

Antibodies with Dual Reactivity to Plasminogen and Complementary PR3 in PR3-ANCA Vasculitis

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ABSTRACT

Patients with inflammatory vascular disease caused by anti-neutrophil cytoplasmic autoantibodies (ANCA) can harbor antibodies not only to the autoantigen proteinase 3 (PR3) but also to complementary PR3 (*cPR3*^{105–201}), a recombinant protein translated from the antisense strand of PR3 cDNA. The purpose of this study was to identify potential endogenous targets of anti-*cPR3*^{105–201} antibodies. Patients' plasmapheresis material was tested for the presence of antigens reactive with affinity-purified rabbit and chicken anti-*cPR3*^{105–201} polyclonal antibodies. Antigen-containing fractions were tested with patients' anti-*cPR3*^{105–201} affinity-purified IgG, and putative protein targets were sequenced by mass spectrometry. Unexpectedly, plasminogen was identified as a target of anti-*cPR3*^{105–201}. Reactivity of affinity-purified antibodies from two patients was lost when plasminogen was converted to plasmin, indicating restricted specificity. Antiplasminogen antibodies from five patients bound plasminogen at a surface-exposed loop structure within the protease domain. This loop contains an amino acid motif that is also found in a portion of recombinant *cPR3*^{105–201}; site-directed mutagenesis of this sequence decreased antibody reactivity by 30%. Functionally, antiplasminogen antibodies delayed the conversion of plasminogen to plasmin and increased the dissolution time of fibrin clots. Serologically, antiplasminogen antibody levels were higher in PR3-ANCA patients ($n = 72$) than healthy control subjects ($n = 63$), myeloperoxidase-ANCA patients ($n = 34$), and patients with idiopathic thrombosis ($n = 57$; $P = 0.001$). Of the patients with PR3-ANCA, nine had documented deep venous thrombosis events, five of whom were positive for antiplasminogen antibodies. In summary, capitalizing on interactions with complementary proteins, specifically complementary PR3, this study identified plasminogen as a previously undescribed autoantigen in PR3-ANCA vasculitis.

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Of the autoimmune diseases studied by our group, one of the most aggressive is glomerulonephritis caused by anti-neutrophil cytoplasmic autoantibodies (ANCA) specific for the neutrophil granule protein proteinase 3 (PR3) or myeloperoxidase (MPO).^{1,2} ANCA activate neutrophils and monocytes, causing inappropriate release of granule constituents, thereby causing injury to vessel walls, in particular, the glomerular capillaries of the kidney and alveolar capillaries of the lung.³ Our serendipitous discovery, that patients with PR3-specific

ANCA (PR3-ANCA) also had antibodies against a protein coded by the antisense strand of the PR3

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cDNA (*cPR3*), led to the proposal of autoantigen complementarity as an underlying mechanism of this autoimmune disease.⁴ The goal of this study was to explore the etiology and functional consequences of anti-*cPR3* antibodies existing in these patients. The data herein describe our findings from screening patients' plasmapheresis material for proteins recognized by these antibodies.

Important concepts that have influenced our ongoing research lie within the principles of complementary protein interactions. Beginning in the mid-1960s, it was postulated that a protein translated 5'-3' from antisense RNA is a *complementary* counterpart of the protein coded by the sense RNA and that these two proteins uniquely interact.^{5,6} Since that time, many investigators have used this concept to identify proteins that interact, such as ligands with receptors, antigens with antibodies, and antibodies with antibodies.⁷⁻¹² It was later shown that antibodies against a sense protein and antibodies against the complement of that sense protein form an idiotypic pair through complementarity of their variable regions.¹³ Idiotypic antibody pairs have been implicated in a number of autoimmune diseases, including myasthenia gravis,¹⁴ Grave's disease,¹⁵ and primary biliary cirrhosis.¹⁶

To gain insight into potential pathologic contributions of anti-*cPR3* antibodies, we sought to identify reactive proteins existing in the circulation of PR3-ANCA patients. Because plasmapheresis is often a treatment of choice, this protein-rich material from acutely active patients provided the needed resources. We report that a protein reactive with patients' affinity-purified anti-*cPR3* antibodies was isolated and identified by mass spectrometry as plasminogen. Anti-plasminogen reactivity was found to be highly restricted to plasminogen and not to plasmin. These anti-plasminogen antibodies delayed fibrin clot dissolution *in vitro* and occurred most commonly in PR3-ANCA small-vessel vasculitis (SVV) patients with coincident thrombotic events.

RESULTS

Identification of a Protein Reactive with Anti-*cPR3* Antibodies

One intervention used in treating ANCA-SVV is a plasma exchange procedure, which removes large amounts of Ig and

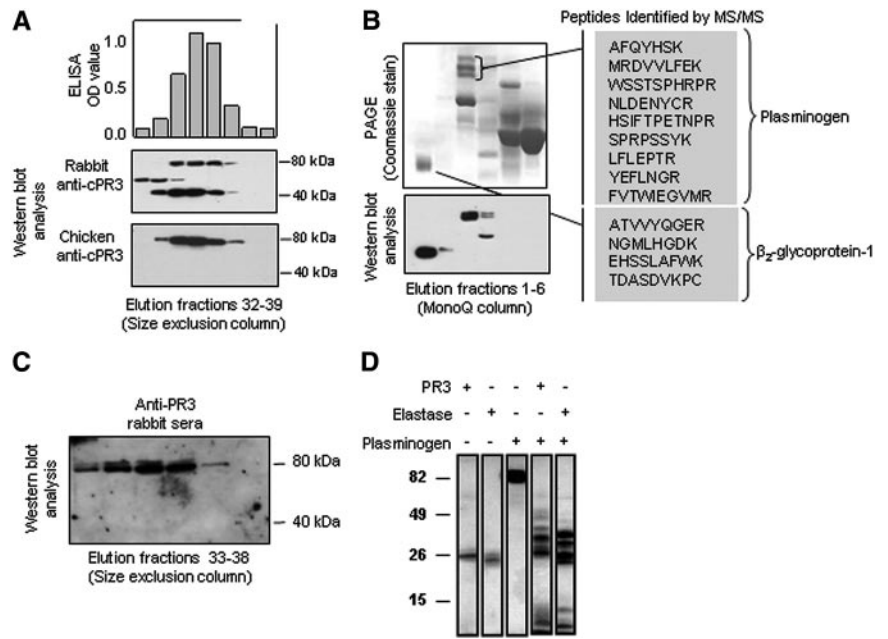


Figure 1. Identification of a protein reactive with anti-*cPR3* antibodies. (A) Plasmapheresis proteins were fractionated by size exclusion chromatography and fractions 32 through 37 contained protein/s reactive with rabbit anti-*cPR3*¹³⁸⁻¹⁶⁹ antibodies by ELISA. Western blot analysis identified three reactive proteins (approximately 80, approximately 50, and approximately 40 kD) using the rabbit antibody; one reactive band was seen using the chicken antibody. (B) Fractions 32 through 37 from the size exclusion column were pooled and further purified on a MonoQ ion exchange column. The approximately 80-, approximately 50-, and approximately 40-kD proteins were again eluted as determined by PAGE and Western blot analysis. The approximately 80-kD protein was identified as plasminogen by in-gel tryptic digest and mass fingerprinting, and the approximately 40-kD protein was identified as β_2 -glycoprotein-1. The approximately 50-kD protein could not be identified on the Coomassie stained gel. (C) For determination of whether native PR3 protein was present in the protein fractions, a Western blot analysis was performed using serum from a rabbit immunized with human PR3. The 30-kD PR3 protein band was not present; however, there was reactivity with an 80-kD band identified as plasminogen. (D) Plasminogen was incubated with PR3 or elastase for 1 h at 37°C. The reaction was stopped by addition of nonreducing SDS buffer, and the samples were separated by SDS-PAGE and stained with Coomassie R-250. The results show cleavage of plasminogen by native PR3.

other proteins from the patient's plasma. We proposed to probe the material removed from the plasma (PLEX) of two PR3-ANCA patients (patient A and patient B) for proteins reactive with anti-*cPR3* antibodies. These antibodies, produced in rabbit and chicken, were raised against a peptide fragment of the complementary-PR3 protein and affinity purified. This fragment, termed *cPR3*¹³⁸⁻¹⁶⁹, corresponds to PR3 residues 138 through 169 and was previously identified as being reactive with PR3-ANCA patient sera in an epitope-mapping experiment using blunt-ended PR3 cDNA fragments in a bacterial expression library. Proteins were separated by size exclusion column chromatography, and resulting fractions were tested for reactivity with anti-*cPR3*¹³⁸⁻¹⁶⁹ antibodies by ELISA (Figure 1A). Positive fractions were further analyzed by Western blot revealing three protein bands reactive with the rabbit anti-

cPR3^{138–169} antibody, whereas the chicken anti-*cPR3*^{138–169} antibody reacted with only one protein band (Figure 1A; data shown are from patient A; patient B results were similar). Proteins were further fractionated by MonoQ ion exchange chromatography and separated by SDS-PAGE (Figure 1B). Bands of interest were subjected to in-gel tryptic digest and analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS).¹⁷ The molecular weights of the tryptic peptides from the larger protein band aligned with plasminogen, whereas those from the lower band aligned with β_2 -glycoprotein-1. The middle protein band could not be positively identified. The implication of these findings is that plasminogen is a protein reactive with anti-*cPR3* antibodies.

We propose the anti-plasminogen/*cPR3*^{138–169} antibody is a product of the anti-idiotypic network. The anti-idiotypic response is bidirectional. Antibodies generated after *cPR3*^{138–169} peptide immunization would lead to the production of anti-PR3 protein antibodies. Likewise, antibodies generated after PR3 protein immunization would lead to production of anti-plasminogen/*cPR3*^{138–169} antibodies. Analysis of sera from a chicken and a rabbit, each immunized with human-*cPR3*^{138–169} peptide, showed the presence of antibodies to human PR3 protein by ELISA (data not shown), and serum from a rabbit immunized with PR3 protein contained antibodies reactive with plasminogen (Figure 1C).

To explore the relationship between PR3 and plasminogen, we determined whether plasminogen is a substrate of PR3. It has been reported that elastase, a close homolog of PR3, cleaves plasminogen.¹⁸ PR3 cleaved plasminogen into discrete fragments (Figure 1D), thus indicating that the two proteins physically interact.

Anti-plasminogen Antibodies in PR3-ANCA-Positive Patients

We next examined whether affinity-purified anti-*cPR3*^{138–169} antibodies from both patient A and patient B were reactive with plasminogen. Each reacted with plasminogen, indicating dual reactivity to both plasminogen and a complementary-PR3-like protein. Specificity was marked by, first, neither reacting with β_2 -glycoprotein-1 or the unidentified 50-kD protein band by Western blot analysis; second, that reactivity was competed with addition of *cPR3*^{138–169} peptide (Figure 2A); and, third, that antibodies recognized only the nonreduced

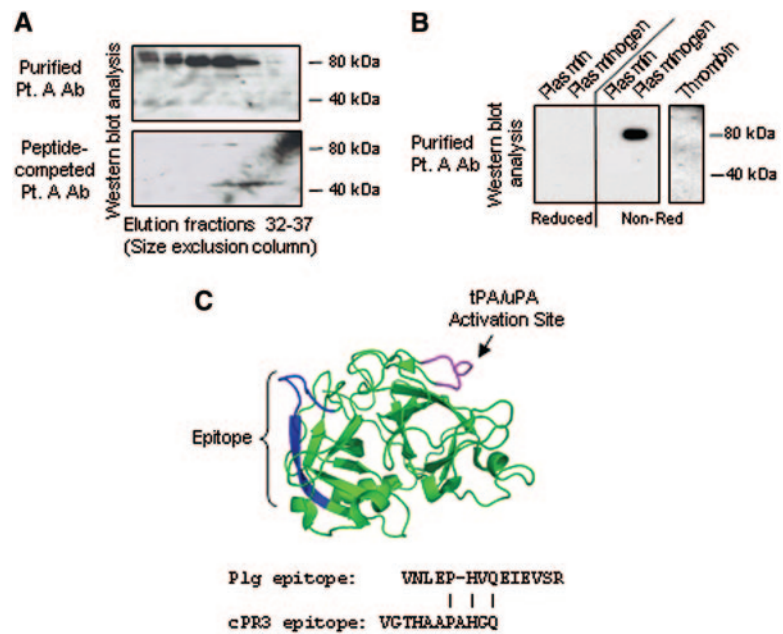


Figure 2. Patient IgG, affinity-purified using a *cPR3*^{138–169} peptide column, reacts with plasminogen. (A) Affinity-purified patient antibody was used for Western blot analysis of protein fractions reactive with the rabbit and chicken anti-*cPR3*^{138–169} antibodies. Plasminogen was recognized by the antibody, whereas β_2 -glycoprotein-1 was not. When *cPR3*^{138–169} peptide was incubated with patient IgG before addition to the nitrocellulose, antibody binding to plasminogen was competed away. (B) Commercially prepared plasminogen, plasmin, and thrombin were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the patient affinity-purified antibody. The patient antibody reacts with nonreduced plasminogen but not with reduced plasminogen, plasmin, or thrombin. (C) Representation of the target epitope of the antiplasminogen antibodies within the catalytic domain of plasminogen (PDB 1DDJ), which is shown in blue. This epitope is shown in relation to the tPA/uPA activation site, which is indicated in purple. An alignment between the sequences for the plasminogen epitope and the target epitope on *cPR3*^{138–169} shows some common residues.

plasminogen protein and did not react with plasmin or with thrombin (Figure 2B).

Using an MS approach, we determined the amino acid sequences of *cPR3* and plasminogen that interacted with affinity-purified anti-*cPR3*^{138–169} antibodies. This method relies on the bound Ig protecting the epitope from proteolytic cleavage after addition of particular proteases. The data revealed the motif of VNLEPHVQEIEVSR as a favored epitope on plasminogen and VGTHAAPAHGQ on *cPR3*^{138–169} peptide. For ensuring that these data were not biased as a result of the use of affinity-purified antibodies, the assay was performed with total IgG fractions. IgG from three additional PR3-ANCA patients contained antibodies that bound the same favored motif on plasminogen. Similar to affinity-purified antibodies from patients A and B, reactivity seemed to be restricted to plasminogen by Western blot analysis, with no detectible reactivity against plasmin or prothrombin (data not shown). The P-HXQ motif seems to be the common epitope between plasminogen and *cPR3*^{138–169} peptide. Site-directed mutagenesis of the PAHGQ motif in complementary PR3 recombinant protein resulted in a 30% decrease in re-

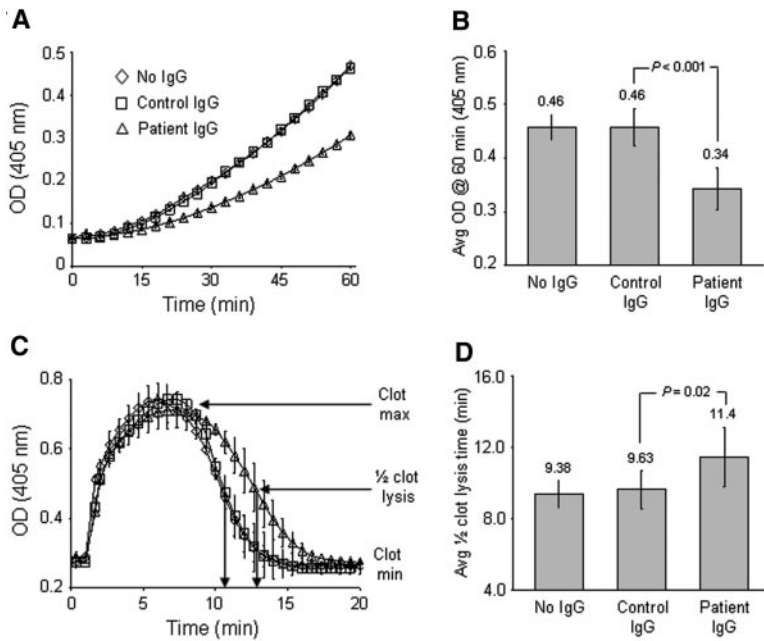


Figure 3. Functional effects of antiplasminogen antibodies. (A) An *in vitro* assay was performed to determine the rate of plasmin formation in the presence of antiplasminogen antibodies by combining plasminogen, uPA or tPA, and a chromogenic substrate with and without control human IgG or affinity-purified antibodies. Shown is the average of two replicates for one patient’s antibodies in the presence of uPA. (B) The average \pm SD absorbance after 60 min is shown from two independent experiments for patients A and B with both uPA and tPA. Antiplasminogen antibodies decreased the conversion of plasminogen to plasmin, when compared with HBS buffer or normal human IgG at the same concentration. (C) An *in vitro* clotting assay examined how antiplasminogen antibodies affect fibrin clot formation and/or dissolution. Normal human plasma was combined with HBS buffer alone, normal human IgG, or patient antiplasminogen antibodies, in the presence of thrombin and uPA. Clot formation and dissolution were monitored by change in absorbance at 405 nm. Shown is the average \pm SD of two replicates for one patient’s antibodies. (D) The average \pm SD 1/2 clot lysis time is shown from two independent experiments for patients A and B. Antiplasminogen antibodies delayed the fibrinolysis of the clot. Statistical analysis done by *t* test.

activity of total IgG from these three PR3-ANCA positive patients.

Assessment of Antiplasminogen Antibodies on Plasminogen Function

The epitope of antiplasminogen antibodies resides in the catalytic domain of plasminogen, which is spatially removed from the tissue-type plasminogen activator (tPA)/urokinase-type plasminogen activator (uPA) cleavage site (Figure 2C). Cleavage by tPA or uPA converts plasminogen into plasmin, an active protease capable of fibrinolysis.¹⁹ We tested whether antibody binding to plasminogen would affect tPA/uPA-induced activation. Conversion of plasminogen was reduced in the presence of affinity-purified anti-cPR3^{138–169} antibodies from patient A (Figure 3A). When the data from all assays performed using affinity-purified antibodies from both patient A and patient B were combined, there was a significant decrease in the conversion of plasminogen to plasmin as compared with addi-

tion of control human IgG ($P < 0.001$; Figure 3B). Next we analyzed the effect of the patients’ antibodies on clot formation using an *in vitro* clotting assay. Normal human plasma was incubated with patients’ affinity-purified anti-cPR3^{138–169} antibodies, normal human IgG, or assay buffer. Thrombin and urokinase were then added, and clot formation and dissolution were monitored by change in absorbance at 405 nm. Antibodies from patient A delayed clot lysis (Figure 3C). An average of 1/2 clot lysis time from testing affinity-purified antibodies from patients A and B in two separate experiments showed a significant increase in lysis time when compared with normal human IgG ($P = 0.02$; Figure 3D). This delay did not involve increased thrombin generation or activation of the thrombin-activatable fibrinolysis inhibitor, because the level of calcium present in the assay was insufficient to cause activation of endogenous clotting factors.²⁰

Prevalence of Antiplasminogen Antibodies

The prevalence of antiplasminogen antibodies in a PR3-ANCA patient population was determined by ELISA analysis (Figure 4A). Demographics of study participants are shown in Table 1. The level of antiplasminogen antibodies was higher in the PR3-ANCA patients (16 [22%] of 72), as compared with four (6%) of 63 healthy control subjects, two (6%) of 34 MPO-ANCA patients, and five (9%) of 57 patients with idiopathic thrombosis ($P = 0.001$).

Focusing on patients with deep venous thrombosis (DVT), we identified nine of 72 PR3-ANCA patients with events (six with Wegener’s granulomatosis and three with microscopic polyangiitis; Figure 4B). Comparisons of the levels of PR3-ANCA and antiplasminogen antibody at the time of the thrombotic events (Table 2) indicated that patients with a positive ANCA also had positive antiplasminogen antibodies except for one 15-yr-old patient. Of the nine PR3-ANCA patients with thrombotic events, five (56%) were positive for antiplasminogen antibodies compared with zero (0%) of four MPO-ANCA patients and five (9%) of 57 patients with idiopathic thrombosis ($P = 0.002$). As assessed by the Birmingham Vasculitis Activity Score, the five antiplasminogen antibody-positive PR3-ANCA patients had active disease, whereas the four negative sera came from patients in remission with a Birmingham Vasculitis Activity Score of 0. An extensive workup for thrombophilic defects in all PR3-ANCA patients with a throm-

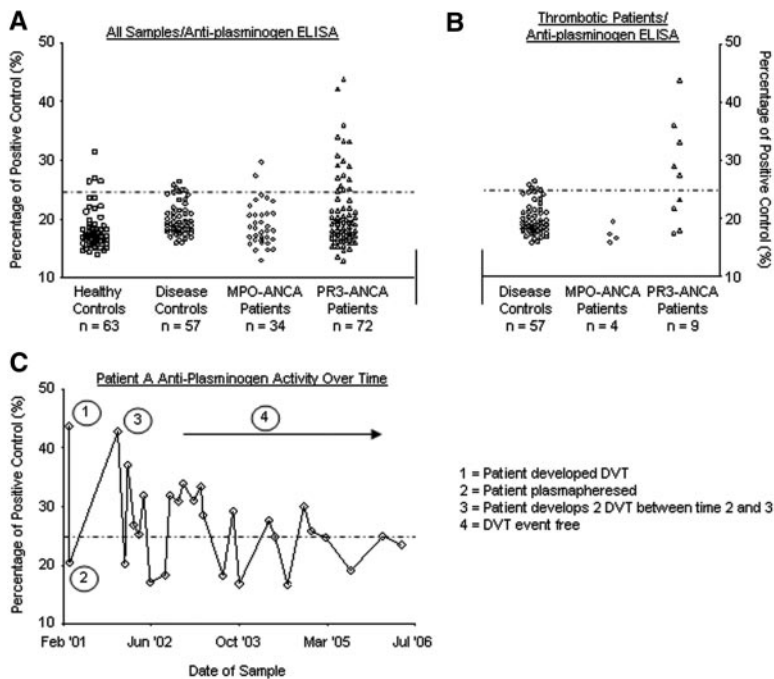


Figure 4. Prevalence of antiplasminogen antibodies. (A) A plasminogen ELISA shows that 16 (22%) of 72 PR3-ANCA patients are positive for antiplasminogen antibodies. This compares with four (6%) of 63 healthy control subjects, five (9%) of 57 patients with idiopathic thrombosis, and two (6%) of 34 MPO-ANCA patients. A positive value is defined as 2 SD above the mean of 63 healthy control subjects (25.6%), as marked by the dashed line. (B) When patients who had thrombotic events are plotted alone, five (56%) of nine PR3-ANCA patients are reactive, compared with five (9%) of 57 disease controls and zero (0%) of four MPO-ANCA patients. (C) Multiple samples from patient A were tested by ELISA to monitor the change in antiplasminogen autoantibody levels compared with DVT events over time. The patient presented with a DVT and had a high antiplasminogen autoantibody level (1). The patient received plasmapheresis treatment, and the antiplasminogen autoantibody level subsequently decreased (2). During the subsequent 9 mo, the patient developed two more DVT, and the next available sample collected shows an increase in the antiplasminogen autoantibody level (3). The patient has not experienced another DVT, and the antiplasminogen autoantibody level has trended downward (4).

botic event revealed no abnormalities. Importantly, none of the PR3-ANCA thrombotic patients had nephrotic-range proteinuria or a history of DVT before the onset of the disease. Four MPO-ANCA patients similarly developed a thrombosis. No differences were found with respect to risk factors for venous thromboembolism among the PR3-ANCA, MPO-ANCA, and the total set of ANCA-SVV patients.

Sera samples from patients collected over a number of years allowed the tracking of autoantibody fluctuations and their association with DVT events. Representative of the data (Figure 4C), a patient with a DVT presented with high levels of antiplasminogen antibodies (number 1). The patient received plasmapheresis treatment, and the antibody titer dropped (number 2). During the next 9 mo, the patient experienced two more DVT events, and the antiplasminogen antibody levels were again very high (number 3). Since that time, the patient has not experienced a DVT, and the antiplasminogen antibody level has trended downward (number 4).

DISCUSSION

Capitalizing on the concept of complementary protein–protein interactions led to the discovery of a previously unidentified autoantigen. This immune response was highly restricted because conformational changes in plasminogen after cleavage of a single bond, by tPA²¹ or uPA²² producing the active form of plasmin, abrogated their reactivity. The antiplasminogen antibodies reacted with a highly restricted motif on plasminogen, also found in *cPR3*^{105–201}. These antibodies were demonstrated to have the potential to alter fibrinolysis and were statistically correlated with thrombotic propensity in PR3-ANCA patients. The data provide sufficient evidence to support the hypothesis that a relationship exists between antiplasminogen antibodies and thrombotic events; however, to establish firm conclusions for causality, additional prospective cross-sectional studies will be required.

Complementary protein pairs have been used to investigate receptor–ligand interactions and for biochemical engineering studies.¹¹ There is no clear explanation of why this interaction occurs, although one group of investigators proposed that the binding is a consequence of opposing hydrophobic profiles.⁷ They furthered this understanding by demonstrating that antibodies raised to complementary proteins had an anti-idiotypic relationship.^{8,13} This relationship was similar to what we previously demon-

strated with PR3-ANCA and antibodies to complementary-PR3.⁴ Yet another investigator posited a variation on these themes by defining a complementary pair as two proteins capable of stereospecific binding, with the stipulation that they induce molecularly complementary antibodies or T cell antigen receptors.²³ We found that rabbits immunized with human PR3 develop antibodies not only to PR3 but also to human plasminogen, and the converse is also true that mice, chickens, and rabbits inoculated with a complementary-PR3 peptide develop antibodies to this peptide as well as PR3. The most logical explanation for this is that the idiotypic network is responsible for the derivation of the secondary antibody response.

That >50% of the PR3-ANCA patients with known thrombotic episodes had antiplasminogen antibodies is intriguing. These data do not prove a relationship between antiplasminogen antibodies and thrombotic events but do point to the necessity to perform a prospective study to examine this relation-

Table 1. Demographics and clinical diagnosis of study participants^a

Characteristic	All ANCA-SVV (n = 106)	PR3-ANCA (n = 72)	MPO-ANCA (n = 34)	PR3-ANCA with Thrombosis (n = 9)	Idiopathic DVT Control Group (n = 57)
Gender (n [%])					
male	56 (52.8)	42 (58.3)	14 (41.2)	6 (66.7)	23 (40.4)
female	50 (47.2)	30 (41.7)	20 (58.8)	3 (33.3)	34 (59.6)
Race (n [%])					
white	89 (84.0)	64 (88.9)	25 (73.5)	7 (77.8)	53 (93.0)
black	10 (9.4)	5 (6.9)	5 (14.7)	2 (22.2)	4 (7.0)
other	7 (6.6)	3 (4.2)	0 (0.0)	0 (0.0)	0 (0.0)
Age (yr)					
mean	53.6 ± 19.0	51.2 ± 19.4	58.2 ± 17.8	51.9 ± 19.2	45.1 ± 15.2
range	13 to 86	13 to 86	17 to 86	15 to 74	16 to 86
Clinical diagnosis (n [%])					
WG	44 (41.5)	38 (52.8)	6 (17.6)	6 (66.7)	
MPA	49 (46.2)	29 (40.3)	20 (58.8)	3 (33.3)	N/A
Churg-Strauss syndrome	4 (3.8)	2 (2.8)	2 (5.9)	0 (0.0)	
renal limited vasculitis	9 (8.5)	3 (4.2)	6 (17.6)	0 (0.0)	

^aMPA, microscopic polyangiitis; WG, Wegener’s granulomatosis.

Table 2. Clinical diagnosis and antibody levels for nine patients with DVT

Patients	Age	Diagnosis	PR3-ANCA Level at Thrombotic Event ^a	Anti-plasminogen Level of Sample Tested ^a
1	15	WG	153.0	Neg
2	46	WG	106.9	29.0
3	50	WG	104.8	43.7
4	64	WG	39.0	33.2
5	63	WG	32.5	36.0
6	57	WG	Neg	27.5
7	51	MPA	Neg	Neg
8	23	MPA	Neg	Neg
9	74	MPA	Neg	Neg

^aThe cutoff for positivity in the plasminogen ELISA was 25.6; the cutoff for positivity in the PR3 clinical test is >20.

ship more closely. Anti-plasminogen antibodies do not seem to be long lasting; therefore, the prevalence of these antibodies in PR3-ANCA patients may be much higher if more samples are evaluated during the course of the disease. It is unlikely that these antibodies are a consequence of a thrombotic event as evidenced by the thrombophilic patient controls who did not have a high prevalence of anti-plasminogen antibodies. None of the ANCA patients had typical risk factors for venous thrombosis or any thrombophilic abnormalities. Our findings may be relevant to a few recent reports that examined venous thrombotic events (VTE) in ANCA vasculitis patients. One study reported 13 of 105 ANCA vasculitis patients developed a VTE.²⁴ Ten of these patients were PR3-ANCA positive and three were MPO-ANCA positive, a percentage quite similar to our findings. A second study showed that 29 of 180 patients in a clinical trial of Wegener’s granulomatosis developed a VTE.²⁵ As a follow-up, those investigators examined the 180 patients for presence of anticardiolipin and anti-β₂-glycoprotein-1 antibodies along with several genetic hypercoagulable factors and found no difference between patients who developed a VTE and those who did not.²⁶

Before firm conclusions can be made about pathogenic epitopes, a comprehensive study of antibody specificity against plasminogen, plasmin, thrombin, prothrombin, and β₂-glyco-

protein-1 is needed. Anti-plasminogen antibodies have been previously reported.²⁷ Anti-plasmin antibodies detected in patients with antiphospholipid antibody syndrome were found to cross-react with plasminogen, but these autoantibodies were reported as nonpathogenic. This was not the case with the antibodies tested here. Other reports have described antibodies that reacted with both prothrombin and plasminogen through their kringle domains.^{28,29}

Some patients in our study had anti-plasminogen antibodies but no clinical evidence for a thrombotic event. One explanation, that takes into consideration the *in vitro* data, is that inadequacies in clot dissolution would be of no consequence unless a clot is forming. A second consideration is that aggressive treatment with immunosuppression and plasmapheresis may reduce the titer of anti-plasminogen antibodies to levels below a threshold required for phenotypic expression. Anti-plasminogen antibodies may increase the likelihood of a thrombotic event but are not sufficient by themselves to cause such an event, as has been speculated for patients with antiphospholipid antibodies but no thrombotic episodes. Anti-plasminogen antibodies were reported in thrombosis patients with systemic lupus erythematosus, but the antibodies had no relationship with the thrombotic events.³⁰

Our studies do not illuminate the cause of PR3-ANCA, al-

though there are a number of microbes linked to the onset of ANCA^{31,32} that bear proteins complementary to PR3.³³ What this study does demonstrate is a novel approach to elucidate new autoantigens; however, they do demonstrate the presence of a novel and important autoantibody system based on the complementary protein interaction hypotheses. Interestingly, antiplasminogen antibodies have also been found in a rat model of human membranous nephropathy known as Heymann nephritis,³⁴ whereby antibodies form to gp330, which belongs to the LDL receptor superfamily and also binds to plasminogen.³⁵ Patients with membranous nephropathy have venous thrombotic episodes, and it would be interesting to determine whether these patients have antibodies to plasminogen as well.

Studying complementary protein interactions provides an elucidation of the perplexing question of why patients with autoimmune diseases have autoimmune responses to structurally different antigens. These studies do provide a novel approach for the discovery of antibodies and autoantigens that may have implications in the broadening field of autoimmunity.

CONCISE METHODS

Study Populations

The 106 patients with ANCA-SVV in this study had pauci-immune necrotizing and crescentic glomerulonephritis and a positive PR3-ANCA ($n = 72$) or MPO-ANCA ($n = 34$) determination. Patients were classified into types of ANCA-SVV as defined by the Chapel Hill nomenclature.³⁶ Patients consented to long-term follow-up in the prospective cohort studies from the time of disease onset (diagnostic entry biopsy) until death. Blood samples were available at the time of clinically indicated diagnostic vasculitis testing and thus not always available at the time of venous thrombotic events.

Control subjects included 57 patients randomly selected from the thrombophilia service with a history of idiopathic DVT or pulmonary emboli of unknown cause. All study participants were evaluated for the presence of classic risk factors for venous thromboembolism. A group of 63 healthy individuals who were either kidney or blood donors constituted a healthy control group. All studies were approved by the University of North Carolina School of Medicine institutional review board.

Antigens/Proteins/Antibodies

Full-length recombinant *cPR3*^{105–201} was produced as described previously.⁴ Site-directed mutagenesis of the *cPR3*^{105–201} protein resulted in residues 46, 48, and 50 (proline, histidine, and glutamine, respectively) being changed to alanines.

Human neutrophil PR3 was obtained from Elastin Products (Owensville, MO). Human plasminogen, plasmin, and thrombin were obtained from Hematologic Technologies (Essex Junction, VT). tPA was supplied by Dr. Alisa Wolberg. uPA and elastase were obtained from Sigma (St. Louis, MO). *cPR3*^{138–169} was produced

by Alpha Diagnostic (San Antonio, TX). Antibodies specific to *cPR3*^{138–169} (Alpha Diagnostic) were separated from immunized chicken egg yolk (IgY), rabbit serum (IgG), and patient total IgG by use of a *cPR3*^{138–169} peptide affinity column (GE Biosciences, Piscataway, NJ). We used rabbit antibody to histidine and goat antibody to plasminogen (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit antisera to PR3 (Weislab, Lund, Sweden); normal human IgG (Bethyl Laboratories, Montgomery, TX); and horseradish peroxidase- and alkaline phosphatase-conjugated secondary antibodies specific for rabbit IgG, human IgG, and chicken IgY (Chemicon, Temecula, CA).

Purification and Identification of Plasmapheresis Proteins

Human serum proteins were purified from patient-derived plasmapheresis material by a series of chromatographic purifications. A 50-ml aliquot of plasmapheresis fluid was centrifuged at 3000 rpm to pellet insoluble material before filtering through a 0.22- μ m filter (Costar, Lowell, MA). IgG was removed with a protein G column (GE Biosciences), the IgG-depleted material was passed over a Superdex 200 size exclusion column (GE Biosciences), and fractions reactive to anti-*cPR3*^{138–169} antibody were further separated on a MonoQ ion exchange column (GE Biosciences). All protein purification was performed on an AKTA FPLC with a Frac-950 fraction collector (GE Biosciences). Protein identification was achieved by in-gel tryptic digestion of SDS-PAGE-separated proteins followed by fingerprint identification with MALDI-MS.

ELISA

ELISA detection of anti-*cPR3*^{105–201} antibodies was performed as described previously.⁴ For ELISA detection of antiplasminogen antibodies, EIA/RIA high-binding 96-well plates (Costar) were coated overnight at 4°C with plasminogen (5 μ g/ml), blocked 1 h in 1% goat serum, and incubated with patient sera (diluted 1:100) for 2 h. Bound IgG was detected by alkaline phosphatase-conjugated goat anti-human IgG antibody. Optical density at 405 nm was measured using a VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA). Positivity was defined as a value >2 SD above the mean of 63 healthy control subjects (25.6%). All available sera samples were tested with the highest value for each patient used in the analysis as a marker of peak antiplasminogen reactivity.

PR3 Proteolysis Assay

For the proteolysis assay, PR3 (3.7 μ M) or elastase (0.4 μ M) was incubated with plasminogen (0.4 μ M) for 90 min at 37°C. The reaction was stopped by addition of SDS buffer. Samples were separated by nonreducing SDS-PAGE and stained with Coomassie R-250. Proteolysis was determined visually by examination of the stained gel.

Epitope Mapping of Antiplasminogen Antibodies

Epitope mapping was performed as described previously.³⁷ Patient antiplasminogen antibodies were coupled to cyanogen bromide-activated Sepharose beads (GE Biosciences). Plasminogen or *cPR3*^{138–169} peptide was incubated with antibody-linked beads for 2 h. Nonpeptide regions were degraded by sequential overnight addition of

Lys-C (Wako Chemicals, Richmond, VA), trypsin-TPCK (Worthington Biochemical, Lakewood, NJ), and aminopeptidase M and carboxypeptidase Y (Roche, Indianapolis, IN). Remaining fragments were analyzed by MALDI-MS. MS/MS analysis confirmed the epitope sequence.

In Vitro Plasminogen Assays

To determine the effect of antiplasminogen antibodies on plasminogen-to-plasmin conversion, plasminogen (15 $\mu\text{g/ml}$) was preincubated with affinity-purified patient antiplasminogen antibodies (30 $\mu\text{g/ml}$), control human total IgG (30 $\mu\text{g/ml}$), or HBS buffer (20 mM HEPES, 150 mM NaCl, and 5 mM Ca^{2+} [pH 7.4]) for 10 min. uPA (3 nM) or tPA (13 $\mu\text{g/ml}$) was combined with Spectrozyme PL (500 μM ; American Diagnostic, Stamford, CT) in HBS buffer and added to the plasminogen/antibody mixture to activate plasminogen. Change in absorbance over time at 405 nm was monitored in duplicate samples.

For determination of the antifibrinolytic effects of the affinity-purified patient antiplasminogen antibodies, normal human plasma (90% final concentration) was preincubated with affinity-purified antibodies (50 $\mu\text{g/ml}$), control human total IgG (50 $\mu\text{g/ml}$), or HBS buffer for 10 min. Plasma was added to a 96-well plate containing thrombin (15 nM) and uPA (30 nM). Clot formation and dissolution were monitored by absorbance at 405 nm.³⁸ Each reaction was performed in duplicate.

Statistical Analysis

Statistical analysis of the *in vitro* plasminogen assays was performed using a *t* test. Antiplasminogen antibodies are plotted as a continuous measure of the percentage of positive control; however, the frequency of positive antiplasminogen antibodies and not the continuous value was the primary measure of interest. Therefore, statistical comparisons between the prevalence of positives between patient groups were evaluated using a two-sided Fisher exact test to accommodate the small number of positive values in several patient groups.

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DISCLOSURES

None.

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