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Antibodies against the Activated Coagulation Factor X (FXa) in the Antiphospholipid Syndrome That Interfere with the FXa Inactivation by Antithrombin

Yao-Hsu Yang,*† Kwan-Ki Hwang,2* John FitzGerald,* Jennifer M. Grossman,* Mihaela Taylor,* Bevra H. Hahn,* and Pojen P. Chen*

Antiphospholipid Ab have been shown to promote thrombosis and fetal loss in the antiphospholipid syndrome (APS). Previously, we found IgG anti-thrombin Ab in some APS patients that could interfere with inactivation of thrombin by antithrombin (AT). Considering that activated coagulation factor X (FXa) is homologous to thrombin in the catalytic domains and is also regulated primarily by AT, we hypothesized that some thrombin-reactive Ab may bind to FXa and interfere with AT inactivation of FXa. To test these hypotheses, we studied reactivity of eight patient-derived monoclonal IgG antiphospholipid Ab with FXa and the presence of IgG anti-FXa Ab in APS patients and investigated the effects of FXa-reactive mAb on AT inactivation of FXa. The results revealed that six of six thrombin-reactive IgG mAb bound to FXa and that the levels of plasma IgG anti-FXa Ab in 38 APS patients were significantly higher than those in 30 normal controls (p < 0.001). When the mean plus 3 SDs of the 30 normal controls was used as the cutoff, 5 of 38 APS patients (13.2%) had IgG anti-FXa Ab. Importantly, three of six FXa-reactive mAb significantly inhibited AT inactivation of FXa. Combined, these results indicate that anti-FXa Ab may contribute to thrombosis by interfering with the anticoagulant function of AT on FXa in some APS patients. The Journal of Immunology, 2006, 177: 8219–8225.

Antiphospholipid syndrome (APS) is characterized by the presence of antiphospholipid Ab (aPL) and the clinical manifestations of thrombosis and/or miscarriage (1, 2). Studies of aPL have shown that they represent a heterogeneous group of immunologically distinct Ab that recognize various phospholipids (PL), PL-binding plasma proteins, and factors involved in hemostasis. The involved proteins include β2-glycoprotein I (β2GPI), annexin V, prothrombin (PT), thrombin, activated protein C, plasmin, and tissue plasminogen activator (tPA) among others (3–14). Importantly, thrombin, activated protein C, plasmin, and tPA are trypsin-like serine proteases (14–17). Although aPL have been shown to promote thrombosis and miscarriage in animal studies (18, 19), the underlying mechanisms remain unclear.

Hemostasis is composed of four major events that occur following the loss of vascular integrity: vascular constriction; platelet activation and aggregation; coagulation cascade and clot formation; and the clot dissolution. The coagulation cascade is initiated by expression of tissue factor (TF) upon vascular injury. TF binds and accelerates the activation of factor VII, and the activated factors VII (VIIa) form complexes with TF (designated TF-VIIa), which, in turn, activate factors IX and X (generating IXa and Xa, respectively). Subsequently, factor Xa (FXa) works with factor Va to convert PT to thrombin, which then converts fibrinogen to a fibrin clot (20, 21).

The coagulation cascade is tightly regulated by several inhibitors including activated protein C as well as antithrombin (AT). AT inactivates thrombin, FXa, and other serine proteases by forming a 1:1 molar complex with a target protease (22). The anticoagulant activity of AT is potentiated by the presence of heparin, which enhances the rate of inactivation up to several thousand times. AT binds to heparin-like glycosaminoglycans, on the surface of vascular endothelial cells (EC) in vivo (23). Inherited heterozygous deficiency in AT increases the risk of thromboembolism by 5-fold or higher, and women with the deficiency are at particularly high risk of abortion during pregnancy (20, 24). Therefore, it is conceivable that interference in the anticoagulant function of AT may promote thrombosis.

Previously, we showed that five of the seven patient-derived monoclonal IgG anti-cardiolipin Ab (aCL) and one monoclonal IgG anti-PT Ab (aPT) bound to thrombin and that three of the six thrombin-reactive mAb (CL1, CL15, and CL24) interfered with the inactivation of thrombin by AT (Table I). In addition, CL15 and CL24 promoted blood clotting in a pinch-induced thrombosis model in mice, suggesting that the thrombin-reactive Ab were prothrombotic (Table I) (25). In contrast, it is known that thrombin is most homologous to FXa structurally and mechanically among all the serine proteases (26, 27). In particular, the catalytic domains of FXa and thrombin share a similarity of 56.4% at the protein level (22). Thus, it is conceivable that some anti-thrombin Ab may also bind to FXa and interfere with the FXa inactivation by AT.

Therefore, we hypothesize that anti-FXa Ab are present in some APS patients, and that some of such autoantibodies interfere with...
AT inactivation of FXa. In this study, we report the reactivity of six patient-derived thrombin-reactive IgG mAb with both FXa and FX, and the detection of anti-FXa Ab in some APS patients. Importantly, of the FXa-reactive mAb, three (CL15, CL24, and IS6) impair the anticoagulant function of AT to inactivate FXa.

Materials and Methods

Patient-derived mAb

Seven IgG monoclonal aCL and one IgG monoclonal aPT derived from patients with APS were analyzed in this study. The aCL were CL1, CL15, CL24, IS1, IS2, IS3, and IS4 (28) and the single aPT was IS6 (29). The generation and characteristics of these mAb have been described previously. Of note, IS1 and IS2 are IgG1 and the other six mAb belong to IgG3 (28, 29).

Patients and healthy controls

Plasma samples were obtained from 38 APS patients (10 males and 28 females) and 30 healthy subjects (12 males and 18 females) at the University of California Medical Centers (Los Angeles and San Diego, CA). Informed consents were obtained and the study was approved by the University of California (Los Angeles, CA; UCLA) Institution Review Committee. All APS patients in this study satisfied the Sapporo classification criteria for definite APS (2). The average ages (in years) at the time of blood sampling from APS patients and healthy controls were 40 (range, 16–64) and 31.4 (range, 20–72), respectively. Medical charts and laboratory test reports for each patient entered in this study were reviewed by a rheumatologist (J. M. Grossman). Patients were then classified as primary APS if they had no associated autoimmune diseases or secondary APS if they had SLE or other criteria for definite APS (2). The average ages (in years) at the time of blood sampling from APS patients and healthy controls were 40 (range, 16–64) and 30 healthy subjects (12 males and 18 females) at the University of California Medical Centers (Los Angeles and San Diego, CA).

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ELISA for Ab against FX and FXa

The ELISA for anti-FX and anti-FXa Ab was performed as follows. Briefly, 96-well high-binding plates (Costar) were coated with 5 μg/ml of either human FX or human FXa (both from Hematologic Technologies) in TBS (0.05 M Tris-HCl and NaCl (pH 7.5)). After incubating overnight at 4°C, plates were blocked with TBS containing 0.5% gelatin. Purified IgG (mAb: 1 μg/ml; which was determined to be in the linear range of titration curves for mAb; IgG fraction: 6.25–100 μg/ml) in TBS/0.1% gelatin was distributed into wells in duplicate and incubated for 1.5 h at room temperature. A pooled normal human IgG (Jackson ImmunoResearch Laboratories), a monoclonal human IgG1 (Sigma-Aldrich), and a monoclonal human IgG3 (Calbiochem/EMD Bioscience) were used as negative controls. After washing with TBS, bound human IgG was detected with HRP-conjugated goat anti-human IgG (γ-chain specific; BioSource International), and peroxidase substrate tetramethylbenzidine (Ketjenblack, E. Merck-Perry Laboratories). Results were read at a wavelength of 450 nm against a background of 650 nm with a Thermomax plate reader (Molecular Devices).

Detection of Ab against FXa in plasma of APS patients and normal controls was done similarly, except that the test plasma samples were diluted at 1/50 in TBS/0.5% gelatin. The IS6 mAb, which was found to be reactive with FXa in our preliminary experiment, was used as a reference Ab, and the OD of each test sample was divided by the OD of IS6 in the same plate, resulting in a reference unit (RU) for the sample. Therefore, 1 RU is equivalent to 1 μg/ml IS6 IgG anti-FXa mAb.

Purification of IgG from plasma samples

IgG was purified from two chosen plasma samples positive for IgG anti-FXa Ab by HiTrap Protein G column (Pharmacia) according to the manufacturer’s instruction.

Functional assays for FXa activity and the inactivation of FXa by AT

The effects of FXa-reactive mAb on FXa activity were evaluated in a buffer containing 20 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂, 0.01% polyethylene glycol, and 0.5% Prowax (Centerchem) (pH 7.4) at 25°C in microtiter plates. Briefly, 25 μl of human FXa (5 nM) was separately incubated with 25 μl of a test mAb (200 μg/ml), a pooled normal human IgG, or an isotype control monoclonal IgG3 for 1 h at room temperature. Then, to each reaction mixture was added 100 μl of the FXa chromogenic substrate S-2765 (N-2-benzoyloxycarbonyl-o-arginyl-L-glutamyl-L-arginine-p-nitroanilide-dihydrochloride, 330 μM, DiaPharma). Generation of p-nitroaniline was monitored by measuring OD at 405 nm by iEMS kinetic microplate reader (Labsystems). The activity of FXa was determined as the rate of hydrolysis of S-2765 from the linear range of absorbance at 405 nm with time. The effects of FXa-reactive mAb on FXa inactivation by AT were studied in a functional assay for the FXa activity in the presence of AT and heparin, according to Wiebe et al. (30) with minor modifications. In particular, human AT (Enzyme Research Laboratories) was used at a concentration that was at least 10-fold higher than that of human FXa, and the experiments were performed in the aforementioned buffer for the FXa activity assay. The assay was initiated by incubating 25 μl of FXa (5 nM) separately with 25 μl of each test mAb (25–400 μg/ml), normal human polyclonal IgG, or the isotype monoclonal IgG3 in duplicate for 1 h at room temperature. Then, to each reaction mixture was added 50 μl of AT (25 nM) in the buffer containing heparin, resulting in a final heparin concentration of 0.05 U. S. Pharmacopeia unit/ml (U/ml) or the indicated concentrations. Subsequently, 50 μl of the chromogenic substrate S-2765 (660 μM) was added, and OD at 405 nm was measured over time. The final
concentrations of FXa, AT, and IgG were 1.25 nM, 12.5 nM, and 33.3 µg/ml (or the indicated concentrations), respectively. The FXa concentration was dictated by the need to determine the initial rate of FXa activity from the linear range of hydrolysis of the substrate with time. The percentage of FXa inactivation by AT was calculated as (1− (the residual FXa activity with AT)/(the initial FXa activity without AT)) × 100%.

The binding affinity of mAb to FX and FXa

A competitive inhibition assay was used to determine binding affinities of the selected mAb to FX and FXa as previously described (10). Briefly, each mAb (1−2 µg/ml) was preincubated for 1.5 h at room temperature with various concentrations of either FX or FXa in TBS/0.1% gelatin. The mixture was then distributed to FX- or FXa-coated wells in duplicate. After incubation, bound IgG was measured. The percentage of inhibition of each test mAb at each inhibitor concentration was calculated as: (OD of the test mAb alone− OD of test mAb with inhibitor)/OD of mAb alone × 100%. Then, the inhibition data of each mAb were used to calculate its relative $K_d$ toward FX and FXa.

Statistical analysis

The comparisons between APS patients and normal controls were conducted using the Mann-Whitney U test. Subsequently, the mean RU plus 3 SD of the 30 normal controls was used as the cutoff, and plasma samples with RU values higher than the cutoff were considered positive. Differences in the test Ab-induced inhibition of FXa activity or the FXa inactivation by AT were analyzed with paired ANOVA followed by the Bonferroni multiple comparison test. A two-tailed p value of <0.05 was considered statistically significant.

Results

All six thrombin-reactive monoclonal IgG aPL that bind to FXa and FX

To test our hypothesis that some of the thrombin-reactive aPL bind to FXa due to the high sequence and structure homology between thrombin and FXa, we examined five thrombin-reactive monoclonal aCL, one thrombin-reactive monoclonal aPT, and two non-thrombin-reactive monoclonal aCL for their reactivity with human FXa and FX. Fig. 1A shows that all six thrombin-reactive mAb (CL1, CL15, CL24, IS3, IS4, and IS6) bind to FXa, while two non-thrombin-reactive mAb (IS1 and IS2) do not bind to FXa. Among the six FXa-reactive mAb, CL1, CL15, and CL24 mAb were derived from a patient with SLE and secondary APS; IS3, IS4, and IS6 mAb were derived from a patient with primary APS. Subsequently, we analyzed these mAb for their reactivity with the zymogen FX. As can be seen in Fig. 1B, all six FXa-reactive mAb also bound to FX, although the binding pattern was not comparable.

Of note, the ODs of CL1 binding to both FXa and FX were low. Thus, the reactivity of CL1 with both FXa and FX might be questionable. In addition, because some reactive mAb were from an APS patient with SLE and some SLE patients are known to have Ab against gelatin, all mAb were analyzed for binding to wells that were not coated with Ags but were blocked with TBS/0.3% gelatin only. The results showed that bindings of all mAb to gelatin were negligible (data not shown).

Presence of IgG anti-FXa Ab in APS patients

Subsequently, to evaluate the clinical relevance of IgG anti-FXa Ab in APS, we searched for the presence of such Ab in 38 patients with primary or secondary APS and 30 normal controls. Because some SLE patients may have Ab that bind to gelatin, plasma samples were analyzed simultaneously for binding to wells that were coated with either FXa or buffer only, and then blocked with TBS/0.3% gelatin. Then, for each test sample, its IgG binding to gelatin-only wells (i.e., coated with buffer only) was subtracted from its IgG binding to FXa-coated wells, and the differential binding was expressed as IgG anti-FXa Ab. The results showed that the levels of plasma IgG anti-FXa in APS patients were significantly higher than those in normal controls (mean ± SD, 1.51 ± 1.27 RU vs 0.72 ± 0.52 RU, p < 0.001, Fig. 2A). When the mean plus 3 SD of normal controls was used as the cutoff, 5 of 38 APS patients (13.2%) were positive for IgG anti-FXa Ab (Fig. 2A). Of note, the patient with the highest IgG anti-FXa Ab had catastrophic APS. FXa contains the γ-carboxyglutamic acid domain that mediates binding to PL (31). Consequently, soluble PL in the test plasma samples may bind to this domain of FXa, thus allowing aPL to bind to FXa-associated PL and to be registered falsely as the Ab against FXa. To rule out this possibility, IgG was purified from two positive plasma samples and analyzed for binding to FXa. Fig. 2B shows that IgG from both patients bound to FXa in a concentration-dependent manner compared with normal human IgG at the same concentrations. Combined, these data demonstrate the presence of IgG anti-FXa Ab in some APS patients.

Functional properties of FXa-reactive mAb on FXa activity and FXa inactivation by AT

We first studied the effects of FXa-reactive mAb on FXa activity using a FXa chromogenic substrate (S-2765). As can be seen in Fig. 3, IS6 reduced FXa activity by 27%, whereas the remaining five mAb did not affect FXa activity.

Thereafter, we studied FXa-reactive mAb for their abilities to interfere with the FXa inactivation by AT in a functional assay, which contained 0.05 U/ml heparin and used AT at a concentration that was at least 10-fold higher than that of FXa. The addition of
heparin is to approximate the in vivo inactivation of FXa by AT, which often binds to heparin-like glycosaminoglycans (such as heparan sulfate) on the EC surface (23). The final concentrations of FXa, AT, and IgG were 1.25 nM, 12.5 nM, and 33.3 g/ml, respectively. Under these conditions, AT inactivated 87% of FXa activity (buffer alone), and the degrees of FXa inactivation by AT were not significantly changed by the presence of either polyclonal human IgG control or monoclonal human IgG3 isotype control (Fig. 4). In contrast, CL24 and CL15 reduced the degrees of FXa inactivation to 12 and 37%, respectively. IS6 showed a moderate inhibitory effect on AT inactivation of FXa, reducing the FXa inactivation to 52%. The remaining three FXa-reactive mAb did not affect the FXa inactivation by AT.

Because CL24 could reduce FXa inactivation from 87 to 12%, the resultant increase in FXa activity over time could present a significant procoagulant effect, as the residual FXa may continue to activate PT to thrombin at a constant rate. To visualize this cumulative effect over time, the amidolytic activity of FXa in the presence of AT, together with CL24, or polyclonal human IgG, or a monoclonal IgG3 isotype control was measured over a period of 5 min. As can be seen in Fig. 5A, accumulated substrate conversion in the presence of CL24 increased significantly and constantly over those in the presence of polyclonal human IgG or IgG3 mAb.

The above experiment was done with IgG and FXa at 33.3 µg/ml (222 nM) and 1.25 nM, respectively, resulting in a molar ratio of 178:1. Assuming that a total plasma IgG concentration is 10 mg/ml and that IgG anti-FXa Ab in an APS patient account for 1% of total IgG (32), the concentration of Ab used in this assay was ~30% of the possible total IgG anti-FXa Ab. Therefore, we studied the concentration-dependent effects of anti-FXa Ab on AT inactivation of FXa. The CL24, polyclonal human IgG, and an IgG3 isotype control were analyzed at a series of 2-fold lower concentrations (from 4.2 to 66.7 µg/ml). The results showed that at a lower concentration of 16.7 µg/ml (resulting in a molar IgG-FXa ratio of 89:1), CL24 reduced the degree of FXa inactivation...
to 56%. Importantly, CL24 at 66.7 μg/ml (resulting in a molar IgG-FXa ratio of 356:1) completely inhibited the anticoagulant function of AT on FXa (Fig. 5B).

Because the plasma concentration of AT is 2 μM (32), and the above experiments were performed with the final concentration of AT at 12.5 nM, we studied the effects of CL24 on FXa inactivation in the presence of AT from 6.25 to 100 nM, due to the prohibitory cost of AT. Fig. 5C shows that the CL24-mediated reduction in AT inactivation of FXa remained similar over the tested range of AT concentrations.

It is known that heparin greatly enhances AT inactivation of FXa. Therefore, we explored the possibility that high concentrations of heparin may neutralize the interfering FXa-reactive Ab. To this end, we evaluated the effects of CL24 over a range of heparin concentrations. The results showed that heparin at 0.2 U/ml completely abolished the procoagulant effect of CL24 in reducing FXa inactivation by AT (Fig. 5D).

The binding properties of three chosen mAb to FXa and FX

Based on functional characteristics of our six FXa-reactive mAb, two mAb (CL15, CL24) with the inhibitory effect on AT inactivation of FXa and one mAb (IS6) that inhibits both AT inactivation of FXa and FXa activity per se were chosen for analysis of their binding affinities to FXa and FX using a competitive inhibition assay. As shown in Fig. 6A, the binding affinity of CL24 to FXa was slightly higher than those of CL15 and IS6, whereas...
CL15 bound to FX with a slightly higher affinity than those of CL24 and IS6 (Fig. 6B). Based on the inhibition data, the relative $K_a$ values of these mAb to FXa were $2.2 \times 10^{-5}$, $1.3 \times 10^{-5}$, and $5.0 \times 10^{-5}$ M for CL15, CL24, and IS6, respectively. The relative $K_a$ values of CL15, CL24, and IS6 to FX were $1.0 \times 10^{-5}$, $1.2 \times 10^{-5}$, and $1.5 \times 10^{-5}$ M, respectively.

**Discussion**

In the present study, we showed that six of six patient-derived, thrombin-reactive mAb bound to both FXa Ab and FX (Fig. 1) and that the levels of plasma IgG anti-FXa in APS patients were significantly higher than those in normal controls ($p < 0.001$, Fig. 2A). Significantly, of the six FXa-reactive mAb, three (CL15, CL24, and IS6) significantly reduced the AT inactivation of FXa (Fig. 1); IS6, however, also inhibited FXa activity per se (Figs. 3 and 4). The effect of CL24 on AT inactivation of FXa was in a concentration-dependent manner and CL24 at 66.7 μg/ml could completely block AT inactivation of FXa (Fig. 5). Considering that the average human plasma IgG concentration is 10 mg/ml (32), the observed inhibition of AT inactivation of FXa by CL24 is likely to be physiologically relevant. In this context, it is important to note that the CL24-mediated dysregulation of the FXa inactivation by AT leads to rapid increase in the cumulative FXa activity over time (Fig. 5). In contrast, in vivo coagulation is influenced by many confounding factors in plasma, such as inflammatory conditions and shear stress. Combined, these data suggest that some anti-FXa Ab in APS patients (like CL24) could interfere with the AT activation of FXa and that the dysregulated FXa activity may contribute toward thrombus formation.

In 1994, it was reported the presence of an autoantibody to FX with lupus anticoagulant activity in a patient with high titer of aCL and a hemorrhagic diathesis (33). Here, when we analyzed plasma samples from 38 APS patients and 30 normal controls for IgG anti-FXa Ab and use the mean plus 3 SD of normal controls as the cutoff, 13.2% (5 of 38) of patients were positive for IgG anti-FXa Ab (Fig. 2). Intriguingly, the highest plasma level of IgG anti-FXa Ab was from an APS patient suffering from rapid and disseminated microthrombosis (catastrophic APS).

During AT inactivation of a target protease, the protruded reactive center loop (RCL) of AT interacts with the active site of the target protease. Subsequently, when the protease cleaves the scissile bond (P1–P1') in the RCL of AT, the protease is covalently linked to P1 of AT and is irreversibly inactivated by AT (34). In this context, the extensive contact between AT and a target protease during the AT inactivation of the protease may make the process sensitive to Ab that bind to or around the active sites of the target proteases. This contention is supported by the present finding on interference of AT inactivation of FXa by the FXa-reactive CL24 mAb, as well as the previous data on inhibition of AT activation of thrombin by the same mAb that also bind to thrombin.

The procoagulant effect of the FXa-reactive CL24 mAb on the AT inactivation of FXa could be decreased and even completely abolished by increasing concentrations of heparin. The similar concentration-dependent neutralizing effect of heparin was also observed previously in the inhibition of AT inactivation of thrombin by CL24 (10). Binding of this pentasaccharide to AT induces a conformational change in the RCL of AT that facilitates its reaction with thrombin and FXa, thereby enhancing the rate of AT inactivation of thrombin and FXa by about two orders of magnitude (35–37). In addition, during the in vitro heparin-dependent AT inactivation of FXa, heparin binds simultaneously to FXa and AT in the presence of Ca$^{2+}$, and thus bridges FXa and AT to form a ternary heparin-AT-FXa complex, leading to an additional ~10-fold increase in the rate of inhibition of FXa (30, 38).

Binding affinities of the above two FXa-reactive, AT-interfering mAb (Fig. 6) to FXa were not significantly different from those of the noninterfering ones (data not shown). The relative $K_a$ values of all FXa-reactive mAb were in the range of $10^{-5}$ M. These data suggest that binding affinities of different FXa-reactive mAb are unlikely to account for their differences on AT inactivation of FXa.

Consequently, it would be fruitless to assess the clinical significance of all anti-FXa Ab in APS patients by association studies of anti-FXa Ab to APS. Instead, it will be necessary to first delineate the FXa epitopes recognized by anti-FXa Ab with different functional activities, such as CL24 (which inhibits AT inactivation of FXa), IS6 (which inhibits FXa activity per se and inhibits AT inactivation of FXa), and CL1 (which neither inhibits FXa activity nor inhibits AT inactivation of FXa). Once the specific epitopes for different anti-FXa Ab are defined, specific assays for each type of anti-FXa Ab may be developed and applied to study functionally distinct anti-FXa Ab in patients.

To elucidate the immunopathogenesis of APS, we have analyzed one mAb against PT and seven mAb against cardiolipin in the presence of bovine serum (28, 29). The accumulated data reveal that six of eight mAb bound to several serine proteases involved in hemostasis including thrombin (10), activated protein C (11), plasmin (13), and tPA (14), and that some of these mAb interfere with different anticoagulant functions and/or fibrinolytic process (Table 1) (10, 11, 13, 14). Importantly, our previous study showed that CL24 could inhibit AT inactivation of thrombin (10). Taken together with the present finding of CL24-mediated interference of FXa inactivation by AT, our data indicate that a single aCL may bind to two different target serine proteases and promote thrombosis at the two sequential steps in the coagulation cascade, which may result in a synergistic, procoagulant effect.

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**Disclosures**

The authors have no financial conflict of interest.

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