Autoantibodies to Proteinase 3 and Myeloperoxidase in Systemic Sclerosis

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ABSTRACT. ***Objective.*** We evaluated the prevalence and clinical significance of proteinase 3 (PR3-) and myeloperoxidase (MPO-) antineutrophil cytoplasmic antibodies (ANCA) in 115 patients with systemic sclerosis (SSc, scleroderma).

Methods. Sera were assayed by 2 independent centers, which used indirect immunofluorescence (IIF) and direct ELISA as screening tests. Inhibition-ELISA for PR3- and MPO-ANCA and PR3 capture-ELISA experiments were also performed. The clinical features of the ANCA positive were compared with those of the ANCA negative scleroderma patients.

Results. The IIF test revealed 5 P-ANCA positive sera (4.34%). Surprisingly, by ELISA 2 of these were PR3-ANCA positive, one was MPO-ANCA positive, and 2 were both PR3- and MPO-ANCA positive. In addition, 3 IIF negative sera were ELISA positive, 2 for PR3- and one for MPO-ANCA. ELISA results were confirmed by fluid phase inhibition experiments. Only 2 out of the 6 PR3-direct ELISA positive sera were positive by PR3-capture ELISA at low titers. Neither PR3- nor MPO-ANCA were significantly associated to any clinical feature of patients with SSc.

Conclusion. As well as the previously described MPO-ANCA, even PR3-ANCA may be detected in some sera from patients with SSc. The IIF pattern and the negative results obtained with PR3-capture ELISA suggest that different epitopes from those recognized by vasculitis sera might be involved with PR3-ANCA in SSc, and show the importance of combining IIF and ELISA tests for ANCA detection. (J Rheumatol 2002;29:918–23)

Key Indexing Terms:
ANTIBODIES TO PROTEINASE 3 — ANTIBODIES TO MYELOPEROXIDASE
ANTINEUTROPHIL CYTOPLASMIC ANTIBODIES — SYSTEMIC SCLEROSIS

Antineutrophil cytoplasmic antibodies (ANCA) are widely considered diagnostic markers for Wegener's granulomatosis (WG), microscopic polyangiitis, Churg-Strauss syndrome, and pauciimmune crescentic necrotizing glomerulonephritis when they are found in patients with signs or symptoms of primary vasculitides[1]. This high diagnostic specificity is not, however, confirmed when sera from patients with a wide range of diseases are tested[2]. ANCA are determined by indirect immunofluorescence (IIF) test and ELISA. Two major fluorescence patterns are described: cytoplasmic (C-ANCA) and perinuclear (P-ANCA) stainings. The former is strongly associated with antibodies to proteinase 3 (PR3) and the latter with antibodies against a number of proteins, among which myeloperoxidase (MPO) is the most frequent[2]. Since 1990[3] ANCA have been investigated in the sera of patients with systemic sclerosis (SSc) or scleroderma, with a prevalence between 0 and 9.09%[3-11], and since 1996 investigators have described patients with ANCA positive SSc in case reports[12-24]. Nonetheless a precise definition of the clinical value of ANCA in scleroderma is needed. Moreover, only P-ANCA pattern and/or MPO-ANCA have been tested in the studies cited, while as far as we know C-ANCA patterns and/or PR3-ANCA have never been described in scleroderma sera.

We evaluated the prevalence of PR3- and MPO-ANCA in a group of patients with SSc, using IIF and ELISA screening tests assessed in 2 independent centers. The clinical value of ANCA in our patients with scleroderma was also determined.

MATERIALS AND METHODS

Patients. We examined 115 patients, 100 women and 15 men (mean age 54.28 yrs ± 12.92 SD, range 23–83), with SSc; 55 had a diffuse and 60 a limited clinical form. All patients fulfilled American Rheumatism Association criteria for scleroderma[25]. Those showing signs or symptoms of other associated diseases were excluded. The following clinical features were evaluated according to described criteria[26]: age, sex, disease duration (months), clinical form of disease, Raynaud's phenomenon, and kidney, lung, heart and esophagus involvement. Renal involvement was investigated by the following measures: hypertension (minimum blood pressure > 100 mm Hg), renal failure (serum creatinine > 1.3 mg/dl), proteinuria (> 0.5 g/24 h), and hematuria. Histological kidney data were available for only 2

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patients with scleroderma renal crisis. Current or previous D-penicillamine treatment was also noted.

Assays for ANCA. ANCA tests were performed by 2 independent centers. Sera from 75 healthy subjects matched for age and sex with our scleroderma patients were used as controls.

IIF on ethanol and formalin fixed human neutrophils. Commercial slides (Menarini, Inova Diagnostics, San Diego, CA, USA) were employed as a substrate by both centers and IIF was performed according to the standard procedure[27,28] using an initial serum dilution of 1:20. The IIF patterns were defined as cytoplasmic (C-ANCA), perinuclear (P-ANCA), atypical, or negative stainings.

ELISA for PR3- and MPO-ANCA. PR3 antigen was purified by Center 1 as described[1,29,30], whereas it was purchased from Wieslab, Lund, Sweden, by Center 2. MPO antigen was purchased by both centers from Calbiochem, UK. The same ELISA method with minor modifications was used for anti-PR3 and anti-MPO antibody assays[29,30]. Results were expressed as arbitrary units by reading off a standard curve, and the normal range for each test was established as the mean plus 2 standard deviations calculated from 100 healthy blood donors. The cutoff values of Centers 1 and 2 for PR3-ANCA were 15 and 30 units (U) and for MPO-ANCA 15 and 14 U. We considered sera as "low positive" when the antibody units were between the cutoff value and 2.5-fold the cutoff value; as "medium positive" between 2.5- and 5-fold the cutoff value; and as "high positive" if greater than 5-fold the cutoff value.

PR3 capture-ELISA. The presence of PR3-ANCA was also assessed by Center 1 in all PR3 direct-ELISA positive samples using a PR3 capture-ELISA (Wieslab). This assay, developed to circumvent the possible partial denaturation of the antigen with alteration of conformational epitopes due to the direct coating of PR3 to plastic, is based on immobilization of the antigen by capturing monoclonal antibody (Mab). This particular Mab was chosen from those antibodies linked to epitopes not usually recognized by vasculitis sera[31].

Inhibition ELISA for PR3- and MPO-ANCA. The ability of fluid phase PR3 and MPO to inhibit the binding of autoantibodies to the respective antigens coated onto microtiter plates was tested by inhibition assays performed on one PR3-ANCA positive and one MPO-ANCA positive scleroderma serum, as described[31,32]. This test was performed only by Center 1.

Detection of other antibodies. Sera were screened for antinuclear antibodies (ANA) by IIF on HEp-2 cells (Immunoconcepts, Sacramento, CA, USA). Anticentromere antibody (ACA) was detected by centromere fluorescence pattern on HEp-2 cells. Precipitating antibodies to topoisomerase I (anti-topo-I) and other extractable nuclear antigens (ENA) such as Sm, U1-RNP, SSA/Ro, SSB/La, PM1, Jo1, PCNA, Ku, and SL were investigated by an in-house counterimmunoelectrophoresis method. Anti-ds-DNA antibodies were assayed by IIF using *Crithidia luciliae* (Immunoconcepts) as substrate.

Statistical analysis. Fisher's exact test was used for comparison of prevalence of clinical features in the PR3 positive and MPO positive patients. It was also applied to the group of patients with any kind of positivity (either PR3 or MPO) and to the group that was negative for both antibody specificities. The Mann-Whitney U test was used for comparison of mean values of age and disease duration in ANCA positive and negative patients. A p value < 0.05 was considered statistically significant.

RESULTS

The combined results of IIF and ELISA from the 2 test centers are given in Table 1. The results reported by the 2 centers regarding IIF were in agreement. When the assay was performed on ethanol fixed neutrophils, 5 sera (4.34%) showed a pattern characterized by fluorescence of the rim of the neutrophil nuclear lobes, which in all cases was associated with a diffuse or speckled nuclear fluorescence (Figure 1). A diffuse grainy or centromere ANA pattern was respectively observed on HEp-2 cells testing the same sera. This perinuclear reaction became more evident as sera were diluted until the nuclear fluorescence reaction faded (Figure 2). When formalin fixed neutrophils were used as a substrate, a diffuse granular cytoplasmic fluorescence was observed (Figure 3). Using international guidelines[2] we defined this pattern as P-ANCA staining. Only nuclear fluorescence, which faded when formalin fixed cells were employed as a substrate, was observed on ethanol fixed neutrophils in 108 sera (93.91%). All these sera were ANA positive in IIF employing HEp-2 cells as substrate. Two sera (1.73%) were negative on both ethanol and on formalin fixed neutrophils. The C-ANCA pattern was never found on ethanol fixed neutrophils.

ELISA results for PR3-ANCA from the 2 test centers differed only with regard to the antibody levels of 2 sera, while ELISA results for MPO-ANCA were in complete agreement. Only PR3-ANCA were detected in 4 cases (3.47%) and were associated with MPO-ANCA in 2 (1.73%). Only MPO-ANCA were found in 2 cases (1.73%) and were associated with PR3-ANCA in 2 (1.73%). IIF test revealed P-ANCA pattern in 2 out of 4 PR3-ANCA positive sera and in one of 2 MPO-ANCA positive sera, and in both PR3-/MPO-ANCA positive sera. ANCA with specificities for PR3 or MPO antigens were not detected in the control sera.

The PR3 capture-ELISA was positive at low titers in only 2 out of the 6 PR3 direct-ELISA positive patients (Patients 1 and 7).

The specificity of anti-PR3 and anti-MPO reactions was confirmed by fluid phase inhibition-ELISA experiments in the sera of Patients 7 and 5, respectively.

ANA were found in 113 scleroderma sera (98.26%), ACA in 38 (33.04%), anti-topo-I in 76 (66.08%), anti-SSA/Ro in 7 (6.08%), anti-SSB/La in one (0.86%), and anti-U1-RNP in one (0.86%). In no case did we find antibodies to other ENA or to dsDNA.

Clinical and serological features of the 8 ANCA positive scleroderma patients are given with IIF and ELISA results in Table 2. The comparison between the clinical and serological features of PR3-ANCA positive patients and MPO-ANCA positive patients gave a result that was not statistically significant. Table 3 outlines the clinical and serological features of ANCA positive and ANCA negative patients. The statistical comparison showed no significant difference between the 2 groups. In particular, no statistically significant association was found between ANCA positivity and kidney involvement, including those cases of normotensive renal failure, as illustrated in Table 4. The 2 patients with scleroderma renal crisis resulting in hemodialysis were both negative to ANCA testing.

DISCUSSION

This study investigated the prevalence of PR3- and MPO-

Table 1. Results of IIF assays and ELISA from the 2 testing centers.

Patient	MPO–ANCA		PR3–ANCA		IIF–ANCA	
	Center 1	Center 2	Center 1	Center 2	Center 1	Center 2
1	–	–	High	High	P-ANCA	P-ANCA
2	–	–	Medium	Medium	–	–
3	–	–	Medium	High	P-ANCA	P-ANCA
4	–	–	Low	Low	–	–
5	Medium	Medium	–	–	P-ANCA	P-ANCA
6	Low	Low	–	–	–	–
7	Low	Low	High	High	P-ANCA	P-ANCA
8	Low	Low	Low	High	P-ANCA	P-ANCA

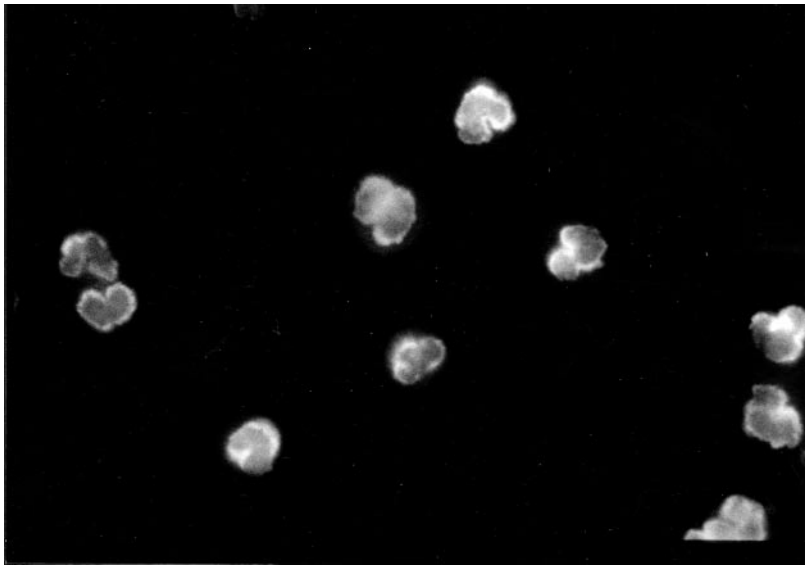

Figure 1. IIF test on ethanol fixed neutrophils shows P-ANCA staining, characterized by fluorescence of the rim

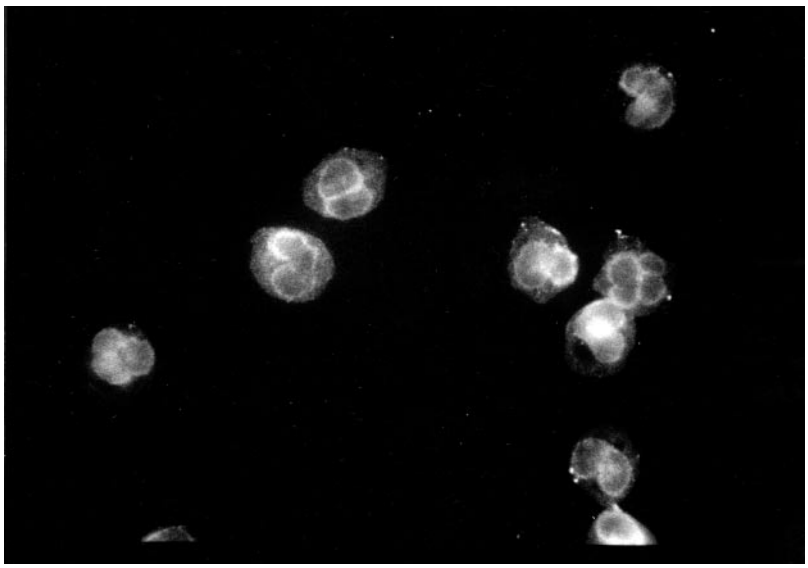

Figure 2. IIF test on ethanol fixed neutrophils using more diluted serum than in Figure 1. The perinuclear reaction is more evident here since diffuse nuclear fluorescence has faded (original magnification ×1000).

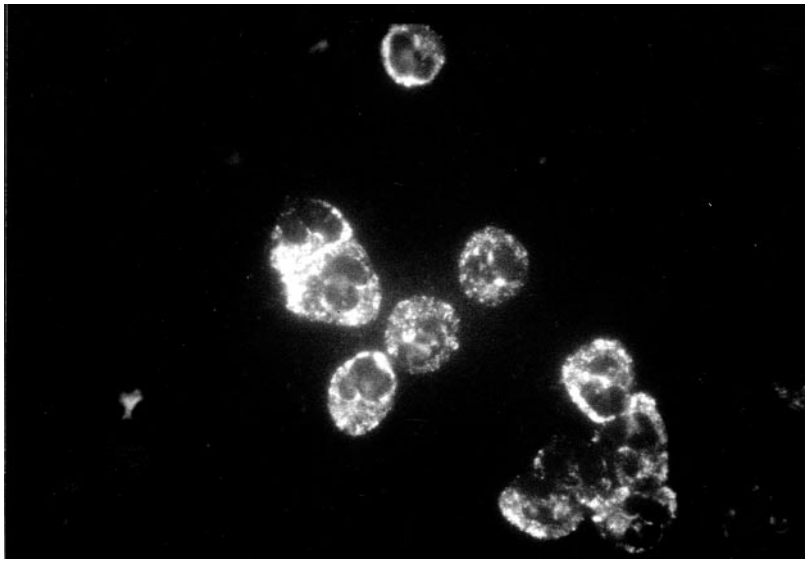

Figure 3. IIF test on formalin fixed neutrophils shows P-ANCA staining, characterized by diffuse granular cytoplasmic fluorescence (original magnification ×1000).

Table 2. Clinical and serological features of ANCA positive patients with scleroderma.

Patient	Sex	Age, yrs	Disease Duration, mo	Diffuse Form	Limited Form	Raynaud's Phenomenon	Lung	Heart	Involvement of Kidney	Esophagus*	D–Penicillamine Treatment	Anti–Topo–I	Anti–Centromere	ANCA ELISA	ANCA IIF
1	M	66	22	+	–	+	–	–	+	+	–	+	–	PR3	P-ANCA
2	F	75	16	–	+	+	+	–	–	+	–	–	+	PR3	–
3	F	34	16	+	–	+	+	–	–		+	+	–	PR3	P-ANCA
4	F	60	102	–	+	+	–	–	–	+	+	+	–	PR3	–
5	F	62	32	+	–	+	+	+	+	+	–	+	+	MPO	P-ANCA
6	F	55	197	–	+	+	+	–	–	–	+	–	+	MPO	–
7	F	41	155	–	+	+	+	+	–	+	+	+	–	PR3 MPO	P-ANCA
8	F	58	12	+	–	+	+	–	–		+	+	–	PR3 MPO	P-ANCA

*Esophagus involvement was studied in 6/8 patients.

Table 3. Comparison of clinical and serological features of ANCA positive and ANCA negative patients with scleroderma.

	ANCA+, n = 8	ANCA–, n = 107	p
Sex, F, M	7,1	93, 14	NS
Mean age, yrs	56.37	54.12	NS
Diffuse form (%)	4 (50)	51 (47.66)	NS
Limited form (%)	4 (50)	56 (52.33)	NS
Disease duration, mean mo	69	87.09	NS
Raunaud's phenomenon (%)	8 (100)	104 (97.19)	NS
Lung involvement (%)	6 (75)	69 (64.48)	NS
Heart involvement	2 (25)	36 (33.64)	NS
Esophagus involvement* (%)	5 (83.33)	59 (71.08)	NS
Kidney involvement (%)	2 (25)	8 (6.95)	NS
D–penicillamine treatment (%)	5 (62.50)	74 (71.15)	NS
Anti-topo–I (%)	6 (75)	70 (65.42)	NS
Anticentromere (%)	3 (37.50)	35 (32.71)	NS

*Esophagus involvement was studied in 6/8 ANCA positive and 83/107 ANCA negative patients.

ANCA in patients exclusively with SSc. Assays for ANCA detection were performed according to international guidelines[2]. In particular the association of IIF and antigen-specific ELISA was used to improve test reliability. Moreover, the tests were performed blindly by 2 independent centers, which obtained similar results. The findings of previous reports were confirmed concerning presence of MPO-ANCA in some patients with SSc[3,5-8,10,11,13-21,23,24]. In addition, PR3-ANCA was observed in some patients. However, these latter differed from PR3-ANCA described in patients with WG in their IIF pattern, which was not C- but P-ANCA in sera with high antibody levels from ELISA. The presence of "true" P-ANCA was confirmed by results obtained with formalin fixation. Moreover, the specificity of these PR3-ANCA was confirmed by inhibition assay. On the other hand, capture-ELISA results suggested that these antibodies to PR3 antigen in most cases might recognize an epitope that was different from that recognized by anti-PR3

Table 4. Clinical and laboratory characteristics of renal involvement in ANCA positive and ANCA negative patients with scleroderma.

ANCA+, n = 2				ANCA –, n = 8			
Hypertension	Renal Failure	Proteinuria	Hematuria	Hypertension	Renal Failure	Proteinuria	Hematuria
–	+	+	+	–	+	+	+
+	+	+	–	+	+	+	–
				–	–	+	+
				–	+	+	–
				–	+	–	+
				+	–	+	–
				+	–	+	–
				+	+	+	+

antibodies detected in patients with WG. Indeed only 2 out of the 6 positive sera were weakly positive in the PR3 capture-ELISA. Since the capture-ELISA utilizes a Mab directed against an epitope usually not recognized by vasculitis sera, it could be speculated that PR3-ANCA positive scleroderma sera are mostly directed against the same epitope (or one similar to it) recognized by the Mab used to immobilize PR3 onto the microtiter plates. As reported for anti-MPO antibodies by Segelmark, *et al*[33], even the different fluorescence pattern of PR3-ANCA may be due to different availabilities of the epitopes in the microenvironment where PR3 is present. In other articles[3-11] and case reports[12-24] on ANCA in scleroderma sera the presence of PR3-ANCA has never been described, probably because ELISA specific for PR3 were rarely performed in these studies. Indeed most of the authors used IIF as a screening test, which detected only the P-ANCA pattern. Only MPO specificity was therefore investigated by ELISA. Following this commonly applied criterion, it would have been impossible to detect PR3-ANCA in scleroderma sera. Instead, testing all the sera not only by IIF and ELISA for MPO-ANCA but also by ELISA for PR3-ANCA, we detected a particular PR3-ANCA associated with the P-ANCA fluorescence pattern or with negative staining. To our knowledge only 3 studies[9,10,16] describe ELISA for anti-PR3 antibodies on scleroderma sera. Merckel, *et al*[9] and Kind, *et al*[16], respectively, tested 45 and 2 sera giving negative results, while Choi, *et al*[10] assayed 27 sera, detecting one positive for PR3-ANCA. However, in their interpretation these authors ruled out the presence of PR3-ANCA in scleroderma sera, due to the lack of a C-ANCA pattern and the negative result of a sandwich anti-PR3 ELISA. These peculiarities of PR3-ANCA detected by Choi, *et al*[10] are similar to those characterizing most of the PR3-ANCA found in our scleroderma sera. The PR3-ANCA result was not significantly associated with any definite clinical feature. Thus it is difficult to explain their significance in scleroderma.

MPO-ANCA have been detected in patients with SSc, with controversial clinical significance. Some case reports[13-15,17-21,23,24] describe their association with normotensive crescentic glomerulonephritis with progressive renal failure, while other studies came to different conclusions. Choi, *et al*[10] and Chikazawa, *et al*[11] found no clinical association; Kiraz, *et al*[7] observed a correlation with severity of scleroderma; and Endo, *et al*[5], Akimoto, *et al*[6], and Locke, *et al*[8] found an association with necrotizing vasculitis. In our scleroderma sera MPO-ANCA occurred with a low prevalence (3.47%), showing a classical P-ANCA pattern at medium antibody levels and negative staining at low antibody levels. Their antigenic specificity was confirmed by inhibition studies. Moreover these antibody results were not significantly associated with any clinical feature, including normotensive renal failure.

The statistical comparison of clinical and serological features in ANCA positive and negative patients showed no significant difference. The lack of significant association, however, may be due to the low number of ANCA positive patients.

We conclude that in addition to MPO-ANCA, PR3-ANCA may also be detected in some patients with scleroderma. The P-ANCA pattern associated with PR3-ANCA and the negative results of the PR3 capture-ELISA raise the possibility that different epitopes from those recognized by vasculitis sera are involved by PR3-ANCA in scleroderma patients, and stress the importance of combining IIF and antigen-specific ELISA tests for ANCA detection.

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