Anti-Scl-70 antibodies detected by immunoblotting in progressive systemic sclerosis: specificity and clinical correlations

A AESCHLIMANN,¹ O MEYER,² P BOURGEOIS,¹ T HAIM,²
N BELMATOUG,¹ E PALAZZO,¹ AND M F KAHN¹

From the ¹Clinique de Rhumatologie, Université Paris VII, Hôpital Bichat, Paris and the ²Laboratoire d'Immuno-Rhumatologie, Hôpital Lariboisière, Paris

SUMMARY One hundred and forty five serum samples from patients with a connective tissue disease and 30 serum samples from healthy blood donors were analysed by immunoblotting. The presence of anti-Scl-70, which seems to discriminate between progressive systemic sclerosis (PSS) and the CREST (calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly, telangiectasia) syndrome, was found in 31/64 (48%) patients with PSS, in 6/55 (11%) patients with systemic lupus erythematosus, in 2/26 (8%) patients with mixed connective tissue disease and in none of 30 healthy blood donors. These data resulted in a specificity of 93% for this antibody in systemic sclerosis. For patients with PSS the duration of disease was significantly shorter in those with anti-Scl-70 antibodies than in those without, whereas the presence of anti-Scl-70 did not correlate with severity of disease. An 82% prevalence of anticentromere antibodies in patients with the CREST variant compared with a 4% prevalence in patients with PSS or with overlap syndrome confirms the high diagnostic value of this autoantibody for the CREST variant of PSS.

Progressive systemic sclerosis (PSS) has been defined as 'a generalised disorder of small arteries, microvessels, and the diffuse connective tissue, characterised by scarring (fibrosis) and vascular obliteration in the skin, gastrointestinal tract, lungs, heart, and kidneys, in which hidebound skin is the clinical hallmark, and organ compromise the prognostic keystone'.¹

Various autoantibodies reacting with nuclear components may be present in the sera of patients with scleroderma. Antibodies that precipitate a soluble 70 000 dalton nuclear protein called Scl-70,² identified recently as topoisomerase I,³ and antibodies directed against the centromere region of chromosomes have been reported to characterise respectively diffuse systemic sclerosis^{4,5} and the CREST variant of systemic sclerosis.⁶ Antibodies to Scl-70, which are considered almost specific for PSS and are also called Scl-86,⁷ are found in only up to

20% of patients with diffuse PSS^{4,5} and therefore are of limited diagnostic value using standard techniques. Anticentromere antibodies, however, are present in 44-98% of patients with the CREST syndrome^{6,8} as opposed to only 6-12% in PSS.^{5,6}

These data were obtained in studies using routine techniques such as counterimmunoelectrophoresis or immunodiffusion. In the past decade detection of antinuclear antibodies by immunoblotting was introduced.¹⁰

The purpose of our study was to analyse the different antinuclear antibodies in a group of 64 patients with PSS from a single centre, to compare the results obtained with different methods, such as immunodiffusion and immunoblotting, for antinuclear antibody determination, and to define the specificity and sensitivity of these autoantibodies and their correlation with the clinical features.

Patients and methods

The study was performed retrospectively. We considered 64 patients with PSS who attended our

Accepted for publication 7 March 1989.

Correspondence to Dr O Meyer, Clinique de Rhumatologie, Université de Paris, Hôpital Bichat, 46 rue Henri-Huchard, 75018 Paris, France.

centre between 1985 and 1987 and for whom serum samples were available. They were classified into three types according to Barnett and Coventry.¹¹ Type I comprised patients with sclerodactyly (n=15), type II those with skin changes involving hands, forearms, face, and neck (n=13), and type III those in whom the skin changes were diffuse and usually most marked on the trunk (n=7). Forty nine of 63 (78%) patients fulfilled the recent criteria of the American Rheumatism Association (ARA) for diagnosis of PSS.¹²

Patients with the CREST variant of the disease⁶ were identified in a separate group (n=11) as were patients with PSS with features of overlap syndrome (n=18). Our CREST population was characterised by the prominence of calcinosis, Raynaud's phenomenon, sclerodactyly, telangiectasia, associated in two patients with oesophageal dysfunction.

The case reports were reviewed for sex, age, clinical manifestations, available laboratory and immunological data, histological and radiological documents, treatment, and outcome.

The control population consisted of 55 patients with systemic lupus erythematosus (SLE), who fulfilled at least four of the ARA 1982 revised criteria for the classification of SLE,¹³ 26 patients with mixed connective tissue disease (MCTD) according to the Alarcon-Segovia classification,¹⁴ and 30 healthy blood donors.

STANDARD TECHNIQUES

Serum samples were collected at the time of clinical examination, stored at -20°C until use, and analysed without knowledge of the clinical data, together with the serum from control groups and healthy blood donors.

Immunofluorescence

Antinuclear antibodies were detected by indirect immunofluorescence on two different substrates: unfixed rat liver sections and acetone treated HEP₂ cell preparations. In each case the conjugate used was a fluorescein labelled polyvalent antihuman immunoglobulin raised in goats.¹⁵

Immunodiffusion

Antibodies to extractable nuclear antigen were assayed by double immunodiffusion in agarose using the Ouchterlony technique against saline soluble antigens either from fresh calf thymus extract (U1RNP, Sm, SSB/La) or from human spleen extract (SSA/Ro).¹⁶ The extract of rabbit thymus powder (Pel-Freez, Rogers Arkansas, USA) used as the source of Scl-70 was made fresh as the Scl-70 antigen is labile. The precipitating reaction was read after a three day incubation at 6°C in a humid

atmosphere and compared with an anti-Scl-70 reference serum.

Immunoblotting

For blotting analyses a total nuclear protein fraction from HeLa cells was prepared.¹⁷ Protein samples were dissolved in 2% sodium dodecyl sulphate, 10% glycerol, 5% β-mercaptoethanol, 0.1 M TRIS HCl pH 6.8. Samples were heated for three minutes at 100°C followed by electrophoresis on 12% polyacrylamide gels. Proteins were loaded over the entire width of the gel and separated according to their molecular weight.¹⁸ After an 18 hour run at 50 mA per gel (50 V/cm) replicas of the gels were made in nitrocellulose by transferring the proteins electrophoretically using a Bio-Rad trans blot system. Transfer was performed for four hours at 40 mA (80 V/cm) and at 25°C in 192 mM glycerol, 25 mM TRIS pH 8.3, and 20% methanol.¹⁰ The protein blots were cut into strips of about 6 mm and treated with preincubation buffer (3% bovine serum albumin, 10 mM NaCl, TRIS HCl pH 7.6, 0.5 mM phenylmethylsulphonyl fluoride (PMSF)) for two hours at room temperature under constant agitation to saturate protein binding sites on the nitrocellulose.

Incubation with diluted patient's serum (1/20) was performed for two hours in the following buffer: 0.3% bovine serum albumin, 150 mM NaCl, 10 mM TRIS HCl pH 7.6, 0.1 mM PMSF, 1% Triton-X 100, 0.5% deoxycholic acid, and 0.1% sodium dodecyl sulphate. After extensive washing with this buffer (5×5 minutes) IgG and IgM immune complexes were detected by incubating the blots for one hour with a horseradish peroxidase conjugated IgG fraction goat antihuman γ chain specific or μ chain specific serum (Cappel Labs, Cochranville, PA), diluted 1:500 or 1:200 in 0.5% bovine serum albumin phosphate buffered saline, 0.5% Triton-X 100. After five additional washes the immunostain reaction was visualised with diaminobenzidine and H₂O₂ as the substrate. A kit of seven coloured protein standards (Amersham-Rainbow marker) was used to determine molecular weights ranging from 200 kilodaltons (myosin) to 14.3 kilodaltons (lysozyme). Anti-Scl-70 antibody was identified as an immunostain reaction at 92 kilodaltons and compared with an anti-Scl-70 reference serum.

STATISTICAL ANALYSIS

χ² Tests, with or without Yates's correction for small groups, Student's *t* test for paired groups, and Fisher's exact test were performed.

Results

Table 1 shows the demographic characteristics of the

patients with CREST, PSS, and overlap syndrome. Ten of 64 (16%) patients were male. The patients with CREST were significantly older than those with overlap syndrome ($p<0.05$). The mean duration of disease was significantly longer in patients with CREST than in patients with PSS type III ($p<0.02$) and patients with overlap syndrome ($p<0.05$).

Antinuclear antibodies as determined by indirect immunofluorescence on rat liver sections were present in the sera of 44/64 (69%) patients. The most common pattern was homogeneous 23/44 (52%), followed by a speckled 15/44 (34%) and a nucleolar pattern 6/44 (14%).

With HEP₂ cells as substrate antinuclear antibodies were detected in 47/64 (73%) patients' sera. Eight of 11 serum samples from patients with CREST were negative on liver sections, seven of these nine showed a speckled pattern on HEP₂ cells characteristic of anticentromere antibodies (Table 2). A comparison of antinuclear antibodies detected on rat liver sections and on HEP₂ cells showed a similar pattern in 31/64 (48%) cases. Antinuclear antibodies were detected in eight cases exclusively on liver section, in seven cases exclusively on HEP₂ cells, and in either one or the other test in 55/64 (86%) patients.

Anticentromere antibodies were found in nine (82%) of the 11 patients with CREST and in two of

Fig. 1 Immunoblot analysis of serum samples from patients with anti-Scl-70 and anticentromere antibodies. Reference sera: anti-SS-B (lane 1), anti-U1RNP (lane 2), anti-Sm (lane 3), anti-Scl-70 (lane 4), anticentromere (lane 5). Patients' sera: progressive systemic sclerosis (lanes 6, 7, and 8), systemic lupus erythematosus (lane 9), CREST (lanes 10, 11, and 12).

Table 1 Demographic characteristics of the patients with systemic sclerosis (n=64)

	CREST (n=11)	PSS*			Overlap syndrome (n=18)
		I (n=15)	II (n=13)	III (n=7)	
Sex ratio (M/F)	0/11	3/12	1/12	3/4	3/15
Mean (SD) age (years)	66.4 (10)	59.4 (15.3)	54.2 (11.3)	48.4 (18.4)	48.4 (13.6)
(Range)	(36-75)	(15-75)	(22-65)	(24-68)	(22-68)
Mean (SD) disease duration (years)	25.9 (16.9)	15.3 (16.1)	11.0 (7.4)	6.7 (6)	13.4 (10.0)
(Range)	(1-52)	(1-49)	(1-31)	(1-16)	(1-27)

*PSS=progressive systemic sclerosis.

Mean age: CREST v overlap syndrome: $p<0.05$.

Mean disease duration: CREST v type III PSS: $p<0.02$; CREST v overlap syndrome: $p<0.05$.

Table 2 Prevalence of antinuclear antibodies on rat liver sections and HEP₂ cells and of anticentromere antibodies in various subtypes of systemic sclerosis. Values show number (%) of patients

	CREST (n=11)	PSS†			Overlap syndrome (n=18)	Total (n=64)
		Type I (n=15)	Type II (n=13)	Type III (n=7)		
ANA+ (liver section)	3 (27)	9 (60)	10 (77)	7 (100)	15 (83)	44 (69)
ANA (HEP ₂ cells)	9 (82)	10 (67)	10 (77)	4 (57)	14 (78)	47 (73)
Anticentromere antibodies	9 (82)*		1 (8)		1 (6)	11 (17)

* $p<0.000001$ ($\chi^2=26$) CREST v PSS.

†PSS=progressive systemic sclerosis; ANA=antinuclear antibodies.

Table 3 Prevalence of serum anti-Scl-70 antibody (anti-Scl-70) detected with immunodiffusion and immunoblotting in 64 patients with systemic sclerosis. Values show number (%) of patients

	CREST (n=11)	PSS* (types I-III) (n=35)	Overlap syndrome (n=18)	Total (n=64)
Anti-Scl-70 detected with:				
Immunodiffusion	0	6 (17)	0	6 (9)
Immunoblotting	2 (18)	22 (63)	7 (39)	31 (48)

*PSS=progressive systemic sclerosis.

the remaining patients, one with PSS type II and one with overlap syndrome (PSS associated with rheumatoid arthritis). With immunoblotting all the patients with anticentromere antibodies also showed the presence of autoantibodies against a 17 kilodalton antigen (Fig. 1).

With immunodiffusion, anti-Scl-70 antibodies were detected in 6/64 (9%) PSS sera. None of the controls (SLE, MCTD, healthy blood donors) was positive. Anti-Scl-70 antibodies were not detected in sera from patients with the CREST variant or with

Table 4 Prevalence of serum anti-Scl-70 antibody (anti-Scl-70) detected with immunodiffusion and immunoblotting in 145 sera from patients with connective tissue disease and in 30 sera from healthy blood donors. Values show number (%) of patients

	Systemic sclerosis (n=64)	SLE* (n=55)	MCTD* (n=26)	HBD* (n=30)
Anti-Scl-70 detected with:				
Immunodiffusion	6 (9)	0	0	0
Immunoblotting	31 (48)	6 (11)	2 (8)	0

*SLE=systemic lupus erythematosus; MCTD=mixed connective tissue disease; HBD=healthy blood donors.

Table 5 Characteristics of 31 anti-Scl-70 antibody positive patients and 33 anti-Scl-70 antibody negative patients with systemic sclerosis. Values show number (%) of patients

	Anti-Scl-70 present (n=31)	Anti-Scl-70 absent (n=33)	Significance
Mean (SD) age (years)	54.4 (12.6)	52.2 (15)	NS
Mean (SD) disease duration (years)	11.5 (9.5)	17.6 (15.6)	p<0.02
Calcinosis	7 (23)	15 (45)	p<0.05
Telangiectasia	14 (45)	19 (58)	NS
Digital pitting ulcers	12 (39)	15 (45)	NS
Arthritis	16 (52)	10 (30)	NS
Myositis	10 (32)	7 (21)	NS
Lung fibrosis	18 (58)	16 (48)	NS
Cardiac involvement	5 (16)	5 (15)	NS
Oesophageal dysfunction	19 (61)	15 (45)	NS
Renal involvement	3 (10)	3 (9)	NS

Discussion

Detection of antinuclear antibodies in 44/64 (69%) of the cases using rat liver section and 47/64 (73%) using HEP₂ cells as substrate is comparable with previously published data.^{19 20} Recently, Giordano *et al* reported that antinuclear antibodies with a diffusely grainy and nucleolar pattern associated with anti-Scl-70 antibodies were significantly more prevalent in a form of PSS they called 'diffuse cutaneous systemic sclerosis',²¹ which was characterised by poor prognosis. This finding has not been confirmed by other authors.²² In our series the different fluorescent nuclear patterns did not correlate with the severity of the disease.

An 82% prevalence of anticentromere antibodies in patients with the CREST variant as compared with a 4% prevalence in patients with PSS or with overlap syndrome confirms the diagnostic value of this autoantibody for the CREST variant of PSS. The prevalence of anticentromere antibodies in patients with CREST has been estimated as between 55% and 96%.^{4 6 23}

Similar results were recently published by Rothfield *et al* using cloned autoantigen CENP-B for enzyme linked immunosorbent assay (ELISA) detection of anticentromere antibodies.²⁴

According to other authors, patients with the CREST variant are characterised by a significantly longer mean duration of disease and less involvement of internal organs.^{25 26} Weiner *et al* did not find that patients with scleroderma with anticentromere antibodies had a longer disease duration nor that these antibodies identify patients with less severe disease.²³ When the mean age at diagnosis is considered the patients with anticentromere antibodies are significantly older than those without. We suggest that variability of serological and clinical characteristics is the consequence of differences in selection and classification of patients.^{1 5}

With immunoblotting anti-Scl-70 were found in 31/64 (48%) patients with PSS, in 6/55 (11%) patients with SLE, in 2/26 (8%) patients with MCTD, and in none of 30 healthy blood donors. The data resulted in a specificity of 93% of this antibody in systemic sclerosis. Anti-Scl-70 antibodies are therefore not absolutely specific for PSS as they are also found in SLE and MCTD. It is possible that reactions with unidentified intracellular peptides with the same molecular weight as Scl-70 have been identified and it is not certain that all the antibodies are truly against Scl-70. The sensitivity of anti-Scl-70 antibody detected by immunoblotting (48%) is significantly higher than that reported in previous studies using immunodiffusion as standard detection technique.^{4 5} In those studies

anti-Scl-70 antibodies were detected in up to 20% of cases.

Our study showed that the specificity of anti-Scl-70 for PSS is lower by immunoblotting than immunodiffusion (93% v 100% in our series). The information contained in both tests is complementary as the source of antigen is not the same. Immunodiffusion is fast and reliable. With immunoblotting it is possible to obtain more precise information about the different antigenic components and, in addition, to detect several other specificities. The prevalence of anti-Scl-70 in the different subgroups was 18% in the CREST syndrome, 39% in scleroderma associated with overlap syndrome, and 63% in types I-III of diffuse scleroderma. These results re-emphasise the diagnostic value of this antibody, which seems able to discriminate between subsets of systemic sclerosis—for example, PSS and the CREST variant. This was first suggested by Tan *et al*.⁴ Comparable results have been published by Weiner *et al*, who found that anti-topoisomerase I was positive in 26% of patients who fulfilled the ARA criteria for systemic sclerosis and in 10% of patients with four clinical features of the CREST syndrome.²³ With immunoblotting Van Venrooij *et al* detected the presence of autoantibodies against an 86 000 mol.wt marker antigen in 13/22 (59%) patients with diffuse scleroderma and in only 1/19 (9%) patients with limited scleroderma.⁷

The presence of anti-Scl-70 antibody in patients with diffuse scleroderma according to the Barnett and Coventry classification does not correlate with the severity of the disease. Our data do not support the conclusion previously published that anti-Scl-70 antibodies are associated with skin ulcerations and cardiac²³ or lung involvement.^{27 28} On the other hand, duration of disease was significantly shorter in patients with anti-Scl-70 antibodies than in those without. This suggests that screening for anti-Scl-70 antibody with immunoblotting is particularly useful in the early stages of the disease. Whether the presence of anti-Scl-70 antibody has any prognostic value so far as severity and progression of disease are concerned will have to be confirmed by prospective longitudinal studies.

We would like to thank the Swiss Rheumatology Society for financial support. The anti-Scl-70 reference serum was a gift from Dr Humbel, Centre Hospitalier, Luxembourg.

References

- 1 Le Roy E C, Krieg T, Black C, *et al*. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* 1988; **15**: 202-5.
- 2 Douvas A S, Achten M, Tan E M. Identification of a nuclear protein (Scl-70) as a unique target of human antinuclear antibodies in scleroderma. *J Biol Chem* 1979; **254**: 10514-22.
- 3 Shero J, Bordwell B, Rothfield N F, Earnshaw W C. High titers

- of autoantibodies to topoisomerase I (Scl-70) in sera from scleroderma patients. *Science* 1986; **231**: 737-40.
- 4 Tan E M, Rodnan G P, Garcia I, Moroi Y, Fritzler M J, Peebles C. Diversity of antinuclear antibodies in progressive systemic sclerosis. Anti-centromere antibody and its relationship to CREST syndrome. *Arthritis Rheum* 1980; **23**: 617-25.
- 5 Catoggio L J, Skinner R P, Maddison P J. Frequency and clinical significance of anticentromere and anti-Scl-70 antibodies in an English connective tissue disease population. *Rheumatol Int* 1983; **3**: 19-23.
- 6 Fritzler M J, Kinsella T D, Garbutt E. The CREST syndrome: a distinct serologic entity with anticentromere antibodies. *Am J Med* 1980; **69**: 520-6.
- 7 Van Venrooij W J, Stapel S O, Houben H, *et al.* Scl-86, a marker antigen for diffuse scleroderma. *J Clin Invest* 1985; **75**: 1053-60.
- 8 Bernstein R M, Steigerwald J C, Tan E M. Association of antinuclear and antinucleolar antibodies in progressive systemic sclerosis. *Clin Exp Immunol* 1982; **48**: 43-51.
- 9 Powell F C, Weinermann R K, Venencie-Lemarchand F, Spurbeck J L, Schroeter A L. The anticentromere antibody: disease specificity and clinical significance. *Mayo Clin Proc* 1984; **59**: 700-6.
- 10 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer to proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**: 4350-4.
- 11 Barnett A J, Coventry D A. Scleroderma: clinical features, course of illness and response to treatment in 61 cases. *Med J Aust* 1969; **1**: 992-1101.
- 12 Subcommittee for scleroderma criteria of the American Rheumatism Association diagnostic and therapeutic criteria committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 1980; **23**: 581-90.
- 13 Tan E M, Cohen A S, Fries J F, *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**: 1271-7.
- 14 Alarcon-Segovia D. Classification and diagnostic criteria for mixed connective tissue disease. In: Kasukawa R, Sharp G C, eds. *Mixed connective tissue disease and antinuclear antibodies*. Amsterdam: Elsevier, 1987: 33-40.
- 15 Miller M H, Littlejohn G O, Jones B W, Strnad H. Clinical comparison of cultured human epithelial cells and rat liver as substrates for the fluorescent antinuclear antibody test. *J Rheumatol* 1985; **12**: 265-9.
- 16 Ouchterlony O, Nilsson L A. Immunodiffusion and immunoelectrophoresis. In: Weir D M ed. *Handbook of experimental immunology*. London: Blackwell Scientific, 1978: 19.1-44.
- 17 Habets W H, De Rooij D J, Salden M H, *et al.* Antibodies against distinct nuclear matrix proteins are characteristic for mixed connective tissue disease. *Clin Exp Immunol* 1983; **54**: 265-76.
- 18 Laemmli U K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1979; **227**: 680-5.
- 19 Jordan R E, Deheet D, Schroeter A, *et al.* Antinuclear antibodies: the significance in scleroderma. *Mayo Clin Proc* 1971; **46**: 111-3.
- 20 Riboldi P, Asero R, Origgi S, *et al.* Antinuclear antibodies in progressive systemic sclerosis. *Clin Exp Rheumatol* 1985; **3**: 205-11.
- 21 Giordano M, Valentini G, Migliaresi S, Picillo U, Vatti M. Different antibody patterns and different prognoses in patients with scleroderma with various extent of skin sclerosis. *J Rheumatol* 1986; **13**: 911-6.
- 22 Burgos-Vargas B, Martinez-Cordero E, Reyes-Lopez P A, Herrera-Esparza R. Antibody pattern and other criteria for diagnosis and classification in PSS. *J Rheumatol* 1988; **15**: 153-4.
- 23 Weiner E S, Earnshaw W C, Senecal J L, Bordwell B, Johnson P, Rothfield N F. Clinical associations of anticentromere antibodies and antibodies to topoisomerase I. A study of 355 patients. *Arthritis Rheum* 1988; **31**: 378-85.
- 24 Rothfield N, Whitaker D, Bordwell B, Weiner E, Senecal J L, Earnshaw W. Detection of anticentromere antibodies using cloned autoantigen CENP-B. *Arthritis Rheum* 1987; **30**: 1416-9.
- 25 McCarty G A, Rice J R, Bembe M L, Barada F A. Anticentromere antibody. Clinical correlations and association with favorable prognosis in patients with scleroderma variants. *Arthritis Rheum* 1983; **26**: 1-7.
- 26 Steen V D, Ziegler G L, Rodnan G P, Medsger T A. Clinical and laboratory associations of anticentromere antibody in patients with progressive systemic sclerosis. *Arthritis Rheum* 1984; **27**: 125-31.
- 27 Catoggio L J, Bernstein R M, Black C M, Hughes G R V, Maddison P J. Serological markers in progressive systemic sclerosis: clinical correlations. *Ann Rheum Dis* 1983; **42**: 23-7.
- 28 Steen V D, Powell D L, Medsger T A. Clinical correlations and prognosis based on serum autoantibodies in patients with systemic sclerosis. *Arthritis Rheum* 1988; **31**: 196-203.