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Identification of Anti-Plasmin Antibodies in the Antiphospholipid Syndrome That Inhibit Degradation of Fibrin¹

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The combined presence of anti-phospholipid Ab (aPL) and thrombosis is recognized as the antiphospholipid syndrome (APS). The aPL represent a heterogeneous group of Ab that recognize various phospholipids (PL), PL-binding plasma proteins, and/or PL-protein complexes. Recently, we found the presence of antithrombin Ab in some APS patients and that some of these antithrombin Ab could inhibit thrombin inactivation by antithrombin. Considering that thrombin is homologous to plasmin, which dissolves fibrin, we hypothesize that some APS patients may have Ab that react with plasmin, and that some anti-plasmin Ab may interfere with the plasmin-mediated lysis of fibrin clots. To test this hypothesis, we searched for anti-plasmin Ab in APS patients and then studied those found for their effects on the fibrinolytic pathway. The results revealed that seven of 25 (28%) APS patients have IgG anti-plasmin Ab (using the mean OD plus 3 SD of 20 normal controls as the cutoff) and that six of six patient-derived IgG anti-thrombin mAb bind to plasmin with relative K_d values ranging from 5.6×10^{-8} to 1×10^{-6} M. These K_d values probably represent affinities in the higher ranges known for human IgG autoantibodies against protein autoantigens. Of these mAb, one could reduce the plasmin-mediated lysis of fibrin clots. These findings suggest that plasmin may be an important driving Ag for some aPL B cells in APS patients, and that the induced anti-plasmin Ab may act either directly, by binding to plasmin and inhibiting its fibrinolytic activity, or indirectly, by cross-reacting with other homologous proteins in the coagulation cascade to promote thrombosis. *The Journal of Immunology*, 2004, 172: 5765–5773.

The antiphospholipid syndrome (APS)⁵ is characterized by the combined presence of anti-phospholipid Ab (aPL) with thrombosis and/or recurrent pregnancy loss (1, 2). The characteristic aPL include lupus anticoagulants (LAC), detected by their abilities to prolong certain in vitro phospholipids (PL)-restricted blood clotting tests, and anti-cardiolipin Ab (aCL) (1–6). Immunological studies show that aPL represent a heterogeneous group of Ab that recognize various PL, PL-binding plasma proteins, and/or PL-protein complexes. The involved

plasma proteins include β_2 -glycoprotein-1 (β_2 GPI), prothrombin (PT), protein C (PC), and protein S (7–12).

Hemostasis consists of primary and secondary components. The primary component involves platelet activation and aggregation, and the secondary component activates the plasma coagulation system and results in fibrin formation. The key coagulation cascade consists of 1) the expression of tissue factor (TF), which binds and activates factor VII (generating factor VIIa); 2) TF-VIIa complexes, which activate factors IX and X; 3) factor Xa, which works with factor Va to convert PT to thrombin, whereas factor IXa works with VIIIa to generate more factor Xa; and 4) thrombin, which converts fibrinogen to fibrin clots. The coagulation cascade is subjected to three major pathways of feedback regulation: 1) the TF pathway inhibitor inhibits TF-VIIa complexes from activating factors IX and X; 2) antithrombin (AT) binds to thrombin and inactivates thrombin; and 3) activated PC (which is generated from PC after a cleavage in the H chain of PC) works with protein S to inactivate factors Va and VIIIa (13, 14). Moreover, there is the fibrinolytic system that generates plasmin, which dissolves fibrin in hemostatic plugs and thrombi. As such, fibrin formation and fibrinolysis are in dynamic balance, and abnormalities in either hemostasis or fibrinolysis may promote thrombosis (14, 15).

Recently, during our studies of anti-PT Ab (aPT), we found that most, but not all, aPT-positive patient plasma samples contained anti-thrombin Ab (16). Moreover, the IS6 monoclonal IgG aPT (derived from a primary APS patient) binds to cardiolipin (CL) and thrombin, and five of seven patient-derived monoclonal IgG aCL cross-react with PT and thrombin (Table I) (16–18). Of these six thrombin-reactive IgG mAb, the CL24 mAb could interfere with inactivation of thrombin by AT (16). When CL24 and two other thrombin-reactive mAb were analyzed for their binding affinities to thrombin and PT by competitive inhibition, the results showed that

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⁵ Abbreviations used in this paper: APS, antiphospholipid syndrome; aCL, anti-cardiolipin Ab; α_2 -AP, α_2 -antiplasmin; aPL, anti-phospholipid Ab; aPT, anti-prothrombin Ab; AT, antithrombin; CL, cardiolipin; 3D, three-dimensional; β_2 GPI, β_2 -glycoprotein-1; LAC, lupus anticoagulant; PAI, plasminogen activator inhibitor; PC, protein C; PL, phospholipid; PT, prothrombin; SLE, systemic lupus erythematosus; TF, tissue factor; TLCK, N^α -tosyl-L-lysine chloromethyl ketone-HCl; t-PA, tissue plasminogen activator.

Table I. Summary of the characteristics of 8 monoclonal IgG aCL from two APS patients

Ags ^a	Ab							
	IS1	IS2	IS3	IS4	IS6	CL1	CL15	CL24
CL/bovine serum	+	+	+	+	+	+	+	+
CL/ β_2 GPI	-	-	+	+	ND	+	+	+
β_2 GPI	-	-	+	+	+ ^b	+	-	+
Thrombin	-	-	+	+	+	+	+	+
PT	-	-	+	+	+	+	+	+
PC	-	-	+	+	+	+	+	+
Activated PC	-	-	+	+	+	+	+	+
Plasmin	-	-	+	+	+	+	+	+

^a Binding to CL and human β_2 GPI is compiled from the report of Zhu et al. (17). Binding to human thrombin, PT, PC, and activated PC is compiled from the report of Hwang et al. (16, 19), and designated + or -. ND, not done. Data for binding to plasmin are from Fig. 3.

^b The initial study showed that IS6 did not bind to β_2 GPI when wells were coated with β_2 GPI in Tris-buffered saline (the buffer for aPT ELISA) (18). However, subsequent study showed that IS6 did bind to β_2 GPI when wells were coated with β_2 GPI in PBS (data not shown).

soluble thrombin was more effective than PT in inhibiting all three mAb binding to either PT or thrombin in the solid phase. Importantly, thrombin could inhibit all tested mAb from binding to thrombin and PT, whereas PT could only inhibit mAb from binding to PT, but not thrombin. These results demonstrated that these three mAb are more specific for thrombin than PT. Based on these inhibition data, the relative K_d values of these anti-thrombin Ab are $\sim 1.7\text{--}7.5 \times 10^{-6}$ M (16).

The discovery of thrombin-reactive aCL raised the possibility that such aCL may also react with PC, which contains a trypsin/thrombin-like serine protease domain and shares a 50.5% similarity with thrombin. The homologous amino acid sequences reside mainly in the trypsin/thrombin-like serine protease domains of thrombin and PC as well as activated PC. This similarity of thrombin to PC and activated PC led us to determine that all six thrombin-reactive monoclonal aCL bind to PC and activated PC (19). Importantly, of these six PC- and activated PC-reactive aCL, one (CL15) shortens the plasma coagulation time in the presence of exogenous activated PC (19). These findings suggest that CL15 inhibits the anticoagulant function of activated PC.

Similarly, it is known that plasmin and thrombin also share homologous trypsin/thrombin-like serine protease domains with a 48% similarity (Fig. 1). Taken together with the discovery of anti-thrombin and anti-activated PC Ab in APS, we hypothesize that some APS patients may have Ab against plasmin and that some of the anti-plasmin Ab may interfere with fibrinolysis and thus promote thrombosis in such patients. In this study we report the detection of Ab against plasmin in APS patients and the inhibitory effects of one patient-derived IgG monoclonal anti-plasmin Ab on the lysis of fibrin clots. These findings define a novel anti-plasmin autoantibody in APS and show that some anti-plasmin autoantibody may interfere with the lysis of fibrin clots in the circulation.

Materials and Methods

Computer sequence analysis and three-dimensional (3D) structure modeling

The amino acid sequence of thrombin (residues 328–622 of PT, with the accession name of tbnh in the Protein Information Resource database) was compared with that of plasmin (residues 97–810 of the plasminogen precursor, pir:plhu) using the Gap program in the Genetics Computer Group software package (20). The Gap program uses a scoring matrix with matches scored as 1.5, and mismatches scored according to the evolutionary distance between the amino acids. The 3D structures of thrombin and plasmin in the ribbon and the space-filling models were generated based,

thrombin	364	I	V	E	G	S	D	A	E	I	G	M	S	P	W	Q	V	M	L	F	R	K	S	P	Q	E	L	L	C	G	A	S	L	I	S	D	R	W	V	L	T	A	A	H	C	L	L	I	P	P	W	413
plasmin	581	V	G	G	C	V	A	H	P	H	S	W	P	W	Q	V	S	L	R	T	R	F	G	M	H	624																										
thrombin	414	D	K	N	F	T	E	N	D	L	L	V	R	I	G	K	H	S	R	T	R	Y	E	R	N	I	E	K	T	S	M	L	E	K	I	Y	I	H	P	R	Y	N	W	R	E	N	L	D	R	I	463	
plasmin	625	E	K	S	P	R	P	S	S	Y	K	V	I	L	G	A	H	Q	E	V	N	L	E	P	H	V	Q	E	T	E	V	S	R	L	F	L	E	P	T	R	K	D	I	666							
thrombin	464	<u>A</u>	<u>L</u>	<u>M</u>	<u>K</u>	<u>L</u>	<u>K</u>	<u>K</u>	<u>P</u>	<u>V</u>	<u>A</u>	<u>F</u>	<u>S</u>	<u>D</u>	<u>Y</u>	<u>I</u>	<u>H</u>	<u>P</u>	<u>V</u>	<u>C</u>	<u>L</u>	<u>P</u>	<u>D</u>	<u>R</u>	<u>E</u>	<u>T</u>	<u>A</u>	<u>S</u>	<u>L</u>	<u>L</u>	<u>Q</u>	<u>A</u>	<u>G</u>	<u>Y</u>	<u>K</u>	<u>R</u>	<u>V</u>	<u>T</u>	<u>G</u>	<u>M</u>	<u>N</u>	<u>L</u>	<u>K</u>	<u>E</u>	<u>T</u>	<u>W</u>	<u>T</u>	<u>A</u>	513			
plasmin	667	<u>A</u>	<u>L</u>	<u>L</u>	<u>K</u>	<u>L</u>	<u>S</u>	<u>S</u>	<u>P</u>	<u>A</u>	<u>V</u>	<u>I</u>	<u>T</u>	<u>D</u>	<u>K</u>	<u>V</u>	<u>I</u>	<u>P</u>	<u>A</u>	<u>C</u>	<u>L</u>	<u>P</u>	<u>S</u>	<u>P</u>	<u>N</u>	<u>Y</u>	<u>V</u>	<u>V</u>	<u>A</u>	<u>D</u>	<u>R</u>	<u>T</u>	<u>E</u>	<u>C</u>	<u>F</u>	...	<u>I</u>	<u>T</u>	<u>G</u>	<u>W</u>	<u>E</u>	<u>T</u>	<u>Q</u>	<u>G</u>	<u>T</u>	<u>F</u>	711					
thrombin	514	N	V	G	K	G	Q	P	S	V	L	Q	V	N	L	P	I	V	E	R	P	V	C	K	D	S	T	R	I	R	I	T	D	N	M	F	C	A	G	Y	K	P	D	E	G	K	R	G	561		
plasmin	712	..	G	A	G	...	L	L	K	E	A	L	P	V	I	E	N	K	V	C	N	R	Y	E	F	L	N	G	R	V	Q	S	T	E	L	C	A	G	H	L	A	G	..	G	T	..	753					
thrombin	562	<u>D</u>	<u>A</u>	<u>C</u>	<u>E</u>	<u>G</u>	<u>D</u>	<u>S</u>	<u>G</u>	<u>G</u>	<u>P</u>	<u>F</u>	<u>V</u>	<u>M</u>	<u>K</u>	<u>S</u>	<u>P</u>	<u>F</u>	<u>N</u>	<u>R</u>	<u>W</u>	<u>Y</u>	<u>Q</u>	<u>M</u>	<u>G</u>	<u>I</u>	<u>V</u>	<u>S</u>	<u>W</u>	<u>E</u>	<u>G</u>	<u>E</u>	<u>C</u>	<u>D</u>	<u>R</u>	<u>D</u>	<u>G</u>	<u>K</u>	<u>Y</u>	<u>G</u>	<u>F</u>	<u>Y</u>	<u>T</u>	<u>H</u>	<u>V</u>	<u>F</u>	<u>R</u>	<u>L</u>	<u>K</u>	611		
plasmin	754	<u>D</u>	<u>S</u>	<u>Q</u>	<u>G</u>	<u>D</u>	<u>S</u>	<u>G</u>	<u>G</u>	<u>L</u>	<u>V</u>	<u>C</u>	<u>F</u>	<u>K</u>	<u>D</u>	<u>K</u>	<u>Y</u>	<u>I</u>	<u>L</u>	<u>Q</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>S</u>	<u>W</u>	<u>G</u>	<u>L</u>	<u>G</u>	<u>C</u>	<u>A</u>	<u>R</u>	<u>P</u>	<u>N</u>	<u>K</u>	<u>P</u>	<u>G</u>	<u>V</u>	<u>V</u>	<u>R</u>	<u>V</u>	<u>S</u>	<u>R</u>	<u>F</u>	<u>V</u>	<u>T</u>	801					
thrombin	612	W	I	613																																																
plasmin	802	W	I	803																																																

FIGURE 1. Amino acid sequence comparison of human thrombin and human plasmin in their trypsin/thrombin-like serine protease domains. The amino acid sequence of thrombin (residues 364–613 of PT, pir:tbnh) was compared with that of Lys-plasmin (residues 581–803 of the plasminogen precursor, pir:plhu) using the Gap program. The pipe characters (|) are placed between identical amino acids in two proteins; colons are between very similar amino acids (in two proteins) whose comparison values are greater or equal to 0.5; and periods are between similar amino acids whose comparison values are ≥ 0.1 . Three regions of the most homologous amino acid sequences are underlined.

respectively, on the coordinates of human thrombin (1A5G) (21) and human plasmin (1BML) (22), using RasMol software (version 2.7; Bernstein & Sons, Bellport, NY).

Patients and healthy controls

Plasma samples were obtained from 25 patients with APS and 20 normal controls at University of California Medical Center (Los Angeles, CA) and University of California Medical Center (San Diego, CA). All APS patients in this study satisfied the Sapporo classification criteria for definite APS (2). Medical charts and laboratory test reports for each patient entered in this study were reviewed by a rheumatologist (J. M. Grossman or B. H. Hahn). Patients were then classified as primary APS if they had no associated autoimmune disease or as secondary APS if they also fulfilled criteria for another autoimmune disease.

Of the 25 APS patients, 11 were primary APS (44%) and 14 were secondary APS (56%); the latter group included 12 patients with systemic lupus erythematosus (SLE), one with SLE-like disease, and one with both SLE and Sjögren's syndrome. Twenty-two APS patients were positive for LAC (88%), and 21 were positive for aCL (88%). LAC were determined by either the dilute Russell's viper venom time test or the rabbit brain neutralization procedure test (23).

Patient-derived monoclonal aCL and aPT

Seven IgG monoclonal aCL and one IgG monoclonal aPT were analyzed in the present study. The aCL included CL1, CL15, CL24, IS1, IS2, IS3, and IS4 (17), and the single aPT was IS6 (18). Their generation and characterization were reported previously (17, 18).

ELISA for Ab against plasmin and plasminogen

The ELISA for anti-plasmin and anti-plasminogen Ab was performed as follows. Briefly, high binding ELISA plates (Costar, Cambridge, MA) were coated with 5 μ g/ml of either human plasmin or human plasminogen (Haematologic Technologies, Essex Junction, VT) in PBS, pH 7.4. After incubating overnight at 4°C, plates were blocked with PBS containing 0.25% gelatin. Then test plasma samples (1/300 dilution) or purified IgG (at the indicated concentrations) in PBS/0.1% gelatin were distributed to wells in duplicate and incubated for 1.5 h at room temperature. The control human IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). After washing with PBS, bound human IgG was detected with HRP-conjugated goat anti-human IgG (γ -chain specific; BioSource International, Camarillo, CA) and the peroxidase substrate tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

A competitive inhibition assay was used to study the binding properties of selected mAb to plasmin and plasminogen. Briefly, each mAb (1 μ g/ml) was preincubated for 1.5 h with various concentrations of either plasmin or

plasminogen in PBS/0.1% gelatin; each mAb at 1 $\mu\text{g}/\text{ml}$ was in the linear range of its titration curve. Then, the mixture was distributed to the plasmin- or plasminogen-coated wells in duplicate. After incubation, bound IgG was measured. The amount of inhibition for an mAb at a given concentration of soluble plasmin was calculated as follows: % inhibition of mAb binding to plasmin = [(OD from a test mAb alone) - (OD from the same mAb plus plasmin at the given concentration)] / (OD from the same mAb alone) \times 100. The inhibition data of each mAb were used to calculate its relative K_d to plasmin and plasminogen (24).

A few studies reported plasmin digestion of some human IgG (25, 26), suggesting that some of the observed plasmin inhibition of mAb binding to plasmin on plates might actually be due to plasmin digestion of mAb. To address this issue, 2 μM plasmin was first incubated with a plasmin inhibitor, 2 μM aprotinin (Sigma-Aldrich, St. Louis, MO), for 30 min. Then, the plasmin-inhibitor mixture was serially diluted 10-fold, and the diluted samples were mixed with a test mAb in the competitive inhibition assay described above.

In addition, plasmin was irreversibly inactivated with N^α -tosyl-L-lysine chloromethyl ketone-HCl (TLCK; Sigma-Aldrich), which forms a chemical bond with His⁶²² (one of the three conserved catalytic residues of all proteases in the trypsin family). Briefly, 12 μM plasmin was first incubated with 6 mM TLCK in PBS (pH 7.4) for 100 min at room temperature, which inhibited >99% of the plasmin amidolytic activity using S-2403. After incubation, the excess TLCK was removed by dialyzing the plasmin-TLCK mixture against PBS at 4°C overnight. Thereafter, the TLCK-inactivated plasmin was mixed with a test mAb in the above-mentioned competitive inhibition assay. The percent inhibition of mAb binding to plasmin was calculated as described above.

Affinity purification of IgG from patients' plasma

IgG was purified from two chosen plasma samples positive for IgG anti-plasmin Ab using a protein G-Sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions.

Effects of anti-plasmin mAb on plasmin activity

The effects of mAb on plasmin activity were first studied using the plasmin chromogenic substrate Spectrozyme-PL (H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide; American Diagnostica, Greenwich, CT) according to the manufacturer's instructions. Briefly, 12.5 μl of human plasmin (120 nM) was mixed separately with 12.5 μl of a test mAb or a control human IgG (all at 300 $\mu\text{g}/\text{ml}$) for 1 h at room temperature. Then, to each reaction mixture was added 50 μl of Spectrozyme-PL (150 μM) in 10 mM HEPES-NaOH and 150 mM NaCl (pH 7.4). The final concentrations of plasmin, mAb, and Spectrozyme-PL were 20 nM, 50 $\mu\text{g}/\text{ml}$, and 100 μM , respectively. Generation of *p*-nitroaniline was monitored by measuring OD at 405 nm.

In addition, the effects of plasmin-reactive mAb on plasmin activity were determined according to the method described by Van Nostrand et al. (27) with minor modifications. Briefly, fibrin was prepared in microtiter wells by incubating 5.4 μM fibrinogen and 1.5 nM thrombin (Haematologic Technologies) in a final volume of 100 μl of 50 mM Tris-HCl, 150 mM NaCl, and 5 mM CaCl_2 (pH 7.5) for 1 h at room temperature.

At the same time, human plasmin (300 nM) and hirudin (6 U/ml; 1 U is defined as the amount of hirudin that will neutralize 1 National Institutes of Health unit of thrombin at 37°C, pH 7.5; Calbiochem, San Diego, CA) were mixed separately with a test mAb or a control human IgG (all at 300 $\mu\text{g}/\text{ml}$) for 1 h at room temperature. Thereafter, 50 μl of each test mixture was added to fibrin wells in duplicate, and plates were incubated with shaking at 37°C in a humidified environment. The extent of fibrinolysis was determined as a reduction in absorbance at 405 nm with time.

Because all six test mAb react with thrombin, and two of six mAb inhibit thrombin activity (16), hirudin (a specific thrombin inhibitor) was added to the fibrinolysis assay to completely stop thrombin activity. This prevented any confusion from possible mAb-mediated reduction of additional fibrin generation. Of note, hirudin had no effect on plasmin activity and did not interfere with binding of mAb to plasmin (data not shown).

Statistical analysis

The mean OD plus 3 SD of the 20 normal controls was used as the cutoff, and the plasma samples with OD values consistently higher than the cutoff in two separate experiments were considered positive. Differences in APS and control groups were analyzed using the unpaired *t* test. Differences in the test Ab-induced inhibition of plasmin activity were analyzed using paired ANOVA, followed by the Bonferroni multiple comparison test. A value of $p < 0.05$ was considered significant.

Results

Detection of anti-plasmin Ab in some APS patients

To test the hypothesis that there are anti-plasmin Ab in APS patients, we developed an ELISA for IgG anti-plasmin Ab and used the assay to analyze plasma samples from 25 APS patients and 20 normal controls. All samples were analyzed at 1/300 dilutions. The results showed that APS patients have more IgG anti-plasmin Ab than normal controls ($p < 0.01$, by unpaired *t* test; Fig. 2A). When the mean + 3 SD of the normal controls was used as the cutoff, IgG anti-plasmin Ab were positive in seven of 25 (28%) APS patients (Fig. 2A).

A concern was raised that the observed IgG anti-plasmin Ab might actually reflect IgG anti- β_2 GPI that bound to β_2 GPI, which

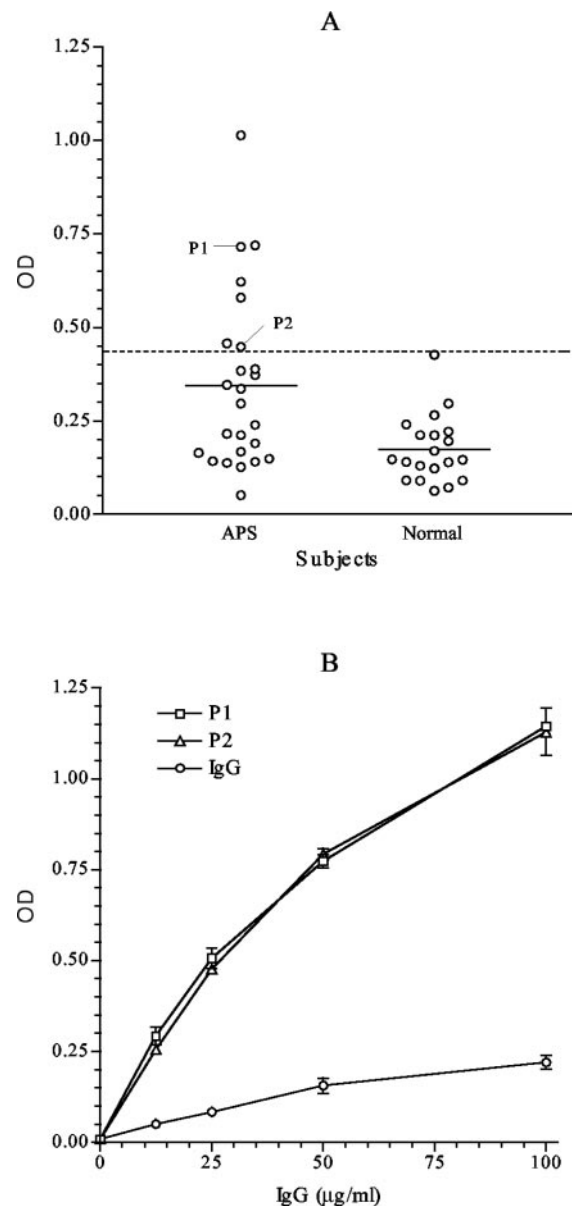


FIGURE 2. Presence of IgG anti-plasmin Ab in some APS patients. *A*, Plasma samples from 25 APS patients and 20 normal controls were analyzed at the 1/300 dilution. The horizontal bars indicate the mean OD for each group; the dashed line represents the cutoff, which is mean OD + 3 SD of the 20 normal controls ($n = 2$). *B*, Affinity-purified IgG from two patient plasma samples (positive for IgG anti-plasmin Ab, designated P1 and P2) or a control normal human IgG were analyzed at the indicated concentrations. A representative result from two experiments is shown.

is bound to plasmin to some extent, as plasmin is responsible for the known cleavage of β_2 GPI. To address this possibility, IgG was purified from two positive plasma samples and analyzed for binding to plasmin. Fig. 2B shows that IgG from both patients bound to plasmin in a concentration-dependent fashion compared with normal human IgG at the same concentrations. Combined, these data demonstrate the presence of IgG anti-plasmin Ab in some APS patients.

Identification of monoclonal anti-plasmin Ab and anti-plasminogen Ab

As polyclonal anti-plasmin Ab in APS patients are likely to be heterogeneous (particularly in their functional activity), it would be important to obtain anti-plasmin mAb and use such mAb to study the functional significance of anti-plasmin Ab in APS. Accordingly, we examined our six patient-derived IgG monoclonal anti-thrombin/aCL Ab for their reactivity with human Lys-plasmin. The results showed that all six anti-thrombin/aCL mAb bound to plasmin, whereas two of two non-thrombin-reactive IgG monoclonal aCL did not react with plasmin (Fig. 3A). The plasmin-reactive mAb included CL1, CL15, CL24, IS3, IS4, and IS6. The first three mAb were from a secondary APS patient with primary SLE, and the latter three mAb were from a primary APS patient (17, 18). Subsequently, we analyzed these mAb for their reactivity with human plasminogen. Fig. 3B shows that all six plasmin-reactive mAb also bound to plasminogen, whereas neither of the two non-thrombin-reactive monoclonal aCL reacted with plasminogen.

Binding properties of mAb to plasmin and plasminogen

As a first step to assess the clinical significance of the above patient-derived mAb against plasmin and plasminogen, we studied the binding affinity of all six mAb to plasmin and plasminogen by competitive and cross-inhibition. The results showed that plasmin was much more effective than plasminogen at inhibiting all mAb binding to the corresponding Ags. Significantly, plasmin at 1 nM inhibited ~70% of binding to plasmin by CL1 and CL24. Based on these inhibition data, the relative K_d values of the plasmin-reactive mAb to plasmin were 3×10^{-10} , 7×10^{-10} , and 4×10^{-10} M for CL1, CL15, and CL24, respectively (Fig. 4A). IS3 and IS4 bound to plasmin with lower affinity, with relative K_d values of 3×10^{-8} and 9×10^{-9} M, respectively.

In contrast to very high affinity binding to plasmin by these patient-derived IgG mAb, the binding to plasminogen by these mAb was of much lower affinity (Fig. 4B). For example, 1 μ M plasminogen could inhibit ~55% of CL24 binding to plasminogen. Based on these inhibition data, the relative K_d values of these mAb binding to plasminogen were 3×10^{-7} and 7×10^{-7} M for CL24 and CL1, respectively, and $\sim 2 \times 10^{-6}$ M for CL15, IS3, and IS6. Considering that the plasma concentration of plasminogen is 2 μ M (15), these six mAb are unlikely to have a meaningful reactivity with plasminogen in plasma.

When these six mAb were analyzed by cross-inhibition, plasmin inhibited six mAb from binding to plasminogen, whereas plasminogen (up to 1 μ M) failed to inhibit six mAb from binding to plasmin, except for CL1 (Fig. 4, C and D). Combined, these results demonstrate that these six mAb are more specific for plasmin than plasminogen and can interact meaningfully with plasmin even in the presence of plasminogen in plasma.

A few studies reported plasmin digestion of some human IgG (25, 26), suggesting that the observed high affinity of mAb binding to plasmin may be due in part to plasmin digestion of mAb. To address this issue, we first repeated the competitive inhibition assay in the presence of a plasmin inhibitor, aprotinin. The results showed that aprotinin (at equal molar concentrations to plasmin)

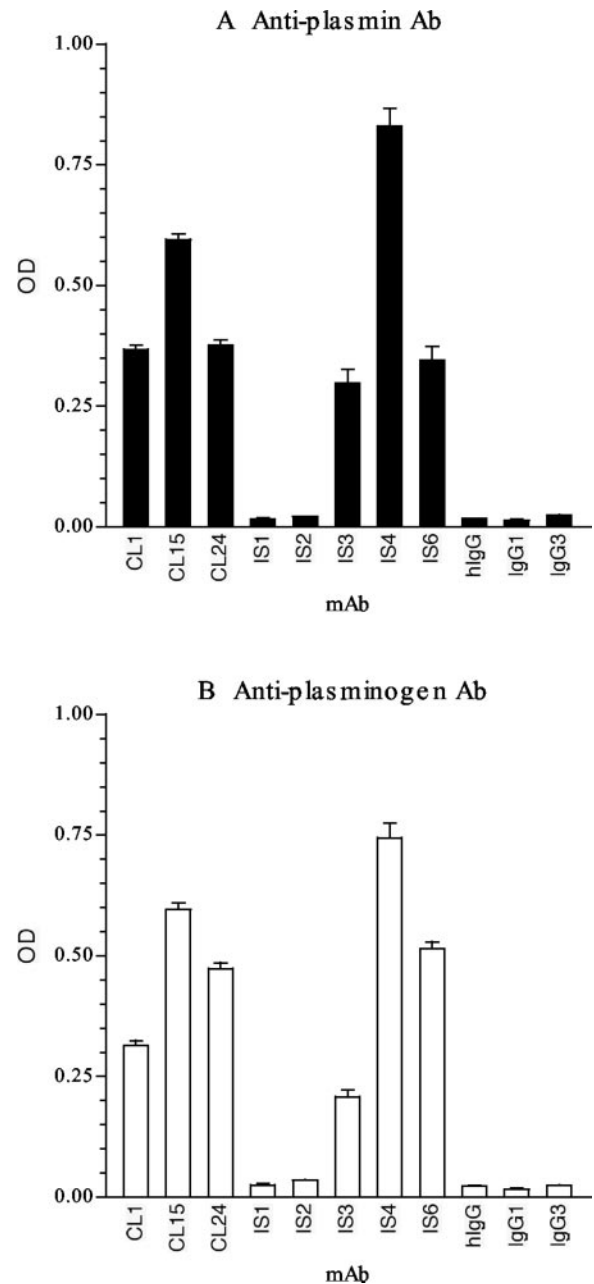


FIGURE 3. Identification of monoclonal anti-plasmin Ab and anti-plasminogen Ab. *A*, Some patient-derived monoclonal aCL bind to plasmin. *B*, All monoclonal anti-plasmin Ab bind to plasminogen. Microtiter wells were coated with plasmin or plasminogen, and the test mAb, normal human IgG, or monoclonal isotype controls (IgG1 or IgG3) were analyzed at 1 μ g/ml. IS1 and IS2 are IgG1, and the other mAb are IgG3. Bound IgG were measured and expressed in OD, and the mean and SEM are given ($n = 2$).

substantially reduced the binding affinity of mAb to plasmin. For example, the relative K_d value of CL15 to plasmin was 7×10^{-7} M in the presence of equimolar aprotinin (data not shown), compared with 7×10^{-10} M in the absence of aprotinin. The findings suggest that plasmin cleaved IgG, resulting in reduced binding of mAb to plasmin on wells and false inhibition. However, these data may also suggest that aprotinin and the plasmin-reactive mAb bind to the same active site of plasmin, leading to aprotinin interference of mAb binding to plasmin. This contention is supported by the report that human autoantibodies to poly(adenosine diphosphate-ribose) polymerase recognized the catalytic site of the enzyme

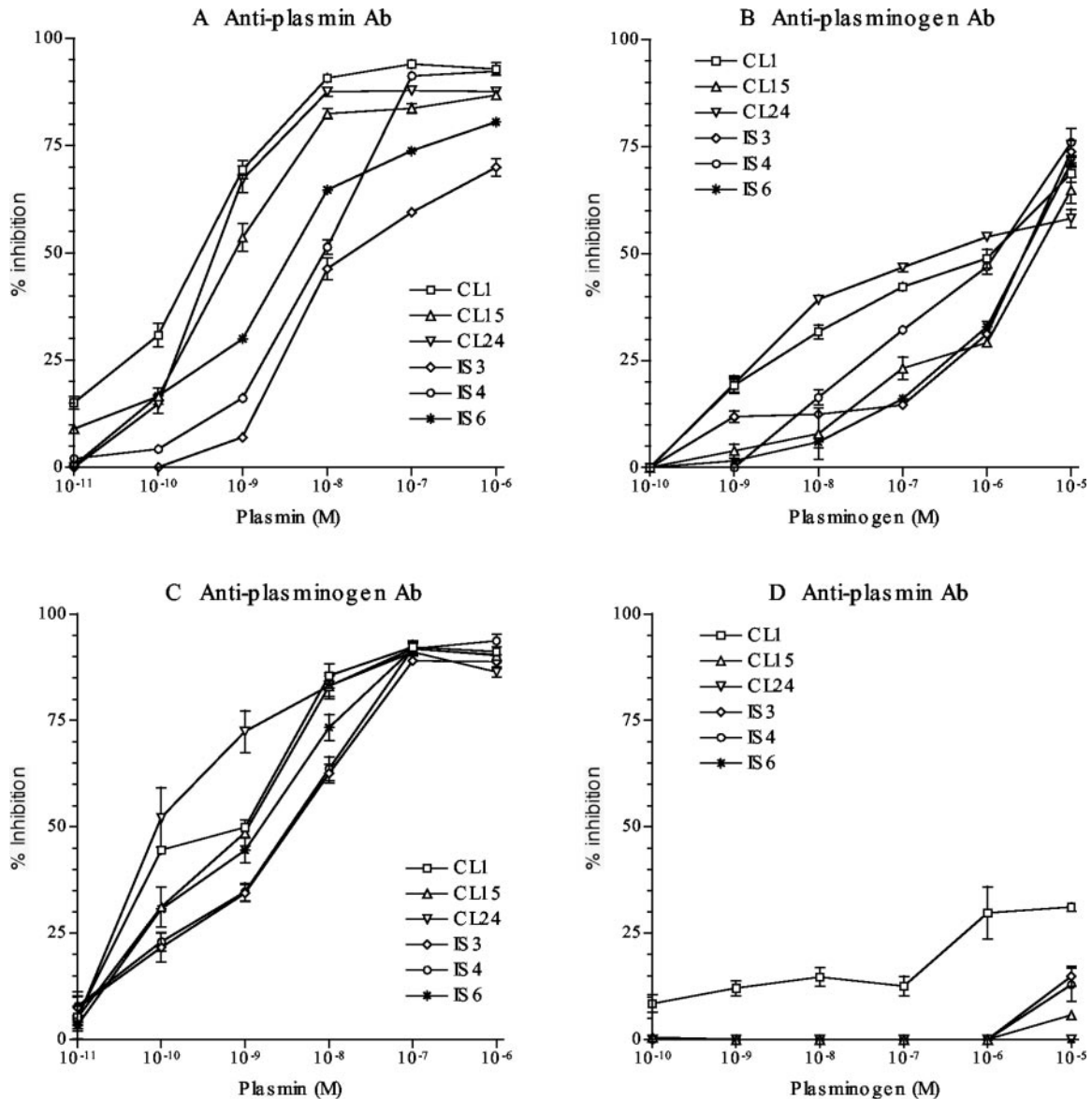


FIGURE 4. Plasmin-reactive mAb bind to plasmin with high affinity. Soluble plasmin in the nanomolar range could inhibit mAb from binding to plasmin (A), whereas soluble plasminogen required the micromolar concentrations to inhibit mAb from binding to plasminogen (B). In cross-inhibition studies, plasmin could inhibit all mAb from binding to plasminogen (C), whereas plasminogen failed to inhibit mAb from binding to plasmin (D). The results are expressed as the percent inhibition, and a representative result from four experiments is shown.

(28), and the high binding affinity of aprotinin to plasmin (with a K_d of 2.3×10^{-10} M) (29).

Second, we preincubated each mAb with plasmin under the same conditions as in the competitive inhibition, and then distributed the mixtures to wells precoated with affinity-purified goat anti-human Ig. After incubation, bound human IgG was detected with HRP-conjugated goat anti-human IgG (γ -chain specific). The results showed that plasmin up to $1 \mu\text{M}$ (that causes 100% inhibition for all six mAb binding to plasmin in solid phase in Fig. 4A) did not cause any detectable reduction in OD of our mAb bound to the capturing Ab (data not shown). These findings suggest that plasmin in the competitive inhibition assay did not cleave mAb to cause false inhibition.

Third, to further address the possibility that extremely high binding affinity of mAb to plasmin may, at least in part, be due to plasmin digestion of IgG, we repeated the competitive inhibition for all six plasmin-reactive mAb with the TLCK-inactivated plasmin. TLCK forms a chemical bond with His⁶²², which is one of the

three conserved catalytic residues of all proteases in the trypsin family. As shown in Fig. 5, the relative K_d values were 5.6×10^{-8} M for CL1, 1×10^{-7} M for CL15, and 1×10^{-6} M for CL24 (Fig. 5). Compared with relative K_d values of mAb determined with plasmin alone in Fig. 4A, these affinities were lowered by 10- to 2500-fold. For example, the relative K_d value for IS3 was reduced from 3×10^{-8} to 2.7×10^{-7} M, whereas the relative K_d value for CL24 was reduced from 4×10^{-10} to 1×10^{-6} M.

Effects of anti-plasmin mAb on plasmin activity

Assuming that anti-plasminogen or anti-plasmin IgG accounts for 1% of the total IgG, the plasma concentration of IgG anti-plasmin or anti-plasminogen Ab will be $\sim 0.7 \mu\text{M}$, based on a plasma IgG concentration of 10 mg/ml (equivalent to $67 \mu\text{M}$). In this context, the high affinity anti-plasmin Ab (with relative K_d values ranging from 10^{-8} to 10^{-10} M) are likely to bind to plasmin to form stable complexes, whereas the low affinity anti-plasminogen Ab (with relative K_d values ranging from 10^{-6} to 10^{-7} M) may not be able

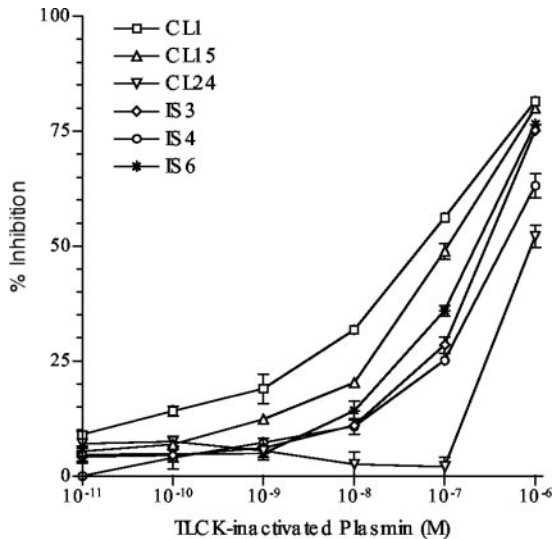


FIGURE 5. CL1 and CL15 bind to plasmin with truly high affinity. To prevent the possible contribution of plasmin proteolytic activity to the binding affinity of mAb to plasmin, plasmin was preinactivated with TLCK (which forms a chemical bond with His⁶²², one of the three catalytic residues of plasmin) and then used as the soluble plasmin inhibitor in the competitive inhibition. The results are expressed as the percent inhibition, and the mean and SEM are given ($n = 2$).

to bind to plasminogen to form stable complexes. Therefore, we studied only the effects of these mAb on plasmin activity. We first examined the effects of these six mAb on the amidolytic activity of plasmin using a small chromogenic substrate of plasmin, Spectrozyme-PL. None of the six mAb affected the amidolytic activity of plasmin (data not shown). As Spectrozyme-PL is a small molecule, the assay might not reflect the inhibitory effects of some plasmin-reactive mAb on lysis of fibrin by plasmin. Accordingly, we examined the effects of plasmin-reactive mAb on plasmin in a fibrinolysis assay, using freshly generated fibrin (from fibrinogen by thrombin) as the substrate for plasmin.

As shown in Fig. 6A, when 100 nM plasmin (final concentration) was added to fibrin in microtiter wells, fibrin was dissolved over a period of 220 min, resulting in a reduction of OD from 0.33 at 0 min to 0 at 220 min. The lysis curve appears to be biphasic. It may be speculated that the biphasic appearance represents a faster dissolution of longitudinal fibers and the subsequent slower cleavage of shorter fibers. However, the precise meaning of this biphasic appearance is not clear at this point. Regardless, based on this lysis curve, we elected to analyze the effects of mAb on plasmin fibrinolytic activity at 90 min, when ~50% of fibrin was dissolved.

Fig. 6, B and C, shows that the control polyclonal human IgG and a monoclonal IgG3 control (both at 100 $\mu\text{g}/\text{ml}$, final concentration) displayed 5 and 10% inhibition of fibrinolysis, respectively. Accordingly, the effects of mAb on plasmin-mediated fibrinolysis were compared with those of the IgG3 control. Under these conditions, only one of six (i.e., CL15) showed a significant inhibition of plasmin activity (Fig. 6B).

Discussion

To test our hypothesis that some APS patients may have Ab against plasmin, which might interfere with fibrinolysis, we searched for the presence of IgG anti-plasmin Ab and studied their effects on plasmin-mediated fibrin dissolution. The results showed that IgG anti-plasmin Ab were detected in seven of 25 (28%) APS patients (Fig. 2), and that six patient-derived IgG mAb bound to

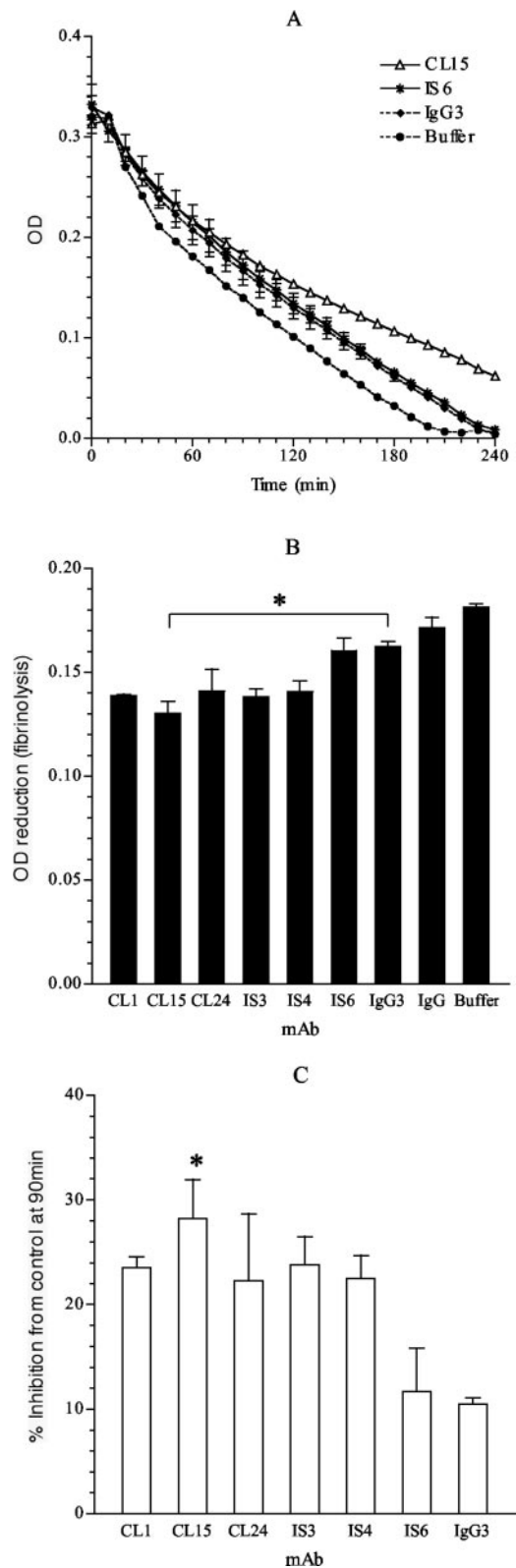
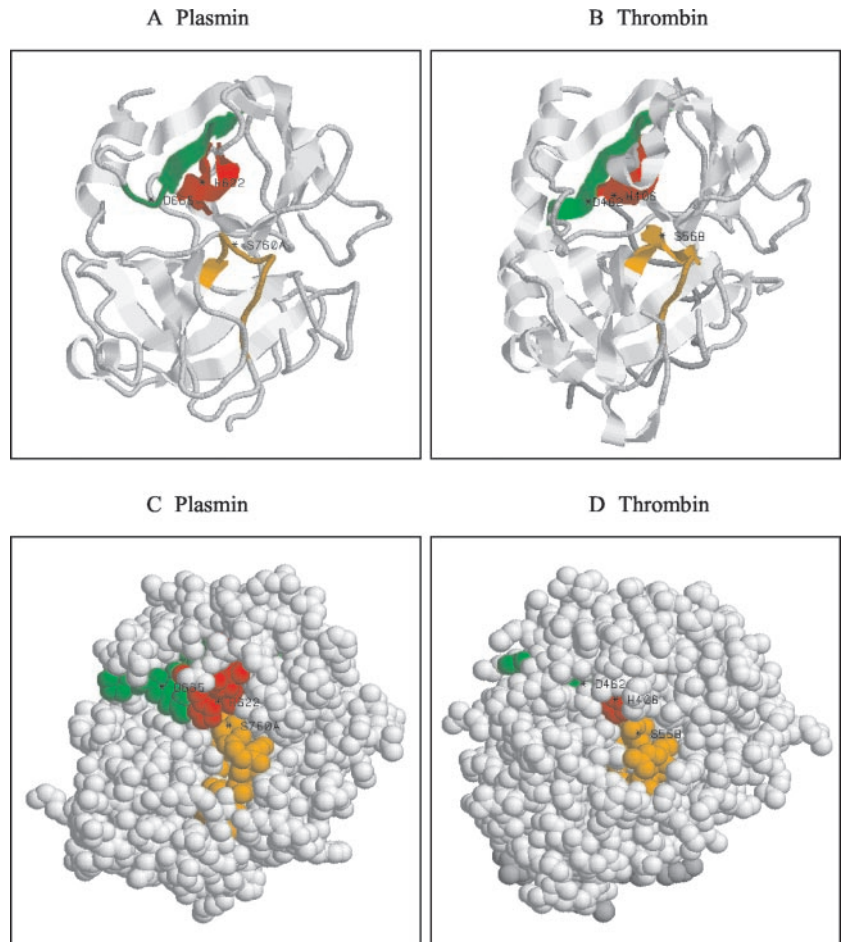


FIGURE 6. The CL15 IgG anti-plasmin mAb prolongs dissolution of fibrin. A, Kinetics of plasmin-mediated lysis of fibrin in buffer or in the presence of a test IgG; B, plasmin-mediated fibrinolysis in the presence or the absence of test IgG at 90 min. C, Ab-mediated effects on fibrinolysis are expressed as the percent inhibition from control at 90 min. Human plasmin was mixed separately with a test mAb or a control human IgG for 1 h at room temperature, and then the mixtures were added to fibrin wells. The final concentrations of plasmin and IgG were 100 nM and 100 $\mu\text{g}/\text{ml}$, respectively. OD at 405 nm was determined every 10 min. Fibrinolysis is given in OD and represents a reduction in OD with time for the assay condition.

FIGURE 7. The potential plasmin and thrombin epitope for the plasmin- and thrombin-reactive mAb may reside in and/or around a surface homologous region (in orange) that is shared between human plasmin and thrombin. The 3D structures of plasmin and thrombin in the ribbon (*A* and *B*) and the space-filling (*C* and *D*) models are given. The homologous regions (in descending order of regional similarity) are shown in red (W616-L624 for plasmin and W400-L408 for thrombin), orange (C756-P763 for plasmin and C564-P571 for thrombin), and green (K664-L671 for plasmin and R461-L468 for thrombin), according to sequence comparison data in Fig. 1. *A*, The ribbon model of plasmin is shown with a direct view into the active site cleft, with the catalytic residues of plasmin (H622, D665, and S760) at the center. *B*, The ribbon model of thrombin is shown with an orientation similar to that of plasmin in *A*. The catalytic residues of thrombin (H406, D462, and S568) are at the center. *D*, The space-filling model of thrombin is shown with a direct view into the active site cleft in the standard orientation (the cleft runs horizontally from *left to right*, with the exosite I on the *right*). *C*, The space-filling model of plasmin is shown with an orientation similar to that of thrombin in *D*. Importantly, one-half of the C756-P763 region of plasmin and the C564-P571 region of thrombin (in orange) are on the surface. Therefore, this homologous region probably represents the potential epitope on plasmin and thrombin for mAb that react with both plasmin and thrombin.



plasmin with high affinity (Fig. 4A). Specifically, the relative K_d values of the plasmin-reactive mAb to plasmin were 3×10^{-10} and 4×10^{-10} M for CL1 and CL24, respectively. As a few studies reported plasmin digestion of some human IgG (25, 26), the competitive inhibition was repeated with either aprotinin or TLCK, protease inhibitors. The relative K_d values of all mAb were increased from the range of 3×10^{-10} to 9×10^{-9} M to the range of 5.6×10^{-8} to 1×10^{-6} M when TLCK-inactivated plasmin was used as the soluble inhibitor (Fig. 5). These findings suggest that plasmin cleavage of IgG may lead to falsely higher affinities than the actual binding affinities of mAb to plasmin. Nevertheless, the relative K_d value of 5.6×10^{-8} M for CL1 and 1×10^{-7} M for CL15 in binding to plasmin (when its proteolytic activity is inhibited by >99%) remains high affinity among human autoantibodies that bind to protein autoantigens.

More importantly, CL15 reduced by ~18% the fibrinolysis mediated by 100 nM plasmin compared with fibrinolysis in the presence of a control IgG3 (Fig. 6, *B* and *C*). Combined, these data show that some IgG anti-plasmin Ab in APS patients (such as CL15 at 100 μ g/ml, ~1% of the plasma IgG concentration) could interfere with plasmin-mediated fibrinolysis.

The fibrinolytic system comprises the proenzyme, plasminogen, which is converted to plasmin mainly by tissue plasminogen activator (t-PA). Activation of plasminogen by t-PA is not efficient in solution, but becomes 400-fold more efficient when both proteins are bound to fibrin (15). The fibrinolytic system is tightly regulated; t-PA is inhibited by plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2), and plasmin by α 2-antiplasmin (α 2-AP). The plasma concentrations of t-PA, PAI-1, plasminogen, and α 2-AP are 80 pM (5 ng/ml), 400 pM (20 ng/ml), 2 μ M (0.2 mg/ml), and

1 μ M, respectively. Thus, most plasmin in the fluid phase is inhibited by α 2-AP, whereas plasmin formed on a fibrin surface is protected and can exert its activity for longer.

Studies have suggested that the fibrinolytic system is an important defense mechanism against thrombosis. The reduction of fibrinolysis may allow the growth and development of thrombi, resulting in a prothrombotic state. Decreased levels of t-PA and/or increased levels of PAI-1 have been reported in patients with deep vein thrombosis (30). Along this line, mice lacking plasminogen were predisposed to severe thrombosis (31). In contrast, venous thrombosis occurs frequently in transgenic mice overexpressing the native form of PAI-1 under control of the metallothionein promoter (32), whereas age-dependent spontaneous coronary arterial thrombosis occurs in mice that express a stable form of human PAI-1 (33). In this context, it is conceivable that CL15 may promote thrombosis in the host patient by inhibiting plasmin-mediated fibrinolysis.

This contention is consistent with some recent studies that suggested defective fibrinolysis in APS. In 1999, Ieko et al. (34, 35) reported that β 2GPI exerted a dose-dependent enhancement of t-PA activity in the presence of PAI-1, and that addition of EY1C8 patient-derived monoclonal aCL reduced t-PA activity by ~45% in a mixture of t-PA (3.6 U/ml), PAI-1 (7.1 ng/ml), and β 2GPI (3.8 μ M). In 2000, Cugno et al. (36) showed that of 39 patients with primary APS, three had high titers of IgG Ab against t-PA, and four had high titers of Ab against fibrin-bound t-PA, which is the physiologically active form of t-PA. Very recently, Kolev et al. (37) reported that IgG from APS patients impaired the fibrin dissolution with plasmin. Interestingly, these investigators noticed that although normal human IgG appeared to slightly retard the

dissolution of fibrin by plasmin, retardation was much stronger with IgG from APS patients. These findings are similar to the effects we observed of normal human IgG and CL15 on plasmin-mediated fibrinolysis (Fig. 6). The slight inhibitory effect of normal human IgG on plasmin-mediated fibrinolysis may be due to some IgG that is bound and cleaved by plasmin (25, 26), resulting in its competition with fibrin for proteolytic activity of plasmin. In addition, IgG from APS patients did not affect the amidolytic activity of plasmin when analyzed with the substrate Spectrozyme-PL (37), which is also similar to our current finding that CL15 failed to inhibit plasmin from digesting Spectrozyme-PL.

Along this line, Takeuchi et al. (38) showed that the EY2C9 IgM anti- β_2 GPI mAb (from an APS patient) suppressed intrinsic fibrinolysis in the presence of β_2 GPI, apparently by enhancing the β_2 GPI-mediated weak suppression of intrinsic fibrinolysis. Similar to intrinsic coagulation, intrinsic fibrinolysis is initiated by the contact activation of coagulation factor XII (FXII). Then, activated FXII (FXIIa) converts prekallikrein to kallikrein, which, in turn, converts prourokinase (single-chain, urokinase-type plasminogen activator) to urokinase-type plasminogen activator. In this context, Takeuchi et al. (38) suggested that β_2 GPI and anti- β_2 GPI may either inhibit FXIIa from activating prekallikrein or suppress kallikrein from activating urokinase-type plasminogen activator, leading to reduced plasmin generation. Taken together with our present findings, these data suggest that there are two kinds of aCL that may inhibit fibrinolysis, one (like EY2C9) suppresses plasmin generation, and one (like CL15) inhibits plasmin activity.

It is noteworthy that plasminogen belongs to the kringle protein family that also includes PT. Each kringle protein consists of a trypsin/thrombin-like serine protease domain and a variable number of loop-like kringle structures. There are extensive sequence homologies among the protease domains and among the kringle domains of different kringle proteins. This understanding led Puurunen et al. (39) to hypothesize that some aPT in patients developing myocardial infarction might cross-react with plasminogen and thus interfere with the fibrinolytic function of plasminogen. These investigators found that soluble PT inhibited up to 50% of aPT binding to immobilized PT in sera from 17 of 17 patients, whereas plasminogen could inhibit aPT binding by at least 20% in nine of 17 (53%) sera (39). These data indicate frequent cross-reactivity of aPT with plasminogen. These findings are now supported further by the present data showing that six of six patient-derived IgG mAb that bind to thrombin and PT also bind to plasmin and plasminogen (Fig. 3, A and B).

What are the likely epitopes on plasmin and thrombin that are recognized by the thrombin- and plasmin-reactive mAb? As all six mAb react with both plasmin and thrombin, it is conceivable that the potential epitopes most likely reside in and/or around the surface homologous regions that are shared by plasmin and thrombin. Fig. 7 shows the homologous structures shared by human plasmin and thrombin. As is evident from their 3D structures in a ribbon model (Fig. 7, A and B), plasmin and thrombin share two α -helices on the left side, four β -strands at the bottom, and a homologous active site at the center that consists of the identical catalytic triad residues (H622, D665, and S760 for plasmin; H406, D462, and S568 for thrombin) (21, 22). Moreover, the space-filling models of both molecules show that many amino acid residues in the homologous regions are on the surface (Fig. 6, C and D). In particular, one-half of the C756-P763 region of plasmin and the C564-P571 region of thrombin (in orange) are on the surface. Therefore, the potential epitope on plasmin and thrombin probably resides in and/or around the C756-P763 region of plasmin and the C564-P571 region of thrombin.

Only one of six plasmin-reactive mAb from APS patients inhibits the fibrinolytic activity of plasmin. Therefore, it may be problematic to assess the clinical significance of all anti-plasmin Ab in APS by association studies of the presence of anti-plasmin Ab to APS, although Fig. 2 does show that IgG anti-plasmin Ab levels in APS were significantly higher than those in normal controls. In addition, it may be problematic to study the functional activities of affinity-purified, polyclonal anti-plasmin Ab from patients. Instead, it will first be necessary to develop an assay to detect CL15-like anti-plasmin Ab (that inhibits plasmin-mediated fibrinolysis) and then use it to study the roles of such anti-plasmin Ab in thrombosis in APS patients.

Our accumulated analyses of seven mAb generated by screening against CL in the presence of bovine serum reveal that five of seven bind to thrombin, PT, plasmin, and plasminogen. Of these five mAb, IS3, IS4, CL1, and CL24 also react with β_2 GPI (17), the major autoantigen or cofactor for autoantibodies detected by the conventional aCL ELISA. Among the five reactive autoantigens, all five mAb bind to plasmin with the highest affinity, suggesting that plasmin may be the key autoantigen that drives the aPL B cells in APS patients, and that some induced autoantibodies may act directly by binding to plasmin and inhibiting fibrinolysis, leading to a prothrombotic state. Alternatively, some induced Ab may cross-react with other autoantigens in the coagulation cascade, such as thrombin, and promote thrombosis (like CL24 in reducing inactivation of thrombin by AT) (16). In the future it will be important to test this hypothesis by immunizing mice with plasmin and then studying the induced Ab. If immunization with plasmin leads to prothrombotic aPL, it will be interesting to explore the possibility of inhibiting aPL production by tolerizing mice with plasmin with or without modification.

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