Published in final edited form as:
Arthritis Rheum. 2007 May ; 56(5): 1638–1647.

Some antiphospholipid antibodies recognize conformational epitopes shared by β_2GPI and the homologous catalytic domains of several serine proteases

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Abstract

Objective—To test the hypothesis that some antiphospholipid antibodies (aPL) in patients with the Antiphospholipid Syndrome (APS) recognize a conformational epitope shared by β_2 glycoprotein I (β_2GPI, the major autoantigen for the antiphospholipid antibodies) and the homologous catalytic domains of several serine proteases (such as thrombin, activated protein C and plasmin) in hemostasis.

Methods—We generated four new IgG monoclonal aPL (including two screened against β_2GPI, one against thrombin and one against protein C) from two APS patients. The monoclonal antibodies (mAb) were analyzed for binding to β_2GPI, thrombin, activated protein C (APC) and plasmin, and for anti-cardiolipin antibody (aCL) activity. To demonstrate a shared epitope between β_2GPI and a serine protease, one mAb was studied by cross-inhibition.

Results—Both IgG anti-β_2GPI mAb bound to thrombin, APC and plasmin. On the other hand, one anti-thrombin mAb and one anti-protein C mAb also bound to β_2GPI. Moreover, the binding of one crossreactive mAb to β_2GPI was inhibited by α-thrombin (that contains only the catalytic domain of thrombin). All four mAb displayed aCL activity.

Conclusion—Taken together with the findings that some aCL bind to several serine proteases that participate in hemostasis and share homologous catalytic domains, these data demonstrate that some aCL in APS patients recognize one or more conformational epitopes shared by β_2GPI and the catalytic domains of disease-relevant serine proteases.

INTRODUCTION

Antiphospholipid antibodies (aPL) are associated with thrombosis and fetal loss in some patients, and their combined presence is recognized as the antiphospholipid syndrome (APS) (1-7). APL include anticardiolipin antibodies (aCL, as detected by enzyme-linked immunosorbent assay) and lupus anticoagulants (LAC, as detected by their abilities to prolong certain *in vitro* phospholipid-restricted blood clotting tests). Immunologic studies of aPL show that aPL represent a heterogeneous group of immunologically distinct antibodies (Ab) that recognize various phospholipids (PL), PL-binding plasma proteins and/or PL-protein complexes (8-13). The involved plasma proteins include β_2 glycoprotein-I (β_2GPI),

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prothrombin (PT), thrombin, protein C (PC), activated PC (APC), protein S, annexin A5, plasminogen, plasmin and tissue-type plasminogen activator (tPA) (9-23). Of these plasma proteins, β_2GPI has emerged to play a major role in aCL activity, serving either as the major autoantigen or as a necessary co-factor. Ab against β_2GPI and its complexes with cardiolipin (CL) probably account for most of the positive findings on tests for aCL in APS (24), while anti-PT Ab (aPT) and anti-β_2GPI Ab are responsible for the majority of the LAC activity (11,25).

On the other hand, thrombin, APC, plasmin and tPA belong to the trypsin-like serine protease superfamily; and the catalytic domains of these four enzymes are homologous (26-29). At the amino acid levels, human thrombin and human APC share a 50.5% similarity, while human thrombin and human plasmin share a 48% similarity (19,20). Recently, we showed that 5/7 patient-derived IgG monoclonal aCL reacted with human thrombin, APC, plasmin and tPA; and that one patient-derived IgG monoclonal aPT also bound to CL, thrombin, APC, plasmin and tPA (Table 1) (17,19,20,23). Moreover, the binding of the CL15 monoclonal antibody (mAb) to tPA could be inhibited by α-thrombin (which contains only the catalytic domain), indicating that the shared homologous catalytic domains of the reactive proteases are the structural basis of the observed crossreactivity (23). Of note, in addition to the catalytic domain, tPA contains two Kringle domains plus two epidermal growth factor (EGF) domains. Furthermore, of the protease-reactive mAb, CL24 could interfere with inactivation of thrombin by antithrombin, while CL15 could inhibit the functional activities of APC, plasmin, and tPA (17,19,20,23). Combined, these data indicate that some aCL bind to the homologous catalytic domains of several serine proteases that are involved in coagulation.

In this context, it was tempting to speculate that some aCL in APS patients recognize a conformational epitope shared by β_2GPI and the homologous catalytic domains of several serine proteases in hemostasis, as there is no meaningful amino acid sequence homology between β_2GPI and the catalytic domain of any reactive serine proteases. Intriguingly, this speculation was supported by the fact that 5/8 aforementioned patient-derived IgG monoclonal aCL/aPT also react with β_2GPI (Table 1). Thus, to test the aforementioned hypothesis, we generated and analyzed four new patient-derived IgG monoclonal aPL, including two mAb that were initially screened against β_2GPI, one against thrombin and one against PC.

MATERIALS AND METHODS

Hybridoma donor patients

Patient #1 was a Hispanic female APS patient with no history of pregnancy. In 1994, she was diagnosed with APS at age 21 when she presented with recurrent episodes of deep venous thromboses, elevated aCL and a positive LAC, as determined by the Kaolin Clotting Time (KCT) test. Her course was complicated by the development of systemic lupus erythematosus (SLE) at age 26 manifested by arthritis, hemolytic anemia, leukopenia, persistently positive LAC (as determined by the dilute Russell's Viper Venom Time - DRVVT test), high titer IgG aCL > 100 GPL (one GPL unit is equivalent to 1μg of affinity purified standard IgG aCL), a positive anti-nuclear Ab (ANA) at 1:160 dilution and elevated anti-DNA Ab. Furthermore, she suffered from recurrent pulmonary emboli and secondary pulmonary hypertension, pulmonary hemorrhage, recurrent subdural hematomas, sinus thrombosis and cerebrovascular accidents. Despite aggressive therapy with anticoagulation, intravenous cyclophosphamide, high dose corticosteroid, Rituximab and plasma exchange, she expired at age 32. At the time of the blood collection (February 2001), she was just on prednisolone at 4.5 mg a day and warfarin.

Patient #2 is a 26-year-old female APS patient with no history of pregnancy. In 1999, at age 20, she developed severe headache, pseudotumor cerebri, optic neuritis and multiple ischemic white matter lesions on her magnetic resonance imaging (MRI) of her brain. Her cerebrospinal

fluid (CSF) was unremarkable with negative cultures, with normal protein levels and cell counts, negative oligoclonal bands, and negative myelin basic protein. Her IgM aCL was elevated at 69 MPL (one MPL unit is equivalent to 1μg of affinity purified standard IgM aCL; normal < 10), and her IgM anti-β_2GPI was at 43, with normal < 10. Her DRVVT was negative. She was treated with a course of high dose steroids tapered over 3 months and anti-coagulation. She has remained on warfarin without recurrent thrombotic events and continues to have persistently elevated IgM aCL and anti-β_2GPI Ab. She donated her blood in January 2001.

Generation and preparation of mAb

Four new mAb were generated as described previously (30). Briefly, peripheral blood mononuclear cells from the patients were transformed with Epstein-Barr virus, and cultured in 96-well plates. Supernatants were screened for desired Ab by appropriate enzyme-linked immunosorbent assays (ELISA) (17,19,30). For anti-β_2GPI Ab (30), high-binding ELISA plates (Costar, Cambridge, MA) were coated with 10μg/ml of human β_2GPI (Haematologic Technologies, Essex Junction, VT) in phosphate-buffered saline (PBS, pH 7.2). After incubating overnight at 4 °C, plates were blocked with PBS containing 0.25% gelatin. Supernatants were distributed to wells in duplicate and incubated for 2 hours at room temperature. After washing with PBS, bound human IgG was detected with affinity purified horseradish peroxidase labeled goat anti-human IgG (γ-chain specific; BioSource International, Camarillo, CA). After an additional incubation for one hour at room temperature, 50μl of the substrate tetramethylbenzidine/H_2O_2 (Kirkegard & Perry Labs, Gaithersburg, MD) was added, and the reaction terminated with 50μl of 1 M H_3PO_4. Results were read at a wavelength of 450 nm against a background of 650 nm in an Emax plate reader (Molecular Devices, Sunnyvale, CA).

The ELISA for anti-thrombin Ab and anti-PC Ab were similar except for the following modifications. For anti-thrombin Ab, wells were coated with human thrombin (Haematologic Technologies) at 5μg/ml in Tris-buffered saline (TBS, 50 mM Tris/150 mM NaCl, pH 7.5), and were blocked with 0.3% gelatin in TBS (17). For anti-PC Ab, wells were coated with 5μg/ml of human PC (Haematologic Technologies) in PBS, and were blocked with 0.25% gelatin in PBS.

Positive cells were subcloned to one cell per well, and then fused with the Oubain resistant K6H6/B5 human-mouse heterohybridoma cell line (31). Positive hybridomas were subcloned twice at 1 cell/well. To ensure the monoclonality of each mAb, their light chain isotypes and IgG subclasses were determined by ELISA using isotype and subclass-specific reagents.

To purify mAb, each hybridoma was first switched to a serum-free culture medium. Culture supernatants were passed through a HiTrap Protein G column (Pharmacia, Piscataway, NJ), and the bound IgG was eluted with 0.1 M glycine HCl (pH 2.8), and dialyzed against PBS (32).

In addition to the four new mAb generated here, seven IgG monoclonal aCL and one IgG monoclonal aPT were analyzed in the present study. The aCL included CL1, CL15, CL24, IS1, IS2, IS3 and IS4 (30,32), and the single aPT was IS6 (33). Their generation and characterization had been reported previously (30,32,33).

ELISA for Ab against APC and plasmin, and competitive inhibition assays

The ELISA for anti-APC Ab was similar to that for anti-PC Ab except that plates were coated with human APC (5μg/ml; Haematologic Technologies) in PBS, pH 7.4. The ELISA for anti-plasmin Ab was done similarly except that plates were coated with human plasmin (5μg/ml; Haematologic Technologies) in PBS (20).

Competitive inhibition assays were used to study the binding properties of mAb to four concerned antigens: β_2GPI, α-thrombin, APC, and plasmin (17,19,20). Briefly, each mAb at the chosen concentration (in the linear range of its titration curve) was preincubated for 1.5 hours at RT with various concentrations of the indicated inhibitor in PBS (for β2GPI, APC, and plasmin) or TBS (for α-thrombin) containing 0.1% gelatin. Of note, we have previously found that the enzymes prepared in the buffer containing 0.1% gelatin remain stable during incubation at RT.

The amount of inhibition for a mAb at a given concentration of soluble inhibitor was calculated as follows: % inhibition of mAb binding to a test antigen = [(OD from a test mAb alone) − (OD from the same mAb plus the same antigen at the given concentration)] /(OD from the same mAb alone) × 100. The inhibition data of each mAb for each test antigen were used to calculate its relative K_d toward this test antigen (34).

For inhibition with APC, the recombinant human APC (rhAPC, Xigris, Eli Lilly, Indianapolis, IN) was used in the competitive inhibition, as it was immunologically indistinguishable from purified human APC (data not shown) but was much cheaper than purified human APC. For inhibition with plasmin, a few studies reported plasmin digestion of some human IgG (35, 36), suggesting that some of the observed plasmin inhibition of mAb binding to plasmin on plates might actually be due to plasmin digestion of mAb. To address this issue, plasmin was irreversibly inactivated with N^{α}-Tosyl-L-lysine chloromethyl ketone-HCl (TLCK, Sigma-Aldrich), which forms a chemical bond with His622 (one of the three conserved catalytic residues of all serine proteases). Briefly, 12μM plasmin was first incubated with 6 mM TLCK in PBS (pH 7.4) for 100 min at RT, which eliminated > 99% of the plasmin amidolytic activity using S-2403. After incubation, the excess TLCK was removed by dialyzing the plasmin-TLCK mixture against PBS at 4 °C overnight. Thereafter, the TLCK-inactivated plasmin was used as a soluble inhibitor. In addition, an active-site blocked thrombin, α-thrombin-diisopropylfluorophosphate (DFP; Haematologic Technologies) was used to study the role of active site in the binding of mAb to α-thrombin. DFP reacts with Ser195 of thrombin, which is one of the conserved catalytic triad.

RESULTS

Generation of four monoclonal IgG aPL from two APS patients

To test the hypothesis that β_2GPI shares a conformational epitope with several serine proteases that are recognized by aPL, we initiated the efforts to generate IgG anti-β_2GPI mAb from APS patients with high titers of anti-β_2GPI Ab. This was in part due to present lack of any patient-derived IgG anti-β_2GPI mAb, considering that the major autoantigen for aPL is thought to be β_2GPI and that the IgG aPL is more disease relevant than the IgM aPL. After extensive efforts, we obtained two IgG anti-β_2GPI mAb from a patient with primary APS by screening the culture supernatants initially for binding to β_2GPI and then subcloning each positive hybridoma to a monoclonal population that secret an IgG anti-β_2GPI Ab. The mAb were designated B1 and B2. Figure 1A shows the binding reactivity of B1 and B2 to β_2GPI.

On the other hand, as noted in Introduction, our recent studies of 7 patient-derived IgG monoclonal aCL revealed broad reactivity with thrombin, APC and plasmin, all share homologous catalytic domains. Although we have 5-crossreactive monoclonal IgG aCL, these mAb were generated by screening initially against CL in the presence of bovine serum and thus might not represent all IgG Ab against the same serine proteases in APS patients. Therefore, we initiated the efforts to generate new IgG mAb by screening initially against the concerned protease autoantigens. We obtained two mAb from an APS patient: one anti-thrombin Ab and one anti-PC Ab; the mAb were designated T1 and P1, respectively. Figure 1 B shows the binding of T1 to thrombin, and Figure 1 C shows the binding of P1 to APC. Of

note, we elect to present the binding data to APC, which is a serine protease, while PC is a zymogen.

Since some autoimmune patients are known to have Ab against gelatin, all mAb were analyzed for binding to wells that were not coated with antigens but were blocked with buffers containing 0.3% gelatin only. The results showed that bindings of all mAb to gelatin were negligible (data not shown).

To ensure the monoclonality of each mAb, the heavy chain subclass and light chain isotype of each mAb were determined. The results showed that each mAb had only one light chain isotype and one IgG subclass. Specifically, B1, and B2 have κ light chains, while P1 and T1 have λ light chains. For heavy chains, B1, P1 and T1 are of the γ1 subclass, while B2 is of the γ3 subclass.

Immunological characterization of four patient-derived IgG monoclonal antibodies

Subsequently, we analyzed reactivity of both anti-β_2GPI mAb toward thrombin, APC and plasmin, as well as the reactivity of the T1 and P1 toward β_2GPI. The results showed that both anti-β_2GPI mAb bound to thrombin, APC, and plasmin (Figure 1 B, C and D, and Table 1). Although the binding activities of B1 to thrombin and plasmin were weak, they were still significantly higher than the IgG1 isotype control (Figure 1 B and D, and Table 1). On the other hand, both T1 and P1 bound to β_2GPI when compared against the IgG1 isotype control (Figure 1A, and Table 1). Moreover, P1 binding to β_2GPI was even stronger than that of the B2 anti-β_2GPI mAb (Figure 1A).

To determine the significance of the above crossreactivity of mAb, we used competitive inhibition to determine binding affinity of mAb to all reactive antigens. When mAb binding to β_2GPI was inhibited by soluble β_2GPI up to 12μM, only B1 could be partially inhibited (about 16%), suggesting that both bound to β_2GPI with low affinity (Figure 2A). Surprisingly, at the same concentration of 12μM, P1 binding to β_2GPI was inhibited by about 47%, indicating that P1 bound to β_2GPI with an affinity that is higher than that of B1 (Figure 2A and Table 2).

When mAb binding to thrombin was inhibited by soluble thrombin, all four mAb, including B1 and B2, were inhibited (Figure 2B). The relative K_d values were 4×10^{-6} M for T1, 8×10^{-6} M for P1, 1×10^{-5} M for B1, and 2×10^{-5} M for B2 (Table 2). Similar to inhibition by thrombin, all four mAb binding to APC, including B1 and B2, were inhibited by soluble APC (Figure 2B). The relative K_d values were 5×10^{-6} M for B2, 7×10^{-6} M for T1, and 9×10^{-6} M for P1 (Table 2).

The competitive inhibition of mAb binding to plasmin was done with the soluble plasmin that had been inactivated with TLCK, which forms a chemical bond with His622 of plasmin (one of the three conserved catalytic residues of all serine proteases). This was because a few studies had reported plasmin digestion of some human IgG (35,36), which would lead to lower bound IgG mAb and inaccurate inhibition data. As can be seen in Figure 2D, all four mAb were inhibited by TLCK-inactivated plasmin. The relative K_d values are 9×10^{-8} M for B2, 1×10^{-7} M for P1, 6×10^{-7} M for T1, 1×10^{-6} M for B1 (Table 2).

Combined, these data show that anti-β_2GPI mAb often bind to various relevant members of serine proteases, while the mAb generated against the concerned serine proteases frequently react with β_2GPI. These findings suggest that β_2GPI shares an epitope with the concerned serine proteases. Of the four studied autoantigens, plasmin displays the highest relative binding affinity to all four patient-derived mAb, with the B2 anti-β_2GPI mAb having the highest binding affinity (relative $K_d = 9 \times 10^{-8}$ M). In contrast, β_2GPI has the lowest relative binding affinity to all four patient-derived mAb.

β_2GP1 shares conformational epitopes with the catalytic domain of thrombin

To test the hypothesis that β_2GPI shares an epitope with the concerned serine proteases, we performed a cross inhibition assay with the P1 mAb, which binds strongly to β_2GPI and all three test serine proteases. As noted in the Introduction, the three test serine proteases are homologous in their catalytic domains, while they differ greatly from each other in other regions. For example, in addition to the catalytic domain, APC contains one Gla (for γ-carboxyglutamate) domain and two EGF domains, while plasmin has 1-5 kringle domains depending on the type of plasminogen from which it originated (whether Glu-1, Lys-77 after a cleavage between K76-K77 by plasmin, or Val-442 after a cleavage by elastase). Therefore, the shared epitope is most likely to reside in the catalytic domains of the reactive serine proteases. Consequently, α-thrombin (that contains only the catalytic domain of thrombin) was used in the cross-inhibition assay.

The results showed that P1 binding to β_2GPI was inhibited by β_2GPI and α-thrombin (Figure 3A); and that P1 binding to α-thrombin was inhibited by α-thrombin and β_2GPI (Figure 3B). Since α-thrombin is a protease, the reduced mAb binding to solid phase antigen in the competitive inhibition assay may, in part, be due to digestion of IgG by α-thrombin. To address this issue, we incubated P1 mAb (10-100μg) with either α-thrombin (10μM) or plasmin (10μM) in PBS for 1 hour at RT, and analyzed the mixtures by SDS-PAGE and a silver staining. The results showed that mixture of P1 plus plasmin contained the expected bands of IgG and plasmin plus additional bands of 10 & 20 KD, while the mixture of P1 plus α-thrombin contained only the expected bands of IgG and α-thrombin (data not shown). The data indicated digestion of IgG by plasmin, but no digestion of IgG by α-thrombin, and thus demonstrated that inhibition by α-thrombin was mainly due to antigenic competition.

Subsequently, to define the epitope on α-thrombin, we performed comparative analyses of P1 reactivity with α-thrombin and the corresponding active site-blocked thrombin, α-thrombin-diisopropylfluorophosphate (DFP, which reacts with Ser195 of the conserved catalytic triad). In the direct binding assay, the binding of P1 to α-thrombin-DFP was significantly weaker than that to α-thrombin, indicating that the reactivity of α-thrombin with P1 was reduced significantly when Ser195 in its active site was modified (Fig. 4). The data suggested that the active site (or its vicinity) of α-thrombin was involved in its reactivity with P1. In the inhibition assay, P1 binding to α-thrombin was not inhibited by α-thrombin-DFP (Fig. 3B), suggesting again that the active site (or its vicinity) was involved in the reactivity of P1 to α-thrombin. Considering that there is no amino acid sequence homology between β_2GPI and α-thrombin, these data indicate that β_2GPI and the relevant serine proteases share conformational epitopes that reside at or around the active site in the catalytic domains of the reactive proteases.

All four patient-derived IgG mAb display standard aCL activity

Subsequently, to determine the clinical relevance of these mAb, we analyzed these mAb for binding to CL in the presence of bovine serum, the standard aCL assay. The results showed that all four mAb bound to CL when compared against the IgG1 and IgG3 isotype controls (Figure 5), although the aCL activities of B2 and P1 were stronger than those of B1 and T1. Since B2 and P1 bound to β_2GPI and strongly to plasmin (Figures 1D and 2D), the data suggested that IgG aCL activity might reflect more the reactivity of the strong plasmin-reactive anti-β_2GPI Ab than that of the weak plasmin-reactive anti-β_2GPI Ab (like B1), and that IgG aCL include mainly Ab that recognize one or more epitopes shared by β_2GPI and plasmin.

DISCUSSION

To test the hypothesis that some aPL in APS patients recognize a conformational epitope shared by β_2GPI and the homologous catalytic domains of several serine proteases in hemostasis, we

generated and analyzed four new IgG monoclonal aPL from two APS patients. Of the four IgG mAb, two were generated by screening against β_2GPI, one against thrombin and one against PC. Importantly, immunological analyses of these four monoclonal aPL showed that both anti-β_2GPI mAb bound to thrombin, APC and plasmin. On the other hand, one anti-thrombin mAb and one anti-PC mAb reacted with β_2GPI (Figure 1 and Table 1). Moreover, the B2 anti-β_2GPI mAb bound to APC and plasmin with affinities that were even higher than those of T1 (anti-thrombin mAb) and P1 (anti-PC mAb), while the P1 mAb displayed the highest affinity toward β_2GPI (Figure 2 and Table 2). Most importantly, P1 binding to β_2GPI was inhibited by α-thrombin (that contains only the catalytic domain of thrombin, Figure 3A). Taken together with the fact that there is no amino acid sequence homology between β_2GPI and the involved serine proteases, these data demonstrate that some aPL in APS patients recognize conformational epitopes shared by β_2GPI and the homologous catalytic domains of several serine proteases in hemostasis.

When the four new IgG monoclonal aPL were analyzed for aCL activity, all bound to CL in the presence of bovine serum (Figure 5). Previously, we generated seven IgG mAb generated from two APS patients by screening against CL in the presence of bovine serum. Interestingly, 5/7 bound to thrombin, APC, plasmin and tPA (Table 1) (23). Of the 5 protease-reactive mAb, CL1, CL24, IS3, and IS4 also bound to β_2GPI (Table 1) (32). In addition, analyses of the IS6 patient-derived IgG monoclonal aPT showed that it displayed aCL activity, bound to all four above serine proteases, and reacted with β_2GPI (Table 1). Combined, these data showed that 10 of 12 IgG monoclonal aPL from four APS patients bound to thrombin, APC, plasmin and tPA (Table 1); and, of the 10 protease-reactive mAb, 9 (90%) also reacted with β_2GPI (Table 1). On the other hand, of the 9 patient-derived β_2GPI-reactive IgG aCL, all interacted with the concerned serine proteases (Table 1). Thus, these data indicate that IgG anti-β_2GPI Ab overlap extensively with Ab against certain serine proteases in hemostasis, and that β_2GPI shares epitopes with these serine proteases. Moreover, considering that 9/12 (75%) patient-derived IgG aCL react with β_2GPI and the concerned serine proteases, these findings also show that the majority of IgG aCL recognize one or more epitopes shared by β_2GPI and the concerned serine proteases.

While the above data are from patient-derived IgG monoclonal aPLs, a similar finding was reported recently among polyclonal aPL in APS patients (37). Specifically, when aPL were studied in 120 APS patients, the presence of IgG anti-β_2GPI Ab was closely related to the presence of IgG anti-thrombin Ab. On the other hand, the contention of a shared epitope between β_2GPI and the relevant serine proteases is supported by our previous analyses of five monoclonal IgG aPL Fab fragments from a phage displayed-Ab library, including B14, B22 and B27 anti-β_2GPI from panning with β_2GPI; and P11 and P13 anti-PT from panning with PT (38). The results revealed that the P11 aPT Fab also reacted with β_2GPI, and had comparable binding affinity to both antigens, with relative K_d values of 3×10^{-6} M for PT versus 2×10^{-6} M for β_2GPI (38). Moreover, similar findings were also reported very recently by other investigators (39). In particular, when 39 single chain FV (scFv) clones from an APS patient were isolated and analyzed, 10 clones were found to react with PT and to crossreact with β_2GPI. Taken together with the present data in Figures 1-3, these data clearly demonstrate that β_2GPI shares epitopes with the homologous catalytic domains of certain serine proteases in hemostasis.

It is interesting to note that, of the four currently studied autoantigens, the mAb bind to plasmin with the highest affinity, with the relative K_d values ranging from 1×10^{-6} M to 9×10^{-8} M (Table 2). Similar high affinity interaction was also observed between plasmin and six other patient-derived, protease-reactive monoclonal IgG aPL (20). Specifically, CL1 and CL15 bind to plasmin with relative Kd values of 6×10^{-8} M and 1×10^{-7} M, respectively. These affinities are significantly higher than the reported affinity of IgG anti-β_2GPI Ab (in 5 APS patients)

toward β_2GPI, with the relative K_d values ranging from 3 to 7×10^{-6} M (40). Viewed as a whole, these data suggest that plasmin may be an important autoantigen that drives certain IgG aPL in some APS patients, and that plasmin may even be the driving autoantigen for some IgG anti-β_2GPI Ab (like the P1 mAb).

Since our seven monoclonal IgG aCL from two APS patients were generated and reported in 1999 (32), they have been studied extensively by several investigators, and the results have contributed significantly toward our understanding of immunopathogenesis of autoantibody-mediated thrombosis and fetal loss in APS. In addition to our analyses of these mAb, Pierangeli and her colleagues examined the prothrombotic potentials and functional activities of these mAb (41). Using a pinch-induced *in vivo* murine thrombosis model, which allowed for continuous and quantitative monitoring of a focally induced non-occlusive mural thrombosis in an exposed femoral vein (42), five aCL (including CL15 and CL24) were found to be prothrombotic (41). In addition, CL15 was shown to induce human umbilical vein endothelial cells to express highest level of E-selectin and vascular cell adhesion molecule-1 (41). Furthermore, Pierangeli and her colleagues employed an *in vivo* microcirculation model to examine aCL-induced leukocyte adhesion to endothelium in venules (41). The results showed that IS2, CL15 and IS4 increased significantly the number of endothelium-adhering leukocytes (41). On the other hand, Rand and his associates studied the effects of these mAb on annexin A5. Using atomic force microscopy, a method previously used to study the crystallization of annexin A5, IS3 together with β_2GPI were shown to disrupt the annexin A5 crystallization pattern over the bilayers and to increase generation of thrombin (43). Along this line, IS4 together with β_2GPI were found to reduce annexin A5 binding to PL and to inhibit the annexin A5 anticoagulant activity (16). Therefore, we expect the availability of four new patient-derived IgG monoclonal aPL will similarly contribute toward our progress in delineating immunopathogenesis of autoantibody-mediated thrombosis and fetal loss in APS.

Acknowledgements

We thank Dr. John FitzGerald for critical review of the manuscript and helpful discussion, and Brian Chung and Tony Hsu for their technical assistance.

This work was supported by a research award from the Alliance for Lupus Research, a grant from the Southern California Chapter of the Arthritis Foundation and a grant AR42506 from the National Institutes of Health. W. Lin was supported by a Research Training grant from the Taiwan government.

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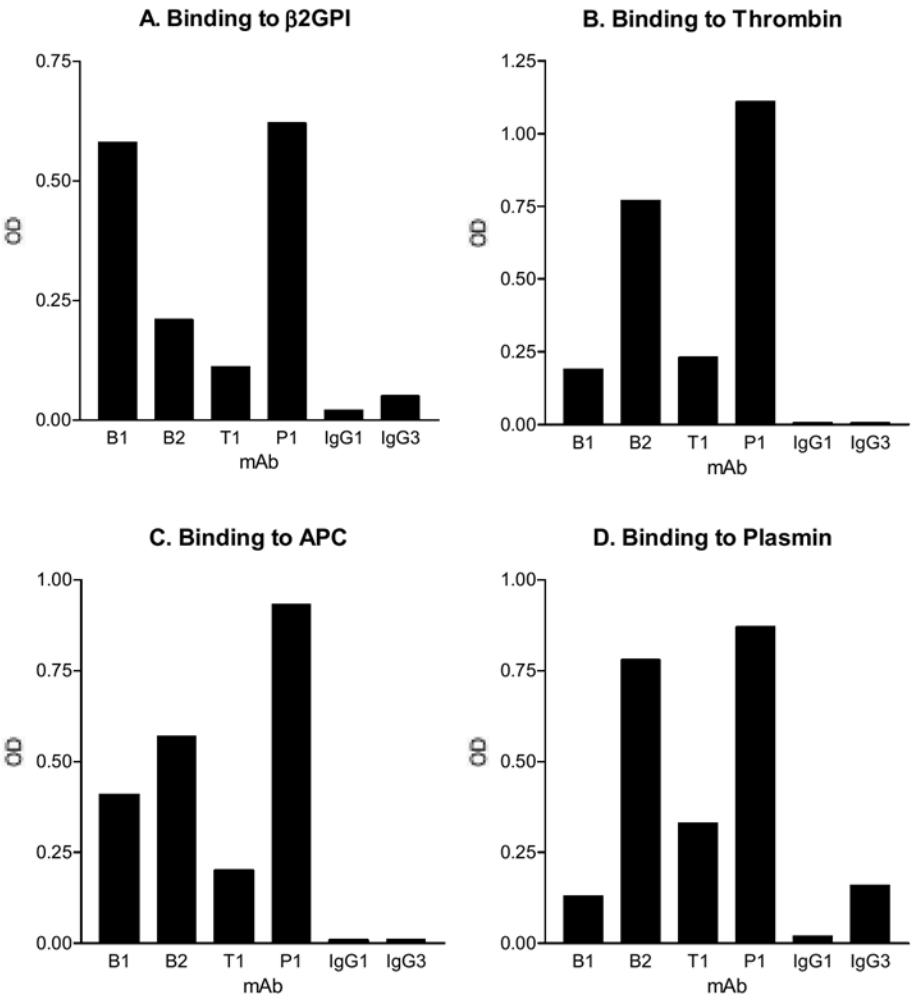

Figure 1. Binding characteristics of four new patient-derived IgG monoclonal aPL

The mAb were analyzed for their binding to β_2GPI (panel A), thrombin (B), APC (C) and plasmin (D). Microtiter wells were coated with the indicated autoantigens, and the test mAb, or the monoclonal isotype controls (IgG_1 or IgG_3) were analyzed at 1μg/ml. Except for B2 (which is IgG_3), all mAb are IgG_1. Bound IgG were measured and expressed in OD. One of two experiments with similar results is shown.

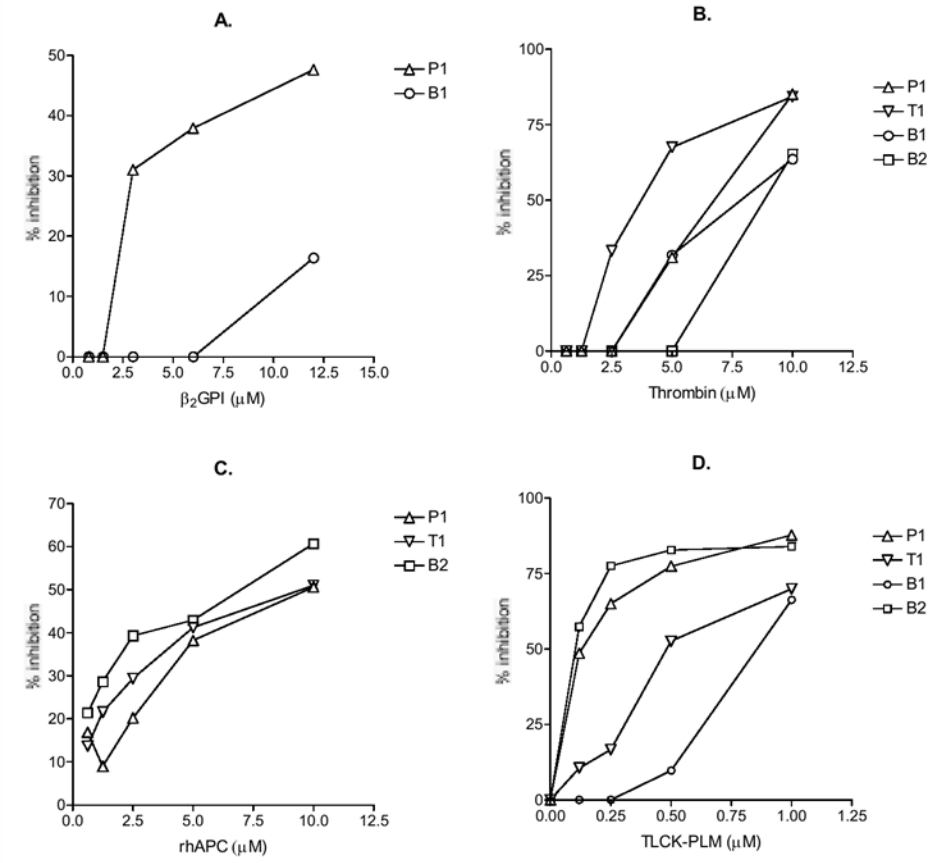

Figure 2. Competitive inhibition of mAb binding to four APS-relevant autoantigens

The test mAb were preincubated with β_2GPI (panel A), thrombin (B), rhAPC (C) or the TLCK-inactivated plasmin (D) at the indicated concentrations, and then the mixtures were distributed to wells coated with the corresponding autoantigens. The results are expressed in % inhibition. One of two experiments with similar results is shown.

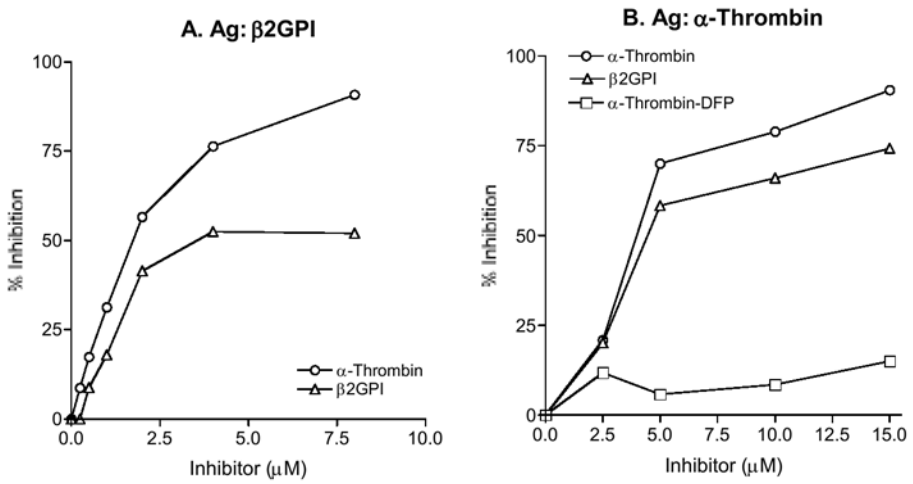

Figure 3. Cross inhibition of P1 binding to β_2GPI (panel A) or α-thrombin (B)

The P1 mAb was preincubated with β_2GPI, α-thrombin or α-thrombin-DFP (an active site-blocked α-thrombin) at the indicated concentrations, and then the mixtures were distributed to wells coated with β_2GPI (panel A) or α-thrombin (B). The results are expressed in % inhibition. A representative of two experiments is shown.

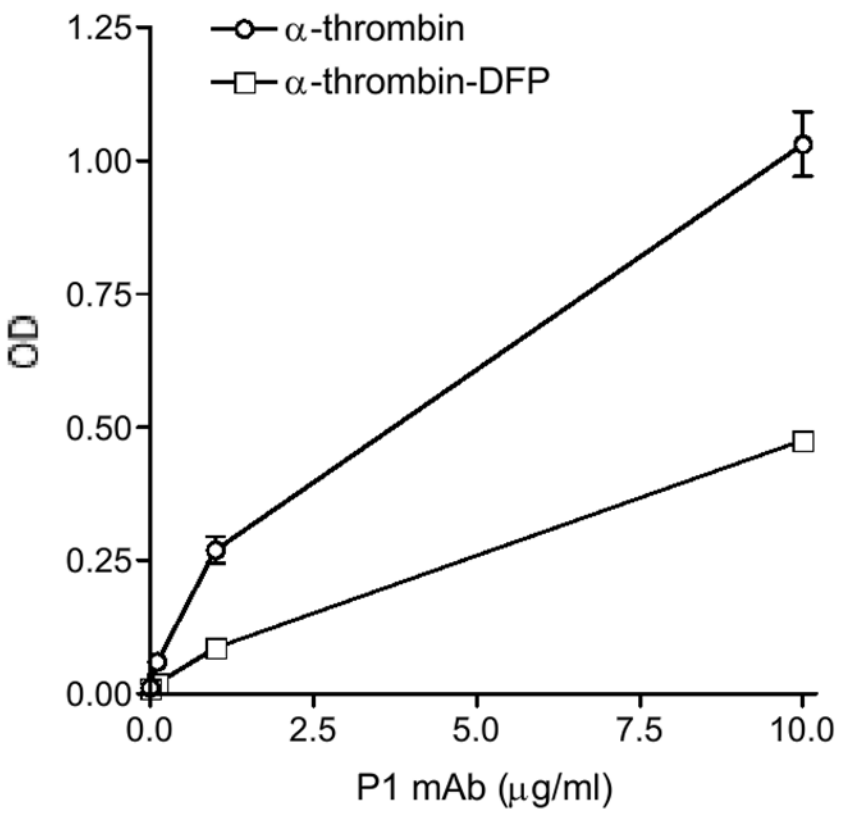

Figure 4. Comparison of P1 binding to α-thrombin and α-thrombin-DFP

Wells were coated with each antigen at 5μg/ml, and P1 mAb was analyzed at the indicated concentrations. Bound IgG were measured and expressed in OD. A representative of two experiments is shown.

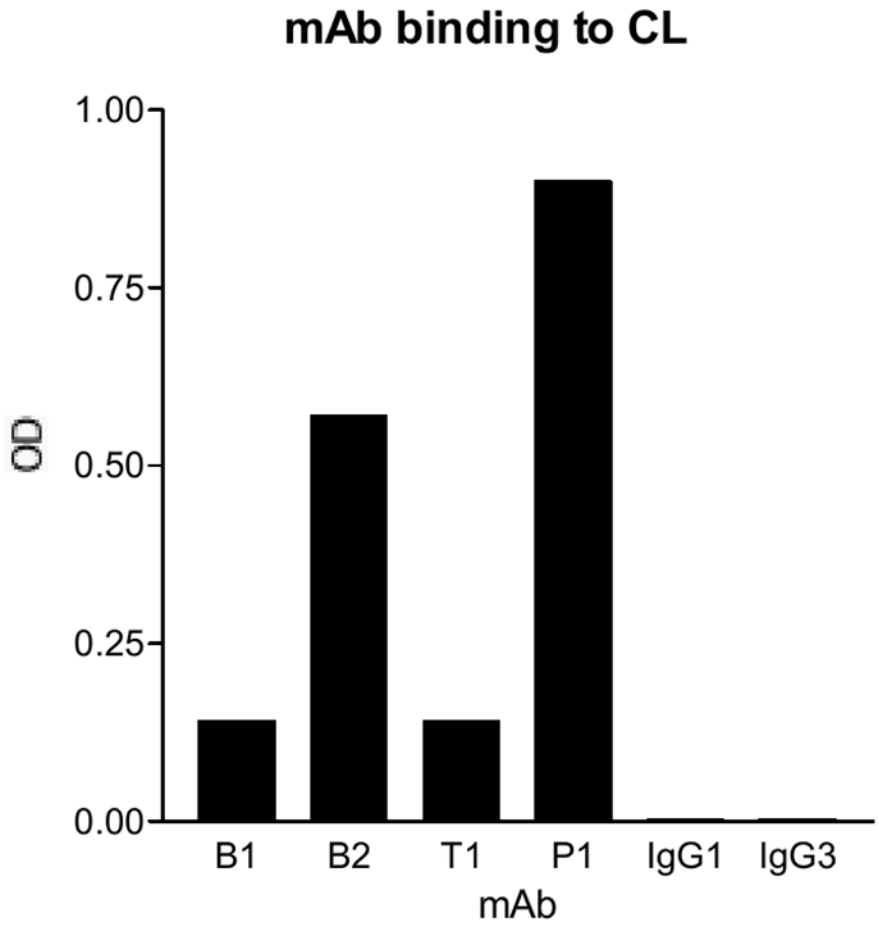

Figure 5. All four new patient-derived IgG monoclonal aPL bind to CL in the presence of bovine serum

The mAb were analyzed for their aCL activity. Microtiter wells were coated with the CL, and the test mAb, or the monoclonal isotype controls (IgG_1 or IgG_3) were analyzed at 1μg/ml. Except for B2 (which are IgG_3), all mAb are IgG_1. Bound IgG were measured and expressed in OD. A representative of two experiments is shown.

Table 1

Summary of 12 monoclonal IgG aPL from four APS patients[a]

Antigens: mAb:	screening	CL/BS	β_2GPI	thrombin	APC	plasmin
IS1	CL/BS	+++	-	-	-	-
IS2	CL/BS	++	-	-	-	-
IS3	CL/BS	+++	++	+++	+++	+
IS4	CL/BS	++	+	++	+++	+++
IS6	prothrombin	++	++	++	++	++
CL1	CL/BS	++	++	+++	+++	+
CL15	CL/BS	++	-	++	+++	++
CL24	CL/BS	++	+	++	+	++
B1	β_2GPI	+	+++	+	+	+
B2	β_2GPI	++	+	++	++	+++
T1	thrombin	+	+	+	+	++
P1	protein C	++	+++	++	++	+++

[a]Binding to all test antigens are expressed in relative term for each antigen by all mAbs. Binding to CL in the presence of bovine serum (BS) and to human β_2GPI are from references (30,32,33) and the present study. Binding to human thrombin, APC, and plasmin are from references (17,19,20) and the present study. Although initial study of IS6 binding to β_2GPI in Tris-buffered saline was negative (33), subsequent study of IS6 binding to β_2GPI in PBS revealed positive interaction (data not shown).

Table 2

Relative Kd of IgG monoclonal aPL toward β_2GPI and relevant serine proteases

mAb: Antigens:	**B1**	**B2**	**T1**	**P1**
β_2GPI	$> 10^{-5}$ M	Not inhibitable	Not inhibitable	10^{-5} M
Thrombin	10^{-5} M	2×10^{-5} M	4×10^{-6} M	8×10^{-6} M
rhAPC	Not inhibitable	5×10^{-6} M	7×10^{-6} M	9×10^{-6} M
TLCK-Plasmin	1×10^{-6} M	9×10^{-8} M	6×10^{-7} M	1×10^{-7} M