Anti–β2-Glycoprotein I (β2GPI) Monoclonal Antibodies with Lupus Anticoagulant-like Activity Enhance the β2GPI Binding to Phospholipids

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Abstract

β2-Glycoprotein I (β2GPI), a plasma glycoprotein with phospholipid-binding property, is known to be the actual target antigen for autoimmune type anticardiolipin antibodies (aCLs). Certain groups of aCLs (anti-β2GPI antibodies) exert lupus anticoagulant (LA) activity and perturb the function of vascular endothelial cells. This investigation aimed at highlighting some insights into the molecular basis by which aCLs exert their biological effects by using anti-β2GPI mAbs with well-characterized epitopes from mice and from patients with antiphospholipid syndrome. Anti-β2GPI mAbs directed against the third domain (Cof-20 and Cof-22) and fourth domain (Cof-21, EY1C8, and EY2C9) of β2GPI inhibited the thrombin generation induced by Russell's viper venom in diluted plasma and that induced by the prothrombinase complex reconstituted with purified clotting factors. This anticoagulant activity was abrogated in the presence of an excess amount of phospholipids, thus resembling the LA activity. In stark contrast, anti-β2GPI mAbs directed against the fifth domain and the carboxy-terminal region of the fourth domain showed no LA-like activity. These findings suggest that the LA activity of anti-β2GPI antibodies depends on their epitope specificity. Experiments carried out to clarify the mechanism of the LA activity showed that anti-β2GPI mAbs with LA-like activity, but not those without this effect, enhance the β2GPI binding to phospholipids. In addition, the $F(ab')_2$ fragment, but not the Fab′ fragment, of the anti-β2GPI mAbs was found to enhance the LA activity and the β2GPI binding to phospholipids, suggesting that anti-β2GPI antibodies induce formation of multiple complexes of β2GPI on the surface of phospholipids because of their bivalent property. This clustering of β2GPI molecules induced by anti-β2GPI antibodies, probably because of their multivalent property and epitope specificity, might hinder the lateral mobility and activation of clotting factors on the surface of phospholipids and thus exert LA activity. Clustering of β2GPI molecules may also explain the molecular mechanism by which anti-β2GPI antibodies alter the function of leukocytes and endothelial cells. The well-documented heterogeneous LA activity of aCLs (anti-β2GPI antibodies) may also be explained by their epitope specificity. (*J. Clin. Invest.* 1997. 99: 2260–2268.) Key words: antiphospholipid • apolipoprotein • autoantibodies • prothrombin • phospholipid

Introduction

β2-Glycoprotein I (β2GPI)[1] is a plasma glycoprotein with an apparent molecular mass of 50 kD (326 amino acids) (1–3). β2GPI is composed of five short-consensus-repeat domains, the so-called sushi domains, often found in the structure of complement factors and selectins (4). β2GPI strongly binds to anionic phospholipids; the fifth sushi domain (domain V) being the major phospholipid-binding region (5–9). The high structural homology among the human, bovine, rat, and mouse β2GPI molecules suggests that β2GPI might play an important physiological role in vivo (10). β2GPI exhibits anticoagulant properties in vitro, such as the inhibition of the intrinsic blood coagulation pathway (11, 12), and the prothrombinase activity of activated platelets (13). Recently, β2GPI became the subject of increasing interest since it was demonstrated that it is the actual target antigen of autoimmune-type anticardiolipin antibodies (aCLs) (14–17, see references 18 and 19 for recent review). We and others have also recently demonstrated that aCLs bind to β2GPI even in the absence of cardiolipin (20, 21).

The presence of antiphospholipid antibodies is defined by the detection of aCL by immunoabsorbent assays or by the detection of lupus anticoagulant (LA) activity (22–24). Bevers et al. (25) reported that the expression of LA activity depends on the potential of LA antibodies to bind to phospholipid-bound prothrombin. However, originally, the term LA was defined as antiphospholipid antibodies that prolong the clotting time of phospholipid-dependent coagulation tests. Based on this definition, LA antibodies may recognize a heterogeneous group of antigens. In fact, certain groups of aCLs also possess LA activity (26). According to the classification by Galli et al. (26), aCLs of type A prolong the phospholipid-dependent clotting time, while those of type B lack this anticoagulant activity. Nevertheless, both types require β2GPI for detection in aCL tests.[2] The molecular basis of the different properties of types A and B aCLs (aCLs with or without LA activity, respectively) is unknown.

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1. *Abbreviations used in this paper:* aCL, anticardiolipin antibody; APTT, activated partial thromboplastin time; β2GPI, β2-glycoprotein I; LA, lupus anticoagulant; RVV, Russell's viper venom.

2. References 25 and 26 use the term LA in a limited fashion to designate antibodies with anticoagulant activity that recognize prothrombin; type A and type B aCL refer to antibodies to β2GPI with and without anticoagulant activity, respectively. In this report, however, we consider type A aCL to be LA.

The presence of antiphospholipid antibodies has been linked to a major risk of developing thromboembolic complications in various autoimmune disorders (27, 28). Numerous studies have been carried out regarding the effect of antiphospholipid antibodies on coagulation factors and inhibitors present in the fluid phase, and on cellular regulators of hemostasis. However, the paradox of the in vitro anticoagulant activity of antiphospholipid antibodies and their relation with increased thrombotic complication in vivo remain unsolved (29). Epidemiological studies described a significant relationship between the presence of aCL (anti-β2GPI antibodies) and the history of thrombosis (30–33). Induction of antiphospholipid syndrome in experimental animals by injecting anti-β2GPI antibodies or by immunizing β2GPI also emphasizes the importance of these antibodies in thrombogenesis (34–37). Moreover, activation or functional perturbation of leukocytes and vascular endothelial cells induced by aCL or anti-β2GPI antibodies also has been recently reported (38, 39).

We have recently developed several monoclonal aCL from lymphocytes of patients with the antiphospholipid syndrome (40) and NZW × BXSB F1 mice, an animal model of human antiphospholipid syndrome (20, 41). Various anti-β2GPI mAbs were also developed in our laboratory by mice immunization, and the domains recognized by these antibodies were determined using recombinant β2GPI mutants produced by a baculovirus/insect cell expression system (42, 43). These newly developed anti-β2GPI mAbs which recognize specific epitopes in the molecule of β2GPI were used in this study to evaluate the molecular basis of the β2GPI-dependent LA activity. The results of the present investigation suggest that anti-β2GPI mAbs exert LA-like activity by inducing a clustering of β2GPI molecules on the surface of phospholipids and that this effect depends on the epitope specificity of the anti-β2GPI mAbs. The clustering mechanism may play an important role in the pathophysiology of the well-recognized cell surface alteration induced by anti-β2GPI antibodies.

Methods

Reagents, proteins, and phospholipid vesicles. Prothrombin and Factor X were isolated from human plasma, as described (44). Factor X was activated by incubating with Russell's viper venom (RVV)-factor X activator coupled to 2-fluoro-1-methylpyridinium toluene-4-sulfonate-activated Cellulofine (Seikagaku Kogyo Co., Tokyo, Japan) (45). Factor V was purified from freshly frozen platelet-free plasma (46, 47), using affinity column chromatography with anti–Factor V murine mAb-coupled Sepharose gel, and was activated with thrombin, as described (46). β2GPI was purified from normal human sera by sequential cardiolipin-affinity, ion exchange and protein A–Sepharose column chromatographies, as described (41). All purified proteins were homogeneous with > 95% of purity, as judged by SDS-polyacrylamide gel electrophoresis followed by silver staining and were stored in small aliquots at −80°C until use. The synthetic chromogenic peptide substrate of thrombin, S-2238 (D-Phe-pipecolyl-Arg-pNA), was purchased from Chromogenix AB (Mölndal, Sweden). Rabbit brain cephalin, ovalbumin, and RVV were from Sigma Chemical Co. (St. Louis, MO). HSA (crystallized) and BSA (crystallized) were from Seikagaku Kogyo Co. Phosphatidylcholine (1-palmitoyl-2-oleoyl) and phosphatidylserine (1-palmitoyl-2-oleoyl) were from Avanti Polar Lipids (Alabaster, AL). Gelatin was from Bio-Rad Laboratories (Richmond, CA). Rabbit brain thromboplastin (Thromboplastin C) and Actin™, consisting of ellagic acid and cephalin, were from Baxter Dade (Miami, FL). Monoclonal mouse anti–human vWf antibody (clone F8/86) was purchased from DAKO A/S (Glostrup, Denmark). The phospholipid mixture used in this study was rabbit brain cephalin containing phosphatidylcholine 30–35%, phosphatidylserine 10–15%, and phosphatidylethanolamine 35–40% (48, 49); this commercially available (Sigma Chemical Co.) phospholipid mixture is often used in clinical laboratory coagulation assays such as activated partial thromboplastin time (APTT) or diluted RVV (dRVV)-test. Sonicated phospholipid vesicles were prepared as described (50). Briefly, the phospholipids (1 mg/ml) were suspended by vortexing in Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.4, 150 mM NaCl), and sonicated using Sonifier 250 (Branson Ultrasonics Corp., Danbury, CT) for 20 min in an ice bath under nitrogen flow at a 20% power setting. After sonication, phospholipid suspensions were centrifuged at 8,000 *g* for 15 min and passed through a 0.22-μm filter (Millex-GV; Millipore, Bedford, MA). About 90% of the initial phospholipid concentration was recovered. When phospholipid vesicles were stored, they were kept at 4°C under nitrogen and resonicated for 5 min immediately before use. Pooled normal plasma was prepared by mixing fresh citrated plasma from normal healthy volunteers and stored at −80°C. All other chemicals and reagents were of the best quality commercially available.

Anti-β2GPI mAbs. Six monoclonal IgGs (Cof-18, Cof-19, Cof-20, Cof-21, Cof-22, and Cof-23) were obtained from BALB/c mice immunized with human β2GPI (20, 42). WB-CAL-1 is a monoclonal aCL IgG obtained from NZW × BXSB F1 mice, an animal model of

Table I. Characteristics of Anti-β2GPI Monoclonal Antibodies

Antibody	Origin of mAb	Reactivity to β2GPI	K_A* (M^{-1})	Subclass	Epitopes‡	References
Cof-18	BALB/c mice immunized with human β2GPI	Reactive with β2GPI coated to non-oxygenated plates or with soluble β2GPI	2.5×10^{9}§	IgG1, κ§	Domain V	42
Cof-19			3.8×10^{9}§	IgG2a,κ§	Domain V	42
Cof-20			9.0×10^{10}§	IgG1, κ§	Domain III	42
Cof-21			1.9×10^{9}§	IgG1, κ§	Domain IV	42
Cof-22			1.2×10^{9}§	IgG1, κ§	Domain III	42
Cof-23			5.8×10^{9}§	IgG1, κ§	Domain IV (close to domain V)	42
WB-CAL-1	NZW × BXSB F1 mice	Reactive with β2GPI coated on oxygenated plates	ND‖	IgG2a, κ	Close to, or in domain IV	41
TM1B3	Lymphocytes from patients with antiphospholipid syndrome		ND	IgM, μ, λ	Domain IV	40, 42
EY1C8			ND	IgM, μ, κ	Domain IV	40, 42
EY2C9			ND	IgM, μ, λ	Domain IV	40, 42
GR1D5			ND	IgM, μ, λ	Domain IV and/or domain V	40, 42

*K_A, affinity constant. §Determined in this study. ‖ND, not determined. ‡See Methods section for details.

human antiphospholipid syndrome (41). WB-CAL-1 binds to human β2GPI coated on polyoxygenated plates, even in the absence of cardiolipin (20). Four monoclonal IgMs (TM1B3, EY1C8, EY2C9, and GR1D5) were produced by hybridomas between lymphocytes isolated from three patients with antiphospholipid syndrome and an SHM-D33 mouse–human heterohybridoma cell line (40). These IgMs also bind to β2GPI immobilized on polyoxygenated plates in the absence of cardiolipin (40). The epitopes for WB-CAL-1, EY2C9, and Cof-series IgGs were mapped using several recombinant deleted mutants of β2GPI produced by a baculovirus/insect cell expression system (42, 43). The epitope for Cof-23 was further determined by competitive binding assays. In these experiments, fluid phase Cof-23 competed with Cof-18 immobilized on microtiter wells for binding to radiolabeled β2GPI, suggesting that the epitope of Cof-23 is closely related to that of Cof-18. The epitopes for TM1B3, EY1C8, and GR1D5 were also analyzed in our laboratory using recombinant deleted mutants of β2GPI (manuscript in preparation). The affinity constants for the interaction between radiolabeled β2GPI and Cof-series mAbs were determined by radioimmunoassay as previously described (51). The isotypes of the Cof-series IgGs were determined by enzyme-linked immunoassays. Characteristics of mAbs used in this study are summarized in Table I. A monoclonal IgM produced by hybridomas between lymphocytes from a healthy volunteer and SHM-D33 hybrid cells was used as control.

Preparation of F(ab′)$_2$ and Fab′ fragments. The F(ab′)$_2$ and Fab′ fragments of IgG were prepared as described (21, 52, 53). Briefly, purified IgG (2 mg/ml) was digested with 0.2 mg/ml pepsin at 37°C for 16 h in 0.1 M sodium citrate, pH 3.5. The reaction was stopped by the addition of 3 M Tris and then gel-filtrated (Sephadex G-150). A small amount of intact IgG in F(ab′)$_2$ fraction was removed by protein A-column chromatography. Purity of the resultant F(ab′)$_2$ fragment was monitored by SDS-PAGE under nonreducing conditions. Fab′ fragments were derived from F(ab′)$_2$ preparations by reducing with 0.01 M DTT for 1 h at room temperature and by alkylation with a 10% molar excess of iodoacetamide for 15 min at room temperature. Reagents were removed using a PD-10 column (Pharmacia Fine Chemicals, Uppsala, Sweden). Intact IgG was also reduced and alkylated under identical conditions. Reduced and alkylated IgG was used for control experiments.

Thrombin generation in plasma. The effect of anti-β2GPI mAbs on prothrombin activation was determined by a modification of the dRVV test (54). A mixture of 20 μl of RVV (1 μg/ml), 40 μl of diluted pooled normal plasma (1:50 in TBS), and 20 μl of cephalin (14 μg/ml) was added to wells of 96-well microtiter plates (Nunc-Immuno BreakApart; Nunc A/S, Roskilde, Denmark), and incubated with either 20 μl of TBS or mAb (0.1 mg/ml) for 10 min at room temperature. Unless otherwise indicated, all material concentrations indicated in brackets were the initial concentrations used in the assays. Microtiter wells used in the assays were precoated with TBS containing gelatin (1 mg/ml), ovalbumin (1 mg/ml), and BSA (10 mg/ml) to avoid adsorption and rapid inactivation of generated thrombin on the microplate surface. Thrombin generation was initiated by the addition of 20 μl of 50 mM $CaCl_2$. After 10 min, 100 μl of TBS containing 50 mM EDTA and chromogenic substrate S-2238 (0.8 mM) were added to stop the reaction and to determine thrombin generation. Change in absorbance at 405 nm produced by *p*-nitroaniline liberation from the substrate was monitored for 20 min, using an EAR 340 microplate reader (SLT-Lab Instruments, Salzburg, Austria). All of the S-2238-cleaving activity measured in plasma in our assays depended on thrombin generation because the activity was completely inhibited by hirudin. The amount of generated thrombin was extrapolated from a standard curve drawn using purified human α-thrombin (2,500 U/mg).

The effect of mAb on thrombin generation was also assessed using coagulation tests for the intrinsic or extrinsic pathway. All reactions were performed in the wells of a 96-well microtiter plate at room temperature. For assaying the intrinsic coagulation pathway, 20 μl of diluted Actin (1:30 in TBS) and 40 μl of diluted pooled normal plasma (1:50 in TBS) were incubated with either 20 μl of TBS or mAb (0.1 mg/ml) at room temperature. After 10 min, 20 μl of 50 mM $CaCl_2$, and 20 min later, 100 μl of S-2238 (0.8 mM) were added. Assay of the extrinsic coagulation pathway was done following a similar procedure but using 20 μl of diluted tissue thromboplastin (Ortho Diagnostic Systems Inc., Raritan, NJ) (1:30 in TBS) instead of Actin.

Plasma clotting time was evaluated according to the diluted APTT (dAPTT) using the KC-10 coagulometer (Heinrich Amelung, Lemgo, Germany). A mixture of 25 μl of pooled normal plasma, 10 μl of Actin, and 65 μl of TBS was incubated with either 50 μl of TBS or mAb (0.25 mg/ml) for 10 min at 37°C. Clotting was then initiated by the addition of 50 μl of 25 mM $CaCl_2$.

Thrombin generation in a reconstituted prothrombinase complex. The effect of anti-β2GPI mAbs, the F(ab′)$_2$ and Fab′ fragments of these mAbs, and reduced and alkylated mAbs on prothrombin activation was determined by evaluating their effects on prothrombinase complex reconstituted using purified factor Xa, factor Va, phospholipids, and Ca^{2+}. A mixture of 20 μl of prothrombin (0.5 mg/ml), 10 μl of factor Va (0.4 μg/ml), 20 μl of cephalin (70 μg/ml), 20 μl of anti-β2GPI mAb (25 μg/ml), and 20 μl of various concentrations of β2GPI (0–1.25 mg/ml) was added to the wells of a 96-well microtiter plate and incubated for 2 min at 25°C. All reagents were diluted in TBS containing gelatin (1 mg/ml), ovalbumin (1 mg/ml), BSA (10 mg/ml), and 2 mM $CaCl_2$. Microwells were precoated with the same solution. Thrombin generation was triggered by the addition of 10 μl of factor Xa (0.1 μg/ml). After 10 min, 100 μl of S-2238 (0.8 mM) was added and absorbance at 405 nm was monitored. The amount of generated thrombin was determined as described above. To evaluate the effect of mAbs and of their F(ab′)$_2$ and Fab′ fragments, various concentrations of intact mAb or derivatives and a fixed concentration of β2GPI (final concentration 50 μg/ml) were used under the same above-mentioned assay conditions.

Iodination of β2GPI. β2GPI was labeled with $Na^{125}I$ (Du Pont-New England Nuclear, Boston, MA), using IODO-BEADS iodinating reagent (Pierce Chemical Co., Rockford, IL). Three beads were washed and added to 0.2 ml of 0.1 M phosphate buffer, pH 6.5, containing 1 mCi of $Na^{125}I$ and allowed to react for 5 min. β2GPI (0.1 mg in 200 μl of the same buffer) was added to the reaction mixture and incubated for 15 min. The reaction was stopped by removing the solution from the beads. Labeling of β2GPI was then resolved by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Unbound radioactive iodine was removed using a PD-10 column. The specific activity of ^{125}I-labeled β2GPI was 1.8×10^5 cpm/pmol.

Binding of β2GPI to phospholipids. Microplate wells were coated with each type of phospholipid (rabbit brain cephalin, pure phosphatidylcholine, or phosphatidylserine/phosphatidylcholine mixture [20%:100%]) by incubating 100 μl of phospholipid vesicles (260 μg/ml) prepared with each type of phospholipids for 5 h at room temperature. After washing and blocking appropriately with TBS containing BSA (0.5 mg/ml) (TBS-B), 100 μl of ^{125}I-β2GPI (0–80 nM) in TBS-B with or without 150 nM of each mAb was applied to the wells and the preparation was incubated for 1 h at 37°C. In separate experiments, 100 μl of various concentrations of ^{125}I-β2GPI (0–180 nM) in TBS-B was incubated with 150 nM of each intact IgG, reduced and alkylated IgG or F(ab′)$_2$ fragments of Cof-21, or 300 nM of Fab′ fragments of Cof-21 in microtiter wells. The wells were then washed three times with TBS-B and the radioactivity was counted in a γ-counter (ARC 6000; Aloka, Tokyo, Japan). Specific binding of ^{125}I-β2GPI to phospholipids was determined by subtracting the nonspecific binding of ^{125}I-β2GPI to pure phosphatidylcholine vesicles from the total binding, since no β2GPI-binding to pure phosphatidylcholine vesicles was reported (9). Specific binding of ^{125}I-β2GPI to phospholipids was also determined as the difference in the binding observed in the absence and in the presence of 10-fold molar excess of unlabeled β2GPI.

The binding of ^{125}I-β2GPI to liposome vesicles in solution phase was determined by the centrifugation procedure (9). 20 nM of ^{125}I-β2GPI was incubated with 250 μg/ml phospholipid vesicles (pure phosphatidylcholine or phosphatidylserine/phosphatidylcholine mixture

[20%:100%]) in 400 μl of TBS-B with or without 150 nM of each mAb for 1 h at 25°C. The mixtures were then centrifuged at 120,000 *g* for 60 min, and the radioactivity in the supernatant was counted in a γ-counter. The amount of liposome-bound ^{125}I-β2GPI was expressed in relation to the amount of ^{125}I-β2GPI in the supernatant observed in experiments carried out in the same assay conditions but in the absence of phospholipids. Specific binding of ^{125}I-β2GPI to phosphatidylserine/phosphatidylcholine mixture (20%:100%) was determined by subtracting the nonspecific binding of ^{125}I-β2GPI to pure phosphatidylcholine vesicles from the total binding.

Results

Effect of anti-β2GPI mAbs on thrombin generation. The effect of anti-β2GPI mAbs on dAPTT clotting time assay was initially evaluated. Of the 11 mAbs listed in Table I, the dAPTT was prolonged by Cof-20 and Cof-21 (the dAPTT of normal control plasma without mAb was 120 s, whereas that in the presence of Cof-20 and Cof-21 was 132 and 147 s, respectively), and slightly by Cof-22 (125 s). The anticoagulant activity of Cof-20, Cof-21, and Cof-22 was more evident in the dRVV-test than that in the dAPTT clotting assay (Fig. 1). Cof-20, Cof-21, and Cof-22 inhibited 69, 90, and 60% of thrombin generation induced by RVV in diluted plasma, respectively. However, none of the remaining anti-β2GPI mAbs significantly affected thrombin generation in the absence of exogenous β2GPI (Fig. 1, *open columns*). The inhibition by Cof-20 and Cof-22 was further enhanced by the addition of 2 μM of β2GPI (Fig. 1, *hatched columns*). Cof-20, Cof-21, and Cof-22 also inhibited thrombin generation induced in diluted plasma by the APTT reagent (25, 36, and 14%, respectively) and by tissue thromboplastin (47, 50, and 27%, respectively).

It was shown previously that the monoclonal aCL (anti-β2GPI) antibodies developed from lymphocytes isolated from patients with antiphospholipid syndrome (TM1B3, EY1C8, EY2C9, and GR1D5) did not show LA activity (40). The results of this previous study were confirmed in the dAPTT clotting time assay (data not shown) and in the dRVV-test (Fig. 1, *open columns*). We found, however, that in the presence of an additional 2 μM of purified β2GPI, EY1C8 or EY2C9 remarkably, and GR1D5 moderately inhibited the RVV-induced thrombin generation in diluted plasma (Fig. 1, *hatched columns*). β2GPI also stimulated the EY1C8- or EY2C9-inhibition of thrombin generation induced by diluted Actin or tissue thromboplastin (data not shown). EY1C8 and EY2C9 mAbs from patients with aCL required an excess amount of β2GPI to express LA activity, suggesting that they have a low affinity for β2GPI.

Effect of anti-β2GPI mAbs on prothrombin activation by the prothrombinase complex. Evaluation of the effect of mAbs on prothrombin activation by the prothrombinase complex reconstituted with purified factor Xa, factor Va, cephalin, and Ca^{2+} showed that β2GPI dose dependently inhibited prothrombinase activity, even in the absence of anti-β2GPI mAb (Fig. 2). However, this β2GPI inhibitory effect was weak; 1 μM β2GPI inhibited only 16% of thrombin generation. As shown in Fig. 2 *A*, although Cof-20, Cof-21, and Cof-22 did not affect the prothrombinase complex activity in the absence of β2GPI, they did induce a marked inhibition of thrombin generation, depending on the concentration of β2GPI. Cof-20, Cof-21, and Cof-22 inhibited 64, 50, and 56% of thrombin generation in the presence of 0.2 μM β2GPI, respectively. As shown in Fig. 2 *B*, among the four mAbs developed from lymphocytes isolated from patients with antiphospholipid syndrome, EY1C8 and EY2C9 markedly inhibited thrombin generation, in a β2GPI dose-dependent manner; EY1C8 and EY2C9 inhibited 56 and 64% of thrombin generation in the presence of 0.2 μM β2GPI, and 87 and 89% in the presence of 1 μM β2GPI, respectively. EY1C8 and EY2C9 required 2 μM exogenous of β2GPI in addition to ~ 32 nM of endogenous β2GPI to express LA activity in the dRVV assay (Fig. 1). The results obtained in this dRVV assay are in accord with those described in Fig. 2 *B*, in which these mAbs inhibit thrombin generation in the presence

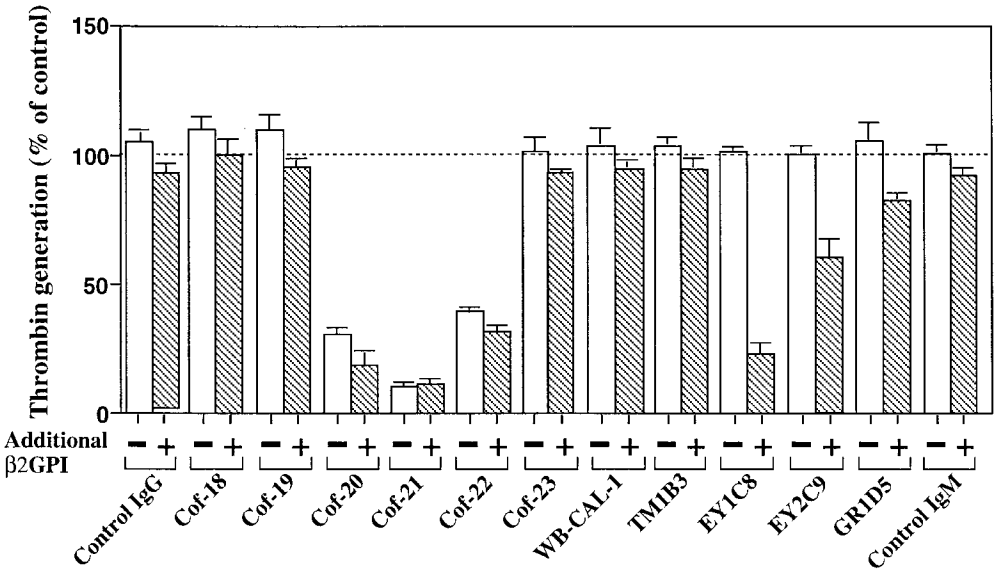

Figure 1. Effect of anti-β2GPI mAbs on thrombin generation induced by RVV in diluted plasma. The assay was performed in the presence (+) or absence (−) of exogenously added β2GPI (2 μM). Assuming a normal β2GPI plasma concentration of ~ 200 μg/ml (4 μM), 40 μl of a 1:50 dilution plasma in a 100 μl final assay volume, the concentration of endogenous β2GPI is ~ 32 nM. Thrombin generation was measured using the chromogenic substrate S-2238. Thrombin generation in the absence of both mAb and exogenous β2GPI was taken as 100%. Anti-β2GPI mAbs used in the assay are listed in Table I. A monoclonal mouse anti–human vWf antibody (clone F8/86) was used as control IgG. A monoclonal IgM produced by hybridomas between lymphocytes from a healthy volunteer and SHM-D33 hybrid cells was also used as controls. Plotted values represent the means of triplicate determinations±SD. Details are described in the Methods section.

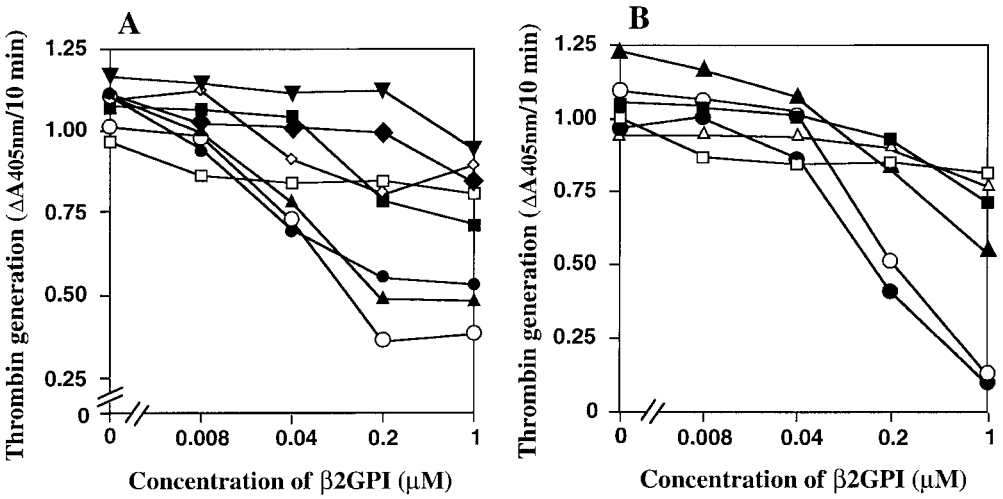

Figure 2. Effect of anti–β2GPI mAbs on prothrombin activation induced by prothrombinase complex reconstituted with purified clotting factors. Prothrombin (0.1 mg/ml) was activated by factor Xa (10 ng/ml) in the presence of factor Va (40 ng/ml), phospholipids (14 μg/ml), 2 mM Ca^{2+}, and various concentrations of β2GPI. Anti-β2GPI mAbs used in the assay are listed in Table I. *A* includes data in the absence (None; □) or presence of mAb; Cof-18 (▼), Cof-19 (◇), Cof-20 (○), Cof-21 (●), Cof-22 (▲), Cof-23 (■), or WB-CAL-1 (◆). *B* includes data in the absence (None; □) or presence of mAb; TM1B8 (■), EY1C8 (○), EY2C9 (●), GR1D5 (▲), or control IgM (△). Thrombin generation was measured by using the chromogenic substrate S-2238. Plotted values are the means of duplicate values and are representative of triplicate experiments.

of ≥ 0.2 μM β2GPI, but not in the presence of 0.04 μM β2GPI.

Taken together, the data in Table I and Figs. 1 and 2 show that anti-β2GPI mAbs directed against the third domain (Cof-20 and Cof-22) and the fourth domain of β2GPI (Cof-21, EY1C8, and EY2C9) inhibited the thrombin generation induced by RVV in diluted plasma and that induced by the prothrombinase complex.

Effects of phospholipid concentration on mAb-induced inhibition of thrombin generation. The effect of phospholipid concentration on the mAb (Cof-20, Cof-21, or Cof-22)-induced inhibition of thrombin generation triggered by RVV in diluted plasma was evaluated. Cof-20, Cof-21, and Cof-22 inhibited thrombin generation in the range of 0.7–22 μg/ml of cephalin; however, higher concentrations of cephalin decreased this inhibition rate: Cof-20, Cof-21, and Cof-22, respectively, inhibited 73, 90, and 50% of thrombin generation in control plasma in the presence of 5.5 μg/ml cephalin, but only 27, 56, and 12% in the presence of 22 μg/ml cephalin. The inhibition of thrombin generation by Cof-20, Cof-21, and Cof-22 was completely abolished in the presence of an excess amount of cephalin (220 μg/ml). Inhibition of thrombin generation by EY1C8 and EY2C9 observed in the presence of exogenous β2GPI (2 μM) was also abrogated by the addition of 220 μg/ml of cephalin. These data clearly indicate that the anticoagulant effect of these mAbs depends on the concentration of phospholipids, thereby suggesting that they are LA-like mAbs.

Effect of anti-β2GPI mAbs on β2GPI binding to phospholipids. In an attempt to clarify the mechanism of the phospholipid-dependent anticoagulant activity of the LA-like anti-β2GPI mAbs, we examined the effect of ^{125}I-β2GPI binding to solid-phase phospholipid vesicles prepared with cephalin (Fig. 3). No binding of ^{125}I-β2GPI was observed in albumin-coated wells both in the presence and in the absence of anti-β2GPI mAb. To determine whether radiolabeling of β2GPI altered the binding affinity to phospholipid vesicles, an assay was performed by using labeled- and unlabeled-β2GPI at varying ratios, keeping constant the total amount of added β2GPI. After measuring the bound radioactivity, a linear correlation between the percentage of added concentration of labeled-β2GPI and the percentage of bound labeled-β2GPI was found, indicating no difference in the binding affinity between labeled- and unlabeled-β2GPI (data not shown). As shown in Fig. 3 *A*, in the absence of anti-β2GPI mAb, ^{125}I-β2GPI bound weakly but in a dose-dependent manner to phospholipid vesicles. In the presence of Cof-20, Cof-21, Cof-22, EY1C8, or EY2C9, the binding of β2GPI to cephalin was significantly enhanced. The ^{125}I-β2GPI binding to pure phosphatidylcholine was almost undetectable in our system, as it was in a previous report (9); thus, in Fig. 3 *A*, the ^{125}I-β2GPI binding to pure phosphatidylcholine was considered as nonspecific binding. The specific binding of ^{125}I-β2GPI to cephalin was determined by subtracting the binding to phosphatidylcholine from the total binding. To check for nonspecific β2GPI binding to cephalin, ^{125}I-β2GPI binding to cephalin was also assayed in the presence of 10-fold molar excess of unlabeled β2GPI. As shown in Fig. 3 *B*, ^{125}I-β2GPI binding to cephalin was significantly inhibited in the presence of unlabeled β2GPI, but it was not inhibited in the presence of 10-fold molar excess of HSA (data not shown), thus confirming the specificity of β2GPI binding to cephalin. The enhanced binding of ^{125}I-β2GPI to cephalin in the presence of each mAb was also significantly inhibited in the presence of unlabeled β2GPI (Fig. 3 *C*). The remaining mAbs, Cof-19, Cof-23, WB-CAL-1, TM1B3, GR1D5, and control IgG (a monoclonal mouse anti–human vWf antibody) and IgM did not significantly affect the β2GPI binding to cephalin (data not shown).

In experiments carried out using phospholipid vesicles in solid phase, we also used a phospholipid mixture containing phosphatidylserine and phosphatidylcholine. Increased concentrations of phosphatidylserine induced increasing bindings of ^{125}I-β2GPI, reaching a maximum binding at a phosphatidylserine/phosphatidylcholine ratio of 50%:50%. Higher concentrations of phosphatidylserine in the phospholipid mixture tended to decrease slightly the β2GPI binding (data not shown). When a phospholipid mixture of phosphatidylserine/phosphatidylcholine (20%:80%), which is physiologically relevant and typical in prothrombinase assays (55), was used as

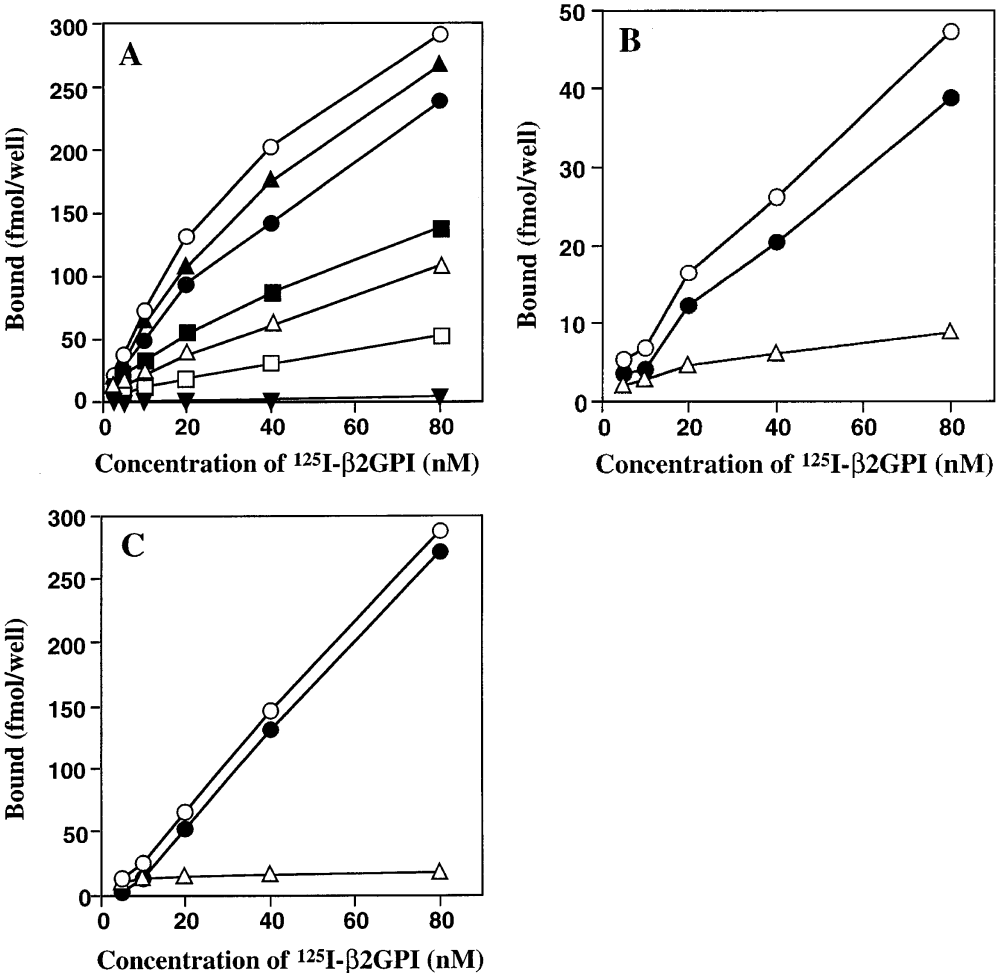

Figure 3. Effect of anti-β2GPI mAbs on the binding of ^{125}I-β2GPI to cephalin. (*A*) Specific ^{125}I-β2GPI binding to cephalin (plotted in this figure) was calculated by subtracting nonspecific ^{125}I-β2GPI binding to pure phosphatidylcholine from the total binding. Binding of ^{125}I-β2GPI to phospholipids in the absence of mAb (□) was enhanced in the presence of 150 nM of LA-like mAbs, Cof-20 (○), Cof-21 (●), Cof-22 (▲), EY2C9 (■), and EY1C8 (△). Cof-18 (▼) inhibited this binding. (*B* and *C*) ^{125}I-β2GPI binding to cephalin in the absence (*B*) and in the presence of Cof-22 (*C*). In *B* and *C*, nonspecific ^{125}I-β2GPI binding to cephalin (△) was determined in the presence of 10-fold molar excess of unlabeled β2GPI. Specific binding (●) was calculated by subtracting nonspecific binding from total binding (○). Plotted values are the means of duplicate values and are representative of triplicate experiments.

solid phase phospholipid vesicles, ^{125}I-β2GPI bound to this phospholipid mixture in the absence of mAb, but the specific binding was < 60% of that observed in the presence of solid-phase cephalin. Cof-20, Cof-21, Cof-22, EY1C8, or EY2C9 potentiated this binding with similar magnitude to that observed between ^{125}I-β2GPI and cephalin (data not shown).

Recently, Hagihara et al. (9) reported β2GPI binding to phospholipid vesicles in equilibrium solution phase assays as a function of phospholipid concentration. We also assessed in solution phase the binding of ^{125}I-β2GPI to phospholipid vesicles containing either a mixture of phosphatidylserine/phosphatidylcholine (20%:80%) mixture, or pure phosphatidylcholine. Our data confirmed the results of previous studies (9): ^{125}I-β2GPI bound to phospholipid vesicles containing phosphatidylserine in a phospholipid-dose dependent manner, but not to pure phosphatidylcholine vesicles (data not shown). In the presence of Cof-20, Cof-21, or Cof-22, the binding of ^{125}I-β2GPI to phospholipid vesicles containing phosphatidylserine/phosphatidylcholine (20%:80%) mixture in solution phase was also enhanced. Taking the binding of ^{125}I-β2GPI (20 nM) to phospholipid vesicles (250 μg/ml) as 100%, the binding was 275, 225, or 342% in the presence of Cof-20, Cof-21, or Cof-22, respectively.

The results obtained both in solid- and solution-phase assays showed that anti-β2GPI mAbs with LA-like activity, but not those without this activity, enhance the β2GPI binding to phospholipid vesicles. On the other hand, Cof-18 completely inhibited the β2GPI binding to phospholipids as compared to control (Fig. 3 *A*). Since Cof-18 binds to domain V of the β2GPI molecule (42), it presumably recognizes the phospholipid-binding region of this domain. The pathophysiological relevance and the therapeutic value of this type of antibody remain to be addressed.

Effects of intact IgG, F(ab′)$_2$ fragment, and Fab′ fragment of anti-β2GPI mAb on the β2GPI binding to phospholipids and on the prothrombinase complex activity. To assess whether the stimulatory effect of anti-β2GPI mAbs on β2GPI binding to phospholipids requires antibody bivalency, the LA-like anti-β2GPI mAbs were proteolytically modified, and their F(ab′)$_2$ (bivalent) and Fab′ (monovalent) fragments were prepared. Fig. 4 *A* shows the effect of F(ab′)$_2$ and Fab′ fragments of Cof-21 on the binding of ^{125}I-β2GPI to solid-phase phospholipid vesicles. F(ab′)$_2$ fragment of Cof-21 potentiated the binding of ^{125}I-β2GPI to phospholipids; however the Fab′ fragment of Cof-21 did not retain this activity. Binding of intact Cof-21 IgG and reduced and alkylated Cof-21 IgG were included as controls to demonstrate that neither digestion nor reduction/alkylation alone adversely affected antibody activity.

Effects of F(ab′)$_2$ and Fab′ fragments on the activity of the prothrombinase complex were then compared with those pro-

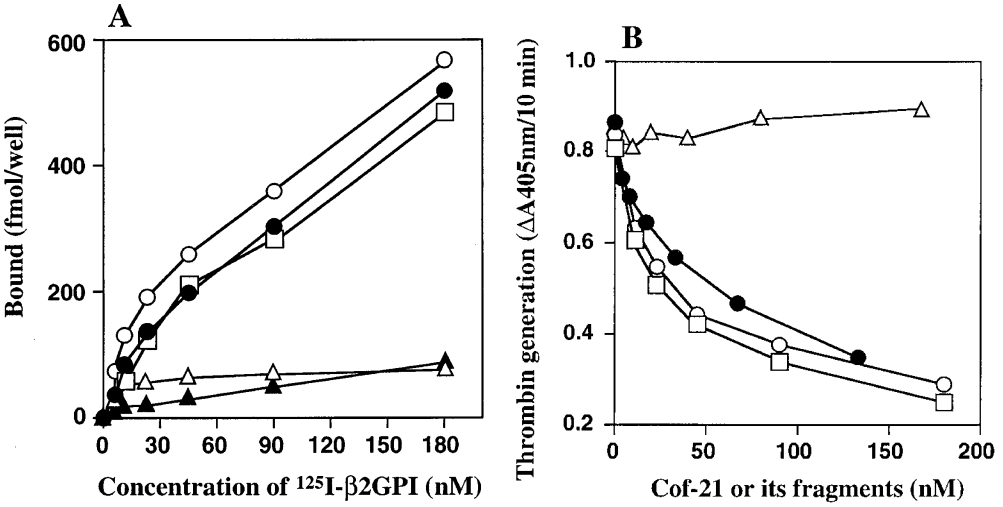

Figure 4. Effect of Cof-21 and its fragments on the ^{125}I-β2GPI binding to phospholipids (*A*) and on prothrombinase activity (*B*). (*A*) Specific ^{125}I-β2GPI binding to cephalin (plotted in this figure) was calculated by subtracting nonspecific ^{125}I-β2GPI binding to pure phosphatidylcholine from the total binding. The ^{125}I-β2GPI binding to cephalin (▲) was potentiated by 150 nM of each intact IgG (●), reduced and alkylated IgG (□), or F(ab′)$_2$ fragments (○) of Cof-21, but not by 300 nM of monovalent Fab′ fragments (△). (*B*) Prothrombin activation induced by prothrombinase complex reconstituted with purified factors was dose dependently inhibited by intact IgG (●), reduced and alkylated IgG (□), or F(ab′)$_2$ fragments (○) of Cof-21, but not by Fab′ fragments (△) of this antibody. Prothrombin (0.1 mg/ml) was activated by factor Xa (10 ng/ml) in the presence of factor Va (40 ng/ml), cephalin (14 μg/ml), 2 mM Ca^{2+}, β2GPI (50 μg/ml), and various concentrations of intact IgG or fragments. Plotted values are the means of triplicate values.

duced by intact IgG (Fig. 4 *B*). The prothrombinase complex activity was inhibited by intact IgG and the F(ab′)$_2$ fragment of Cof-21, in a dose-dependent manner. The prothrombinase complex activity was also inhibited by reduced and alkylated Cof-21 IgG, but not by the monovalent Fab′ fragment of Cof-21. F(ab′)$_2$ fragments of Cof-20 and Cof 22, but not their Fab′ fragments, also potentiated the ^{125}I-β2GPI binding to solid-phase phospholipids and the inhibition of the prothrombinase complex activity (data not shown). These findings suggest that the LA-like activity of anti-β2GPI mAbs, which is the result of the potentiation of β2GPI binding to phospholipids, depends on the multivalency of these mAbs.

Discussion

The presence of antiphospholipid antibodies is defined by the detection of aCL (anti-β2GPI-cardiolipin complex antibodies) by immunoabsorbent assays or by the presence of LA activity. Although conflicting observations exist regarding the relationship between LA and aCL (anti-β2GPI antibodies) (56–60), recent investigations showed that part of the LA activity observed in patients with antiphospholipid antibodies depends on the presence of anti-β2GPI antibodies (type A aCL) and β2GPI (26, 61). However, there is a certain group of aCL that does not possess anticoagulant activity (type B aCL). In accord with this, findings of the current study showed that a group of anti-β2GPI mAbs (Cof-20, Cof-21, Cof-22, EY1C8, and EY2C9) had LA-like activity (type A aCL), whereas another group of anti-β2GPI mAbs (Cof-18, Cof-19, Cof-23, WB-CAL-1, and TM1B3) did not show inhibitory activity on thrombin generation. This difference in the LA activity appears to depend on their ability to enhance the β2GPI-binding to phospholipid vesicles, and on their epitope specificity. The results of this study demonstrated, for the first time, that the heterogeneity of anti-β2GPI mAbs or aCL, in terms of their function and epitope specificity, might explain their distinct LA activity reported in previous studies. However, it is worthy to note that, presently, there are no data showing that polyclonal antibodies from patient plasma exhibit such epitope restriction (62). Thus, it may be difficult to extrapolate mAbs to the polyclonal autoantibodies in patient plasma. An alternative explanation is that the conditions of the coagulation assays used to determine LA activity may not be optimal for some or all type B aCL. For instance, EY1C8, EY2C9, and GR1D5 were originally reported not to have LA activity.

In addition, the molecular target of aCL, β2GPI per se, has been described to possess anticoagulant activity under physiological conditions. β2GPI binds to negatively charged surfaces, and inhibits the contact phase coagulation activation in plasma and the prothrombinase complex activity of platelets possibly by displacing coagulation factors from phospholipid surfaces (11–13). Anti-β2GPI antibodies may accelerate the clustering of β2GPI molecules on the surface of phospholipids (Fig. 3), and may thereby enhance the anticoagulant activity of β2GPI. As shown in Fig. 2, in contrast to the inhibition of prothrombinase complex activity (16% of control) by a high concentration of β2GPI (1 μM), even low concentrations of β2GPI exert strong anticoagulant activity in the presence of anti-β2GPI mAbs. The most likely explanation for the minimal anticoagulant effect of physiological concentrations of β2GPI alone, but the marked effect of β2GPI in the presence of antibodies, is that the β2GPI-phospholipid interaction is weak as compared to that of coagulation proteins such as prothrombin, factor Xa, and factor Va. Antibody cross-linked membrane-bound β2GPI would be expected to have significantly tighter binding and would successfully compete with coagulation proteins for anionic phospholipid binding sites. This potential mechanism is supported by a recent study reported by Willems et al. (63). Alternatively, the anticoagulant activity of the complex between anti-β2GPI antibodies and β2GPI may be attributed to induction of rigidness of the phospholipid surface and/or a conformational change of phospholipid vesicles (13). These phospholipid changes may provoke a fencing effect by which anti-β2GPI antibody/β2GPI complexes would interfere with

the lateral transport of clotting factors toward the prothrombinase complex on the phospholipid surface (55). Currently, this hypothesis is under investigation in our laboratory.

In this study, we demonstrated that several anti-β2GPI mAbs enhance the β2GPI binding to phospholipids. The data shown in Fig. 3 were evaluated in a nonequilibrium solid-phase assay. This simple approach is useful to examine the effect of antibodies on the β2GPI binding to phospholipids, but it should be mentioned that binding under this condition may differ from that observed in solution phase. However, many factors may increase difficulty of interpreting results obtained in experiments done in solution phase. For instance, based on the data reported by Hagihara et al. (9), it appears that the affinity of β2GPI for phospholipids depends on the content of anionic phospholipids present in solution phase. In addition, the composition and the physical characteristics of phospholipids (size, form, and surface curvature of vesicles) may also influence the binding ability of β2GPI to phospholipid vesicles (our unpublished data).

The results of our investigation suggest that bivalency of the anti-β2GPI antibodies is required for enhancing the β2GPI binding to phospholipids and for the expression of LA-like activity. A possible interpretation for these observations is that bivalent antibodies engage one molecule of β2GPI bound to phospholipids and another β2GPI molecule from the fluid phase. However, if this were the only interpretation, the increased β2GPI binding to phospholipids induced in the presence of Cof-20, Cof-21, and Cof-22 (up to five times of that in the absence of mAb) might not be completely explained. Thus, it is conceivable that an additional mechanism such as a self-association of β2GPI molecules might be also involved in the increased β2GPI binding to phospholipid surface induced by the anti-β2GPI antibodies.

Increased risk for developing thrombosis has been reported in patients with aCL. The paradox of the in vitro anticoagulant activity of anti-β2GPI antibodies and their relation with increased thrombogenic events in vivo is unclear. Activation or perturbation of the functions of leukocytes or endothelial cells by aCL or anti-β2GPI antibodies has been reported to be important for their thrombogenic activity in vivo (38, 39). The precise mechanisms by which anti-β2GPI antibodies perturb the function of these cells are unknown. The results of this study suggest that the clustering of β2GPI molecules on leukocyte or endothelial cell membrane induced by anti-β2GPI antibodies may injure the plasma membrane of these cells directly or may alter the function of these cells through the cellular Fc receptor (29). Anti-β2GPI antibodies may also cause clinical thrombosis by interfering with the anticoagulant protein C system, by inhibiting either the thrombomodulin-mediated protein C activation or the phospholipid-dependent anticoagulant activity of activated protein C (APC). In this regard, we reported recently that β2GPI itself functions as an inhibitor of phospholipid-dependent anticoagulant activity of APC (49). The clustering mechanism proposed in this study may also explain the anti-APC activity of anti-β2GPI antibodies observed in previous studies (64–66), and indeed, anti-β2GPI mAbs used in the current study were found to have a potent inhibitory activity against the anticoagulant activity of APC (our unpublished data).

In conclusion, our study shows that anti-β2GPI mAbs exert LA activity by enhancing the binding of β2GPI to phospholipids. This clustering of β2GPI molecules on the surface of phospholipids might lead to rigidness of this phospholipid surface and to subsequent reduction of the lateral mobility of coagulation factors. The epitope specificity and the multivalency of the anti-β2GPI antibodies may be critical for their LA activity. The clustering effect may also account for the molecular basis by which aCL perturbs the function of endothelial cells and leukocytes and interferes with the anticoagulant protein C system observed in the previous studies.

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