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Interference of Wegener's granulomatosis autoantibodies with neutrophil Proteinase 3 activity

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SUMMARY

Classic anti-neutrophil cytoplasmic autoantibodies (C-ANCA) are disease-specific markers of Wegener's granulomatosis (WG). The possible pathogenetic role of these autoantibodies, which are directed against Proteinase 3 (PR3), is not yet clear. We studied the effect of C-ANCA on PR3 proteolytic activity and on the complexation of PR3 with alpha$_1$-antitrypsin (α_1AT). C-ANCA IgG from eight patients with active WG significantly inhibited PR3 proteolytic activity, particularly towards elastin (median 84·2% inhibition). C-ANCA IgG significantly inhibited the complexation of PR3 with α_1AT (median 58·8% inhibition). Moreover, addition of purified PR3 to C-ANCA-positive sera from WG patients yielded less complexes with α_1AT (median 44·8%) compared with sera containing perinuclear anti-neutrophil cytoplasmic autoantibodies (P-ANCA) or ANCA-negative sera. These findings indicate the existence of a hitherto unknown property of C-ANCA, which may be of importance in the pathogenesis of WG.

Keywords C-ANCA Proteinase 3 alpha$_1$-antitrypsin

INTRODUCTION

Classic anti-neutrophil cytoplasmic autoantibodies (C-ANCA) are sensitive and specific markers for the diagnosis of active Wegener's granulomatosis (WG) and have been suggested to be responsible for the development of WG [1–4]. In addition, C-ANCA titre recurrence or increase often precedes relapses of WG and treatment based on changes in serum C-ANCA titre prevents disease relapses [5,6]. C-ANCA are specifically directed against Proteinase 3 (PR3), a neutral serine protease present in the azurophil granules of neutrophils [7–10]. However, the exact role of C-ANCA in the pathogenesis of WG is not yet clear. It has been suggested that activation of primed neutrophils by C-ANCA might play a role [11]. Alternatively, C-ANCA may interfere with PR3 biological activity. *In vitro*, PR3 degrades a variety of matrix proteins including elastin, and it induces emphysema upon intratracheal insufflation in hamsters [12,13]. Stimulation of cytochalasin B-treated neutrophils with fMLP induces release of PR3 which indicates that *in vivo* C-ANCA may interact with PR3 after its release upon inflammatory stimulation [7]. We have studied the effect of C-ANCA on the proteolytic activity of PR3 and on the complexation of PR3 with alpha$_1$-antitrypsin (α_1AT), the major inhibitor of PR3 [13].

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MATERIALS AND METHODS

Sera

Serum samples were collected from eight patients with active and proven WG. All eight sera were C-ANCA-positive as determined by standard immunofluorescence (IF) [14] and reacted specifically with PR3 as determined by antigen capture ELISA [7,15]. Furthermore, seven sera containing perinuclear anti-neutrophil cytoplasmic autoantibodies (P-ANCA) with different specificities (five anti-myeloperoxidase (MPO), two antineutrophil elastase (NE)) and five ANCA-negative sera were included in the study.

Purification of IgG

IgG was purified from all collected sera by affinity chromatography using protein A Sepharose CL-4B (Pharmacia, Uppsala, Sweden). All purified IgGs contained no detectable functional α_1AT as determined by a radioimmunoassay (RIA), described by Abbink *et al.* [16]. IgGs purified from C-ANCA-positive sera (C-ANCA IgG) specifically reacted with PR3, IgGs from P-ANCA-positive sera (P-ANCA IgG) specifically reacted either with MPO or NE as determined by solid phase ELISA (see below). ANCA titre in the C-ANCA IgGs was determined by standard IF.

Solid phase ELISA for determining IgG specificity

Purified PR3 (see below), NE and MPO (both obtained from Elastin Products Co., Pacific, MO) were coated overnight at 4°C at protein concentrations of 1 μg/ml in 100-μl volumes containing PBS on flat-bottomed microtitre plates (Microelisa, M129B, Dynatech, Plochingen, Germany). All subsequent incubations were performed in 100-μl volumes of 1 h at room temperature. Between all incubation steps, the plates were washed five times with PBS containing 0·02% (v/v) Tween-20. After coating, residual binding sites were blocked with PBS containing 1% (w/v) bovine serum albumin (BSA). Subsequently, purified IgG samples diluted in PBS containing 0·02% (v/v) Tween-20 and 0·2% (w/v) gelatin (PTG) were incubated. Bound IgG was detected by incubation with horseradish peroxidase-conjugated mouse monoclonal anti-human IgG (CLB MH-16-01-ME, prepared in our institute) diluted 1:1000 in PTG. The plates were developed with 3,3′,5,5′-tetramethylbenzidine (Merck, Darmstadt, Germany) at 100 μg/ml in 0·11 M sodium acetate pH 5·5 containing 0·003% (v/v) H_2O_2. After termination of colour development with 2 M H_2SO_4, absorbance was measured in a Titertek Twinreader (Flow Laboratories, Irvine, UK) at 450 nm. Antibody positivity was defined as 2 s.d. above the mean of a panel of sera from healthy bloodbank donors. Antibody activity in each ELISA was expressed in arbitrary units by reference to a standard sample containing ANCA with the concerned specificity.

Purification of Proteinase 3

PR3 was purified polymorphonuclear leucocyte granules as described by Kao *et al.* [12]. The PR3 preparation contained no detectable NE and cathepsin G as measured by antigen capture ELISA [7,17]. The preparation appeared to be homogeneous by SDS–PAGE analysis and PR3 migrated as a 29-kD triplet [17]. PR3 protein concentration was measured according to Bradford [18] using BSA as a standard.

Active site titrations

Active site titrated bovine chymotypsin (CT) was purchased from the National Institute for Biological Standards and Control (Herts, UK). The activity of α_1AT (Calbiochem, San Diego, CA) was determined essentially as described by Beatty *et al.* [19] using CT as the primary standard and MeO-Suc-Arg-Pro-Tyr-pNA (S2586, KabiVitrum, Stockholm, Sweden) as substrate. The active protein concentration of α_1AT was found to be 62·5%. This α_1AT was used as a secondary standard for determining the activity of PR3 and NE according to Rao *et al.* [13] using MeO-Suc-Ala-Ala-Pro-Val-pNA (M4765, Sigma Chemical Co., St Louis, MO) as substrate. The preparations of PR3 and NE were found to be 90% and 85% active, respectively. In all experiments total protein concentrations for PR3 and NE and active protein concentrations for α_1AT were used.

Proteolytic assays

Proteolysis of elastin (elastinolysis) by PR3 and NE was assayed using FITC-labelled elastin (ICN Biochemicals Inc., Cleveland, OH) according to Campanelli *et al.* [20]. Proteolysis of the synthetic substrate MeO-Suc-Ala-Ala-Pro-Val-pNA was assayed in a 200-μl total volume consisting of 2 mM substrate, PBS with 0·1% (v/v) Tween-80, 1·25% (v/v) dimethylsulphoxide and 25 nM PR3 or 1 nM NE at 37°C in microtitre plates. MeO-Suc-Ala-Ala-Pro-Val-pNA proteolysis was determined by continuously measuring the change of absorbance at 405 nm during a 1-h period using a Titertek Twinreader. To study the effect of C-ANCA on PR3 elastinolysis and MeO-Suc-Ala-Ala-Pro-Val-pNA proteolysis, 114·9 and 25 nM PR3 respectively were incubated with a 45-fold molar excess of C-ANCA IgG for 30 min at room temperature. Subsequently, elastinolysis and MeO-Suc-Ala-Ala-Pro-Val-pNA proteolysis were measured. In addition, after incubation of 25 nM PR3 with a 45-fold molar excess of C-ANCA IgG, α_1AT was added in a 28-fold molar excess (to PR3). After 30 min incubation at room temperature, MeO-Suc-Ala-Ala-Pro-Val-pNA proteolysis was measured and compared with PR3 proteolytic activity incubated only with C-ANCA IgG.

RIA for the detection of NE-α_1AT and PR3-α_1AT complexes

NE-α_1AT complexes were detected using a RIA as described by Nuijens *et al.* [21]. PR3-α_1AT complexes were detected using a similar RIA. Briefly, test samples were incubated with Sepharose-bound MoAb 12.8 (anti-PR3) [7]. Bound PR3-α_1AT complexes were detected by subsequent incubation with ^{125}I-labelled MoAb AT15 which is specific for complexed α_1AT [16]. Levels of PR3-α_1AT complexes were calculated by reference to a standard dose-response curve of preformed complexes prepared by incubating purified PR3 with purified α_1AT. The detection level for PR3-α_1AT complexes was 0·5 nM. To study the effect of C-ANCA on the complexation of PR3 with α_1AT, 23 nM PR3 was first incubated with a 45-fold molar excess of C-ANCA IgG for 30 min at room temperature and subsequently, a 28-fold molar excess of α_1AT (to PR3) was added. After 30 min incubation at room temperature, PR3-α_1AT complexes were measured. The formation of PR3-α_1AT complexes in serum was studied by measuring PR3-α_1AT complexes in C-ANCA-positive, P-ANCA-positive and ANCA-negative serum samples (using 1:40 dilution) to which 34·5 nM purified PR3 had been added, after 30 min incubation at room temperature.

Statistical analysis

Pairwise comparisons between the subject groups (C-ANCA-, P-ANCA- and ANCA-negative) were analysed with the Wilcoxon–Mann–Whitney test. Correlations were analysed with the Spearman rank correlation analysis. Two-tailed *P*-values of less than 0·05 were considered to indicate a significant difference or correlation. All calculations were performed on a IBM-compatible computer using SPSS statistical software.

RESULTS

Effect of C-ANCA IgG on PR3 proteolytic activity

C-ANCA IgG inhibited elastinolysis with a median inhibition of 84·2% (range 70–100; $P=0·0032$) compared with IgG from ANCA-negative sera (controls) as shown in Fig. 1a. MeO-Suc-Ala-Ala-Pro-Val-pNA proteolysis was also inhibited by C-ANCA IgG with a median inhibition of 63·3% (range 16·3–98·4; $P=0·0281$) compared with the controls (Fig. 2a). With one C-ANCA IgG an increase in MeO-Suc-Ala-Ala-Pro-Val-pNA proteolysis by PR3 (53·5% compared with the controls) was observed while the IgG preparation itself contained no proteolytic activity. ANCA-negative IgG had no effect on PR3 proteolytic activity towards both substrates compared with PR3 activity in the absence of IgG (results not shown). A clear dose-dependent effect of (the three strongest inhibiting) C-ANCA

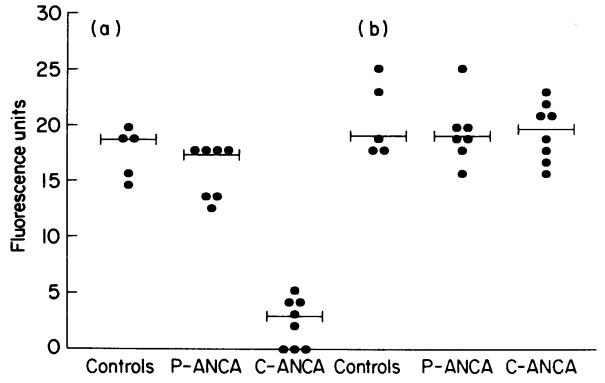

Fig. 1. Effect of classic anti-neutrophil cytoplasmic autoantibodies (C-ANCA) IgG on Proteinase 3 (PR3) (a) and neutrophil elastase (NE) (b) elastinolysis. PR3 (114·9 nM) or NE (55·5 nM) was incubated with a 45-fold molar excess of C-ANCA IgG, perinuclear anti-neutrophil cytoplasmic autoantibodies (P-ANCA) IgG and ANCA-negative IgG (controls) for 30 min at room temperature. Subsequently, elastinolysis was assayed using FITC-labelled elastin and expressed in arbitrary units of fluorescence measured fluorospectrophotometrically. Results represent the mean of two experiments and the median value of each subject group is shown.

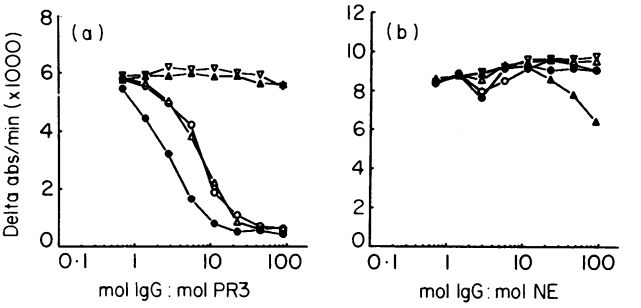

Fig. 3. Effect of various concentrations of classic anti-neutrophil cytoplasmic autoantibodies (C-ANCA) IgG on MeO-Suc-Ala-Ala-Pro-Val-pNA proteolysis by Proteinase 3 (PR3) (a) and neutrophil elastase (NE) (b). PR3 (25 nM) or NE (1 nM) was incubated with increasing concentrations of three C-ANCA IgGs, one perinuclear anti-neutrophil cytoplasmic autoantibody (P-ANCA) IgG (anti-NE) and one ANCA-negative IgG (indicated on the abscissa as molar ratio of IgG to PR3 and NE respectively). Subsequently, proteolytic activity was measured spectrophotometrically at 405 nm and expressed as change of absorbance per min (delta absorbance/min) × 1000. Results represent the mean of two experiments. ○, C-ANCA 1; ●, C-ANCA 2; △, C-ANCA 3; ▲, P-ANCA; ▽, ANCA negative.

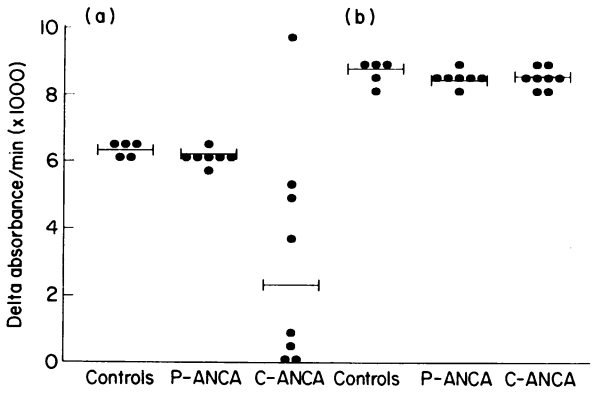

Fig. 2. Effect of classic anti-neutrophil cytoplasmic autoantibodies (C-ANCA) IgG on MeO-Suc-Ala-Ala-Pro-Val-pNA proteolysis by Proteinase 3 (PR3) (a) and neutrophil elastase (NE) (b). PR3 (25 nM) or NE (1 nM) was incubated with a 45-fold molar excess of C-ANCA IgG, perinuclear anti-neutrophil cytoplasmic autoantibodies (P-ANCA) IgG and ANCA-negative IgG (controls) for 30 min at room temperature. Subsequently, proteolytic activity was measured spectrophotometrically at 405 nm and expressed as change of absorbance per min (delta absorbance/min) × 1000. Results represent the mean of two experiments and the median value of each subject group is shown.

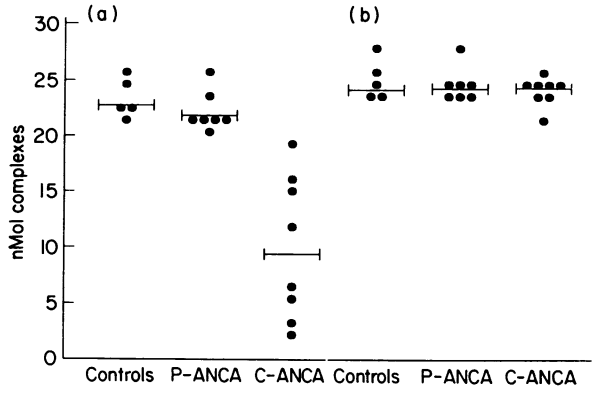

Fig. 4. Effect of classic anti-neutrophil cytoplasmic autoantibodies (C-ANCA) IgG on Proteinase-3-alpha$_1$-antitrypsin (PR3-α_1AT) (a) and neutrophil elastase (NE)-α_1AT (b) complexation. Complexation of PR3 with α_1AT was assayed by RIA. PR3 (23 nM) or NE (23 nM) was first incubated with a 45-fold molar excess of C-ANCA IgG, perinuclear anti-neutrophil cytoplasmic autoantibody (P-ANCA) IgG and ANCA-negative IgG (controls) for 30 min at room temperature. Subsequently, a 28-fold molar excess of α_1AT (to PR3 and NE respectively) was added. After 30 min incubation at room temperature, formed complexes were measured by RIA and expressed in nMol concentrations. Results represent the mean of two experiments and the median value of each subject group is shown.

IgGs on the inhibition of MeO-Suc-Ala-Ala-Pro-Val-pNA proteolysis by PR3 was observed when the molar ratio of C-ANCA IgG to PR3 was varied (Fig. 3a). Half-maximal inhibition was observed at relatively small molar excesses of IgG to PR3 (ranging from approximately 3- to 9-fold) with the three strongest inhibiting C-ANCA IgGs. The residual activity of PR3 towards MeO-Suc-Ala-Ala-Pro-Val-pNA, observed with some C-ANCA IgGs, was completely abolished after the addition of α_1AT (results not shown). However, with the C-ANCA IgG which caused an increase in MeO-Suc-Ala-Ala-Pro-Val-pNA proteolysis by PR3, only 45·3% inhibition of PR3 proteolytic activity after the addition of α_1AT was observed. No significant inhibition of PR3 proteolytic activity towards both substrates was observed by P-ANCA IgG. The inhibiting effect of C-ANCA IgG was not due to contamination by α_1AT (see Materials and Methods). Moreover, no effect of C-ANCA IgG was observed on NE proteolytic activity (see below).

Effect of C-ANCA IgG on PR3-α_1AT complexation

As shown in Fig. 4a, C-ANCA IgG inhibited the formation of PR3-α_1AT complexes with a median inhibition of 58·8% (range

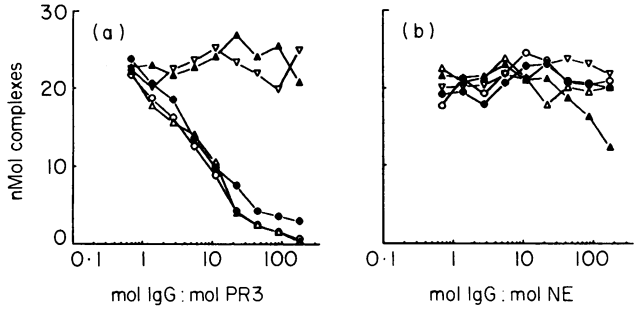

Fig. 5. Effect of various concentrations of classic anti-neutrophil cytoplasmic autoantibodies (C-ANCA) IgG on proteinase-3 alpha$_1$ -antitrypsin (PR3-α_1AT) (a) and neutrophil elastase (NE)-α_1AT (b) complexation. PR3 (23 nM) or NE (23 nM) was incubated with increasing concentrations of three C-ANCA IgGs, one perinuclear anti-neutrophil cytoplasmic autoantibody (P-ANCA) IgG (anti-NE) and one ANCA-negative IgG (indicated on the abscissa as molar ratio of IgG to PR3 and NE respectively) for 30 min at room temperature. Subsequently, a 28-fold molar excess of α_1AT (to PR3 and NE respectively) was added. After 30 min incubation at room temperature, formed complexes were measured by RIA and expressed in nMol concentrations. Results represent the mean of two experiments. ○, C-ANCA 1; ●, C-ANCA 2; △, C-ANCA 3; ▲, P-ANCA; ▽, ANCA negative.

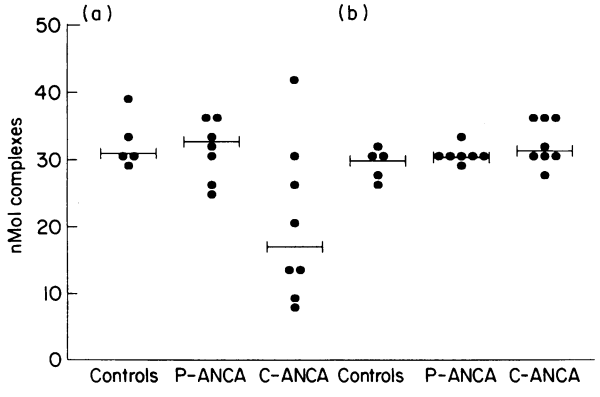

Fig. 6. Proteinase-3-alpha$_1$ -antitrypsin (PR3-α_1AT) (a) and neutrophil elastase (NE)-α_1AT (b) complexation in classic anti-neutrophil cytoplasmic autoantibody (C-ANCA)-positive sera. PR3 or NE was added to C-ANCA-positive sera, perinuclear anti-neutrophil cytoplasmic autoantibody (P-ANCA)-positive sera and ANCA-negative sera (controls) for 30 min at room temperature (yielding final concentrations of 34·5 nM for both PR3 and NE and final serum dilutions of 1:40). Subsequently, formed complexes were measured by RIA and expressed in nMol concentrations. Results represent the mean of two experiments and the median value of each subject group is shown.

16·7–90·3; $P=0·0034$) compared with the controls (ANCA-negative IgG, which had no effect on PR3-α_1AT complexation compared with PR3-α_1AT complexation in the absence of IgG, results not shown). C-ANCA IgG inhibited PR3-α_1AT complexation in a clear dose-dependent manner (Fig. 5a). Half-maximal inhibition was observed with approximately an average of a 10-fold molar excess of IgG to PR3 with the three strongest inhibiting C-ANCA IgGs. No significant inhibition was observed by P-ANCA IgG.

PR3-α_1AT complexation in C-ANCA-positive sera

Whereas in P-ANCA-positive sera PR3-α_1AT complexes were formed at levels comparable to those formed in ANCA-negative sera (controls), formation of PR3-α_1AT complexes in C-ANCA-positive sera was reduced, median 44·8% (range 1·9–74·4; $P=0·057$) compared with the controls (Fig. 6a). In one C-ANCA-positive serum an increase in PR3-α_1AT complexation (35·3% compared with the controls) was observed. However, complexation of PR3 with purified α_1AT was inhibited by the purified IgG from this particular serum. The α_1AT concentrations of all sera fell within normal range values as determined by nephelometry (1·7–3·2 mg/ml). Functional activity of α_1AT in each serum was likely to be sufficient for complete inactivation of PR3 since NE-α_1AT complexation in C-ANCA sera (to which purified NE had been added) did not differ from the complexation in ANCA-negative sera (see below).

Control experiments with NE

To exclude aspecific measurements in the experiments described above, identical experiments were performed with NE instead of PR3. They showed that C-ANCA IgG did not affect either the proteolytic activity of NE (Figs 1b and 2b) nor the complexation of NE with α_1AT (Fig. 4b). Likewise, complexation of NE with α_1AT in C-ANCA-positive sera to which purified NE had been added, did not differ from the complexation in ANCA-negative sera (Fig. 6b). P-ANCA IgG had no effect on the proteolytic activity of NE (Figs 1b and 2b) nor on the complexation of NE with a$_1$AT (Fig. 4b). One P-ANCA IgG with specificity against NE caused a limited inhibition of both the proteolytic activity of NE towards MeO-Suc-Ala-Ala-Pro-Val-pNA (Fig. 3b) and the complexation of NE with α_1AT (Fig. 5b) if the molar ratio of IgG to NE exceeded 45. Complexation of NE with α_1AT in P-ANCA-positive sera to which purified NE had been added did not differ from the complexation in ANCA-negative sera (Fig. 6b).

Relation of C-ANCA inhibitory activity with IF titre

Spearman rank correlation analysis was performed for determining correlations between C-ANCA inhibitory activity and C-ANCA titres in purified IgG and sera as determined by standard IF. C-ANCA titres in C-ANCA IgG correlated significantly with both the inhibition of PR3 elastinolysis ($r=0·85$, $P=0·0067$) and the inhibition of PR3-α_1AT complexation ($r=0·83$, $P=0·011$), but not with the inhibition of PR3 MeO-Suc-Ala-Ala-Pro-Val-pNA proteolysis ($r=0·28$, $P=0·5$). C-ANCA titres in C-ANCA sera had no significant correlation with the reduction in PR3-α_1AT complexation in C-ANCA sera ($r=0·37$, $P=0·36$).

DISCUSSION

Because C-ANCA titres correlate with disease activity it has been suggested that C-ANCA are directly involved in the pathogenesis of WG [1–4]. Activation of primed neutrophils by C-ANCA has been observed *in vitro* and this may be an important mechanism in the development of the disease [11]. Our findings indicate that C-ANCA also have other effects which may be of pathogenetic importance as well. In this study, we have demonstrated that C-ANCA significantly inhibit the proteolytic activity of PR3, particularly towards elastin. In addition, C-ANCA interfere with the complexation of PR3 with α_1AT. The inhibition by C-ANCA of PR3-α_1AT complexation was observed with purified IgG but also after the addition of PR3 to C-ANCA-positive sera. The latter experiment more

closely reflects the physiologic situation and suggests that also *in vivo* C-ANCA may inhibit the complexation of PR3 with α_1AT. These effects of C-ANCA on PR3 were specific since C-ANCA had no effect on NE proteolytic activity and the complexation of NE with α_1AT, and since P-ANCA had no effect on the proteolytic activity of PR3 and the complexation of PR3 with α_1AT. The limited amount of C-ANCA IgG necessary to cause the observed effects on PR3 suggests unusual high proportions of specific anti-PR3 IgG in WG patients (up to 16% considering bivalent binding by IgG), but this seems unlikely. Alternative mechanisms may be involved, for example modification of the active site of PR3 by C-ANCA followed by dissociation of C-ANCA from PR3. Further studies are clearly necessary to clarify possible mechanisms. A significant correlation was found between C-ANCA titres in purified IgG determined by standard IF and C-ANCA inhibitory activity towards PR3 elastinolysis and PR3-α_1AT complexation. However, no significant correlation was found between C-ANCA titres in sera and the reduction in PR3-α_1AT complexation in sera. This discrepancy might possibly be explained by differences in the total amount of IgG or α_1AT in the sera. Previous studies showed that C-ANCA predominantly belong to the IgG1, IgG3 and IgG4 subclass [22,23]. Since protein A chromatography, which has a low affinity for IgG3, was used for the purification of IgG, the contribution of this particular subclass to C-ANCA (inhibitory) activity might have been overlooked. However, no differences in C-ANCA inhibitory activity were found between protein A purified IgG and protein G purified IgG from three C-ANCA-positive sera (results not shown).

PR3 activity *in vivo* is controlled by the proteinase inhibitors α_1AT and alpha$_2$-macroglobulin [13]. Presumably, inhibition of PR3-α_1AT complexation by C-ANCA implicates defective inactivation of PR3 by α_1AT. The same C-ANCA IgGs inhibited PR3 proteolytic activity, and therefore act like alternative inhibitors. In most cases tested, residual proteolytic activity of PR3 after complexation with C-ANCA IgG was completely inhibited by α_1AT. However, one C-ANCA IgG prevented complexation with α_1AT, but did not interfere with PR3 proteolytic activity. In this way PR3 might escape from its physiologic control, which emphasizes the possible pathogenetic role of C-ANCA. The different effects of the C-ANCA IgGs are probably due to differences in epitope specificities.

In vivo, PR3 might be accessible to circulating C-ANCA either by its presence on the outer membrane of primed neutrophils or by its release upon activation of neutrophils [7,24]. Subsequently, upon binding of PR3 C-ANCA could cause derailment of the inflammatory process by a number of different mechanisms. The inhibition of PR3 complexation with α_1AT by C-ANCA might interfere with the clearance of PR3 released from activated neutrophils. Presumably, protease-serpin complexes are cleared from the circulation by binding of the serpin moiety to specific receptors present on hepatocytes, as has been described for NE-α_1AT complexes [25]. Persistence of antibody-protease complexes in the tissues may lead to damage either through residual antibody-bound PR3 activity or through dissociation of active PR3 from the complex. The inhibition of PR3 proteolytic activity by C-ANCA might lead to persistence of inflammatory stimuli, resulting in granuloma formation. Alternatively, C-ANCA might serve as an alternative inhibitor for PR3 instead of α_1AT, especially in the microenvironment of activated neutrophils where α_1AT can be inactivated by reactive oxygen derivates, and thus protect against tissue damage [26]. Possible other biological functions of PR3, for example its antimicrobial activity observed in *in vitro* experiments [20], are not well defined, but could also be influenced by C-ANCA.

The pathophysiological meaning of C-ANCA is thus difficult to assess. Both transversal and longitudinal studies should be undertaken to investigate the correlation between the effects of C-ANCA on different functions of PR3 and the clinical manifestations of WG patients. One out of two P-ANCA IgGs with specificity against NE showed limited inhibition of NE proteolytic activity and complexation of NE with α_1AT. Whether this finding has pathogenetic importance in case of anti-NE associated disease should be studied.

We found marked effects of C-ANCA on the activity of its target antigen PR3 with sera from eight WG patients. We suggest that this may be of importance in the pathogenesis of WG.

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REFERENCES

1 Van der Woude FJ, Rasmussen N, Lobatto S *et al.* Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. Lancet 1985; i:425–9.

2 Savage COS, Winearls CG, Jones S, Marshall PD, Lockwood CM. Prospective study of radioimmunoassay for antibodies against neutrophil cytoplasm in diagnosis of systemic vasculitis. Lancet 1987; i:1389–93.

3 Lüdemann G, Gross WL. Autoantibodies against cytoplasmic structures of neutrophil granulocytes in Wegener's granulomatosis. Clin Exp Immunol 1987; **69**:350–7.

4 Kallenberg CGM, Cohen Tervaert JW, van der Woude FJ, Goldschmeding R, von dem Borne AEGKr, Weening JJ. Autoimmunity to lysosomal enzymes: new clues to vasculitis and glomerulonephritis? Immunol Today 1991; **12**:61–64.

5 Cohen Tervaert JW, van Woude FJ, Fauci AS *et al.* Association between active Wegener's granulomatosis and anticytoplasmic antibodies. Arch Int Med 1989; **149**:2461–5.

6 Cohen Tervaert JW, Huitema MG, Hené RJ, Sluiter WJ, The TH, van der Hem GK, Kallenberg CGM. Prevention of relapses in Wegener's granulomatosis by treatment based on antineutrophil cytoplasmic antibody titre. Lancet 1990; ii:709–11.

7 Goldschmeding R, van der Schoot CE, ten Bokkel Huinink D, Hack CE, van den Ende ME, Kallenberg CGM, von dem Borne AEGKr. Wegener's granulomatosis autoantibodies identify a novel diisopropylfluorophosphate-binding protein in the lysosomes of normal human neutrophils. J Clin Invest 1989;**84**:1577–87.

8 Niles JL, McCluskey RT, Ahmad MF, Arnaout MA. Wegener's granulomatosis autoantigen is a novel neutrophil serine proteinase. Blood 1989; **74**:1888–93.

9 Lüdemann J, Utecht B, Gross WL. Anti-neutrophil cytoplasm antibodies in Wegener's granulomatosis recognize an elastinolytic enzyme. J Exp Med 1990; **171**:357–62.

10 Jennette JC, Hoidal JR, Falk RJ. Specificity of anti-neutrophil cytoplasmic autoantibodies for Proteinase 3. Blood 1990; **75**:2263–5.

11 Falk RJ, Terrell RS, Charles LA, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals *in vitro*. Proc Natl Acad Sci USA 1990; **87**:4115–9.

12 Kao RC, Wehner NG, Skubitz KM, Gray BH, Hoidal JR. Proteinase 3. A distinct human polymorphonuclear leukocyte

proteinase that produces emphysema in hamsters. J Clin Invest 1988;**82**:1963–73.

13 Rao NV, Wehner NG, Marshall BC, Gray WR, Gray BH, Hoidal JR. Characterization of Proteinase-3 (PR-3), a neutrophil serine proteinase. Structural and functional properties. J Biol Chem 1991; **266**:9540–8.

14 Wiik A. Delineation of a standard procedure for indirect immunofluorescence detection of ANCA. APMIS 1989; **97**(Suppl. 6): 12–13.

15 Cohen Tervaert JW, Goldschmeding R, Elema JD *et al.* Autoantibodies against myeloid lysosomal enzymes in crescentic glomerulonephritis. Kidney Int 1990; **37**:799–806.

16 Abbink JJ, Kamp AM, Swaak AJG, Hack CE. Production of monoclonal antibodies against inactivated α_1-antitrypsin: cross-reactivity with complexed α_1-antitrypsin and application in an assay to determine inactivated and complexed α_1AT in biological fluids. J Immunol Methods 1992; **143**:197–208.

17 Kam C-M, Kerrigan JE, Dolman KM, Goldschmeding R, von dem Borne AEGKr, Powers JC. Substrate and inhibitor studies on Proteinase 3. FEBS Lett. 1992; **297**:119–23.

18 Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein. Anal Biochem 1976; **72**:248–54.

19 Beatty K, Bieth J, Travis J. Kinetics of association of serine proteinases with native and oxidized α_1-Proteinase Inhibitor and α_1-Antichymotrypsin. J Biol Chem 1980;**255**:3931–4.

20 Campanelli D, Detmers PA, Nathan CF, Gabay JE. Azurocidin and a homologous serine protease from neutrophils. Differential antimicrobial and proteolytic properties. J Clin Invest 1990; **85:** 904–15

21 Nuijens JH, Abbink JJ, Wachtfogel YT *et al.* Plasma elastase-α_1-antitrypsin and lactoferrin in sepsis: evidence for neutrophils as mediators in fatal sepsis. J Lab Clin Med. 1992; **119:** 159–68.

22 Brouwer E, Cohen Tervaert JW, Horst G, Huitema MG, van der Giessen M, Limburg PC, Kallenberg CGM. Predominance of IgG1 and IgG4 subclasses of anti-neutrophil cytoplasmic autoantibodies (ANCA) in patients with Wegener's granulomatosis and clinically related disorders. Clin Exp Immunol 1991; **83**:379–86.

23 Jayne DRW, Weetman AP, Lockwood CM. IgG subclass distribution of autoantibodies to neutrophil cytoplasmic antigens in systemic vasculitis. Clin Exp Immunol 1991; **84**:476–81.

24 Csernok E, Lüdemann J, Gross WL, Bainton DF. Ultrastructural localization of Proteinase 3, the target antigen of anti-cytoplasmic antibodies circulating in Wegener's granulomatosis. Am J Pathol 1990; **137**:1113–20.

25 Perlmutter DH, Joslin G, Nelson P, Schasteen C, Adams SP, Fallon RJ. Endocytosis and degradation of α_1-antitrypsin-protease complexes is mediated by the Serpin-Enzyme Complex (SEC) receptor. J Biol Chem 1990; **265**:16713–6.

26 Weiss SJ. Tissue destruction by neutrophils. N Engl J Med 1989; **320**:365–76.