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

Robert A. S. Roubey,* Charlotte W. Pratt,‡ Jill P. Buyon,§ and John B. Winfield*

**Division of Rheumatology and Immunology, Department of Medicine, Thurston Arthritis Research Center, and ‡Department of Pathology, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599; and §Department of Rheumatic Diseases, Hospital for Joint Diseases, New York University School of Medicine, New York 10003*

Abstract

It has been reported that antiphospholipid autoantibodies do not recognize phospholipid alone, but rather the plasma protein β_2-glycoprotein I (β2GPI), or a β2GPI-phospholipid complex. In vitro β2GPI 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 β2GPI 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 β2GPI. As expected, anticardiolipin reactivity associated with autoimmune disease was β2GPI dependent. In contrast, IgG from a patient with syphilis recognized cardiolipin alone and binding was inhibited by β2GPI. Autoimmune antiphospholipid antibodies prolonged the dilute Russell viper venom time of normal plasma, but had no effect on β2GPI-depleted plasma. Antiphospholipid antibodies associated with syphilis had no anticoagulant effect. RP-1, an anti-β2GPI 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 β2GPI. These antibodies and RP-1 appear to amplify the anticoagulant effect of β2GPI 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 (β2GPI, also designated apolipoprotein H) or with a complex antigen comprised of both phospholipid and β2GPI (2, 3).

While its physiological role is not known, β2GPI binds to anionic phospholipids (4) and exhibits anticoagulant properties in vitro. For example, β2GPI 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). β2GPI is thought to inhibit the prothrombinase activity of platelets or phospholipid vesicles via binding to anionic phospholipids expressed on these membranes (7). β2GPI 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 β2GPI, $\sim$ 200 μg/ml in normal individuals, is under genetic control (9–12). Although the in vitro data cited above imply an in vivo anticoagulant role for β2GPI, 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 β2GPI levels, although this appeared to be an acquired phenomenon associated with the coagulopathy (5). Slightly increased to normal levels of β2GPI have been observed in a small group of patients with SLE (13).

The finding that β2GPI 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 β2GPI in a modified anticardiolipin ELISA and in a lupus anticoagulant assay, the dilute Russell viper venom time (dRVVT). Additionally, the anticoagulant properties of β2GPI itself and of a murine mAb to β2GPI, RP-1, were investigated.

Address correspondence to Dr. R. A. S. Roubey, Division of Rheumatology and Immunology, CB #7280, Room 932 FLOB, University of North Carolina, Chapel Hill, NC 27599-7280.

Received for publication 30 March 1992 and in revised form 21 May 1992.

1. *Abbreviations used in this paper:* aPL, antiphospholipid antibodies; β2GPI, β_2-glycoprotein I; dRVVT, dilute Russell viper venom time.

J. Clin. Invest.

0021-9738/92/09/1100/05 $2.00
Volume 90, September 1992, 1100–1104

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 β2GPI by ELISA.

Purification of β2GPI. β2GPI 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 β2GPI were identified by SDS-PAGE, pooled, dialyzed against 50 mM Tris, pH 9.0, applied to DEAE-Sephacel (Pharmacia-LKB, Piscataway, NJ) and eluted with 50 mM Tris-phosphoric acid, pH 6.0. β2GPI 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 NH_2-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 β2GPI were produced by immunizing BALB/c mice with a crude β2GPI 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 β2GPI 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 isotyped (HyClone Laboratories, Inc., Logan, UT) as IgG1. RP-1 did not bind to solid-phase cardiolipin in the absence of human β2GPI. 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 β2GPI (~ 2 μg/ml, assuming sera are diluted 1:100), and 10% FCS/PBS, which contains bovine β2GPI. In contrast, the modified assay uses purified IgG samples, containing < 100 ng/ml β2GPI, 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 β2GPI. Purified human β2GPI, 30 μg/ml, was added to the samples in certain experiments, as indicated.

β2GPI ELISA. β2GPI was quantitated in a sandwich ELISA. Microtiter plates (ICN/Flow Laboratories, Inc., McLean, VA) were coated with goat anti-human β2GPI IgG (Atlantic Antibodies, Scarborough, ME), 10 μg/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 β2GPI bound to the wells was quantitated by the addition of RP-1, 2 μg/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 β2GPI, 0.2 ng/ml to 1 μg/ml, were included on each plate.

Preparation of β2GPI-depleted plasma. Anti-β2GPI 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 column 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. β2GPI concentration of depleted plasma was < 1 μg/ml by ELISA.

Dilute Russell viper venom time. The effect of antibodies and β2GPI on the dRVVT of platelet-poor plasma was assessed (21). 100 μl normal or β2GPI-depleted plasma, 50 μl Thrombofax (Ortho Diagnostic Systems, Raritan, NJ), diluted 1:4, 50 μl Russell viper venom (Wellcome Diagnostics, Dartford, UK), diluted 1:100, and 100 μl HNP buffer containing antibodies and/or $CaCl_2$ purified β2GPI were incubated 30 s at 37°C. 100 μl 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 (≤ 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 β2GPI (Fig. 1). In the absence of β2GPI, 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 β2GPI. In the presence of β2GPI, 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 β2GPI. The binding of normal IgG was higher in the absence of β2GPI, implying that β2GPI 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 β2GPI, cannot be excluded.) In no instance was binding detected to solid-phase β2GPI 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-β2GPI complex, while the latter recognizes cardiolipin alone and is inhibited by β2GPI.

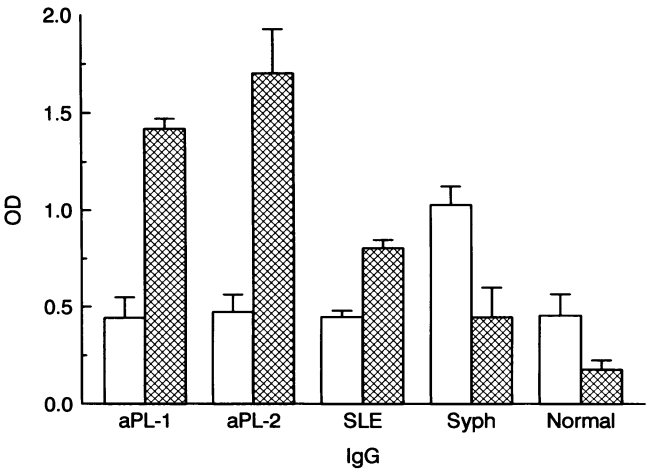

Figure 1. Binding of autoimmune aPL IgG to cardiolipin requires the presence of β2GPI. Purified IgG, 50 μg/ml in 0.3% gelatin, from patients with the primary aPL syndrome (aPL-1 and aPL-2), SLE, primary syphilis (Syph), and from a normal individual were assayed. Each IgG was tested in the absence (*open bars*) or presence (*cross-hatched bars*) of 30 μg/ml human β2GPI. Results are the mean±SD of three samples. □, No β2GPI; ■, + β2GPI.

In view of this differential reactivity with β2GPI, 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 β2GPI-cardiolipin complex, but not reactivity with cardiolipin alone, was correlated with anticoagulant activity.

To determine the role of β2GPI in the lupus anticoagulant activity of autoimmune aPL, the effect of these antibodies on the dRVVT of normal plasma, β2GPI-depleted plasma, and β2GPI-depleted plasma partially reconstituted with β2GPI was studied. The β2GPI 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 β2GPI-depleted plasma. Partial reconstitution of depleted plasma with purified human β2GPI restored the anticoagulant effect of autoimmune aPL. Depletion of β2GPI from normal plasma did not in itself significantly affect the dRVVT.

To investigate further the anticoagulant activity of antibodies reactive with β2GPI, RP-1, a murine mAb to β2GPI, 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 β2GPI. In a representative experiment RP-1, 62.5 μg/ml, prolonged the dRVVT of normal plasma by 53% (41.5 s vs. 27.2 s), had no affect on the dRVVT of β2GPI-depleted plasma (27.7 s vs. 27.3 s), and prolonged the dRVVT of reconstituted plasma (β2GPI-depleted plasma + 24 μg/ml β2GPI) by 36% (36.9 s vs. 27.2 s). While depletion of β2GPI from normal plasma did not significantly affect the dRVVT, addition of supraphysiological concentrations of exogenous β2GPI prolonged the dRVVT in a dose-dependent fashion, as shown in Fig. 4. Fig. 4 also demonstrates that the anticoagulant effects of RP-1 and exogenous β2GPI 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).

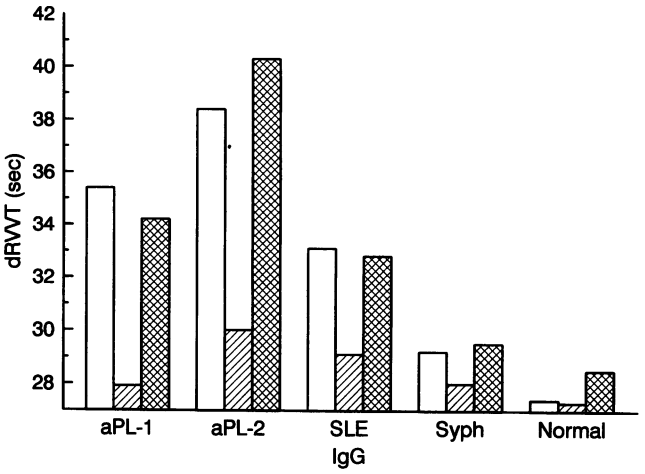

Figure 3. Anticoagulant activity of autoimmune aPL IgG is dependent upon β2GPI. The dRVVT assay was performed using normal plasma (*open bars*), β2GPI-depleted plasma (*single-hatched bars*), and β2GPI-depleted plasma partially reconstituted with 40 μg/ml β2GPI (*cross-hatched bars*). Patient and normal IgG were tested at 1 mg/ml. Data shown are representative of at least three independent experiments. □, Normal plasma; ▨, depleted plasma; ■, depleted plasma + β2GPI.

Discussion

These data demonstrate a critical role for β2GPI in the pathophysiology of lupus anticoagulant activity. Antibodies from pa-

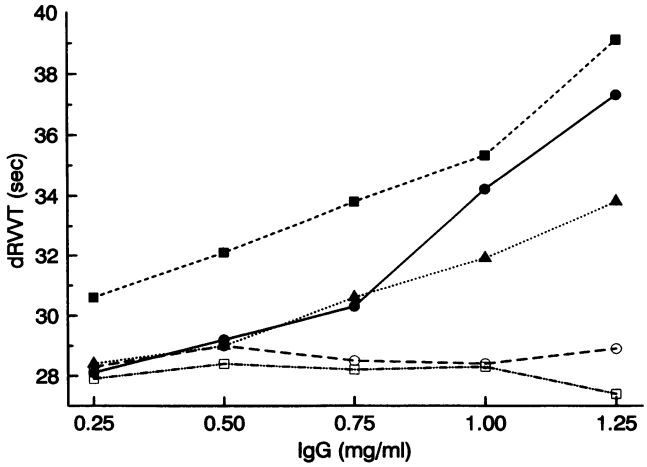

Figure 2. Prolongation of the dRVVT of normal plasma by autoimmune aPL IgG. Samples tested are the same as in Fig. 1. Data shown are representative of at least two independent experiments. — ● —, aPL-1; - - - ■ - - -, aPL-2; · · · ▲ · · ·, SLE; – – ○ – –, primary syphilis; – · · – □ – · · –, normal.

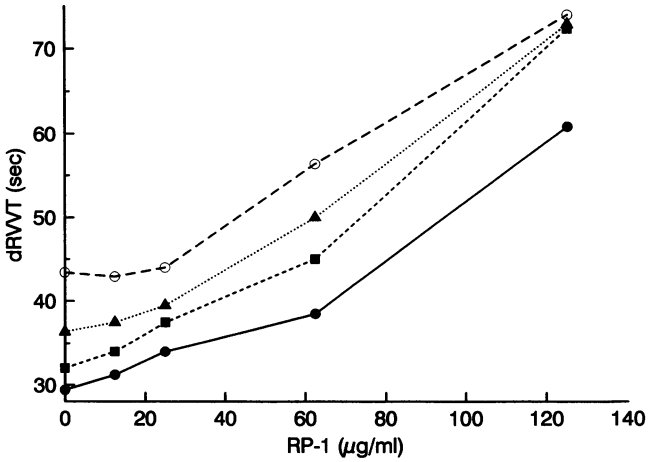

Figure 4. Effects of mAb RP-1 and exogenous β2GPI on the dRVVT of normal plasma. The solid line represents the effect of mAb RP-1 on normal plasma without any exogenous β2GPI. As indicated, exogenous β2GPI was added to normal plasma at final concentrations of 35, 105, and 175 μg/ml. The final concentration of endogenous β2GPI was ~ 50 μg/ml. Isotype control MPOC-21 and anti-C5 mAb 792 did not affect the dRVVT (not shown). Data shown are representative of at least two independent experiments. Exogenous β2GPI (μg/ml): — ● —, none; - - - ■ - - -, 35; · · · ▲ · · ·, 105; – – ○ – –, 175.

tients with the aPL syndrome or SLE reacted with a β2GPI-cardiolipin complex in ELISA and exhibited β2GPI-dependent anticoagulant activity. In contrast, aPL associated with syphilis reacted with cardiolipin and had no anticoagulant effect. Additionally, RP-1, an anti-β2GPI 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 β2GPI 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 β2GPI 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 β2GPI-dependent lupus anticoagulant activity.

Based on our data, the anticoagulant effects of autoimmune aPL and anti-β2GPI mAb are mediated by the interaction of three molecular species: immunoglobulin, β2GPI, and phospholipid. Autoimmune aPL and mAb RP-1 appear to exhibit lupus anticoagulant activity by amplifying the anticoagulant activity of β2GPI. These antibodies may act either via a steric effect, i.e., blocking phospholipid catalytic sites in addition to those blocked by β2GPI, or by increasing the affinity of the β2GPI-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-β2GPI/phospholipid antibodies, augment the interaction of β2GPI with anionic phospholipid surfaces, possibly by cross-linking bound β2GPI 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 β2GPI plays a physiological role as an anticoagulant, then autoimmune aPL could act to decrease the plasma level of β2GPI thereby causing a thrombotic tendency. However, inherited low or absent plasma levels of β2GPI have not been associated with thrombosis, and the patients with primary aPL syndrome and SLE reported in this study had normal levels of β2GPI by ELISA (data not shown). Even if autoimmune aPL do not lower β2GPI levels, these antibodies could theoretically inhibit β2GPI function in vivo. Our in vitro data, however, suggest the contrary, that autoimmune aPL enhance the function β2GPI.

A second hypothesis to explain the paradoxical in vivo procoagulant effect of autoimmune aPL is that β2GPI may affect phospholipid-dependent reactions of the hemostatic system in addition to the prothrombinase activity measured in lupus anticoagulant assays. For example, β2GPI 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 β2GPI 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 β2GPI-phospholipid interactions by autoimmune aPL would favor thrombosis. β2GPI, 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 β2GPI 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 β2GPI with phosphatidylcholine/phosphatidylserine vesicles and observed structural alteration of the phospholipids but not β2GPI. On the other hand, Galli et al. (2) reported that anticardiolipin antibodies bound to solid-phase β2GPI alone and speculated that the antigenic epitope on β2GPI might be present only when the molecule is bound to phospholipid or absorbed to plastic. Moreover, mAb RP-1, which mimics autoimmune aPL, recognizes β2GPI alone. Our data and that of others (3, 30) indicate that autoimmune aPL bind to a β2GPI-phospholipid complex and not to solid-phase β2GPI alone. This discrepancy may be due to differences in immobilization of the antigen, i.e., some methods may cause β2GPI to assume a configuration similar to that of phospholipid-bound β2GPI 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 β2GPI with phospholipid. Further elucidation of the physiological functions of β2GPI should be helpful in understanding the pathophysiology of the clinical phenomena associated with "antiphospholipid" antibodies, e.g., thrombosis and fetal loss.

Acknowledgments

The authors thank James Meade, Ph.D., of the Clinical Coagulation Lab, University of North Carolina Hospitals, for technical assistance, Dr. Jay Lozier for helpful suggestions, and Dr. Gilbert C. White for a thoughtful critique of this manuscript.

This research was supported in part by National Institutes of Health grants AR-30863, AR-7416, AR-30701, and HL-06350, and by a Biomedical Research Center grant from the Arthritis Foundation.

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