The effect of platelet activation on the hypercoagulability induced by murine monoclonal antiphospholipid antibodies

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ABSTRACT

Background

To identify the mechanisms of the hypercoagulability associated with antiphospholipid antibodies, we investigated antibody-mediated platelet activation and interference of antibodies with phospholipid-dependent reactions.

Design and Methods

We used two murine monoclonal antibodies, one against β_2-glycoprotein I (7F6G), the other against prothrombin (28F4). Platelet activation was assessed by phospholipid-related platelet procoagulant activity. Endogenous thrombin potential without activated protein C (ETP_0) and the activated protein C concentration that reduced the ETP_0 by 50% (IC_{50}-APC) were determined by calibrated automated thrombography.

Results

Both monoclonal antibodies mimicked the effect of IgG in 11 out of a series of 40 patients with antiphospholipid antibodies in thrombography. In the presence of their target, 7F6G and 28F4 at 200 μg/mL exhibited comparatively low and high binding to platelets and elicited low and high levels of procoagulant phospholipids on platelet surface, respectively. In platelet-poor plasma, these antibodies induced a 1.6 and >12-fold increase in IC_{50}-APC, respectively, thus providing evidence for a procoagulant effect independent of platelet activation. The 84% decrease in ETP_0 indicated that 28F4 also displayed an anticoagulant effect. In platelet-rich plasma, this anticoagulant effect was significantly less (23% decrease in ETP_0), demonstrating that a high increase in procoagulant surfaces by platelet activation significantly antagonizes the anticoagulant effect of antiphospholipid antibodies. In both types of plasma, the inhibition of thrombin generation (reduced ETP_0) was less than the inhibition of activated protein C activity (increased IC_{50}-APC).

Conclusions

Our findings show that platelet activation reinforces the hypercoagulability induced by competition between antiphospholipid antibodies/target complexes and pro- and anticoagulant complexes for phospholipid surfaces.

Key words: antiphospholipid antibodies, thrombin generation, activated protein C resistance, platelet activation, murine monoclonal antibodies.

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The online version of this article contains a supplemental appendix.

Introduction

Antiphospholipid antibodies (aPL) represent a heterogeneous group of auto-antibodies directed mainly against β2-glycoprotein I (β2GPI) and prothrombin (factor II)[1,2] and are associated with thrombosis and/or pregnancy morbidity.[3] It has been established that the strongest risk factor for thrombosis is lupus anticoagulant (LA)[4,5] with either β2GPI and/or prothrombin binding antibodies.[6,7] Two mechanisms, not mutually exclusive, have been identified to account for the thrombotic tendency associated with aPL: (i) cellular activation and (ii) interference with the activated protein C (APC) system.[1,2] While some evidence exists that aPL may trigger platelet aggregation and secretion,[8,9] opposite results have been reported regarding the aminophospholipid exposure on membranes.[10,11] Interference of aPL with the APC system has been investigated using traditional clotting assays.[12] Experiments in reconstituted systems have demonstrated that aPL inhibit the inactivation of activated factor V by APC.[13,14] Arguments have been made in favor of selective inhibition of APC activity.[15] Using thrombography, we demonstrated that APC resistance of thrombin generation is partially offset by an anticoagulant effect in aPL patients. The net result of these opposite effects is hypercoagulability associated with an increased risk of thrombosis.[16]

The mechanisms leading to interference of aPL in the presence of their target (aPL/target complexes) with pro- and anticoagulant pathways remain to be elucidated. One limitation is the heterogeneity among aPL auto-antibodies and the co-existence of several subsets of auto-antibodies in the same patient. We, therefore, used murine monoclonal antibodies directed to β2GPI and prothrombin. First, we examined whether these antibodies elicit exposure of aminophospholipid at the platelet surface. Second, we determined whether interference of aPL/target complexes with pro- and anticoagulant reactions on phospholipid surfaces may account for the hypercoagulability associated with aPL independently of platelet activation. Finally we examined the interplay between platelet activation and interference with pro- and anticoagulant reactions on the platelet surface.

Design and Methods

More details on the design and methods are given in the *Online Supplementary Section.*

Antibodies

Murine monoclonal antibodies (IgG_1) were a generous gift from S. Krilis (anti-β2GPI directed against domain I: 7F6G) and J. Arnout (anti-prothrombin: 28F4). Polyclonal control murine IgG (Calbiochem, San Diego, USA) was used as a negative control and the murine monoclonal antibody ALB6 (Immunotech, Marseille, France) as a platelet-activating antibody. All antibodies were dialysed against 20 mM Hepes, 140 mM NaCl, pH 7.35 (HBS).

Platelet procoagulant activity

The chromogenic assay measuring the phospholipid-related platelet procoagulant activity (PPA) in human washed platelet preparations was adapted from Warner *et al.*[11] The platelet suspension (35 µL at $1080x10^9$ platelets/L) was added to each murine monoclonal antibody (5 µL at a final concentration of 200 µg/mL) and their specific antigen (at the plasma concentration) in 96-well Polysorp plates and incubated for 1 hour at 22°C. For prothrombin, an equimolar amount of hirudin was added to neutralize trace amounts of thrombin. The blank well was washed platelets incubated with HBS containing 5 g/L bovine serum albumin (BSA). The reaction was stopped by 200 µL of 50 mM Tris, 175 mM NaCl, pH 7.9 containing 2 mg/mL BSA. Then, 50 µL of platelet mixtures were transferred to wells containing 50 µL of factor Xa (1.2 nM), factor Va (2.4 nM), $CaCl_2$ (15 mM) and 50 µL of purified bovine prothrombin (6 µM) and S2238 substrate (0.6 mM). After 5 min in the dark at 22°C, the absorbance change was read at 405 nm. Phospholipid concentration was estimated from the initial rate of thrombin formation by reference to a standard curve constructed with a mixture of phosphatidyl-serine, –ethanolamine and –choline (20:20:60 mole%), and expressed as phosphatidylserine equivalents for $150x10^9$ platelets.[17] The mean platelet procoagulant activity for 17 unstimulated platelet preparations was 0.22±0.03 µM phosphatidylserine equivalents.

Fluorogenic measurement of thrombin activity

Calibrated automated thrombography was performed as previously described.[18,19] Thrombin generation curves were recorded either with platelet-rich plasma (PRP), or with platelet-poor plasma (PPP 13000 g) with platelet microvesicles (PMV) at 4 µM phosphatidylserine equivalents as a source of cellular surfaces (PPP/PMV), in the absence or presence of various concentrations of home-made APC[20] or commercial thrombomodulin. Total generated thrombin activity (endogenous thrombin potential, ETP) was assessed as the area under the curve. The APC and thrombomodulin concentrations that reduced the baseline endogenous thrombin potential (ETP_0) by 50% were defined as IC_{50}-APC and IC_{50}-TM, respectively.[19]

Statistical analysis

Data are expressed as the mean±SEM. Continuous variables were compared to controls by the Mann-Whitney test. *p* values less than 0.05 are considered statistically significant.

Results

Effect of human aPL autoantibodies and murine monoclonal antibodies on thrombin generation and inhibition

First we confirmed that the phenotype in aPL patients was due to antibodies. As shown in Figure 1A (top), the time to thrombin burst was longer and ETP_0 was lower (1264 nM.min) with the LA-positive PRP than with the normal PRP (1721 nM.min). In addition, the LA-positive PRP displayed a dramatic decrease in sensitivity to APC: endogenous thrombin potential in the presence of 13.9 nM APC was 1128 nM.min compared with 581 nM.min for the normal PRP (Figure 1A bottom). Addition of total IgG isolated from the patient to the normal PRP induced the two opposite effects observed with the patient's PRP (Figure 1A), i.e. an anticoagulant effect (delayed thrombin burst and low ETP_0) and impaired sensitivity to APC.

Two different profiles of endogenous thrombin potential as a function of APC concentration (Figure 1B) were obtained with PRP from six aPL-patients (two LA-negative anti-β_2GPI-positive, two LA-positive anti-β_2GPI-negative and two LA-positive anti-β_2GPI-positive). Compared with the mean values calculated with PRP from 19 healthy subjects, the two LA-positive anti-β_2GPI-positive patients exhibited low ETP_0 and marked APC resistance while the two LA-negative anti-β_2GPI-positive patients displayed only impaired sensitivity to APC. The two LA-positive anti-β_2GPI-negative patients exhibited a lower ETP_0 than the LA-positive anti-β_2GPI-positive patients. For an overall evaluation of the response to APC, we used IC_{50}-APC; values were 9.1, 13.2, >65 and >65 nM for mean normal PRP, LA-negative anti-β_2GPI-positive patients, LA-positive anti-β_2GPI-negative patients and LA-positive anti-β_2GPI-positive patients, respectively. To ensure that murine monoclonal antibodies are suitable models to study the influence of aPL on thrombin activity, experiments were performed with normal PRP supplemented with murine monoclonal antibodies. As expected, the addition of murine IgG had neutral effects and thus patterns observed with murine monoclonal antibodies were compared with those observed with murine IgG. Upon addition of murine monoclonal antibodies at a final concentration of 200 µg/mL, profiles were similar to those observed for aPL patients (Figure 1C), both regarding ETP_0 and IC_{50}-APC values. Low ETP_0 and markedly high IC_{50}-APC (>65 nM) were obtained with 28F4, as observed for the two categories of LA-positive patients (anti-β_2GPI-positive or anti-β_2GPI-negative). Addition of 7F6G resulted in increased IC_{50}-APC (23.4 *vs.* 16.3 nM for murine IgG) without an effect on ETP_0, as for the LA-negative anti-β_2GPI-positive patients. The endogenous thrombin potential in the presence of any concentration of APC was higher in the presence of 28F4 and 7F6G than in the presence of murine IgG, revealing hypercoagulability. These findings were confirmed with PRP from another healthy donor (*not shown*).

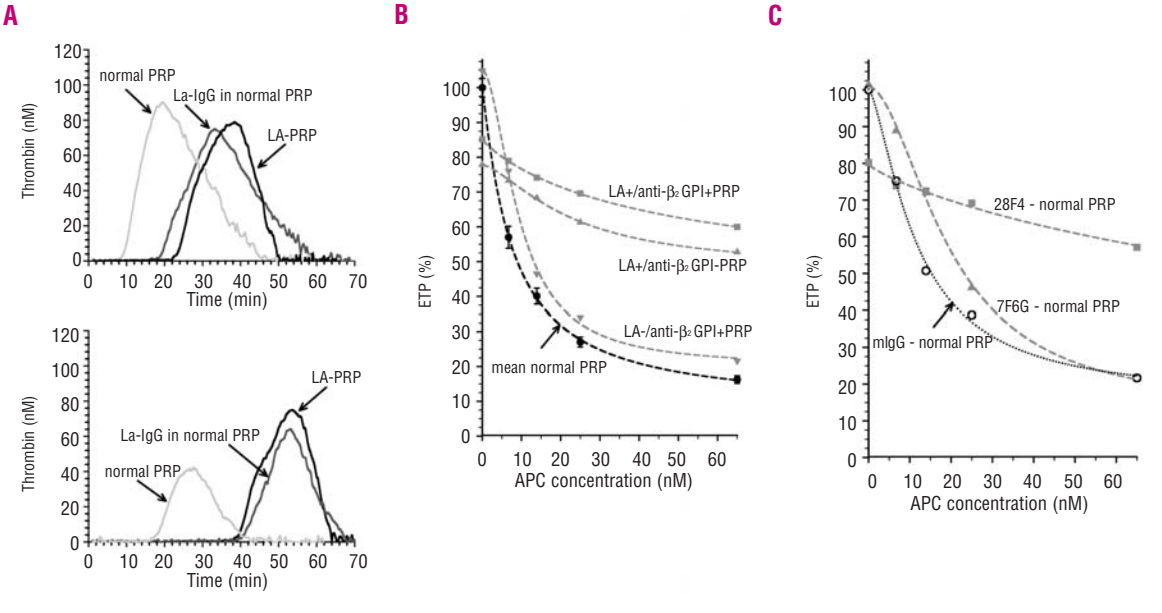

Figure 1 (**A**) **Effect of human antiphospholipid antibodies on generated thrombin activity. Thrombin generation curves obtained in the absence (top) and in the presence (bottom) of APC at a final concentration of 13.9 nM are represented for a normal PRP from a LA-positive patient and normal PRP supplemented with total purified IgG from this patient. (B) and (C) Patterns of endogenous thrombin potential values as a function of increasing concentrations in APC in the presence of human antiphospholipid antibodies and murine monoclonal antibodies to prothrombin and β_2GPI. (B) Mean values obtained with PRP from 19 healthy subjects are displayed as control. For patients, each curve is the mean value obtained with PRP from two patients. (C) Profiles obtained with normal PRP supplemented with 28F4 and 7F6G at 200 µg/mL, and control murine IgG (mIgG) as a negative control. The results in panel B and C are expressed in percentages relative to control values.**

Effects of murine monoclonal antibodies on platelets

The effects of murine monoclonal antibodies on platelet activation were investigated using washed platelets from a healthy donor (Arg131His). In the resting state, less than 5% of the CD42b-positive population expressed P-selectin or CD63 and annexin V-labeled platelets accounted for 2-3% in flow cytometry. We first determined the ability of murine monoclonal antibodies alone or in combination with their target to bind to platelets using surface plasmon resonance binding studies. No binding of annexin V at 100 µg/mL to the platelet surface could be detected, ascertaining the absence of phosphatidylserine exposure at the surface of immobilized platelets. A low signal was observed upon injection of β_2GPI at 200 µg/mL (123 RU). No appreciable binding was detected with prothrombin at 100 µg/mL (-2 RU) or with 7F6G or 28F4 at 200 µg/mL (28 and -60 RU, respectively) whereas combinations of each murine monoclonal antibody with its target resulted in significant responses (Figure 2A and B). Although similar binding of the two complexes would be expected based on their similar molecular masses, the level of binding of 28F4 and prothrombin (925 RU) was about 3-fold higher than that of 7F6G and β_2GPI (297 RU).

We then investigated whether washed platelets incubated with murine monoclonal antibodies alone or in the presence of their target supported thrombin generation. As shown in Figure 2C, prothrombin alone had no effect whereas platelet procoagulant activity values were statistically reduced upon addition of β_2GPI (p=0.01), in line with the reported anticoagulant effect of this latter protein.[21] Platelet procoagulant activity values obtained with murine IgG, 28F4 or 7F6G were not different from those of the control (unstimulated washed platelets). In the presence of their target, murine monoclonal antibodies induced a significant increase in platelet procoagulant activity compared with the antibody alone. This increase was modest for 7F6G (0.74±0.30 *vs.* 0.31±0.09 phosphatidylserine equivalents) and marked for 28F4 (7.04±0.96 *vs.* 0.41±0.14 phosphatidylserine equivalents) reaching the value obtained with the positive control, ALB6. Values reached with 28F4 in the presence of its antigen were markedly higher than those of 7F6G in the presence of its antigen (p=0.002). These results, obtained with the high platelet concentration originally used,[11] were confirmed with a physiological concentration (210x10^9 platelets/L). Moreover, platelet activation was also induced by addition of murine monoclonal antibodies to PRP, but comparatively less than that observed with washed platelets (*Online Supplementary Table S1*). Similar platelet procoagulant activity values were obtained with two other donors (His131His): 1.53 and 1.02 for 7F6G + β_2GPI, 7.36 and 7.16 for 28F4 + prothrombin and 8.04 and 6.89 for ALB6). To further evaluate the platelet response induced by immune complexes, thromboxane synthesis was quantified and found to parallel platelet procoagulant activity (*Online Supplementary Table S1*).

Interference of aPL/target complexes with pro- and anticoagulant reactions occurs independently of platelet activation

To determine the role of interference of aPL/target complexes with pro- and anticoagulant reactions on phospholipid surfaces independently of platelet activation, we monitored thrombin activity in PPP/PMV. As expected, ETP_0 and IC_{50}-APC values remained unchanged upon addition of murine IgG or ALB6 (Figure 3A). When compared with murine IgG, 28F4 decreased ETP_0 (312±45 *vs.* 1917±18 nM.min, p<0.0001; 84% decrease), and increased IC_{50}-APC (>65 *vs.* 5.4±0.6 nM, p=0.02; >12-fold increase) whereas 7F6G influenced only IC_{50}-APC (8.5±0.6 *vs.* 5.4±0.6 nM, p=0.02; 157% increase). A similar distinct effect on APC sensitivity was observed with the addition of thrombomodulin to PPP/PMV. To exclude an artifact due to the phospholipid content, which may depend on the agonist used for the preparation of PMV, and to assess the contribution of

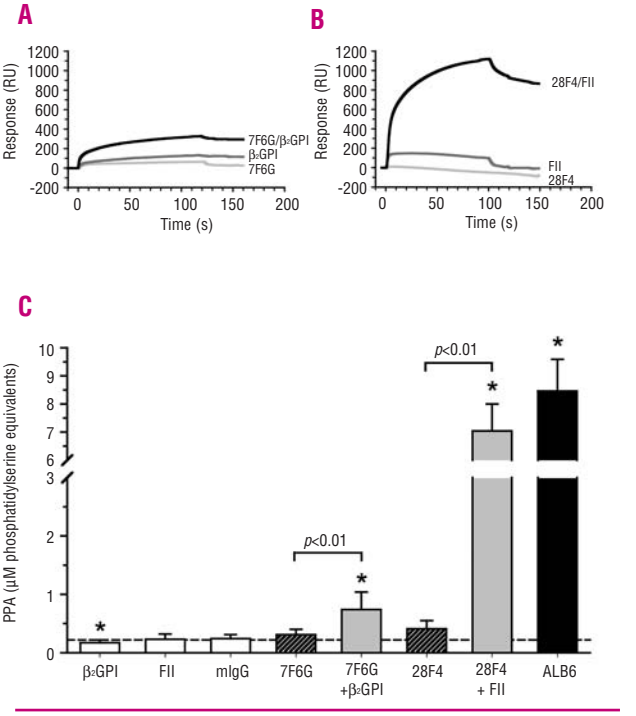

Figure 2. (A) and (B) Binding of murine monoclonal antibodies and their target to washed platelets immobilized on a L1 sensor chip using surface plasmon resonance. Binding is measured as resonance units (RU) as a function of the interaction time. (A) Binding of β_2GPI at 200 µg/mL (dark gray), 7F6G at 200 µg/mL (gray) and a mixture of 7F6G at 200 µg/mL and β_2GPI at 200 µg/mL (black) is shown. (B) Binding of prothrombin (FII) at 100 µg/mL (dark gray), 28F4 at 200 µg/mL (gray) and a mixture of 28F4 at 200 µg/mL and FII at 100 µg/mL (black) is shown. (C) Platelet activation induced by murine monoclonal antibodies. Washed platelets were incubated with or without murine monoclonal antibodies at 200 µg/mL or ALB6 at 10 µg/mL, in the absence or presence of their antigen at their plasma concentrations (200 µg/mL for β_2GPI and 100 µg/mL for prothrombin). Phospholipid-related platelet procoagulant activity was assessed as the ability of these mixtures to support thrombin generation. Results (means ± SEM of six experiments) are expressed as micromolar phosphatidylserine equivalents for 150x10^9 platelets. The dashed line (mean PPA values in 17 unstimulated platelet preparations) indicates the absence of platelet activation, *p<0.05 mixtures vs unstimulated platelets.

cellular membrane proteins, experiments were repeated with PPP enriched with PMV obtained by incubating PRP with collagen, and to PPP 13000 g with pure phospholipid mixtures (phosphatidyl-serine, -ethanolamine and -choline; 20:20:60 mole%). Similar 82% and 86% decreases in ETP_0 and clear APC resistance (IC_{50}-APC >65 nM) were obtained with these two plasma specimens.

Platelet activation reinforces the hypercoagulability induced by interference of aPL/target complexes with thrombin generation and inhibition

To investigate the contribution of immune-mediated platelet activation, experiments similar to those described above were performed with PRP. The addition of ALB6 induced a significant increase in ETP_0 (2076±43 vs 1649±29 nM.min, $p<0.0001$) and APC resistance (IC_{50}-APC values: 42.5±8.4 *vs.* 16.3±0.5 nM, $p=0.01$) (Figure 3B). ETP_0 was unaffected by 7F6G (1683±30 vs 1649±29 nM.min) but IC_{50}-APC was higher than with murine IgG (23.4±3.0 *vs.* 16.3±0.5 nM, $p=0.02$); similar increases were observed for PRP and PPP/PMV (140% and 160%, respectively). The addition of 28F4 resulted in a lower ETP_0 value compared to that with murine IgG (1277±30 *vs.* 1649±29 nM.min, $p<0.0002$). ETP_0 was reduced to a lesser extent in PRP than in PPP/PMV (23% and 84%, respectively). In the presence of 28F4, the IC_{50}-APC value was >65 nM, as was observed with PPP/PMV. APC-resistance was confirmed with thrombomodulin added to PRP. These data were confirmed using another healthy donor.

The platelet-activating 28F4 murine monoclonal antibody was studied at different concentrations ranging from 2 to 200 µg/mL (Table 1). As described previously, ETP_0 was higher and IC_{50}-APC lower in PPP/PMV than in PRP.[18,19] Addition of 28F4 to PPP/PMV affected ETP_0, thrombin peak and IC_{50}-APC in a concentration-dependent manner; at any concentration of

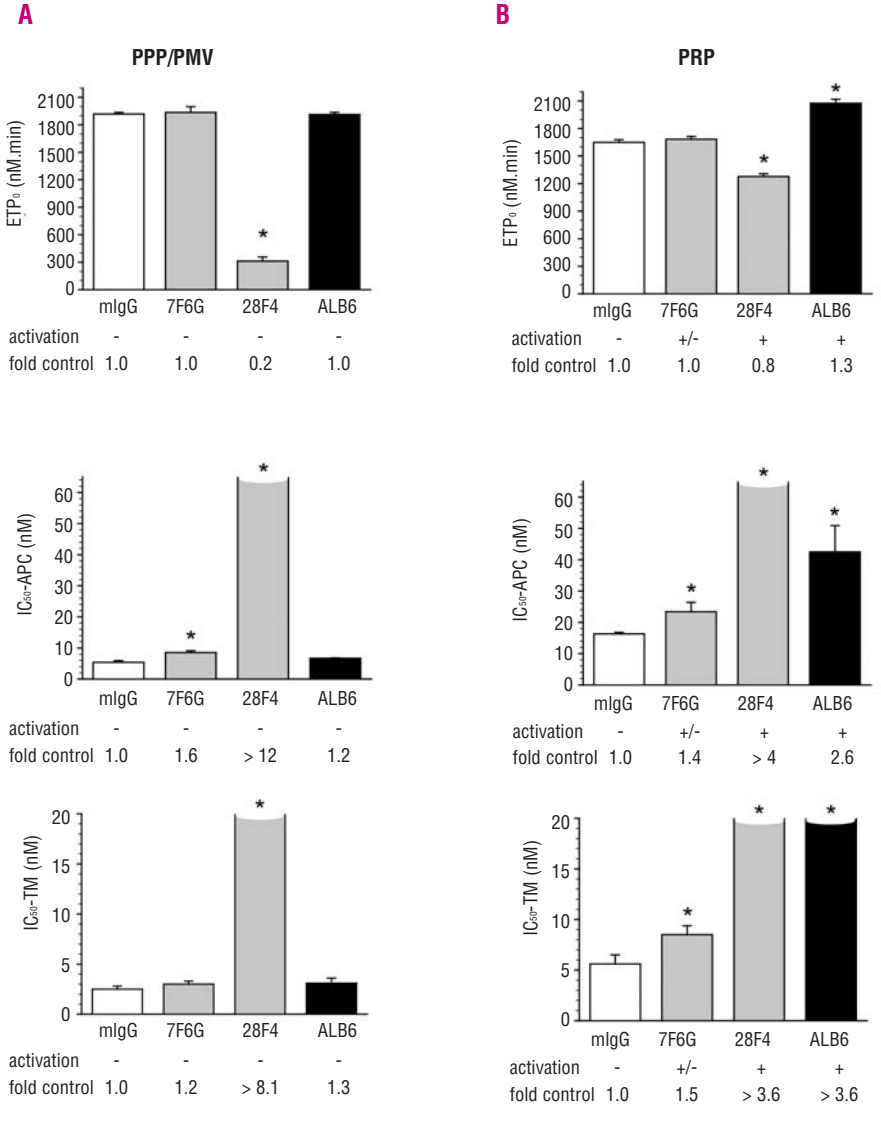

Figure 3. Influence of platelet activation and interference with pro- and anticoagulant reactions on the generated thrombin activity. Panel (A) displays values obtained with platelet-poor plasma in which platelet microvesicles were added at a final concentration of 4 µM (PPP/PMV) and panel (B) values obtained with platelet-rich plasma (PRP). Results are means ± SEM of at least seven values for ETP_0 (endogenous thrombin values without APC) and at least three values for IC_{50}-APC and IC_{50}-TM (APC and thrombomodulin concentrations reducing ETP_0 by 50%). *$p<0.05$ plasma with murine monoclonal antibody vs plasma with murine IgG. "Fold control" indicates ratios of ETP_0, IC_{50}-APC or IC_{50}-TM in the presence of murine monoclonal antibodies vs values in plasma with murine IgG (mIgG).

28P4, ETP_0 and the thrombin peak were significantly lower and IC_{50}-APC was significantly higher than with murine IgG. In PRP, ETP_0 in the presence of 2 and 10 µg/mL of 28F4 was not significantly different from that with murine IgG while it was decreased when 200 µg/mL 28F4 was present. At any concentration, the thrombin peak was significantly lower and IC_{50}-APC higher compared with murine IgG. 28F4 at 2 µg/mL behaved like 7F6G at 200 µg/mL: no anticoagulant effect, and similar, moderate inhibition of APC function. Comparison of the two plasma specimens (PPP/PMV and PRP) indicated that the anticoagulant effect (decrease in ETP_0) was dominant in PPP/PMV whereas the procoagulant effect (increase in IC_{50}-APC) was the main phenomenon in PRP integrating both platelet activation and interference with phospholipid-dependent reactions. Thus, platelet activation partially offset the anticoagulant effect of 28F4 and reinforced its procoagulant effect in a concentration-dependent manner.

Effects of platelet inhibitors on generated thrombin activity

To delineate the mechanisms by which 28F4 partially antagonized the anticoagulant effect seen on ETP_0, PRP was preincubated with platelet inhibitors as reported previously.[9,22,24] Table 2 shows that none of these agents alone, except milrinone, inhibited ETP_0 although abciximab reduced the thrombin peak. Blocking thromboxane production with acetylsalicylic acid had no effect on the ETP_0 decrease in the presence of 28F4. Raising the levels of cAMP or cGMP, by addition of milrinone or dipyridamole, abolished the platelet activating effect of 28F4: the 84% and 72% decreases in ETP_0 were comparable to those observed in PPP/MVP (84%). The $\alpha_{IIb}\beta 3$ blocker abciximab, but not the anti-FcγRIIa IV.3 or the GPIbα blocker AK2, markedly reduced the activating property of 28F4. As a control, IV.3 inhibited the effect of ALB6 (*not shown*).

Discussion

In the present study, using two murine monoclonal antibodies as models of aPL, we demonstrated that (i) impairment of APC anticoagulant activity by aPL occurs independently of platelet activation; (ii) immune-mediated platelet activation, when leading to high levels of exposed procoagulant phospholipids, antagonizes the anticoagulant effect of aPL and thus contributes to reinforcing the hypercoagulability due to APC resistance.

Most researchers have used thromboxane synthesis to assess platelet-activating properties of aPL. Our study provides evidence that murine monoclonal antibodies can also promote platelet procoagulant activity in the presence of their target. The clinical relevance of murine monoclonal antibody-induced platelet procoagulant activity is supported by increased circulating levels of PMV in patients with aPL.[25-27] The coagulation reactions require electronegative surfaces to function and the antibody-mediated increase in platelet procoagulant activity provides a rational explanation for the acceleration of thrombin production.[28,29] Consistent with this, ALB6 elicited high levels of platelet procoagulant activity and increased the total amount of thrombin generated. The absence of effect of the anti-β_2GPI murine monoclonal antibody 7F6G on endogenous thrombin potential and the lower levels of platelet procoagulant activity achieved with this antibody compared with ALB6 suggest that a platelet procoagulant activity threshold might be required.

Our data show that both procoagulant and APC-dependent anticoagulant reactions on membrane surfaces can be inhibited by the anti-prothrombin antibody 28F4 in PPP/PMV. Platelet activation cannot be involved in the antibody-mediated effects observed in PPP/PMV since: (i) residual platelets would have been eliminated by centrifugation at 13000 g and (ii)

Table 1. Influence of murine antibody concentration on generated thrombin activity. ETP_0 is the endogenous thrombin potential value without APC; IC_{50}-APC is the APC concentration reducing ETP_0 by 50%. Values are mean ± SEM (n = 4-10). *$p<0.05$ vs plasma with murine IgG.

		Platelet-poor plasma with PMV			*Platelet-rich plasma*		
Antibodies	*Concentration (µg/mL)*	*ETP_0 (nM.min)*	*Thrombin peak (nM)*	*IC_{50}-APC (nM)*	*ETP_0 (nM.min)*	*Thrombin peak peak (nM)*	*IC_{50}-APC (nM)*
Control murine IgG	10	1845 ± 32	239 ± 15	6.4 ± 0.6	1658 ± 44	120 ± 3	12.9 ± 1.9
	200	1917 ± 18	238 ± 12	5.4 ± 0.6	1649 ± 29	114 ± 3	16.3 ± 0.5
28F4	2	1707 ± 29 *	159 ± 12 *	10.2 ± 0.7 *	1576 ± 76	70 ± 3 *	26.7 ± 4.1 *
	10	1174 ± 51 *	48 ± 5 *	27.0 ± 1.3 *	1556 ± 28	73 ± 2 *	> 65 *
	200	312 ± 45 *	9 ± 1 *	> 65 *	1277 ± 30 *	37 ± 2 *	> 65 *
7F6G	200	1935 ± 62	252 ± 11	8.5 ± 0.6 *	1683 ± 30	121 ± 5	23.4 ± 3.0 *

microvesicles are the end-product of platelet activation. Platelets in PRP can be challenged by aPL/target complexes and this leads to an increase in ETP_0 as demonstrated with ALB6. The greater inhibition of ETP_0 in PPP/PMV (84%) than in PRP (23%) leads us to conclude that platelet activation could partially offset the antibody-mediated inhibition of procoagulant reactions. This supposition is supported by the similar decrease in ETP_0 in PRP preincubated with dipyridamole and PPP/PMV. Reduction of the platelet-dependent effect of 28F4 by abciximab but not by IV.3 suggests involvement of the αIIbβ3 integrin, which binds prothrombin,[30] but not of the FcγRIIa receptor. This agrees with the reported FcγRIIa-independent prothrombotic action of β_2GPI-antibody complexes.[31]

In both plasma specimens, the reduction in ETP_0 was low compared with the increase in IC_{50}-APC. Therefore, APC anticoagulant activity was more potently inhibited than prothrombin activation. Addition of thrombomodulin rather than APC in thrombography might enable a closer evaluation of the *in vivo* balance between anticoagulant and procoagulant effects of aPL since it allows participation of the complete protein C system. Our observation of similar hypercoagulable phenotypes in the presence of APC and thrombomodulin ruled out disturbances of protein C activation.

Differences in binding kinetics and avidity[32] between pro- and anticoagulant factors and aPL immune complexes may account for a selective inhibition of the APC pathway. Overlapping values of avidity for phospholipids have been reported for aPL/target complexes and prothrombin alone and this may account for impaired initial thrombin formation. When the prothrombinase complex assembles, its high avidity can displace aPL/target complexes resulting in incomplete steric hindrance by aPL/target complexes and thus activation of prothrombin. Overall, this may account for the anticoagulant effect of antibodies. The low avidity of protein C for phospholipid surfaces prevents its binding in the presence of aPL/target complexes and this leads to APC-resistance. This hypothesis is supported by higher binding on platelets, using surface plasmon resonance, for 28F4/prothrombin compared with 7F6G/β_2GPI complexes at the same murine monoclonal antibody concentration. At a high concentration of 28F4, the 28F4/prothrombin complexes occupy sufficient phospholipid surface to compete with both procoagulant and anticoagulant complexes whereas at a 100-fold lower concentration, only APC binding was impaired as was seen for 7F6G/β_2GPI complexes.

Although the avidities of murine monoclonal antibodies for their targets are often reported to be higher than those of autoantibodies, the clinical relevance of our work was supported by the demonstration that the studied murine monoclonal antibodies had effects similar to IgG from several patients. This is in line with recent data suggesting that the clinical profiles of patients with LA and prothrombin binding antibodies were characterized by thrombotic events, in particular venous events, whereas the profiles of patients with LA and β_2GPI were characterized by total thrombotic events and specifically arterial events.[6]

It should, however, be realized that our results cannot be generalized since we cannot infer that differences mainly rely on the target, its conformation/orientation and the kinetics of the antigen/antibody interaction. The previously reported mechanism based on a similar phosphatidylethanolamine requirement for APC and aPL/target complexes[15] is unlikely to explain our results since APC-resistance is not restricted to anti-β_2GPI antibodies, and prothrombin is known to interact with phosphatidylserine.

In conclusion, platelet activation reinforces the hypercoagulability induced by interference of aPL/target complexes with coagulation reactions on membrane surfaces, providing a pathogenic mechanism in the antiphospholipid syndrome. These findings may open the way for the design of selective antithrombotic therapies.

Table 2. Effects of platelet inhibitors on generated thrombin activity in the presence of 28F4. PRP was preincubated with the indicated inhibitor for 10 min at room temperature. ETP_0 are presented as percentages relative to the control condition without 28F4. Values are mean ± SEM (n = 3). #$p<0.05$ vs control without 28F4; *$p<0.05$ *vs.* control with 28F4.

Inhibitor	*ETP_0*	
	without 28F4	*with 28F4*
Control	100	76 ± 3
acetylsalicylic acid (100 µM)	101 ± 2	70 ± 5
dipyridamole (40 µM)	101 ± 2	28 ± 3 *
milrinone (40 µM)	89 ± 1 #	16 ± 2 *
IV.3 (2 µg/mL)	98 ± 2	74 ± 6
abciximab (20 µg/mL)	99 ± 2	35 ± 3 *
AK2 (85 nM)	101 ± 7	75 ± 4

Authorship and Disclosures

AM: study design, data collection, interpretation of results, drafting the manuscript; DW: study design, statistical analysis, drafting the manuscript; VL-C and J-PM: data collection and revision of the manuscript; PL: interpretation of results and revision of the manuscript for important intellectual content; TL: study design, interpretation of results and revision of the manuscript for important intellectual content; VR: study design, interpretation of results and drafting the manuscript.

The authors reported no potential conflicts of interest.

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