES, 150 mM NaCl, 0.1% polyethylene glycol, pH 7.4) and stored at –20°C. A single protein band was observed by SDS-PAGE and Coomassie blue staining. The identity of the protein was confirmed by NH₂-terminal amino acid sequencing (12 cycles) (20) performed by the Protein Chemistry Laboratory, The University of North Carolina at Chapel Hill.

Monoclonal antibody preparation. mAbs to β 2GPI were produced by immunizing BALB/c mice with a crude β 2GPI preparation, boosting twice, and performing the fusion as described by Harlow and Lane (18) using X63Ag8.653 myeloma cells. Colonies were screened for binding to solid-phase β 2GPI in an ELISA. Positive clones were subcloned and expanded. mAbs were purified from cell culture supernatants by affinity chromatography on immobilized protein A (Beckman Instruments, Inc., Fullerton, CA) and stored in HNP buffer. The mAb of highest affinity, RP-1, was isotypized (HyClone Laboratories, Inc., Logan, UT) as IgG1. RP-1 did not bind to solid-phase cardiolipin in the absence of human β 2GPI. Murine myeloma protein MOPC-21 (Organon Teknika, Durham, NC) and mAb 792 to complement component C5 (provided by Dr. William Kolb, Quidel, San Diego, CA) were used as isotype controls.

Modified anticardiolipin antibody ELISA. The ELISA for antibodies to cardiolipin was performed with modifications similar to those of McNeil et al. (3). The standard anticardiolipin ELISA (17) uses serum samples, which contain endogenous human β 2GPI (~2 μ g/ml, assuming sera are diluted 1:100), and 10% FCS/PBS, which contains bovine β 2GPI. In contrast, the modified assay uses purified IgG samples, containing < 100 ng/ml β 2GPI, and 0.3% gelatin (Sigma Chemical Co., St. Louis, MO)/PBS as the blocking agent and sample diluent. Neither FCS nor BSA were present in the assay to avoid contamination with bovine β 2GPI. Purified human β 2GPI, 30 μ g/ml, was added to the samples in certain experiments, as indicated.

β 2GPI ELISA. β 2GPI was quantitated in a sandwich ELISA. Microtiter plates (ICN/Flow Laboratories, Inc., McLean, VA) were coated with goat anti-human β 2GPI IgG (Atlantic Antibodies, Scarborough, ME), 10 μ g/ml in PBS, and blocked with 1% BSA/PBS. Samples diluted in 1% BSA/PBS were applied to the wells and incubated for 2 h at room temperature. The amount of β 2GPI bound to the wells was quantitated by the addition of RP-1, 2 μ g/ml, followed by alkaline phosphatase-conjugated goat anti-mouse IgG (Zymed) and *p*-nitrophenyl phosphate (Sigma). Optical density at 405 nm was measured with an Emax microplate reader and SOFTmax analysis software (Molecular Devices Corp., Menlo Park, CA). Standard concentrations of β 2GPI, 0.2 ng/ml to 1 μ g/ml, were included on each plate.

Preparation of β 2GPI-depleted plasma. Anti- β 2GPI mAb RP-1 was coupled to Affi-Gel Hz hydrazide gel (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's protocol. The RP-1 col-

umn was equilibrated with PBS and normal platelet-poor plasma was infused at 0.1 ml/min. Peak effluent fractions were collected with minimal dilution, pooled, and stored at –20°C. β 2GPI concentration of depleted plasma was < 1 μ g/ml by ELISA.

Dilute Russell viper venom time. The effect of antibodies and β 2GPI on the dRVVT of platelet-poor plasma was assessed (21). 100 μ l normal or β 2GPI-depleted plasma, 50 μ l Thromboplastin (Ortho Diagnostic Systems, Raritan, NJ), diluted 1:4, 50 μ l Russell viper venom (Wellcome Diagnostics, Dartford, UK), diluted 1:100, and 100 μ l HNP buffer containing antibodies and/or CaCl₂ purified β 2GPI were incubated 30 s at 37°C. 100 μ l 25 mM CaCl₂ (Organon Teknika) was added and the clotting time measured using a fibrometer (Fibrosystem; BBL Microbiology Systems, Becton, Dickinson, and Co., Cockeysville, MD). All assays were performed in duplicate (\leq 5% variability).

Results

In initial studies the anticardiolipin reactivity of purified IgG from patients with the aPL syndrome, SLE, and syphilis was characterized with respect to the requirement for β 2GPI (Fig. 1). In the absence of β 2GPI, the binding of IgG from patients with the aPL syndrome (aPL-1 and aPL-2) and SLE did not differ from the normal control, in agreement with previous reports (2, 3). In contrast, IgG from a syphilitic patient recognized cardiolipin in the absence of β 2GPI. In the presence of β 2GPI, aPL-1 and aPL-2 IgGs bound strongly, and SLE IgG bound moderately, to cardiolipin. The anticardiolipin reactivity of the syphilis IgG was partially inhibited by β 2GPI. The binding of normal IgG was higher in the absence of β 2GPI, implying that β 2GPI is providing additional blocking of the ELISA plate. (An alternative interpretation, that the higher "background" of normal IgG represents low level specific binding that can be inhibited by β 2GPI, cannot be excluded.) In no instance was binding detected to solid-phase β 2GPI alone (data not shown). These data suggest that the "anticardiolipin" reactivity of IgG from patients with autoimmune disease is significantly different from that of patients with syphilis. The former apparently binds to a cardiolipin- β 2GPI complex, while the latter recognizes cardiolipin alone and is inhibited by β 2GPI.

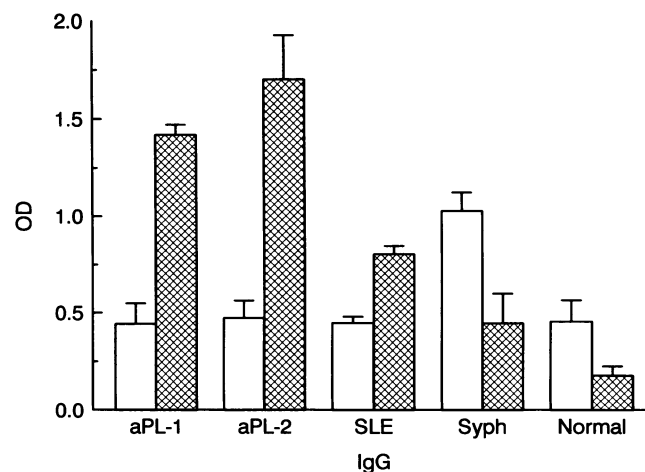


Figure 1. Binding of autoimmune aPL IgG to cardiolipin requires the presence of β 2GPI. Purified IgG, 50 μ g/ml in 0.3% gelatin, from patients with the primary aPL syndrome (aPL-1 and aPL-2), SLE, primary syphilis (Syph), and from a normal individual were assayed. Each IgG was tested in the absence (open bars) or presence (cross-hatched bars) of 30 μ g/ml human β 2GPI. Results are the mean \pm SD of three samples. □, No β 2GPI; ■, + β 2GPI.

In view of this differential reactivity with β 2GPI, the lupus anticoagulant activity of IgG from patients and controls was assessed in a modified dRVVT assay. As shown in Fig. 2, IgG from patients with the aPL syndrome and SLE demonstrated lupus anticoagulant activity when added to normal plasma in a dose-dependent fashion. As expected, IgG from syphilitic or normal individuals did not exhibit an anticoagulant effect. Thus, antibody reactivity with the β 2GPI-cardiolipin complex, but not reactivity with cardiolipin alone, was correlated with anticoagulant activity.

To determine the role of β 2GPI in the lupus anticoagulant activity of autoimmune aPL, the effect of these antibodies on the dRVVT of normal plasma, β 2GPI-depleted plasma, and β 2GPI-depleted plasma partially reconstituted with β 2GPI was studied. The β 2GPI dependence of lupus anticoagulant activity is demonstrated in Fig. 3. Antibodies from patients with autoimmune disease prolonged the dRVVT of normal plasma but had little or no anticoagulant effect when added to β 2GPI-depleted plasma. Partial reconstitution of depleted plasma with purified human β 2GPI restored the anticoagulant effect of autoimmune aPL. Depletion of β 2GPI from normal plasma did not in itself significantly affect the dRVVT.

To investigate further the anticoagulant activity of antibodies reactive with β 2GPI, RP-1, a murine mAb to β 2GPI, was studied. RP-1 was found to have anticoagulant properties similar to those of autoimmune aPL. RP-1 prolonged the dRVVT of normal plasma in a dose-dependent fashion (Fig. 4, *solid line*). Isotype control myeloma protein MOPC-21 did not affect the dRVVT (data not shown). The anticoagulant effect of RP-1 was dependent upon the presence of β 2GPI. In a representative experiment RP-1, 62.5 μ g/ml, prolonged the dRVVT of normal plasma by 53% (41.5 s vs. 27.2 s), had no effect on the dRVVT of β 2GPI-depleted plasma (27.7 s vs. 27.3 s), and prolonged the dRVVT of reconstituted plasma (β 2GPI-depleted plasma + 24 μ g/ml β 2GPI) by 36% (36.9 s vs. 27.2 s). While depletion of β 2GPI from normal plasma did not significantly affect the dRVVT, addition of supraphysiological concentrations of exogenous β 2GPI prolonged the dRVVT in a dose-dependent fashion, as shown in Fig. 4. Fig. 4 also demon-

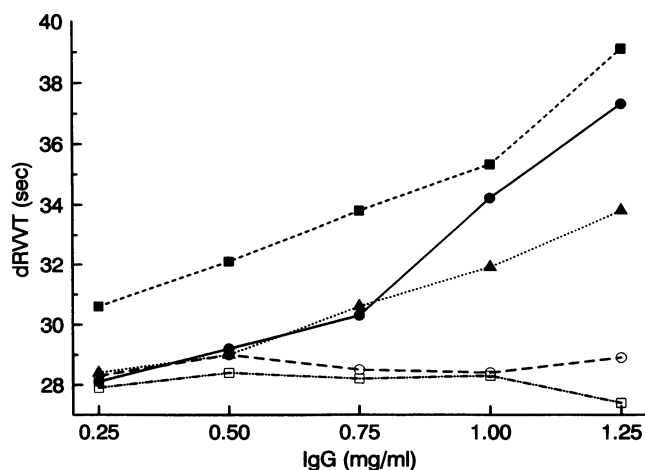


Figure 2. Prolongation of the dRVVT of normal plasma by autoimmune aPL IgG. Samples tested are the same as in Fig. 1. Data shown are representative of at least two independent experiments. —●—, aPL-1; ---■---, aPL-2; ···▲···, SLE; - - -○- - -, primary syphilis; - - -□- - -, normal.

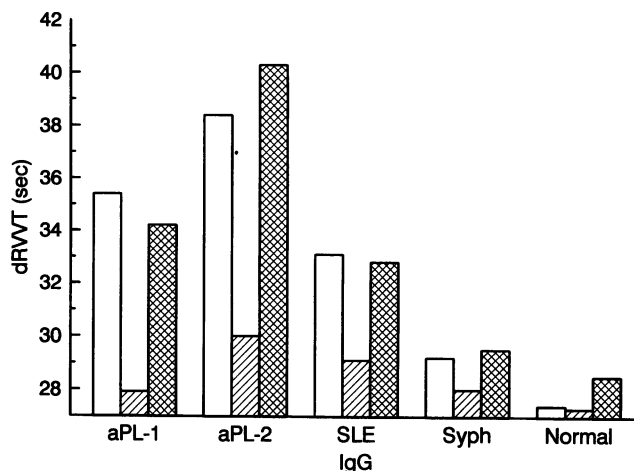


Figure 3. Anticoagulant activity of autoimmune aPL IgG is dependent upon β 2GPI. The dRVVT assay was performed using normal plasma (open bars), β 2GPI-depleted plasma (single-hatched bars), and β 2GPI-depleted plasma partially reconstituted with 40 μ g/ml β 2GPI (cross-hatched bars). Patient and normal IgG were tested at 1 mg/ml. Data shown are representative of at least three independent experiments. □, Normal plasma; ▨, depleted plasma; ▩, depleted plasma + β 2GPI.

strates that the anticoagulant effects of RP-1 and exogenous β 2GPI were approximately additive. Prolongation of the dRVVT was not thought to be due to a nonspecific immune complex effect as mAb 792 to another plasma protein, complement component C5, had no anticoagulant activity (data not shown).

Discussion

These data demonstrate a critical role for β 2GPI in the pathophysiology of lupus anticoagulant activity. Antibodies from pa-

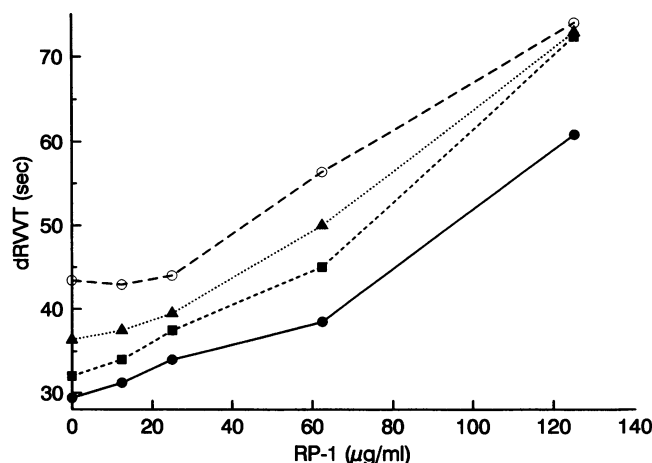


Figure 4. Effects of mAb RP-1 and exogenous β 2GPI on the dRVVT of normal plasma. The solid line represents the effect of mAb RP-1 on normal plasma without any exogenous β 2GPI. As indicated, exogenous β 2GPI was added to normal plasma at final concentrations of 35, 105, and 175 μ g/ml. The final concentration of endogenous β 2GPI was \sim 50 μ g/ml. Isotype control MOPC-21 and anti-C5 mAb 792 did not affect the dRVVT (not shown). Data shown are representative of at least two independent experiments. Exogenous β 2GPI (μ g/ml): —●—, none; ---■---, 35; ···▲···, 105; - - -○- - -, 175.

tients with the aPL syndrome or SLE reacted with a β 2GPI-cardiolipin complex in ELISA and exhibited β 2GPI-dependent anticoagulant activity. In contrast, aPL associated with syphilis reacted with cardiolipin and had no anticoagulant effect. Additionally, RP-1, an anti- β 2GPI mAb, demonstrated lupus anticoagulant-like activity. These findings provide an explanation for the clinical observation that autoimmune aPL, but not those associated with syphilis, are associated with the lupus anticoagulant (22). Although these experiments were not performed using affinity-purified antibodies, the clear requirement for β 2GPI in both the modified anticardiolipin ELISA and the dRVVT suggests that the antibodies possessing anticardiolipin activity and lupus anticoagulant activity are either the same or very closely related. Previous data suggesting different antibody specificities (23, 24) may need to be reinterpreted with regard to the presence of β 2GPI in the respective assays. A recent report by Bevers et al. (25) indicates that there may be two types of lupus anticoagulants. In 11 of 16 patients studied, incubation of plasma with liposomes, followed by centrifugation, reduced the anticardiolipin antibody titer of the plasma supernatants without affecting lupus anticoagulant activity. This anticoagulant activity was attributed to antibodies reactive with lipid-bound prothrombin. In the remaining five patients, lupus anticoagulant activity co-sedimented with the anticardiolipin antibodies. These patients may be similar to those in our study, with β 2GPI-dependent lupus anticoagulant activity.

Based on our data, the anticoagulant effects of autoimmune aPL and anti- β 2GPI mAb are mediated by the interaction of three molecular species: immunoglobulin, β 2GPI, and phospholipid. Autoimmune aPL and mAb RP-1 appear to exhibit lupus anticoagulant activity by amplifying the anticoagulant activity of β 2GPI. These antibodies may act either via a steric effect, i.e., blocking phospholipid catalytic sites in addition to those blocked by β 2GPI, or by increasing the affinity of the β 2GPI-phospholipid interaction. The latter mechanism is supported by the observation that "true" aPL associated with syphilis bind phospholipids but do not act as anticoagulants. It is therefore postulated that autoimmune antiphospholipid antibodies, or more precisely anti- β 2GPI/phospholipid antibodies, augment the interaction of β 2GPI with anionic phospholipid surfaces, possibly by cross-linking bound β 2GPI molecules, further inhibiting phospholipid-dependent reactions.

Two alternative hypotheses are proposed to explain the strong association of aPL and the lupus anticoagulant with thrombosis in vivo. First, if one assumes that β 2GPI plays a physiological role as an anticoagulant, then autoimmune aPL could act to decrease the plasma level of β 2GPI thereby causing a thrombotic tendency. However, inherited low or absent plasma levels of β 2GPI have not been associated with thrombosis, and the patients with primary aPL syndrome and SLE reported in this study had normal levels of β 2GPI by ELISA (data not shown). Even if autoimmune aPL do not lower β 2GPI levels, these antibodies could theoretically inhibit β 2GPI function in vivo. Our in vitro data, however, suggest the contrary, that autoimmune aPL enhance the function β 2GPI.

A second hypothesis to explain the paradoxical in vivo procoagulant effect of autoimmune aPL is that β 2GPI may affect phospholipid-dependent reactions of the hemostatic system in addition to the prothrombinase activity measured in lupus anticoagulant assays. For example, β 2GPI may inhibit the thrombin/thrombomodulin-dependent activation of protein C (26)

or the protein C/protein S inactivation of Factors V_a and VIII_a. In such instances β 2GPI would exert a procoagulant and/or antifibrinolytic effect by binding to phospholipid surfaces, the same mechanism by which it inhibits prothrombinase activity. In the protein C pathway, enhancement of β 2GPI-phospholipid interactions by autoimmune aPL would favor thrombosis. β 2GPI, therefore, may inhibit both procoagulant and anticoagulant/fibrinolytic pathways, with the predominant clinical effect of autoimmune aPL being on the latter pathway. Inhibition of the protein C/protein S system by autoimmune aPL has been proposed as a mechanism of thrombosis (27-29).

The precise nature of the antigen targeted by autoimmune aPL remains to be determined. These antibodies may recognize an epitope comprised of both β 2GPI and cardiolipin or a cryptic epitope on one of the molecules expressed only when the two are bound together. Nimpf et al. (7) studied the interaction of β 2GPI with phosphatidylcholine/phosphatidylserine vesicles and observed structural alteration of the phospholipids but not β 2GPI. On the other hand, Galli et al. (2) reported that anticardiolipin antibodies bound to solid-phase β 2GPI alone and speculated that the antigenic epitope on β 2GPI might be present only when the molecule is bound to phospholipid or absorbed to plastic. Moreover, mAb RP-1, which mimics autoimmune aPL, recognizes β 2GPI alone. Our data and that of others (3, 30) indicate that autoimmune aPL bind to a β 2GPI-phospholipid complex and not to solid-phase β 2GPI alone. This discrepancy may be due to differences in immobilization of the antigen, i.e., some methods may cause β 2GPI to assume a configuration similar to that of phospholipid-bound β 2GPI whereas others may not, or to true differences of antigenic specificity among the patients studied.

In summary, our data suggest that autoimmune aPL express in vitro lupus anticoagulant activity by enhancing the interaction of β 2GPI with phospholipid. Further elucidation of the physiological functions of β 2GPI should be helpful in understanding the pathophysiology of the clinical phenomena associated with "antiphospholipid" antibodies, e.g., thrombosis and fetal loss.

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