Bactericidal/Permeability-Increasing Protein and Cathepsin G Are the Major Antigenic Targets of Antineutrophil Cytoplasmic Autoantibodies in Systemic Sclerosis

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ABSTRACT. ***Objective.*** To study the prevalence and antigenic specificity of antineutrophil cytoplasmic autoantibodies (ANCA) in patients with systemic sclerosis (SSc).

Methods. Sera from 68 patients with SSc were screened for ANCA by indirect immunofluorescence (IIF) assay and for antibodies to myeloperoxidase (MPO) by ELISA. All sera positive for ANCA on IIF were analyzed for reactivity against antigenic targets other than MPO [bactericidal/permeability-increasing protein (BPI), cathepsin G, lysozyme, elastase, PR3, and lactoferrin]. Twenty-three sera negative for ANCA were also tested for antibodies to BPI and cathepsin G using ELISA.

Results. The study included 33 patients with diffuse and 35 with limited SSc. ANCA was detected in 24 of the 68 sera (35.3%). In these 24 sera the antigenic targets were BPI in 14, cathepsin G in 13, and MPO in 8. Sera of 11 patients had reactivity against both BPI and cathepsin G. In sera, that were negative for ANCA, antibodies to BPI (4/23), cathepsin G (3/23), and MPO (1/44) were found in a small proportion of patients. Patients with antibodies to BPI had lower skin score, whereas no patient with antibodies to MPO had renal disease.

Conclusion. BPI and cathepsin G are the major antigenic targets of ANCA seen in patients with SSc. Patients with antibodies to BPI had lower skin scores. (J Rheumatol 2003;30:1248–52)

Key Indexing Terms:
AUTOANTIBODIES SCLERODERMA CONNECTIVE TISSUE DISEASE

Systemic sclerosis (SSc) is a chronic inflammatory disease characterized by excessive fibroblast proliferation in skin, lungs, and gastrointestinal tract. Microvascular injury is the major factor responsible for uncontrolled fibrogenesis. In SSc, serum concentrations of cell adhesion molecules like E-selectin, P-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 are elevated, and these correlate with *in situ* expression on the surface of the endothelial cells and with disease activity[1]. This overexpression of adhesion molecules on endothelial cells may lead to accumulation of activated leukocytes and release of cytokines and growth factors leading to endothelial cell injury, ischemia, and fibrosis.

Vasculitis associated with antineutrophil cytoplasmic autoantibody (ANCA) has a similar pathogenetic mechanism, i.e., vascular injury mediated by enhanced expression of adhesion molecules on endothelial cells, leading to neutrophil adhesion and activation[2,3]. Thus, it is possible that ANCA has a role in the pathogenesis of SSc.

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Data on the presence of ANCA in patients with SSc are sparse[4-7]. A few case reports suggest an association of anti-myeloperoxidase (anti-MPO) antibodies with crescentic glomerulonephritis in patients with SSc[8-11]. In addition, the detailed antigenic specificities of ANCA in SSc have not been elucidated.

We studied the seroprevalence and antigenic specificities of ANCA in patients with SSc. As MPO is the antigenic target reported in a few patients with SSc, anti-MPO antibodies were analyzed in all patients, whereas other antigenic specificities were looked for only in ANCA-positive patients and in a proportion of patients with absence of ANCA.

MATERIALS AND METHODS

Sixty-eight patients with SSc fulfilling the American College of Rheumatology criteria[12] were studied. Of these, 33 had diffuse disease and 35 had limited SSc. Serum samples were obtained from these patients on their first visit and stored at –70°C until further use. No patient had any evidence of infection at the time of sample collection. Clinical details like age at onset of disease, duration of disease, extent of skin involvement, presence of pitting scars, ulcers, gangrene and internal organ involvement, and serological findings were recorded.

The median age of our 68 patients (60 women) was 32 years (range 14–63 yrs) and the median duration of disease was 3 years (range 0.25–20 yrs). The number of patients with different organ system involvement was as follows: interstitial lung disease 43, musculoskeletal 39, esophageal dysfunction 20, pulmonary hypertension 5, cardiac 2, renal one (biopsy proven crescentic glomerulonephritis). Cutaneous findings included pitting scars in 34, ulcers in 32, and gangrene in 8. Fourteen patients were receiving d-penicillamine prior to collection of sample. Antinuclear antibodies were present in 40 patients.

IIF assay for ANCA. This was done according to the method described by Roberts, *et al*[13], with minor modifications. Briefly, for preparation of neutrophil smears, drops of fresh blood were placed on slides coated with 4% bovine serum albumin and incubated at 37°C for 30 min in a moist chamber. The slides were then washed to remove the clot, dried, and fixed in 100% ethanol for 5 min. Twenty-five microliters of serum (diluted 1:40) in phosphate buffered saline (0.15 M, pH 7.2; PBS) was spread on ethanol-fixed neutrophil smears, and the slides were incubated at 37°C for 30 min in a moist chamber. The slides were then washed 3 times for 10 min each in PBS and incubated at 37°C for 30 min with FITC conjugated anti-human IgG (Sigma Chemical, St. Louis, MO, USA; diluted 1:200). The slides were again washed 3 times in PBS, mounted in glycerol, and examined under an epifluorescent microscope (Olympus, Japan). Positive and negative controls were run with each batch of samples. Immunofluorescence patterns were defined as pANCA (perinuclear distribution of staining), cANCA (cytoplasmic distribution), and atypical ANCA (staining did not conform to either of the 2 patterns).

Each sample was tested 3 times; those testing positive on 2 or more occasions were considered positive. All positive samples were then tested in doubling dilutions to determine the end titer. Sera from 100 healthy individuals were tested to define the normality. Of these, 2 were found to be positive.

ELISA for MPO. MPO is the most commonly described antigenic target for ANCA in SSc. All 68 samples were tested using commercial enzyme immunoassay (ELISA) kits for detection of anti-MPO antibodies (BL Diagnostika, city, Germany). Optical density (OD) was measured at 450 nm using an ELISA reader. Specimens with absorbance value > 5 U/ml, as suggested by the manufacturer, were considered positive. Anti-MPO antibodies were also determined in sera of 20 healthy controls.

ELISA for other antigenic specificities. An indirect ELISA was done for the determination of specificities of ANCA in sera positive by IIF. This commercial kit detected antibodies against PR3, BPI, cathepsin G, lysozyme, lactoferrin, and elastase (COMBI ELISA kit, BL Diagnostika, Mainz, Germany). An OD quotient was calculated based on the OD reading of the test sample and the standard, according to the manufacturer's instructions. Values > 1 were considered positive.

Since antibodies to BPI and cathepsin G were found most frequently in ANCA positive sera, we tested ANCA negative samples to find the frequency of these antibodies in ANCA negative sera. Antibodies for these 2 specificities were tested using commercial ELISA kits (BL Diagnostika, Mainz, Germany), according to manufacturer's instructions. Sera of 20 healthy individuals served as controls. The mean + 2 SD of 20 healthy controls for antibodies to BPI was 4.43 + (2 × 1.98) = 8.39 and for cathepsin G was 18.09 + (2 × 8.80) = 35.70.

RESULTS

ANCA positivity was observed in 24 of the 68 patients. In 22, the pattern was atypical, whereas 2 had a perinuclear pattern. No patient had the cANCA pattern. Of these 24 patients, 9 had high (> 1:80) titer of ANCA using the endpoint dilution method (Figure 1).

In the 24 ANCA positive serum specimens, reactivity to BPI was present in 14, and that to cathepsin G in 13, with 11 patients showing reactivity to both. Antibodies to MPO, PR3, lactoferrin, lysozyme, and elastase were present in 8, 4, 2, 2, and 0 patients, respectively (Table 1). In 6 sera, despite ANCA positivity by IIF, no target antigen could be identified.

Anti-MPO antibodies were detected in 9 patients; of these, 8 were positive for ANCA by IIF. In most patients the titer of anti-MPO antibodies was low (< 7 units) (Figure 2). The patient with crescentic glomerulonephritis did not have detectable anti-MPO antibodies in her serum.

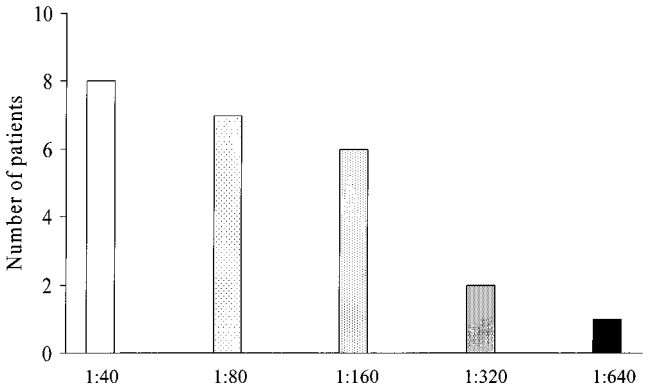

Figure 1. Endpoint dilution titer of ANCA in ANCA-positive sera from patients with SSc.

Among 23 ANCA negative samples, antibodies to BPI were present in 4, whereas 3 had antibodies to cathepsin G. When patients with antibodies to BPI were compared to those not having these antibodies with regard to clinical features, the only difference was in the skin score (11.72 vs 17.0; $p < 0.05$) (Table 2). There was no difference between patients with or without antibodies to cathepsin G.

Antibody reactivity was more frequent in patients with limited SSc compared to diffuse SSc; however, this did not reach statistical significance (Table 3).

DISCUSSION

ANCA was present in about one-third of patients with SSc, and showed a predominantly atypical pattern of staining. The major antigenic targets were BPI and cathepsin G.

The prevalence of ANCA we found in patients with SSc is higher than the previously reported figures of 2.5% to 15.6%[4,6,7,9]. We considered 2 possible explanations for this. First, D-penicillamine has been shown to induce anti-MPO antibodies in patients with SSc[7,14]. However, this is an unlikely explanation, since a similar proportion of our ANCA positive (6/24) and ANCA negative (8/44) patients were receiving D-penicillamine, and only one patient in the ANCA positive group had anti-MPO antibody. Second, autofluorescence can occur in stored sera; however, the presence of specific reactivity to defined neutrophil cytoplasmic antigens on ELISA argues against this possibility.

MPO has been defined as the target antigen in a few patients with SSc with normotensive renal failure associated with crescentic glomerulonephritis, distinct from the hypertensive scleroderma renal crisis[8-11]. Pulmonary hemorrhage has also been described in a few patients[9,15]. We found low titers of anti-MPO antibodies. In addition, in no case was MPO positivity associated with renal disease. Similarly poor association has been reported in a series of 7 patients where only one patient had systemic necrotizing angiitis at autopsy[6]. This could be due to the low frequency of renal disease in Indian patients with SSc[16]. One patient was MPO

Table 1. Antigenic specificities of ANCA-positive sera. Values represent OD quotients; values ≥ 1.0 are considered positive.

Patient	BPI	Cathepsin G	PR3	Lactoferrin	Lysozyme	Elastase	MPO, IU/ml
1	**2.222**	**1.465**	**1.087**	0.584	0.753	0.432	**6.3**
2	**2.117**	0.594	0.407	0.481	0.543	0.386	4.1
3	**1.430**	**1.142**	0.829	0.587	0.567	0.242	5.0
4	0.977	**1.222**	0.974	0.630	0.643	0.310	3.8
5	0.800	0.610	0.440	0.660	0.528	0.208	4.0
6	0.901	**1.215**	0.788	**1.255**	0.664	0.525	**6.6**
7	**1.614**	**1.959**	**1.798**	0.549	0.587	0.285	**8.6**
8	**1.674**	0.876	0.646	0.701	0.741	0.231	4.4
9	**1.392**	0.920	0.800	0.627	0.658	0.794	4.4
10	**1.231**	**1.750**	0.694	0.670	0.649	0.345	**6.2**
11	**1.241**	**3.103**	0.519	0.708	0.572	0.572	4.0
12	**1.323**	**2.041**	0.505	0.992	0.519	0.660	**6.0**
13	**2.769**	**5.751**	0.710	**1.157**	0.800	0.270	**5.6**
14	**2.060**	**3.366**	0.322	0.611	0.733	0.922	**6.2**
15	**2.815**	**4.825**	0.651	0.742	0.684	0.422	5.0
16	0.445	0.796	0.234	0.493	0.305	0.233	3.8
17	0.950	0.892	0.221	0.348	0.550	0.136	3.6
18	**1.001**	**4.764**	0.355	0.481	0.663	0.345	0
19	0.484	0.503	**4.271**	0.267	0.389	0.481	3.1
20	0.676	0.821	0.516	0.611	0.564	0.381	0
21	**1.424**	**1.807**	**1.411**	0.611	**2.599**	0.812	**6.3**
22	0.609	0.848	0.535	0.439	0.844	0.423	2.6
23	0.616	0.912	0.792	0.494	**1.019**	0.456	0
24	0.591	0.823	0.740	0.461	0.983	0.490	1.5

Values in bold type represent positive results. BPI: bactericidal/permeability-increasing protein; PR3: serine proteinase 3, MPO: myeloperoxidase.

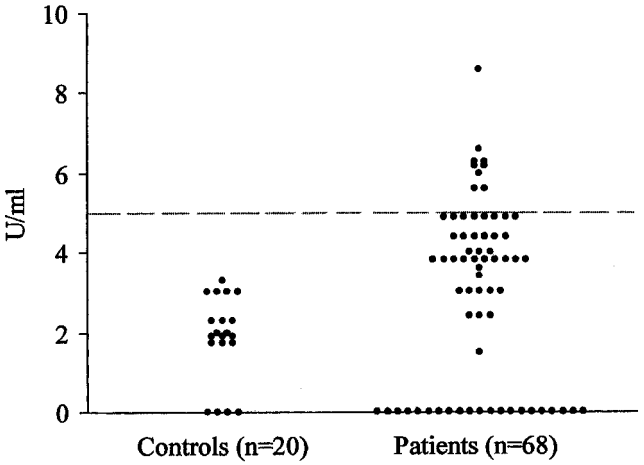

Figure 2. Level of anti-MPO antibodies in controls and patients. Broken line represents the cutoff value for positivity.

positive on ELISA but negative for ANCA by IIF, reflecting the higher sensitivity of ELISA.

Since the majority of the ANCA positive sera had an atypical pattern, we investigated these sera for reactivity against antigens like BPI, cathepsin G, lactoferrin, lysozyme, and elastase. Interestingly, a large proportion of these sera showed reactivity to BPI and cathepsin G. To our knowledge, this is the first report of antibodies to BPI and cathepsin G in SSc.

BPI, a constituent of azurophilic granules of neutrophils, is a 55 kDa membrane associated protein[17] that binds to lipopolysaccharide and exerts bacteriostatic and bactericidal effects against a wide range of gram-negative bacteria[18]. BPI has been shown as an antigenic target for ANCA in patients with systemic vasculitis[19], rheumatoid arthritis (RA), systemic lupus erythematosus, mixed connective tissue disease[20], reactive arthritis[21], inflammatory bowel diseases[20,22], cystic fibrosis[23], primary sclerosing cholangitis, and autoimmune hepatitis[24]. It also has a close relationship with chronic airway infections such as pseudomonas aeruginosa in patients with cystic fibrosis[25]. Presence of interstitial lung disease in SSc increases the risk of lung infections and this could possibly have a role in inducing anti-BPI antibodies. The role of antibodies to BPI in the pathogenesis of SSc is unclear. In a recent study[26], BPI was shown to inhibit angiogenesis via induction of apoptosis of endothelial cells. Angiogenesis is an important sequel of vascular injury in patients with SSc. Anti-BPI antibodies may inhibit the antiproliferative effect of BPI on the vascular endothelium, leading to increased angiogenesis. However, *in vitro* studies are needed to test this hypothesis.

Cathepsin G is a neutrophil protease. It plays a role in platelet activation and degranulation, inhibits thrombin-induced increase in platelet adhesion molecules, and acts as a monocyte chemoattractant at sites of inflammation. It also downregulates adhesion molecules on the neutrophil surface, leading to a decrease in neutrophil endothelial cell

Table 2. Clinical features in patients with and without antibodies to BPI.

Feature	Antibodies to BPI Positive (n = 18)	Antibodies to BPI Negative (n = 29)
Diffuse limited, n	6:12	12:17
Duration of disease, yrs	5.1	5.3
Mean skin score	11.7	16.9*
Pitting scars/digital ulcers, n	8	17
Gangrene, n	3	4
ILD, n	11	16
Arthritis, n	12	15
Reflux esophagitis, n	6	10
ANA+, n	11	19

* $p < 0.05$. ILD: Interstitial lung disease, ANA: antinuclear antibody.

Table 3. Antibodies to ANCA, MPO, and BPI in patients with diffuse and limited SSc.

Antibodies	Diffuse SSc	Limited SSc
ANCA	9/33	15/35
MPO	3/33	6/35
BPI	6/18	12/29
Cathepsin G	5/18	11/29

adhesion[27]. Cathepsin G as a target antigen for ANCA has been found in patients with primary biliary cirrhosis, sclerosing cholangitis, inflammatory bowel disease[28], RA, lupus nephritis[29], and reactive arthritis.

Presence of antibodies to BPI in a significant proportion of our patients, usually in association with antibodies to cathepsin G, suggests that these antigens may be cross-reactive. A protein data bank search (National Center for Biotechnology Information, Bethesda, MD, USA) showed no sequence homology between cathepsin G and BPI. Competitive inhibition ELISA using purified antigens would have helped resolve the issue of cross-reactivity. As noted, antibodies to BPI and cathepsin G have been reported in conditions characterized by increased fibrosis like biliary cirrhosis, sclerosing cholangitis, and cystic fibrosis. Further, in sclerosing cholangitis, simultaneous presence of these 2 antibodies has been associated with presence of cirrhosis[30], another condition associated with fibrosis. Experimental studies are necessary to clarify whether ANCA plays a pathogenic role in causation of SSc or represents a mere epiphenomenon.

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