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Novel Autoantibodies against the Activated Coagulation Factor IX (FIXa) in the Antiphospholipid Syndrome That Interpose the FIXa Regulation by Antithrombin

Yao-Hsu Yang,† Daniel Chien,‡ Meifang Wu,§ John Fitzgerald,* Jennifer M. Grossman,* Bevra H. Hahn,* Kwan-Ki Hwang,* and Pojen P. Chen**

We previously reported that some human antiphospholipid Abs (aPL) in patients with the antiphospholipid syndrome (APS) bind to the homologous enzymatic domains of thrombin and the activated coagulation factor X (FXa). Moreover, some of the reactive Abs are prothrombotic and interfere with inactivation of thrombin and FXa by antithrombin (AT). Considering the enzymatic domain of activated coagulation factor IX (FIXa) is homologous to those of thrombin and FXa, we hypothesized that some aPLs in APS bind to FIXa and hinder AT inactivation of FIXa. To test this hypothesis, we searched for IgG anti-FIXa Abs in APS patients. Once the concerned Abs were found, we studied the effects of the Ab on FIXa inactivation by AT. We found that 10 of 12 patient-derived monoclonal IgG aPLs bound to FIXa and that IgG anti-FIXa Abs in APS patients were significantly higher than those in normal controls ($p < 0.0001$). Using the mean + 3 SD of 30 normal controls as the cutoff, the IgG anti-FIXa Abs were present in 11 of 38 (28.9%) APS patients. Importantly, 4 of 10 FIXa-reactive monoclonal aPLs (including the B2 mAb generated against $\beta_2$-glycoprotein I) significantly hindered AT inactivation of FIXa. More importantly, IgG from two positive plasma samples were found to interfere with AT inactivation of FIXa. In conclusion, IgG anti-FIXa Ab occurred in ~30% of APS patients and could interfere with AT inactivation of FIXa. Because FIXa is an upstream procoagulant factor, impaired AT regulation of FIXa might contribute more toward thrombosis than the dysregulation of the downstream FXas and thrombin. * The Journal of Immunology, 2009, 182: 1674–1680.

Patients with antiphospholipid syndrome (APS) are characterized by the clinical manifestations of thrombosis and/or fetal loss and the presence of heterogeneous antiphospholipid Abs (aPL; Refs. 1–4). The hallmark aPLs recognize a variety of Abs including various phospholipids (PL), PL-binding proteins, and protein-PL complexes, as well as other factors related to hemostasis (4–7). The involved proteins include $\beta_2$-glycoprotein I ($\beta_2$-GPI), prothrombin (PT), protein C (PC), protein S, annexin V, thrombomodulin, thrombin, tissue factor pathway inhibitor, activated PC, endothelial PC/activated PC receptor, plasmin, tissue plasminogen activator, annexin 2, and the activated coagulation factor X (FXa) (5–17). Functional studies of aPLs have shown that different aPLs may promote thrombosis through different pathways (4, 18, 19). For example, anti-$\beta_2$GPI Ab could enhance the expression of tissue factor (TF) on the surface of endothelial cells (EC) and monocytes (19–22), whereas anti-activated PC Ab could inhibit the anticoagulant activity of activated PC (12, 23). Additionally, aPL could activate the complement pathway, leading to inflammatory-mediated fetal loss and thrombosis (24, 25). To date, although our understanding of aPLs and their prothrombotic mechanisms has advanced, the pathogenesis of APS remains unclear.

The coagulation cascade is initiated by expression of TF upon vascular injury. TF binds and accelerates the activation of factor VII, and the activated factor VII form complexes with TF (designated TF-activated factor VII), which, in turn, activate factor IX and, to a lesser degree, factor X (generating factors FIXa and FXa, respectively). Subsequently, FIXa works with activated factor VIII to generate more FXa, and FXa with activated factor V to convert PT to thrombin, which then converts fibrinogen to a fibrin clot (26, 27). Patients with congenital FIX deficiency exhibit the life-threatening hemophilia B (28). Conversely, high FIX levels are associated with increased risk of venous and arterial thromboembolism (29). These findings indicate that FIXa is a major procoagulant enzyme in the coagulation cascade.

Therefore, FIXa is tightly regulated by antithrombin (AT) in normal hemostasis. 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, such as heparan sulfate on the surface of vascular ECs (30). 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 (26, 31). Taken together, it is conceivable that interference of AT inactivation of FIXa may promote thrombosis.

We previously reported that some Abs derived from APS patients bind to the homologous enzymatic domains of several serine

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3 Abbreviations used in this paper: aCL, anticardiolipin Ab; aPL, antiphospholipid Ab; PC, protein C; APS, antiphospholipid syndrome; AT, antithrombin; $\beta_2$GPI, $\beta_2$-glycoprotein I; EC, endothelial cell; FIX, coagulation factor IX; FXa, activated coagulation factor IX; PL, phospholipid; PT, prothrombin; RU, reference unit; SLE, systemic lupus erythematosus; SP, serine proteases; TF, tissue factor; TBS, 0.05 M Tris-HCl, NaCl (pH 7.5).

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FIGURE 1. Detection of the FIXa-reactive monoclonal aPL and the IgG anti-FIXa Ab in APS. For binding of patient-derived IgG monoclonal aPL to FIXa (A) and FIX (B), the test mAb, polyclonal human IgG, or the monoclonal isotype controls (IgG1 or IgG3) were analyzed at 1 μg/ml. IS1, IS2, B1, T1, and P1 are IgG1, and the other mAb is IgG3. C, Plasma samples from 38 APS patients and 30 normal controls were analyzed at 1/50 dilution for IgG anti-FIXa Ab. The IS6 mAb was used in each plate as the reference Ab, and the OD of each test sample was divided by that of the IS6 in the same plate, resulting in a RU for the sample. ---, mean value for each sample group; –——, cutoff (mean RU + 3 SD of 30 normal controls). *, p < 0.0001. D, Purified IgGs from three patient plasma samples (P7, P57, and P64) and control IgG were analyzed at the indicated concentrations for binding to FIXa. Values are expressed as means and SEM (n = 2).

proteases (SP) involved in coagulation, such as thrombin, activated PC, plasmin, tissue plasminogen activator, and FXa (10, 12, 14, 15, 17). Moreover, some of these autoantibodies are prothrombotic and interfere with different anticoagulation pathways and/or fibrinolytic process (10, 12, 14, 15, 17, 32). FIXa also belongs to the SP family and its enzymatic domain is homologous to those of thrombin and FXa (33). Specially, at the protein level, the catalytic domains of FIXa and thrombin share a similarity of 52.7% and the catalytic domains of FIXa and FXa share a similarity of 53.6%.

We thus hypothesized that some aPLs in APS bind to FIXa and interpose AT inactivation of FIXa. Here, we report the reactivity of some patient-derived IgG monoclonal aPL with FIXa and the detection of IgG anti-FIXa Ab in plasma samples from 11 of 38 (28.9%) APS patients. Four FIXa-reactive monoclonal aPLs (CL15, CL24, IS6, and B2) and purified IgG from two anti-FIXa plasma samples (P7 and P46) interpose FIXa inactivation by AT.

Materials and Methods

Patient-derived IgG monoclonal aPLs

Twelve IgG monoclonal aPLs derived from four APS patients were analyzed in this study. These included: seven anticardiolipin Abs (aCL), CL1, CL15, CL24, IS1, IS2, IS3, and IS4; one anti-PT Ab, IS6; two anti-β2-GPI Abs, B1 and B2; one anti-thrombin Ab, T1; and one anti-PC Ab, P1 (34–36). The generation and characterization of these mAbs have been described previously. IS1, IS2, B1, T1, and P1 are IgG1, and the other 7 mAbs are IgG3.

Patients and healthy controls

The studies had been reviewed and approved by the Institutional Review Board at the University of California, Los Angeles. Plasma samples were obtained from 38 APS patients (10 men and 28 women) and 30 healthy subjects (12 men and 18 women) at the University of California Medical Centers (Los Angeles, CA, and San Diego, CA). All patients satisfied the Sapporo classification criteria for definite APS (37). Medical charts and laboratory test reports for each patient enrolled in this study were reviewed by a rheumatologist (J.M.G.). 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. Of the 38 APS patients, 31 (82%) were positive for aCL, 16 (42%) were positive for anti-β2-GPI Ab, and 26 (68%) were positive for lupus anticoagulants. In addition, 15 (39%) were primary APS and 23 (61%) secondary APS; the latter group included 19 patients with systemic lupus erythematosus (SLE), 1 with SLE-like disease, and 3 with autoimmune thyroiditis. All patients suffered thrombosis; 5 had both arterial and venous thrombosis, 15 had only arterial thrombosis, and 18 had only venous thrombosis. Of the 28 female patients, 3 had fetal losses.

ELISA for Ab against FIXa and FIX

The ELISA for anti-FIXa and anti-FIX Ab was performed as follows. Briefly, 96-well high-binding plates (Costar) were coated with either human FIXa or FIX (both from Hematologic Technologies) at a concentration of 5 μg/ml in 0.05 M Tris-HCl, NaCl (pH 7.5; TBS). After incubation overnight at 4°C, plates were blocked with TBS containing 0.3% gelatin. Then, mAb (1 μg/ml, which was determined to be in the linear range of titration curves) in TBS containing 0.1% gelatin were 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 IgG (Sigma-Aldrich), and a monoclonal human IgG3 (Calbiochem/EMD Biosciences) were used as negative controls at the same concentration of 1 μg/ml. After a washing with TBS, bound human IgG was detected with HRP-conjugated goat anti-human IgG (γ-chain specific; BioSource International), and the peroxidase substrate tetramethylbenzidine (Kirkegaard & 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 anti-FIXa Ab in plasma or purified IgG of APS patients and normal controls was performed similarly, except that the test plasma samples were diluted at 1/50 in TBS containing 0.3% gelatin and the purified IgG were tested at a concentration of 25 μg/ml (or indicated concentrations). Both IS6 and CL1 were found to be reactive with FIXa in our preliminary experiment. IS6 was used at 1 μg/ml in each ELISA plate to serve as a reference Ab in the plasma assays, whereas CL1 was used at 1 μg/ml in the purified IgG assays. To standardize ELISA results from different assays at different times, the OD of each test sample was divided by the OD of IS6 or CL1 on the same plate and expressed in
reference units (RU). Therefore, 1 RU is equivalent to 1 µg/ml IS6 or CL1 IgG anti-FIXa mAb.

**Purification of IgG from plasma samples**

Polyclonal IgG was purified from three chosen patients’ plasma samples, which were positive for IgG anti-FIXa Ab, using HiTrap Protein G columns (Pharmacia) according to the manufacturer’s instructions.

**Functional assays for FIXa activity and the inactivation of FIXa by AT**

The effects of FIXa-reactive Ab on FIXa activity were evaluated in a buffer (pH 7.4) containing 20 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂, 30% ethylene glycol, 0.01% polyethylene glycol, and 0.5% Prionex (Centrachel) at 25°C in 96-well microtiter plates. Briefly, 20 µl of human FIXa (20–160 nM) were separately incubated with 20 µl of a test mAb (200 µg/ml), purified IgG (1.8 mg/ml), a pooled normal human IgG, or an isotype control monoclonal IgG for 1 h at room temperature. Then, to each reaction mixture were added 80 µl of the FIXa chromogenic substrate Pefa-3107 (CH₂SO₂-(D)-CHG-Gly-Arg-pNA-AcOH, 1.5 mM; Pentapharm). Generation of p-nitroaniline was monitored by measuring OD₄₀₅ over time with an iEMS kinetic microplate reader (Labsystems). The activity of FIXa was determined as the rate of hydrolysis of Pefa-3107 in the linear range of OD₄₀₅.

The effects of FIXa-reactive Ab on FIXa inactivation by AT were studied in a functional assay for the FIXa activity in the presence of AT and heparin, according to the report of Wiebe et al. (38) 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 FIXa, and the experiments were performed in the above buffer for the FIXa activity assay. The assay was initiated by incubating 20 µl of FIXa (20–160 nM) separately with 20 µl of each test mAb (18.75–300 µg/ml), purified IgG (1.8 mg/ml), normal human polyclonal IgG, or the isotype monoclonal IgG3 for 1 h at room temperature. Then, to each reaction mixture were added 40 µl of AT (100–800 nM) in the buffer containing heparin, resulting in a final heparin concentration of 0.025 USP U/ml or the indicated concentrations. Subsequently, 40 µl of the chromogenic substrate Pefa-3107 (3 mM) were added, and OD₄₀₅ was measured over time. The FIXa concentration was dictated by the need to determine the initial rate of FIXa activity from the linear range of hydrolysis of the substrate with time. The percentage of FIXa inactivation by AT was calculated as [1 − (the residual FIXa activity with AT)/(the initial FIXa activity without AT)] × 100.

The binding properties of Ab to FIXa

The binding affinities of the selected mAb and purified IgG to FIXa were determined using a competitive inhibition assay as previously described (30). Briefly, each mAb (1–2 µg/ml) or purified IgG (12.5–25 µg/ml) was preincubated with various concentrations of FIXa for 1.5 h at room temperature. The above concentrations of mAb or purified IgG were in the linear range of their titration curves. The mixture was then distributed to wells that were coated with either FIXa or buffer only and preincubated with various concentrations of FIXa for 1.5 h at room temperature.

**Statistical analysis**

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

**Results**

**Detection of the FIXa-reactive monoclonal IgG aPL and the IgG anti-FIXa Ab in some APS patients**

To test our hypothesis that some aPLs from APS patients may bind to FIXa, we first analyzed 12 patient-derived IgG monoclonal aPLs for their reactivity with human FIXa. The aPLs include 7 aCL (CL1, CL15, CL24, IS1, IS2, IS3, and IS4), 1 anti-PT mAb, 2 anti-β₂GPI mAb (B1 and B2), 1 antithrombin mAb (T1), and 1 anti-PC mAb (P1). These 12 monoclonal IgG aPLs were generated from 4 APS patients, including 2 secondary APS patients with primary SLE. Fig. 1A shows that 10 aPLs bound to FIXa. Of these 10 FIXa-reactive mAbs, B1 and T1 displayed only weak binding to FIXa (Fig. 1A). We also analyzed these mAb for their reactivity with the zymogen FIX. As can be seen in Fig. 1B, all FIXa-reactive mAbs also bound to FIX. However, the binding pattern was not comparable. For example, B2 bound well to FIXa, but only weakly to FIX (Fig. 1B).

Subsequently, we searched for the IgG anti-FIXa Ab in APS patients. Because SLE patients are well known to have all kinds of autoantibodies, it is conceivable that some SLE patients may have Abs that bind to ELISA plates and/or gelatin in the blocking buffer. Therefore, we simultaneously analyzed plasma samples for binding to wells that were coated with either FIXa or buffer only and then blocked with TBS containing 0.3% gelatin. Then, for each test sample, its IgG binding to the buffer-only wells was subtracted from that to the FIXa-coated wells, and the differential binding was considered to be the IgG anti-FIXa Ab. The results showed that IgG anti-FIXa Ab in APS patients were significantly higher than those in normal controls (mean ± SD, 0.27 ± 0.17 RU vs 0.11 ± 0.08 RU, p < 0.0001; Fig. 1C). Using the mean RU plus 3 SD of normal controls as the cutoff, IgG anti-FIXa Ab were found in 11 of the 38 (28.9%) APS patients.
FIXa contains the γ-carboxyglutamic acid domain that mediates binding to PL (39). Therefore, a concern was raised that the observed IgG anti-FIXa Ab might reflect IgG aPLs that bound to PLs, which bound to FIXa. To address this possibility, IgG was purified from three positive plasma samples (identified as P7, P57, and P64) and analyzed for their binding to FIXa. Fig. 1D shows that IgG from these three patients bound strongly to FIXa in a concentration-dependent manner. Together, these findings indicate the presence of IgG anti-FIXa Ab in some APS patients.

Functional properties of the FIXa-reactive monoclonal aPL on FIXa activity and FIXa inactivation by ATs

To determine the pathogenic significance of the newly found IgG anti-FIXa Ab in APS, we first studied the effects of eight FIXa-reactive monoclonal aPLs on FIXa activity using a FIXa chromogenic substrate (Pefa-3107). As shown in Fig. 2A, none of these mAb affected FIXa activity.

Thereafter, we studied the FIXa-reactive monoclonal aPLs for their abilities to interpose FIXa inactivation by AT. In this functional assay, the final concentrations of FIXa, AT, heparin, and IgG were 40 nM, 400 nM, 0.025 U/ml, and 33.3 g/ml (222 nM), respectively. The addition of heparin was to approximate the in vivo inactivation of FIXa by AT, which binds to heparin-like glycosaminoglycans (such as heparan sulfate) on the EC surface (30). Under these conditions, AT inactivated 94.7% of FIXa activity (buffer alone; Fig. 2B, far right bar). Similar degrees of FIXa inactivation by AT were observed in the presence of normal human IgG, a monoclonal human IgG1, or a monoclonal human IgG3 (Fig. 2B). In contrast, the presence of mAbs B2, CL15, and CL24 reduced AT inactivation of FIXa to 5.5, 18.9, and 29.1%, respectively (Fig. 2B). IS6 showed a small but significant inhibitory effect, reducing FIXa inactivation to 81.8%.

Once AT inactivation of FIXa was inhibited by some FIXa-reactive mAb, the resultant increase in FIXa activity over time could result in a significant procoagulant effect. To visualize this cumulative effect over time, we measured the amidolytic activity of FIXa in the presence of AT and IgG over a period of 10 min. Compared with the IgG3 control, Fig. 3A shows that the Ab-mediated hindrance of FIXa inactivation (by AT) results in a substantial increase in accumulated FIXa activity.

To further determine the pathological significance of aPL-mediated hindrance of FIXa inactivation by AT, we analyzed two chosen mAb (plus an IgG3 isotype control) at a series of 2-fold lower concentrations (from 3.1 g/ml to 50 g/ml) in the presence of FIXa at 40 nM (resulting in a molar IgG-FIXa ratio from 0.5:1 to 8.3:1). The results showed that at 50 g/ml, both CL15 and CL24 reduced the degrees of FIXa inactivation to 9.3 and 14.7%, respectively. Importantly, at 12.5 g/ml, both mAb still showed a small but significant inhibitory effect on AT inactivation of FIXa, reducing the FIXa inactivation to 68.5 and 72.7%, respectively (Fig. 3B). Based on the normal plasma IgG concentration of 10 mg/ml, 12.5 g/ml is equivalent to 0.13% of plasma IgG.

Heparin greatly enhances the AT inactivation of the target SP (40). Therefore, we studied the effects of CL15 and CL24 on AT inactivation of FIXa in the presence of various concentrations of heparin. The results showed that heparin at 0.2 U/ml completely neutralized the procoagulant effect of CL15 and CL24 in reducing FIXa inactivation by AT (Fig. 3C).

**Functional properties of IgG anti-FIXa Ab from APS patients**

To generalize the pathological significance of the IgG anti-FIXa Ab in hindering FIXa inactivation by AT, we investigated the functional properties of the purified IgG from APS patients. The functional assays were done similarly as the above experiments,
except that IgG, FIXa, and AT were used at final concentrations of 300 μg/ml, 5 nM, and 50 nM. This was based on the assumption that ~1% of total IgG is specific for FIXa, resulting in ~3 μg/ml (20 nM), and a desired molar Ab-FIXa ratio of 4, which led to ~50% inhibition of FIXa inactivation by AT (Fig. 3B).

For the effects of IgG anti-FIXa Ab on FIXa activity, Fig. 4A shows that FIXa activity is significantly reduced by P64, but not by P7 and P57. Thereafter, we studied the IgG anti-FIXa Ab for their abilities to interpose FIXa inactivation by AT. In the absence of test Ab, AT and heparin inactivated 84.2% of FIXa activity. When FIXa was first incubated with a test Ab, P7 and P64 reduced FIXa inactivation to 55.2 and 40%, respectively (Fig. 4B); P57 had no effect on FIXa inactivation by AT. Importantly, although P7-reduced FIXa inactivation only from 84.2% to 55.2%, the unchecked FIXa activity over time led to substantially increased substrate conversion (Fig. 4C), symbolizing substantially increased FXa generation and a heightened procoagulant state in the host patient of the P7 IgG anti-FIXa Ab whenever the coagulation cascade is activated.

The binding affinities of FIXa-reactive Ab to FIXa

Based on the above data, four likely procoagulant FIXa-reactive monoclonal aPLs (CL15, CL24, IS6, and B2) and all three purified IgG samples (positive for anti-FIXa Ab) were analyzed for their binding affinities to FIXa by a competitive inhibition assay. The results showed that soluble FIXa could inhibit all mAbs (Fig. 5A) and purified IgG (Fig. 5B) from binding to FIXa. Based on these inhibition data, the relative $K_d$ values of these FIXa-reactive Ab were $2.2 \times 10^{-6}, 4.9 \times 10^{-6}, 7.8 \times 10^{-7}, 1.3 \times 10^{-6}, 2.3 \times 10^{-6}, 1.1 \times 10^{-6}$, and $1.0 \times 10^{-6}$ M for CL15, CL24, IS6, B2, P7, P57, and P64, respectively.

FIGURE 5. Binding properties of FIXa-reactive Ab to FIXa. A. Competitive inhibition of the binding of patient-derived mAb to FIXa. B. Competitive inhibition of the binding of polyclonal IgG to FIXa. Each Ab was preincubated with indicated concentrations of FIXa in TBS containing 0.1% gelatin for 1.5 h; then, the mixtures were analyzed for binding to FIXa by ELISA. Results are expressed as the percentage of inhibition. Values are the means and SEM ($n = 2$).
Discussion
To test the hypothesis that some APS patients may have aPLs against FIXa that interpose FIXa inactivation by AT, we first found that 10 of 12 patient-derived IgG monoclonal aPLs bound to FIXa (Fig. 1A). Significantly, of the 10 FIXa-reactive mAb, 4 (CL15, CL24, IS6, and B2) significantly interposed AT inactivation of FIXa (Fig. 2B). The effects of CL15 and CL24 on AT inactivation of FIXa were concentration dependent (Fig. 3B). Moreover, the CL15- and CL24-mediated dysregulation of the FIXa inactivation by AT led to substantial increase in the cumulative FIXa activity over time (Fig. 3A), which would be equivalent to heightened FXa generation and might contribute toward thrombus formation in the host patient.

In the process of mAb generation, EBV was first used to transform B cells. Because the virus also activates B cells (41), it is possible that some or all FIXa-reactive hybridomas were generated from the resting B cells, which normally do not produce anti-FIXa Ab in patients. Therefore, to determine the pathological relevance of the above findings, we analyzed the plasma samples and purified IgG from APS patients for IgG anti-FIXa Ab. The results showed that plasma levels of IgG anti-FIXa Ab were significantly higher in APS patients than those in normal controls (Fig. 1D), indicating that the observed plasma anti-FIXa Ab were due to direct binding of the IgG anti-FIXa Ab to FIXa, but not indirect binding of aPL to PL that first bound to the ϒ-carboxyglutamatic acid domain of FIXa on ELISA plates. Furthermore, the binding affinities of FIXa-reactive mAb and polyclonal IgG anti-FIXa Ab were similar (Fig. 5), with relative \( K_d \) values in the ranges of \( \sim 10^{-6} \) M. Combined, these data clearly demonstrate the presence of authentic IgG anti-FIXa Ab in some APS patients.

Among the likely procoagulant anti-FIXa Ab, the effective concentrations of mAb and IgG were 33.3 and 300 \( \mu \)g/ml, respectively. Considering that the human plasma concentration of IgG is 10 mg/ml (42), the observed Ab-mediated dysregulation of AT inactivation of FIXa is physiologically relevant. Moreover, the unchecked FIXa led to a substantially increased FXa generation over time (Figs. 3A and 4C), resulting in a heightened procoagulant state.

It is interesting to note that the most potent aPL in hindering AT inactivation of FIXa is the B2 mAb, which was generated from a primary APS patient by screening against \( \beta_2 \)GPI (36). Moreover, 9 of 10 FIXa-reactive monoclonal aPLs bind to \( \beta_2 \)GPI (Table I).

During the last decade, intensive studies of anti-\( \beta_2 \)GPI Ab in APS patients showed that the major prothrombotic mechanism of these Abs was by binding to and activating EC, monocytes, and platelets (19–22). The present data of the B2-mediated dysregulation of AT inactivation of FIXa reveals an equally important mechanism by which anti-\( \beta_2 \)GPI Abs promote thrombosis in APS patients. Moreover, this new mechanism acts at a different point in the hemostasis from the above major mechanisms of anti-\( \beta_2 \)GPI Ab and thus provides an additional insight for the potent prothrombotic property of the anti-\( \beta_2 \)GPI Ab in APS patients.

Of the 11 APS patients (including 8 women) positive for IgG anti-FIXa Ab, 7 (64%) had venous thrombosis, and 5 (46%) had arterial thrombosis (including 1, who also had venous thrombosis), and 1 (9%) who had fatal loss. It had been reported that congenital, heterozygous deficiency in AT increases the risk of venous thrombosis about 5- to 10-fold (26, 43, 44). Interestingly, patient 7 (whose IgG significantly interferes with AT inactivation of FIXa in Fig. 4B) had suffered venous thrombosis.

Anti-FIX/FIXa Ab have been found in some hemophilia B patients receiving FIX infusion (28). However, such anti-FIX/FIXa Ab generally inhibit FIX/FIXa, resulting in a serious complication for infusion of FIX (28). In contrast to the anti-FIX/FIXa Ab in some hemophilia B patients, there appear to at least three different kinds of IgG anti-FIXa Ab in APS. One interposes FIXa inactivation by AT (like the B2 mAb and the P7 IgG), another inhibits both FIXa per se and FIXa inactivation by AT (like the P64 IgG), and the third affects neither FIXa per se nor FIXa inactivation by AT (like the IS3 mAb and the P57 IgG). Therefore, although 28.9% of APS patients had IgG anti-FIXa Ab, it would be problematic to ascertain its clinical significance through the association study of the presence of total IgG anti-FIXa Ab to APS. To study the roles of procoagulant anti-FIXa Ab in APS patients, it will be necessary to first identify the differential epitopes that are only recognized by the procoagulant IgG anti-FIXa Ab (like the B2 mAb and the P7 IgG), and then develop the more specific assay for the procoagulant IgG anti-FIXa Ab in APS patients.

As noted in Introduction, we previously reported that some aPLs bound to thrombin and FXa and hindered their inactivation by AT (Table I). Taken together with the present findings that some aPLs also interpose AT inactivation of FIXa, some aPLs could interfere with AT inactivation of all three procoagulant factors. Therefore, it is conceivable that such Abs might result in a synergistically procoagulant effect. Moreover, considering that the sequential activation of these procoagulant factors leads to an amplification at...
each step, the aPL-mediated dysregulation of the most upstream FIXα is likely to be more prothrombotic than the aPL-mediated dysregulation of the downstream thrombin. Furthermore, the FIXα-reactive aPLs bind to FIXα with higher affinities than the binding of the similar aPL to thrombin and FXα (Table I). This might reflect more affinity maturation for anti-FIXα Ab due to the lower plasma concentration of FIX (~89 nM) than those of PT and factor X (~1.4 μM and 136 nM, respectively). Viewed as a whole, the FIXα-reactive aPLs are likely to be the most important aPLs for dysregulation of the downstream thrombin. Furthermore, the FIXα-reactive aPLs bind to FIXα with higher affinities than the antiphospholipid syndrome that interfere with the FIXα inactivation by antithrombin. J. Immunol. 177: 8219–8225.


