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Inhibition of proteinase 3 by ANCA and its correlation with disease activity in Wegener's granulomatosis

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Inhibition of proteinase 3 by ANCA and its correlation with disease activity in Wegener's granulomatosis. Detection of circulating anti-neutrophil cytoplasmic antibodies (ANCA) to the neutrophil serine proteinase, proteinase 3 (PR3), has proven valuable for the diagnosis of Wegener's granulomatosis (WG). However, the importance of these autoantibodies in the pathogenesis of WG remains unknown. It was recently reported that anti-PR3 autoantibodies (PR3-ANCA) from some patients with WG inhibit the proteolytic activity of PR3 and interfere with the inactivation of PR3 by the physiologic inhibitor, α_1-proteinase inhibitor (α_1-PI). We have studied the effect of PR3-ANCA on the enzymatic activity of PR3 and its correlation with disease activity in patients with WG. We purified IgG from 21 PR3-ANCA positive sera obtained from 17 patients with WG, and determined its effect on the esterolytic and proteolytic activity of purified human PR3 using Boc-Ala-O-Nitrophenyl ester and fluoresceinated-elastin as enzyme substrates. Controls included seven sera containing anti-MPO autoantibodies (MPO-ANCA) from patients with systemic vasculitis and seven ANCA-negative sera obtained from healthy individuals. We found that PR3-ANCA from 9 of the 17 patients significantly inhibited the activity of PR3. There was no correlation between the titers of PR3-ANCA and their inhibitory activity. For one extensively characterized autoantibody, the inhibition reached 70 to 95% at 20-fold molar excess of IgG to enzyme, with an apparent $K_i app$ of 56.5 μM. This inhibition was non-competitive in nature, and was additive to that produced by α_1-PI. A review of the clinical histories of the patients revealed a strong association between active WG and inhibitory autoantibodies. PR3-ANCA from eight of 10 patients with active disease showed significant inhibition of PR3 activity, whereas only one of seven patients in remission had inhibitory PR3-ANCA. These results suggest that the inhibitory profile of PR3-ANCA may be more reflective of disease activity in patients with WG than the absolute titer, and suggest possible alternative mechanisms for the role of these autoantibodies in the pathogenesis of WG.

Wegener's granulomatosis (WG) is a systemic disease of unknown etiology which is characterized by necrotizing granulomas in the upper and lower respiratory tract, vasculitis that can affect multiple organ systems, and necrotizing glomerulonephritis [1, 2]. Although limited forms of the disease with no renal involvement are commonly present early on, the disease usually becomes generalized with time and is invariably fatal if left untreated [1]. Immunosuppressive therapy with cyclophosphamide and corticosteroids is generally effective in inducing remissions. The disease, however, has a tendency to relapse when therapy is discontinued and long-term treatment is associated with severe side effects [3]. Precise monitoring of disease activity in WG is, therefore, of great importance so that the immunosuppressive therapy can be tailored according to the patient's need.

The recently discovered association of anti-neutrophil cytoplasmic antibodies (ANCA) with WG and other forms of systemic necrotizing vasculitis, such as microscopic polyangiitis (MPA) and renal-limited vasculitis (idiopathic necrotizing/crescentic glomerulonephritis), has significantly aided the diagnosis of these disorders [4–7]. ANCA have most commonly been detected by indirect immunofluorescence assay using ethanol-fixed human neutrophils as substrate. Two characteristic staining patterns have been described: cytoplasmic or classic pattern (c-ANCA), produced by antibodies against the myeloid lysosomal serine proteinase, proteinase 3 (PR3) [8–10], and perinuclear pattern (p-ANCA), caused by antibodies against myeloperoxidase (MPO) [6]. C-ANCA (PR3-ANCA) have been shown to be both sensitive and specific for active WG [5, 7, 11].

The pathogenesis of WG is poorly understood. Some available information suggests that ANCA may be involved in the pathogenesis of the disease. Several longitudinal studies have shown a positive correlation between ANCA titers and disease activity, and relapses are often preceded by a rise in ANCA titer [11–13]. Furthermore, treatment based on increase in ANCA titer has been reported to prevent relapse [12]. These findings are supported by *in vitro* data showing that binding of ANCA to surface expressed PR3 (or MPO) can activate primed neutrophils, indicated by the release of oxygen radicals and lysosomal enzymes [14, 15], and this can result in endothelial cell injury [16]. The *in vivo* significance of this observation is unclear, however, because neutrophil activation generally does not result in vasculitis.

Numerous studies have focused on PR3, the Wegener's autoantigen, and its potential role in the pathogenesis of WG. PR3 is a neutral serine proteinase of 29 kD with potent tissue-destructive capacity and is found in the primary granules of neutrophils and monocytes [17]. In addition to the proteolytic activity, PR3 has anti-bacterial activity [18] and appears to have growth-promoting effects on myeloid cell precursors [19]. Following activation of neutrophils, PR3 is expressed on the cell surface and released into the extracellular fluid (ECF) [20, 21]. A major physiologic inhibitor of PR3 in the ECF is α_1-proteinase inhibitor (α_1-PI) [22].

Recent work has shown that c-ANCA from patients with WG interfere with PR3 proteolytic activity as well as with the binding

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of α_1-PI to PR3 which was reported to correlate with disease activity [23, 24]. The authors proposed that the interference of PR3-α_1-PI binding by PR3-ANCA may prolong the half-life of PR3 in tissues, thereby resulting in enhanced proteolytic activity [23, 24]. However, other investigators did not find that PR3-ANCA prevent the binding of α_1-PI to PR3 [25].

We have investigated the effect of PR3-ANCA on the esterolytic and proteolytic activity of purified PR3, and its inhibition by α_1-PI. We have also studied the correlation of this effect of PR3-ANCA with the clinical activity of WG. We found in several patients with WG that PR3-ANCA significantly inhibit, in a non-competitive manner, the esterolytic and proteolytic activity of PR3. This inhibitory phenotype was mostly restricted to patients with active disease, suggesting a possible role for antibody mediated inhibition of PR3 in the disease process.

Methods

Serum samples

Twenty-one serum samples from 17 patients with an established diagnosis of WG were obtained from the Clinical Immunopathology Laboratory at the Massachusetts General Hospital. All 21 sera were positive for PR3-ANCA. The ANCA titers were determined as part of the routine diagnostic workup of these patients using antigen-specific radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) as previously described [26, 27]. In this assay, the titers are expressed in arbitrary units based on a standard laboratory positive serum. Values above a cutoff of 5 units were considered positive [26]. The immunoglobulin G (IgG) levels in these sera were either normal or slightly elevated. None of the sera had detectable anti-MPO autoantibodies (MPO-ANCA), anti-nuclear antibodies (ANA), anti-glomerular basement membrane (GBM) antibodies or rheumatoid factors (RF). Control sera consisted of seven serum samples from patients with MPO-ANCA positive vasculitis, as well as seven serum samples obtained from healthy laboratory volunteers, that tested negative for ANCA by ELISA. The sera were stored at −20°C until purification of the IgG fraction was performed and PR3 activity assayed.

Patients

The clinical findings and histologic data for each of the 17 patients were obtained from their medical records without knowledge of the effect of PR3-ANCA from the patients on the catalytic function of PR3. All 17 patients had clinical features consistent with the diagnosis of WG [1]. In 9 of the 17 patients, the diagnosis of WG was confirmed by histologic data that revealed necrotizing granulomatous inflammation. In the remaining 8 patients, the diagnosis of WG was based on clinical features and a positive test for PR3-ANCA. Disease activity at the time the serum samples were obtained was established according to published criteria [1, 28]. Active disease was defined as progressive disease affecting such organ systems as the upper or lower respiratory tract, kidneys, skin, eyes or nervous system, or constitutional symptoms that could not be attributed to processes other than WG. Remission was defined as complete absence of evidence for active disease, with a stabilized or improved renal function with no further evidence of active sediment (although proteinuria may persist for months or years in the presence of completely inactive renal disease) and complete resolution of pulmonary infiltrates, or evidence of stable scarring without signs of active inflammation.

Isolation of serum IgG

The sera were fractionated by 50% ammonium sulfate. The precipitates were redissolved in 10 mM sodium phosphate/150 mM sodium chloride, pH 7.4 (PBS) and dialyzed against the same buffer with two exchanges [29]. The IgG fractions were further affinity-purified on protein-G agarose columns. Bound IgG was then acid-eluted and the eluate immediately neutralized. Antibody concentrations were determined spectrophotometrically at A_{280} [29]. The homogeneity of the purified IgG fractions was confirmed in each case by SDS-PAGE (8.25%) as described by Laemmli [30], followed by Coomassie staining. Under reducing conditions, only two bands of 50 kD and 25 kD were seen, representing the heavy and light chains of IgG respectively (data not shown).

Enzymatic reactions

Reactions using N-t-Boc-L-Alanine p-Nitrophenyl ester (Boc-Ala-ONp) as substrate [22, 31] were initiated by adding the desired amount of the substrate to a 3 ml cuvette that contained 0.51 μg of enzyme in reaction buffer. The reactions were monitored in a Pharmacia-LKB spectrophotometer at 347 nm and 25°C. OD readings were taken at 1, 3, 5, 7, 10 and 15 minutes and adjusted for the spontaneous hydrolysis of Boc-Ala-ONp by simultaneously running a control sample containing only buffer and substrate. After establishing the baseline activity of PR3, esterolysis was assayed in the presence of antibody or α_1-PI. The enzyme was preincubated with the antibody or α_1-PI, in one ml of the same reaction buffer at 25°C for 30 minutes, then buffer was added to a final volume of 3 ml. All experiments were done at least in duplicate.

Proteolysis of elastin was assayed by adding 1.9 μg of PR3, or 1.0 μg of human neutrophil elastase (Sigma Chemicals, St. Louis, MO, USA) to 300 ml of reaction buffer containing 0.5 mg FITC-elastin, and the reaction monitored as previously described [18].

Reagents

Human neutrophil PR3 was purified by affinity chromatography using monoclonal antibody 1E8 coupled to sepharose as we previously described [9]. Purity was assessed by SDS polyacrylamide gel electrophoresis and by N-terminal sequencing. N-t-Boc-L-Alanine p-Nitrophenyl ester was purchased from Bachem Inc. (Torrance, CA, USA) in powder form and dissolved in dimethyl formamide (DMF) to a 300 mM stock solution. FITC-elastin 200–400 mesh was purchased from ICN Biochemicals (Cleveland, OH, USA) and suspended in 50 mM sodium phosphate buffer, pH 5.5, to a final concentration of 1.67 mg/ml. Protein G was purchased from Pharmacia-LKB (Piscataway, NJ, USA) as Hi-Trap protein G-sepharose minicolumns, and from BRL-GIBCO (Bethesda, MD) as protein G-agarose suspension. Alpha-1-proteinase inhibitor (α_1-PI) was purchased from Calbiochem (La Jolla, CA, USA), and dissolved to 10 mg/ml stock solution in 50 mM sodium phosphate buffer, pH 7.5, with 0.05% Triton X-100 (reaction buffer).

Computational methods

Initial reaction velocities were obtained from slopes determined by linear regression of OD readings, A_{347} versus time graphs using CricketGraph software. OD readings were all adjusted for background representing the spontaneous hydrolysis of Boc-Ala-ONp absorption at A_{347}. Actual velocities were determined based on the extinction coefficient $\epsilon = 5.5 \times 10^3$ M^{-1} cm^{-1} [22]. PR3 activity in the presence of antibody or α_1-PI was calculated as $(V_{exp}/V_{base}) \times 100$ where V_{exp} is the experimental velocity, that is, a reaction containing IgG or α_1-PI, and V_{base} is the velocity of a reaction containing only PR3. Kinetic constants were determined using Lineweaver-Burk, Woolf-Augustinsson-Hofstee, and Dixon plots [32–36]. Descriptive statistics yielding values for the mean and standard error, and Student's *t*-test analyses were calculated with the use of StatWorks computer software. Reaction slopes of all samples containing PR3-ANCA and MPO-ANCA were analyzed against the mean slope of 14 reactions containing enzyme and IgG from seven normal subjects and inhibition was considered significant when $P < 0.05$ on the Student's *t*-test.

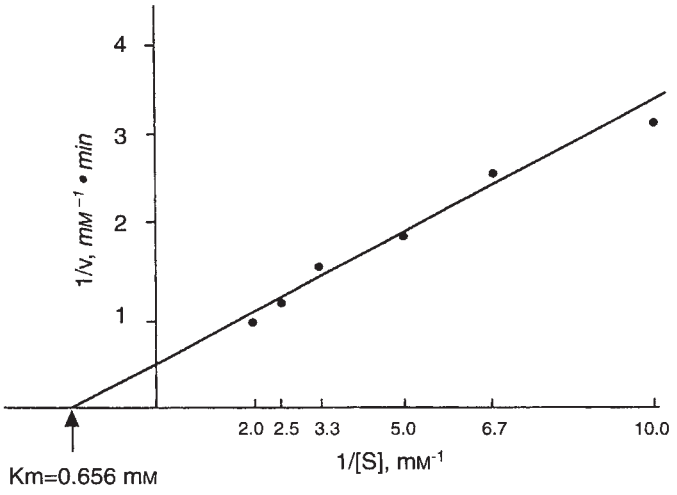


Fig. 1. *Lineweaver-Burke plot of PR3 activity using Boc-Ala-ONp as substrate over a concentration range of 0.1–0.5 mM.* Reaction velocities were calculated by linear regression as explained under **Methods**.

Results

Steady state kinetics of PR3

It was previously shown that PR3 effectively hydrolyzes the p-nitrophenyl ester of alanine [22]. We therefore used Boc-Ala-ONp as a substrate to study the esterolytic activity of the enzyme. Under the conditions described above, the hydrolysis reaction reached its steady-state plateau in 15 minutes at 25°C, and the optimal substrate concentration range was between 0.1 and 0.5 mM. Moreover, the rate of hydrolysis increased linearly with increased substrate concentration, indicating that it had no allosteric effect on the activity of the enzyme. In addition, because of a previous report [37] showing a stimulating effect of increasing hydrophobicity on elastase function, the effect of the solvent, DMF, was studied and was found to have no effect on the enzyme's activity over a concentration range of 10 to 30% (data not shown). The $K_m app$ for the substrate Boc-Ala-ONp was found to be 0.656 mM as determined from a Lineweaver-Burk plot (Fig. 1). This value is in agreement with that derived by Rao et al [22].

Effect of PR3-ANCA #1 and α_1-PI on PR3 esterolytic activity

The initial studies analyzing the effects of PR3-ANCA on PR3 activity were performed using purified IgG from serum #1 (subsequently referred to as PR3-ANCA #1), because large amounts of this serum were available. This antibody was preincubated with PR3 in varying molar ratios followed by measurements of the esterolytic activity of PR3. At a constant Boc-Ala-ONp substrate concentration of 0.20 mM, a clear inhibitory pattern of the enzyme was observed in a dose-dependent pattern (Fig. 2A). Maximal PR3 inhibition was observed at an enzyme to IgG molar ratio of 1:20 and ranged between 70 and 95% in various enzyme preparations. At 1:20 ratio, a control serum IgG containing MPO-ANCA was non-inhibitory (Fig. 2A). This ratio was therefore subsequently used for all other antibodies tested. A Dixon plot analysis performed at variable substrate and PR3-ANCA #1 concentrations showed an apparent non-competitive pattern of inhibition of this antibody with a $K_i app$ of 56.5 μM (Fig. 2B).

To compare these values with those obtained by the physiologic inhibitor α_1-PI, this inhibitor was incubated with PR3 under similar conditions and its inhibitory profile analyzed. As shown in Figure 3A, α_1-PI also produced a dose-dependent inhibition of PR3 catalytic activity. This inhibition was independent of the substrate concentration used (0.1–0.5 mM range, not shown), reached a plateau at an α_1-PI:PR3 molar ratio of ~15:1 and was further enhanced by 70% upon addition of PR3-ANCA #1. Inhibition by α_1-PI was competitive in nature (Fig. 3B) as previously shown [22] with a mean apparent $K_i app$ of 0.40 μM. The differences in $K_i app$ between the PR3-ANCA and α_1-PI may be more apparent than real since total IgG rather than only anti-PR3 antibody was used in this analysis. These data also suggest that both inhibitors, the PR3-ANCA and the α_1-PI, inhibit PR3 function by binding to different regions of the enzyme.

PR3-ANCA from other WG patients also inhibit PR3 activity in vitro

The effect of purified IgG from 20 additional PR3-ANCA positive sera, obtained from 16 patients with WG, on PR3 activity, was studied next. IgG was preincubated with PR3 at an enzyme to IgG molar ratio of 1:20. Controls consisted of IgG from seven MPO-ANCA positive patients with systemic vasculitis, and IgG from seven healthy subjects that tested negative for ANCA. The results are shown in Figure 4A. IgG from the normal subjects had no effect on PR3 activity. The mean of 14 reactions containing PR3 and IgG from the seven normal individuals is displayed in Figure 4A. PR3-ANCA from five of the sixteen patients (PR3-ANCA #2–6), inhibited PR3 esterolytic activity almost completely (mean 70 to 95% inhibition, $P < 0.05$), similar to that observed with PR3-ANCA #1. In follow-up samples available on two of these patients, #2F and #5F, the inhibitory activity had decreased to statistically insignificant levels. In three additional patients (#7–9), the degree of inhibition was less severe (41 to 58%), although statistically significant ($P < 0.05$). The follow-up serum (#7F) from patient #7, obtained only one day after the initial sample, showed persistent inhibition. In the remaining eight patients, PR3 inhibition was variable but did not reach statistical significance ($P > 0.05$). Among the seven MPO-ANCA positive sera, only one (MPO-ANCA #7, Fig. 4A), showed moderate,

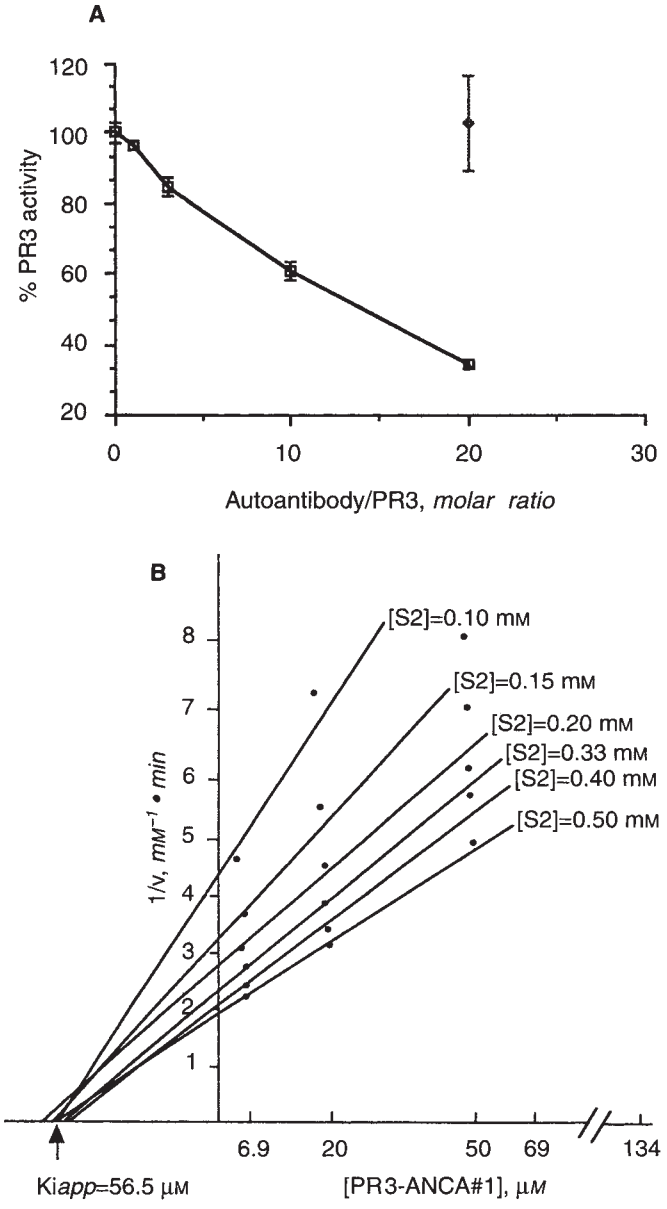


Fig. 2. *Inhibition of the esterolytic activity of PR3 by PR3-ANCA #1.* (**A**) Dose-response curve showing the inhibitory activity of PR3-ANCA #1 (□). No such activity was seen in purified serum IgG from a patient with circulating MPO-ANCA (♦) at a PR3-IgG molar ratio of 1:20. Values represent the mean ± SEM of two independent experiments, each carried out in duplicate. A regression analysis of these points confirms the validity of the antibody's inhibitory effect as reflected by the negative slope (r = 0.986; y = 97.317 − 3.2496x). (**B**) Dixon plot analysis of PR3 activity with increasing PR3-ANCA #1 concentration from 6.9 to 134 μM based on the same PR3-IgG molar ratio calculations that were used in panel A. Boc-Ala-ONp concentration range is again from 0.1 to 0.5 mM. Note that the intersection site of the reaction lines (arrow) suggests a noncompetitive pattern of inhibition according to Dixon [35, 36]. This experiment is one of three independent experiments and shows an apparent Ki ($K_i app$) of 56.5 μM (range 50 to 63 μM).

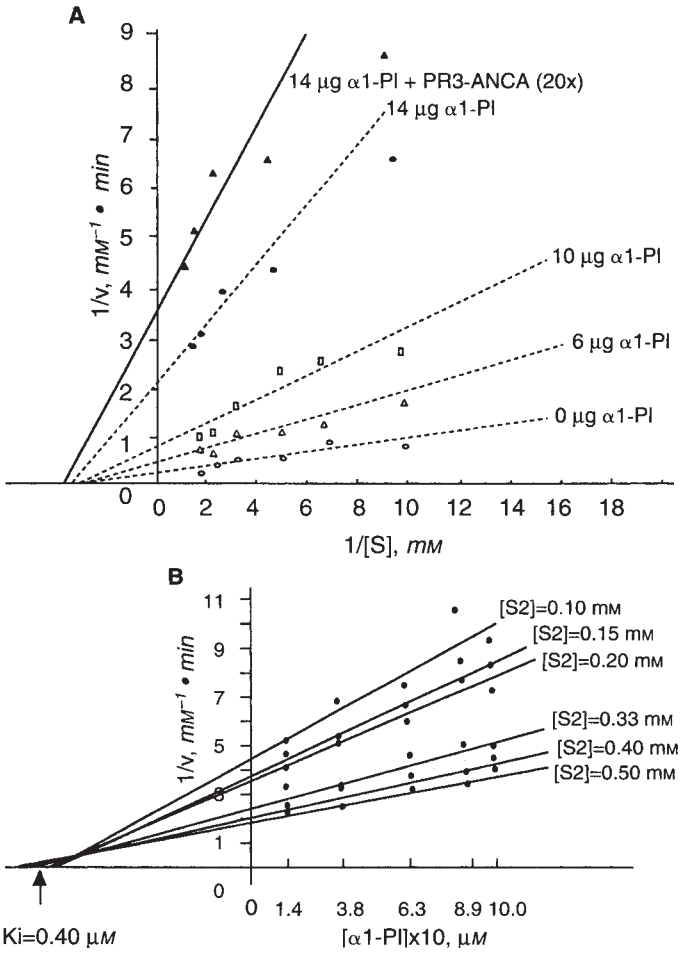
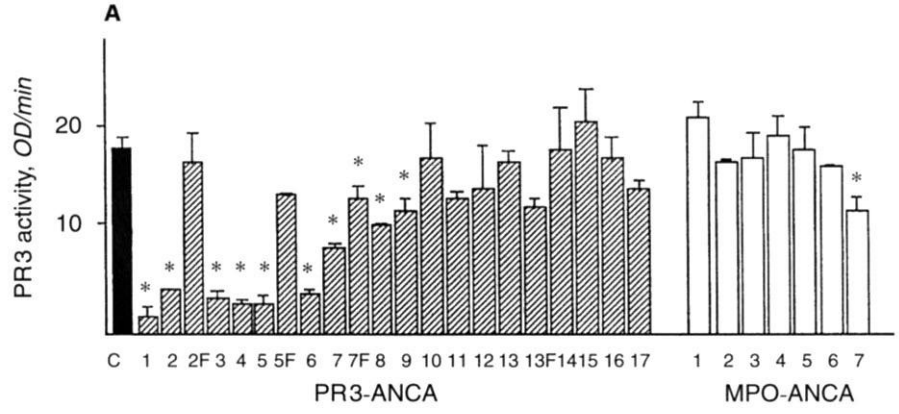


Fig. 3. *Inhibition of PR3 by α_1-PI.* (**A**) Lineweaver-Burk analysis of PR3 activity showing the inhibition of PR3 by α_1-PI. The enzyme was preincubated with the inhibitor for 30 minutes at 25°C in 1 ml of reaction buffer. Subsequently 2 ml of reaction buffer were added and the reaction was initiated by addition of substrate and monitored as described in **Methods**. Inhibition reached a plateau at 14 μg of α_1-PI per reaction and was further increased by addition of PR3-ANCA #1 (1:20 enzyme:IgG molar ratio). (**B**) Dixon plot analysis of the PR3 esterolytic activity with increasing α_1-PI concentration from 1.4 to 10 μM, and a substrate concentration of 0.1 to 0.5 mM. In three independent experiments, one of which is shown here, the $K_i app$ ranged from 0.35 to 0.45 μM. Here, the reaction line intersection (arrow) suggests a competitive inhibition pattern [35, 36].

albeit significant ($P < 0.05$) degree of PR3 inhibition. No correlation was found between the PR3-ANCA titers (quantified using antigen-specific ELISA) and their inhibitory activity (r = 0.051; Fig. 4B).

PR3-ANCA inhibit the elastinolytic activity of PR3

Our analysis of the effects of the anti-PR3 autoantibodies on the esterolytic function of PR3 was extended to the proteolysis of elastin, a known protein substrate for PR3 which may account for its potent capacity of inducing emphysema in hamster lungs *in vivo* [17]. Figure 5 shows a dose-response curve of the inhibitory effect of PR3-ANCA #1 on PR3 activity determined by a fluorometric assay using FITC-elastin as substrate. In contrast, no inhibition of PR3-ANCA #1 on human neutrophil elastase activity was observed (data not shown), further indicating that the activity observed was directed specifically against PR3 and that the purified IgG fraction was not contaminated by natural serum proteinase inhibitors such as α_1-PI or α_2-macroglobulin. The



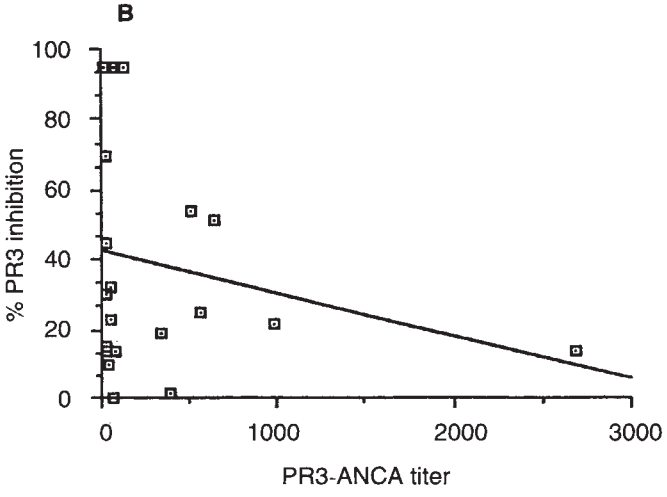


Fig. 4. *Effect of PR3-ANCA and MPO-ANCA on the esterolytic activity of PR3.* (**A**) Each histogram represents PR3 activity in the presence of purified IgG from PR3-ANCA positive patients with WG (indicated by numbers below each bar) and MPO-ANCA positive controls. Also displayed is the mean PR3 activity obtained from 14 reactions containing enzyme and IgG from 7 normal subjects (C). Histograms represent the mean ± SEM of two independent experiments. An asterisk (*) indicates that the sample's activity is significantly ($P < 0.05$) different from that seen with IgG from normal subjects. None of the MPO-ANCA positive sera is positive for PR3-ANCA as determined by ELISA. (**B**) Lack of correlation between PR3-ANCA titers and inhibitory profile. A linear regression analysis of PR3-ANCA titer vs. PR3 inhibition shows no correlation ($r = 0.051$; y = 43.012 − 1.2269e-2x).

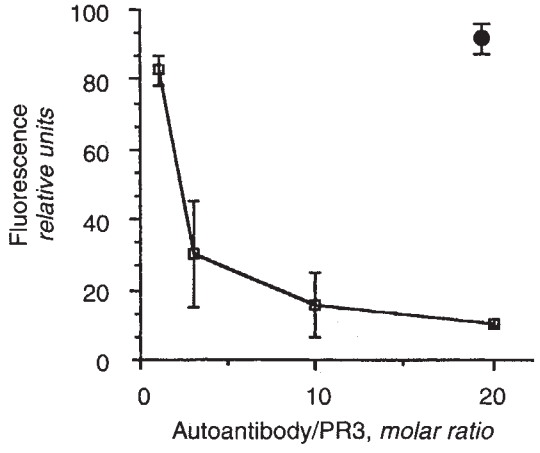


Fig. 5. *Inhibition of the elastinolytic activity of PR3 by PR3-ANCA #1 (□).* No inhibition was observed using an MPO-ANCA positive IgG (●). A dose-response curve shows the inhibition of PR3-initiated proteolysis of the fluoresceinated FITC-elastin by that autoantibody. Values represent the mean ± SEM of two independent experiments.

PR3-ANCA inhibitory pattern paralleled closely its effect on the esterolytic activity of PR3. Moreover, all seven of the inhibitory PR3-ANCA tested also inhibited the elastinolytic activity of the enzyme, and none of three control IgG samples, two containing MPO-ANCA and one negative for ANCA, had any inhibitory effects (data not shown).

Clinical characteristics and disease activity in the patient population

After determining the effects of PR3-ANCA on the activity of PR3, the clinical and histologic findings were reviewed and the disease activity at the time of serum collection was determined. The clinical manifestations, the date of the most recent episode of active disease (initial diagnosis or relapse), the presence or absence of therapy (cyclophosphamide with or without corticosteroids) and its duration, PR3-ANCA titer and PR3 activity in the presence of PR3-ANCA are shown in Table 1. The inhibitory effect of PR3-ANCA is displayed as a mean value of at least two determinations and expressed as % of "normal" PR3 activity (mean PR3 activity when tested in the presence of IgG from 7 normal individuals). Table 1 shows that PR3-ANCA with PR3 inhibitory activity represent mainly patients with active disease. Eight of the 10 patients with active disease showed statistically significant inhibition of PR3, whereas two patients did not. One of the two patients (#10) had a long (13 year) history of WG and was thought to have smoldering disease, that is, a stable form of mild, active disease. The other patient (#14) had made significant recovery after six weeks of immunosuppressive therapy although he had not yet reached complete remission. In two patients, #2 and #5, follow-up samples (#2F and #5F) revealed that the

Table 1. Demographics

Serum #	Sex	ANCA date	Disease state	Date of Dx or last relapse (Re)		Disease duration at time of ANCA determination	Organ involvement at Dx or Re			Therapy *duration*	PR3-ANCA titer	PR3 activity *% of NI*
1	F	8/2/90	Active	7/17/90	(Dx)	2 weeks	L, K	(Dx)	+	(2 wks)	7	7.5[a]
2	F	5/5/89	Active	5/6/89	(Dx)	0 days	ENT, L, K, M, J	(Dx)	−		128	20.3[a]
2F		6/7/89	Active	—		1 month	−		+	(1 month)	31	85.8[a]
3	M	12/26/91	Active	1/2/92	(Dx)	0 days	ENT, L, K, J, PNS	(Dx)	−		64	15.1[a]
4	F	5/26/89	Active	5/5/89	(Re)	16 months	ENT, L, K	(Dx)	+	(3 wks)	29	15.4[a]
							Fever, anemia	(Re)				
5	M	9/20/91	Active	9/20/91	(Dx)	0 days	ENT, K, EY, S, J	(Dx)	−		83	15.4[4]
5F		10/14/91	Active	—		24 days	—		+	(10 days)	76	67.6[a]
6	M	3/18/91	Remission	12/88	(Dx)	27 months	L, K	(Dx)	−		17	19.6[a]
7	M	7/22/91	Active	7/22/91	(Dx)	0 days	ENT, K, EY	(Dx)	−		512	41.1[a]
7F		7/26/91	Active	—		4 days	—		+	(1 day)	563	67.4[a]
8	M	4/25/90	Active	12/7/89	(Dx)	4.5 months	ENT, EY	(Dx)	+	(4.5 mos)	28	52.2[a]
							EY	(Re)				
9	M	1/31/91	Active	10/90	(Re)	22 months	ENT, L, EY, S, J	(Dx)	+	(3 mos)	642	57.7[a]
							Ey	(Re)				
10	M	11/13/91	Active	1/91	(Re)	13 years	ENT, L, K	(Dx)	+	(22 mos)	56	70.3
							ENT, L	(Re)				
11	F	8/15/90	Remission	11/88	(Dx)	21 months	ENT, L, K, EY	(Dx)	+	(21 mos)	20	77.3
12	M	1/17/91	Remission	11/78	(Dx)	13 years	ENT, L, K	(Dx)	−		988	86.4
13	M	6/27/91	Remission	3/5/90	(Dx)	15 months	ENT, L	(Dx)	+	(15 mos)	25	63.0
13F		10/31/91	Remission	—		19 months	—		+	(19 mos)	57	90.8
14	M	3/12/91	Active	2/1/91	(Dx)	6 weeks	ENT, L	(Dx)	+	(6 wks)	38	113.1
15	F	10/15/91	Remission	3/88	(Re)	4.5 years	ENT, L	(Dx)	−		62	86.0
16	M	1/24/91	Remission	12/89	(Dx)	13 months	L, EY, S, J	(Dx)	+	(13 mos)	2688	88.9
17	F	9/25/91	Remission	3/89	(Re)	11 years	ENT	(Dx)	−		398	63.2

Summary of clinical characteristics of PR3-ANCA positive patients whose sera were studied. ANCA date refers to the date that ANCA determination was performed. Dx refers to diagnosis and Re to relapse. Disease duration refers to the time that elapsed from initial diagnosis until the time of ANCA determination. Organ involvement includes: ENT, ear, nose and throat; L, lung; K, kidney; Ey, eye; S, skin; M, muscle; J, joints; and PNS, peripheral nervous system.

inhibitory activity had decreased to nonsignificant levels after immunosuppressive therapy of 1 month and 10 days duration, respectively. Patient #2 did also receive plasma exchange therapy, which may have accounted for the fourfold decrease in PR3-ANCA titer observed in the follow-up sample, suggesting that depletion of PR3-ANCA may have contributed to the rapid decline in inhibition. In the follow-up sample from patient #5, however, the inhibitory activity was reduced in the absence of a significant change in PR3-ANCA titer. The follow-up sample (#7F) from patient #7 was taken only one day after initiating immunosuppressive therapy. One patient, who was in complete remission, showed statistically significant inhibition of PR3. Data on the subsequent course of that patient are lacking so we do not know whether the patient suffered a clinical relapse during the ensuing months. All the six remaining patients were in remission at the time of ANCA testing and none had statistically significant inhibition.

Discussion

The detection of ANCA directed against PR3 has proven valuable for the diagnosis of WG [5, 7, 11]. The strong association between PR3-ANCA and WG suggests that the presence of ANCA is not merely an epiphenomenon but rather has a role in the pathogenesis of the disease.

The data presented in this paper show a strong correlation between inhibition of PR3 caused by PR3-ANCA and disease activity in patients with WG (Table 1). In contrast, we found little correlation between PR3-ANCA titer and disease activity. Although several studies have shown a strong correlation between ANCA titers and disease activity in WG, it is well known that clinical recovery is not always accompanied by a decrease in ANCA titer and persistently high titers are observed in some patients with inactive disease, as is demonstrated by patients #12, 16, and 17 in our study. Moreover, a recent report by Kerr et al showed that changes in ANCA titers correlated with changes in disease status in only 64% of patients and that increase in ANCA titer preceded relapse in only 24% [28]. Our data suggest that PR3-ANCA-mediated inhibition of PR3 may be a better marker for active WG than the PR3-ANCA titer. A prospective study involving a larger group of patients will be needed to confirm this finding.

In one extensively characterized PR3-ANCA, the inhibition of PR3 appeared to be non-competitive in nature and additive to that observed with the natural inhibitor α_1-PI, suggesting that the inhibitors act at different sites on the enzyme. This finding differs from other investigators [23] who reported that in addition to inhibiting PR3 activity, PR3-ANCA from patients with WG also interferes with the binding of α_1-PI to PR3, measured by RIA. Subsequent work by the same group [24] showed that the latter phenomenon correlated with disease activity, leading the authors to postulate that this interference may enhance the proteolytic activity of PR3 by increasing its half-life in the ECF. In that study however, the median inhibition of PR3-α_1-PI complex formation in patients with active WG was only 22%. The authors did not report functional data showing the effect of this interference on PR3 activity. They also found in plasma samples from their

patients that the level of PR3-α_1-PI and HLE-α_1-PI complexes were within the range of those in normal healthy controls. Gross, Schmitt and Csernok could not confirm that PR3-ANCA interferes with the binding of α_1-PI to PR3 [25]. Except for PR3-ANCA #1, we did not have enough serum available to to analyze in detail the effect of the remaining PR3-ANCA on the binding of α_1-PI to PR3.

Our data indicate that the inhibition of PR3 is the result of a highly specific interaction between certain PR3-ANCA and PR3. First, no inhibition was observed when PR3 activity was assayed in the presence of seven ANCA-negative and six out of seven MPO-ANCA IgG. The only exception was one MPO-ANCA, which showed moderate PR3 inhibition. It is possible that this serum did in fact contain PR3-ANCA that escaped detection in the ELISA because of a lower avidity for the antigen. Reactivity to both PR3 and MPO in patients with systemic vasculitis has been reported in rare cases [38]. Secondly, several PR3-ANCA, some with very high titers (all significantly higher than the titer of PR3-ANCA #1), had no inhibitory effects on PR3, again suggesting that the inhibition is not due to a non-specific effect of antibody-antigen complex formation, but rather to the interaction of certain autoantibodies with one or more functional epitopes on PR3. Finally, none of the inhibitory PR3-ANCA inhibited HLE despite its high homology with PR3 (54% sequence identity) [39].

Our data also show that the inhibitory phenotype of the autoantibody may change rapidly during the course of the disease, perhaps in response to therapy. We are not aware of any reports of such a phenomenon in other human autoimmune diseases. In experimental models of autoimmune diseases, such as experimental allergic encephalomyelitis and insulin-dependent diabetes mellitus, an "evolution" of the T cell receptor repertoire to an autoantigen has been demonstrated [40, 41]. In these experimental animals, T cell clones react initially with a confined region of the autoantigen and, in a relatively short period of time (days), expand to involve additional determinants. An analogous immune "maturation" response has been described for autoantibodies against the ribonucleoprotein La(SSB) in patients with Sjögren's syndrome [42]. We speculate that a similar phenomenon may occur in response to immunosuppressive therapy, changing the autoantibody profile from an inhibitory to a non-inhibitory one as was observed in patient #5 (Table 1). Epitope heterogeneity among PR3-ANCA has recently been shown, using overlapping 7-amino acid peptides based on the sequence of PR3 [43]. This analysis also showed that certain autoantibodies recognize part of the catalytic triad and the surrounding region. This suggests that steric as well as allosteric mechanisms may underlie the inhibitory phenotype of various PR3-ANCA.

It has previously been reported for several autoimmune diseases that autoantigens that are enzymes are inhibited by their respective autoantibodies. Anti-mitochondrial antibodies in patients with primary biliary cirrhosis are directed against the pyruvate dehydrogenase complex and inhibit its enzymatic function [44, 45]. Autoantibodies to RNA-polymerase I, found in small number of patients with scleroderma, inhibit the synthesis of 28S and 18S RNA [46]. Autoantibodies to tRNA synthetases from patients with polymyositis inhibit tRNA aminoacylation [47, 48]. The significance of the autoantibody mediated inhibition in these disorders is unclear, since these enzyme targets are solely expressed intracellularly and, therefore, are not accessible to the autoantibodies. This may explain why none of these autoantibodies have been found to be pathogenic or shown to be strongly associated with disease activity. In contrast, an inhibitory autoantibody directed against the plasma serine proteinase thrombin was recently reported in a young man with a symptomatic bleeding disorder who suffered a fatal cerebral hemorrhage, indicating a direct pathogenic role of the anti-thrombin antibody [49].

The inhibition of PR3 activity by certain PR3-ANCA may be an epiphenomenon of no physiologic significance. It is well established that autoantibodies in human autoimmune diseases react predominantly with highly conserved epitopes, which often represent functional or catalytic domains of the target antigens [50]. As discussed above, in several diseases autoantibodies have been found to inhibit the function of their enzyme autoantigens without a clear relationship to the disease process. Since the catalytic site of a serine proteinase is highly conserved [51], it is not surprising that this region may be the target of autoantibodies. It is possible that the close correlation between active disease and inhibitory autoantibodies observed here could be a manifestation of the primary autoimmune response, and may suggest that the disease process is of a relatively short duration with the autoantibody repertoire still directed against the conserved immunodominant epitope of the autoantigen, similar to what has been described for the T cell receptor repertoire in early experimental EAE and IDDM [40, 41]. In fact, six of eight patients with active disease and inhibition of PR3 in our study were in the early stages of their disease.

Alternatively, an inhibitory autoantibody may play a pathophysiologic role by interfering with an essential down-regulatory function of PR3 in the inflammatory response that may be particularly relevant in certain tissues such as the lung. It is intriguing that in the respiratory tract where the early lesions in WG frequently occur, epithelial secretions are relatively lacking in specific PR3 inhibitors [22]; the circulating α_1-PI has relatively low avidity for PR3, and the bronchial secretory leukoprotease inhibitor, a potent inhibitor of elastase, does not inhibit PR3 [22]. ANCA have been detected in bronchoalveolar lavage fluid from patients with WG and there is evidence that ANCA are produced in the respiratory tract of these patients [52]. Testing the validity of this hypothesis will require an elucidation of the physiologic substrates of PR3 *in vivo*. Inhibition of PR3 by autoantibodies as a potential mechanism of injury in WG does not exclude other mechanisms of tissue injury, such as autoantibody-induced neutrophil activation [14, 15], and T cell-mediated cytotoxicity [53].

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