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The combined presence of anti-phospholipid (PL) Ab, including lupus anticoagulants (LAC) and/or anticardiolipin Ab (aCL), and thrombosis is recognized as the antiphospholipid syndrome (APS). LAC are detected as an inhibitory effect on PL-restricted in vitro blood coagulation tests, and are comprised mainly of Ab against β2 glycoprotein I and prothrombin (PT). Recently, anti-PT Ab (aPT) were found to be associated with thrombosis by some investigators, although this is not confirmed by others. Considering that aPT are heterogeneous in patients and that PT is converted into thrombin, we hypothesize that certain aPT in patients may bind to thrombin, and that some of such anti-thrombin Ab may interfere with thrombin-antithrombin (AT) interaction and thus reduce the AT inactivation of thrombin. To test this hypothesis, we searched for anti-thrombin Ab in APS patients and then studied those found for their effects on the AT inactivation of thrombin. The results revealed that most, but not all, aPT-positive patient plasma samples contained anti-thrombin Ab. To study the functional significance of these Ab, we identified six patient-derived mAb that bound to both PT and thrombin. Of these mAb, three could reduce the AT inactivation of thrombin, whereas others had minimal effect. These findings indicate that some aPT in patients react with thrombin, and that some of such anti-thrombin Ab could inhibit feedback regulation of thrombin. Because the latter anti-thrombin Ab are likely to promote clotting, it will be important to develop specific assays for such Ab and study their roles in thrombosis in APS patients. The Journal of Immunology, 2001, 167: 7192–7198.

Coagulation abnormalities, including thrombosis and recurrent fetal loss, have emerged as important clinical complications in systemic lupus erythematosus (SLE); Refs. 1–3). Patients with SLE and acquired coagulation abnormalities often have anti-phospholipid (PL) Ab (aPL). These include lupus anticoagulants (LAC), as detected by their abilities to prolong certain in vitro PL-restricted blood clotting tests, and anticardiolipin Ab (aCL; Refs. 2 and 4–8). Because LAC is neutralized by addition of excess PL, it was suggested that the LAC Ab might interact with PL and thus interfere with blood coagulation on the limited PL surface in the in vitro test. Therefore, LAC and aCL are generally referred to as aPL, and the association of thrombosis and fetal loss with LAC and aCL is recognized as antiphospholipid syndrome (APS; Refs. 2 and 8).

Accumulated studies show that aPL represent a heterogeneous group of immunologically and functionally distinct Ab that recognize various PL, PL-binding plasma proteins, and/or PL-protein complexes. The involved plasma proteins include plasma protein β2 glycoprotein-1 (β2GPI), prothrombin (PT), protein C, and protein S (9–14). To date, the Ab against β2GPI and its complexes with cardiopulmonary probably account for most of the positive findings on tests for aCL in APS (15, 16), whereas Ab against PT and β2GPI are responsible for the majority of the LAC activity (11, 17–19).

Recently, increasing attention has been paid to anti-PT Ab (aPT) and their roles in thrombosis in APS patients (18, 20–29). The prevalence of aPT in patients varies among different studies, ranging from 30 to 60% in APS patients when tested by ELISA using immobilized human PT on activated polyvinyl chloride plates (20, 23, 25). However, aPT were found to be associated with thrombosis (21, 25, 28), although this is not confirmed by other investigators (23). These conflicting data may reflect the heterogeneity of aPT present in individual patient sera and different sets of these autoantibodies in clinically diverse patient populations in different studies.

To understand the functional and pathogenic property of aPT, Rao and coworkers (11, 18) affinity purified IgG aPT and found that the purified Ab bound to immobilized phosphatidylserine in the presence of Ca2+ and PT. These results suggested that IgG aPT cross-linked PT molecules, and thus increased the valence of interactions between PT and phosphatidylserine. Subsequently, these investigators showed that IgG purified from a LAC-positive plasma sample (designated LAC IgG; from a patient with hypoprophosphatemia) enhanced the binding of PT to HuVEC and increased conversion of PT to thrombin on the surface of HuVEC (22).

Thrombin is a key effector enzyme in the coagulation cascade. It converts fibrinogen to fibrin, leading to the formation of fibrin clots. It also feedback amplifies the cascade by activating factors V and VIII, which in turn, enhance conversion of PT to thrombin (30). Therefore, once thrombin is generated in vivo, it is tightly
regulated by antithrombin (AT) that binds to thrombin in the presence of heparin-like glycans in the endothelial cell (EC) surface and inactivates the enzyme irreversibly (30–32). Considering that thrombin is derived from the zymogen PT, it is conceivable that some aPT may bind to thrombin at a site where thrombin interacts with AT, and therefore inhibit AT inactivation of thrombin. In this study, we report the detection of Ab against thrombin in APS patients and the inhibitory effects of three patient-derived IgG monoclonal anti-thrombin Ab on the AT inactivation of thrombin. These findings define a novel anti-thrombin autoantibody in APS and they show that such Ab may interfere with negative feedback regulation of thrombin in circulation, and thus contribute to thrombosis.

**Materials and Methods**

**Patients and healthy controls**

Plasma samples were obtained from 13 patients and 5 normal controls at University of California, Medical Center (Los Angeles, CA) and University of California Medical Center (San Diego, CA). All 13 APS patients satisfied the Sapporo classification criteria for definite APS (8). The diagnosis of APS was confirmed by a medical record review.

Of the 13 patients, there were 8 primary APS (62%) and 5 secondary APS (38%). Three with secondary APS had SLE, one had SLE and Sjogren’s syndrome, and the fifth had undifferentiated connective tissue disease. All were positive for aCL and 10 were positive for aPT. For clinical data, two of the six patients had had one or more unexplained fetal losses. The ethnicity and gender for primary APS were six Caucasians and two Hispanics, and six females and two males. All of the secondary APS were Caucasians with a gender ratio of two females/three males. The average age (in years) at diagnosis for primary APS was 43.3 (range of 17–63) and that for secondary APS was 26.8 (range of 12–41).

Five healthy donors were recruited as normal controls and were designated N1 to N5. Their ethnicity was two Asians and three Caucasians, and gender was three females and two males. Their average age at the time of donation of their sample was 33.6 years (range of 21–53).

**ELISA for Ab against PT and thrombin**

The ELISA for aPT was done as described previously (33). Briefly, high-binding ELISA plates (Costar, Cambridge, MA) were coated with 10 μg/ml of human PT (Enzyme Research Laboratories, South Bend, IN) in TBS (0.05 M Tris-Cl and 0.15 M NaCl, pH 7.5). After incubating overnight at 4°C, plates were blocked with TBS containing 0.3% gelatin. Then test plasma samples (1/100 dilution) or mAb (1.0 μg/ml) in TBS/0.1% gelatin were distributed to wells in duplicate and were incubated for 1.5 h at room temperature. The IS6 monoclonal IgG aPT was used as a positive control and as a reference aPT in all assays (33). After washing with TBS, bound human IgG and total Ig (denoted as IgG) were detected, respectively, with HRP-conjugated goat anti-human IgG (γ-chain specific; BioSource International, Camarillo, CA), anti-human IgG (all isotypes; Jackson ImmunoResearch Laboratories, West Grove, PA), and peroxidase substrate tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The ELISA for anti-thrombin Ab was done similarly except that plates were coated with 10 μg/ml of human α-thrombin (Hematologic Technologies, Essex Junction, VT). Of note, the IS6 monoclonal aPT was found to react with thrombin during the initial study of anti-thrombin Ab (see Results) and thus was used as a reference Ab in all subsequent anti-thrombin Ab ELISA. The results were expressed in arbitrary units (AU) with 1 AU equivalent to the OD of the IS6 mAb at 2 μg/ml.

A competitive inhibition assay was used to study the binding properties of selected mAb to PT and thrombin. Briefly, each mAb (1.5 μg/ml) was preincubated for 1.5 h with various concentrations of either PT or thrombin. Then, the mixture was distributed to the PT- or thrombin-coated wells in duplicate. After incubation, bound IgG was measured. The inhibition data of each mAb was expressed as percentage inhibition of each isotype control, which was preincubated without PT or thrombin. Differences in the percentage inhibition of IgG binding were calculated as (1 – (Isotype control binding to PT or thrombin)/(Isotype control binding to PT or thrombin)) × 100%.

**Statistical analysis**

The mean AU plus 3 SD of the five normal controls was used as the cutoff, and the plasma samples with AU values consistently higher than the cutoff in two separate experiments were considered positive. Differences in the test Ab-induced inhibition of thrombin activity or the thrombin inactivation by AT were analyzed using paired ANOVA followed by the Bonferroni multiple comparison test. Values of p < 0.05 were considered significant.

**Results**

**Detection of anti-thrombin Ab in some APS patients**

To test the hypothesis that some aPT in patients may bind to thrombin, we developed an ELISA for anti-thrombin Ab and used the assay to analyze plasma samples from 13 APS patients (designated P1 to P13) and 5 healthy normal controls (designated N1 to N5); multiple plasma samples from 3 patients were available and were all analyzed. All samples were analyzed at the 1/100 dilution. The results showed that using mean plus 3 SD of the normal controls as the cutoff, anti-thrombin Ab were detected in 10 of 13 patients, and IgG anti-thrombin Ab were detected in 3 of 13 patients (Fig. 1, A and B). Patient P1 had the highest titer of anti-thrombin Ab, but had no detectable IgG anti-thrombin Ab. In contrast, patient P4 had the highest titer of IgG anti-thrombin Ab, but only a medium titer of anti-thrombin Ab. Of the three patients with multiple plasma samples, their anti-thrombin Ab titer fluctuated over time, which paralleled the previously reported fluctuation of aCL titers in APS patients. Specifically, only one of three samples from patient P1 had anti-thrombin Ab, and only one of three samples from patient P6 had IgG anti-thrombin Ab.

To determine the relationship between aPT and anti-thrombin Ab, we analyzed the same samples for aPT. As can be seen in Fig. 1, C and D, aPT were found in 10 of 13 patients and IgG aPT was found in 5 of 13 patients. Except for the P2c plasma sample, there was a good quantitative correlation between aPT and IgG aPT titers; noticeably, patient P5 had abundant aPT and IgG aPT. Of the three patients with multiple plasma samples, their aPT and IgG aPT titers fluctuated over time, similar to the above fluctuation in anti-thrombin Ab titers.

When the presence of aPT and anti-thrombin Ab in patient samples were compared, 11 of 13 aPT-positive patient plasma samples had anti-thrombin Ab, and three of the seven IgG aPT-positive samples had IgG anti-thrombin Ab. These data suggest that many aPT (including the IgG isotype) may also react with thrombin, or that two different Ab for each Ag often occur together, similar to...
autoantibodies against Sjogren syndrome Ag A and B in SLE (1). In contrast, sample P1b had high titers of anti-thrombin Ab, but no aPT, indicating that some anti-thrombin Ab in APS patients do not react with PT and that anti-thrombin Ab are heterogeneous in patients.

Identification of monoclonal aPT and anti-thrombin Ab

Because anti-thrombin Ab are heterogeneous in patients, it would be important to obtain monoclonal anti-thrombin Ab and use such mAb to study the functional significance of anti-thrombin Ab in APS. Accordingly, we searched for patient-derived monoclonal aPT and anti-thrombin Ab. Previously, we found that the IS6 monoclonal aPT cross-reacts with cardiolipin (33), and that the P11 monoclonal aPT F(ab')2 (isolated by panning a phage display Ab library on PT) reacts with both PT and β2GPI, with relative Kd values of $3.2 \times 10^{-6}$ M for PT vs $1.6 \times 10^{-6}$ M for β2GPI (36). These data raised the possibility that some of our monoclonal aCL/anti-β2GPI Ab might reciprocally cross-react with PT. Therefore, we screened seven patient-derived monoclonal IgG aCL (37) for binding to PT; IS1 and IS2 are IgG1, and the other mAb are IgG3. The results showed that five of seven mAb reacted with PT (Fig. 2A). Moreover, the aPT activities of CL1, CL15, and IS3 were even better than that of the IS6 monoclonal IgG aPT (33) at the same concentration.

Subsequently, all monoclonal aPT were analyzed for binding to thrombin. As can be seen in Fig. 2B, all six aPT (CL1, CL15, CL24, IS3, IS4, and IS6) bind to thrombin. Interestingly, their relative binding pattern to thrombin is similar to their binding to PT.

Effects of anti-thrombin mAb on thrombin activity and thrombin inactivation by AT

To study the effects of anti-thrombin mAb on thrombin activity, thrombin was incubated separately with test mAb for 1 h, and then the thrombin chromogenic substrate activity was assessed. As can be seen in Fig. 3, CL15 and IS3 slightly reduced thrombin activity, whereas the other four anti-thrombin mAb did not affect thrombin activity.

Thereafter, we studied thrombin-reactive mAb for their abilities to interfere with AT inactivation of thrombin in a functional assay, which contained 0.1 U/ml of heparin and used AT at a concentration that was at least 10-fold higher than that of thrombin. The addition of heparin is to approximate the in vivo inactivation of
to 61%, the resultant increase in thrombin activity over time could not affect AT inactivation of thrombin. The remaining three other monoclonal anti-thrombin Ab did not affect on AT inactivation of thrombin, reducing thrombin inactivation and 71%, respectively. CL1 showed a small but significant inhibitory effect on AT inactivation of thrombin, reducing thrombin inactivation to 78%. The remaining three other monoclonal anti-thrombin Ab did not affect AT inactivation of thrombin.

Although CL24 reduced thrombin inactivation only from 88% to 61%, the resultant increase in thrombin activity over time could have a significant prothrombotic effect, as the residual thrombin continues to convert fibrinogen into fibrin at a constant rate. To visualize this rapid cumulative effect over time, overall conversion of a thrombin substrate in the absence or presence of test mAb or an isotype control mAb was measured over a period of 5 min. As can be seen in Fig. 4B, accumulated substrate conversion in the presence of CL24 increases dramatically over those in the absence of CL24 in 5 min.

Because the plasma concentration of AT is 2 μM (38) and the above experiments were done with the final concentration of AT at 67 nM, we studied the effects of CL24 on thrombin inactivation in the presence of AT from 67 to 533 nM, due to the prohibitory cost of AT. The results showed that the CL24-mediated reduction in AT inactivation of thrombin remained constant over the tested range of AT concentrations (Fig. 4C). Subsequently, we studied the effects of CL24 and CL15 over a range of heparin concentrations that had been used by other investigators (39). The results show that CL24 and CL15 significantly reduced AT inactivation of thrombin in the presence of heparin concentrations from 0.025 to 0.2 U/ml (final concentrations in the thrombin and AT mixture), and the Ab-mediated reduction disappeared when heparin concentration reached 0.4 U/ml (Fig. 4D). A maximal reduction in the thrombin inactivation by AT occurred at 0.05 U/ml of heparin, reducing the inactivation of thrombin from 76% in the presence of the control IgG3 to 30% in the presence of CL24 (Fig. 4D). In contrast, IS6 did not significantly affect the AT inactivation of thrombin.

The binding properties of three chosen mAb to PT and thrombin

Based on the above data, three representative mAb were chosen for analysis by competitive and cross inhibition. These were CL24 (which inhibits AT inactivation of thrombin), IS3 (which inhibits thrombin per se), and CL15 (which inhibits both thrombin per se and the AT inactivation of thrombin). The results showed that soluble thrombin is more effective than PT in inhibiting all three mAb binding to either PT or thrombin (Fig. 5). Importantly, PT could only inhibit binding of all tested mAb to the immobilized PT but not thrombin. These results demonstrate that these three mAb are more specific for thrombin than PT. Based on the thrombin inhibition data for binding to thrombin, the relative $K_i$ values of these Ab to thrombin were calculated to be $7.5 \times 10^{-6}$, $1.7 \times 10^{-6}$, and $7.4 \times 10^{-6}$ M for CL15, CL24, and IS3, respectively.
FIGURE 4. Some anti-thrombin Ab inhibit thrombin inactivation by AT. A, CL1, CL15, and CL24 significantly reduce the thrombin inactivation by AT. Thrombin was preincubated with test mAb. Then, AT was added to reaction mixtures (in the presence of heparin), which was followed immediately by the addition of the thrombin chromogenic substrate S-2238. OD after 1 min was measured. The results are expressed as the percentage of thrombin inactivation by AT. An asterisk denotes $p < 0.05$. B, The effect of CL24 on accumulated thrombin activity in the presence of AT and heparin. The experiment was performed in a similar manner to that in A except that generation of p-nitroaniline was monitored continuously for 5 min. A representative result from three experiments is given. C, CL24 reduces the thrombin inactivation by AT over a range of AT concentrations. The experiment was performed in a similar manner to that in A except that AT was used at the indicated final concentrations. D, CL15 and CL24 reduce thrombin inactivation by AT over a range of heparin concentrations. The experiment was performed in a similar manner to that in A except that heparin was used at the indicated concentrations (0–0.4 U/ml). The mean and the range are given for A, C, and D ($n = 2$).

Preliminary characterization of thrombin epitope recognized by CL24

As a first step to define the epitope recognized by CL24, we comparatively analyzed the effects of CL24 on $\alpha$-thrombin and $\gamma$-thrombin, a proteolytic variant of $\alpha$-thrombin with impaired exosite I. The exosomes are patches of positively charged amino acid residues on the thrombin surface that interact with the thrombin substrate, inhibitor, and modifier (i.e., thrombomodulin; Ref. 40). As can be seen in Fig. 6, AT alone inactivated 67–69% of $\gamma$-thrombin activity in the presence of either polyclonal human IgG or a monoclonal human IgG3 isotype control. Under the same conditions, CL24 reduced the degree of $\gamma$-thrombin inactivation to 40%, equivalent to a 42% reduction (based on the 69% for the monoclonal human IgG3 isotype control) in AT inactivation of thrombin. Importantly, this CL24-induced reduction on AT inactivation of $\gamma$-thrombin is similar to the 42% reduction of AT inactivation of the $\alpha$-thrombin by CL24 (decreasing from 88% AT inactivation of $\alpha$-thrombin to 51%). The results suggest that CL24 is unlikely to react with thrombin at its exosite I and/or the surrounding regions.

Discussion

To test our hypothesis that some thrombogenic aPT may bind to thrombin and thus interfere with thrombin inactivation by AT, we searched for the presence of anti-thrombin Ab and studied their effects on the AT inactivation of thrombin. The results showed that anti-thrombin Ab were detected in several APS patients (Fig. 1, A and B), and several patient-derived IgG mAb bound to thrombin (Fig. 2B). Importantly, of the six monoclonal anti-thrombin mAb, CL15 and IS3 inhibited thrombin activity per se (Fig. 3), whereas CL1, CL15, and CL24 significantly reduced the AT inactivation of thrombin in the presence of heparin (Fig. 4A), and CL24 at 0.7 µg/ml could inhibit AT inactivation of thrombin in the presence of AT at 533 nM (Fig. 4C). Note, the human plasma concentrations of AT and IgG are 2 µM and 10 mg/ml, respectively (38). Moreover, although the reduction of AT inactivation was only moderate, the effect of such an Ab on cumulative thrombin cleavage products increases rapidly over time (Fig. 4B). In addition, the binding of three functionally representative mAb (i.e., CL15, CL24, and IS3) to PT and thrombin was inhibited more effectively by soluble thrombin than by PT (Fig. 5), indicating that these three mAb are more specific for thrombin than PT. Combined, these data show that some anti-thrombin Ab in APS patients (like CL24 at ~0.1% of the plasma IgG concentration) could interfere with the AT inactivation of thrombin, thus allowing for prolonged coagulation in blood once clotting is initiated in patients carrying such autoantibodies. Obviously, prolonged and unchecked thrombin activity is most likely to promote and/or sustain thrombosis.

Fig. 4D shows that CL24-mediated inhibition of AT inactivation of thrombin was decreased by increasing concentrations of heparin. As noted earlier, the addition of heparin is to approximate the in vivo inactivation of thrombin by AT, which often binds to anticoagulantly active heparan sulfate proteoglycans on the vascular endothelium (31, 41). During the in vitro heparin-dependent AT inactivation of thrombin, heparin binds simultaneously to thrombin and AT, and thus bridges thrombin and AT to form a trimolecular complex of heparin-thrombin-AT, leading to a several
thousand-fold increase in the rate of AT inactivation of thrombin (42). In this context, the above data suggest that CL24 may bind to thrombin’s heparin binding site (the exosite II) and thus interfere with the binding of heparin to thrombin, and this effect is overcome by increased concentrations of heparin, which could compete for the same binding sites. This contention is consistent with the finding that CL24 also inhibited the AT inactivation of γ-thrombin, which contains the intact exosite II but impaired exosite I (Fig. 6).

Similar to the in vitro heparin-mediated enhancement of AT inactivation of thrombin, it is thought that in vivo acceleration of AT inactivation of thrombin occurs through the binding of AT and thrombin to the anticoaguantly active endothelial glycosaminoglycans (41). Although the effective activity of anticoaguantly active heparan sulfate proteoglycans on the EC surface is unknown, it can be speculated that in some in vivo circumstances, anti-thrombin Ab similar to CL24 may interfere with the binding of thrombin to these heparan sulfates and may therefore inhibit the formation of thrombin-heparan sulfate-AT complexes and the accelerated AT inactivation of thrombin.

Of the above three likely prothrombotic anti-thrombin Ab, CL15 also inhibits thrombin activity (Fig. 3). In contrast, IS3 did not reduce AT inactivation of thrombin, but did inhibit thrombin activity per se (Fig. 3). The observed inhibitory activity of these two anti-thrombin mAb was similar to a previously reported monoclonal IgG anti-thrombin Ab that apparently caused severe bleeding in a patient with monoclonal gammopathy (43). This latter mAb did not bind to PT, and thus was similar to the anti-thrombin Ab observed in plasma sample P1b (Fig. 1).

In light of patient-derived anti-thrombin mAb with different (and even opposite) functional activities, it would be fruitless to assess the clinical significance of all anti-thrombin Ab in APS by association studies of the presence of anti-thrombin Ab to APS, or to study the functional activities of affinity-purified polyclonal anti-thrombin Ab.
from patients. Instead, it will be first necessary to delineate the thrombin epitopes recognized by various anti-thrombin Ab with different functional activities such as CL24 (which inhibits AT inactivation of thrombin), IS3 (which inhibits thrombin activity), and IS4 (which binds to thrombin but neither inhibits thrombin activity nor interferes with AT inactivation of thrombin) (Figs. 3 and 4). If anti-thrombin Ab with different functional activities are found to recognize different thrombin epitopes, then specific assays for each type of anti-thrombin Ab may be developed and used to study the roles of CL24-like anti-thrombin Ab in thrombosis in APS patients, as well as the roles of IS3-like Ab in Ab-mediated bleeding disorders.

It is intriguing that five of seven mAb generated by screening against cardiolipin in the presence of bovine serum bind to PT and thrombin. Of these five mAb, IS3, IS4, CL1, and CL24 also react with β2-GPI, the major autoantigen or cofactor for autoantibodies detected by the conventional aCL ELISA (37). Viewed as a whole, these data suggest that the latter four mAb may recognize an epitope shared by PT and β2-GPI, and that such an epitope may be analogous to the one revealed by P11 monoclonal aPT (F(ab′)2) isolated by panning a phage display Ab library on PT (36). However, CL24, IS3, and IS4 differ in their effects on thrombin activity and AT inactivation of thrombin. In this context, the combined data may suggest that there is more than one thrombin epitope that is shared among PT, thrombin, and β2-GPI. Clearly, further experimentation to test these hypotheses is warranted.

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References


