Autoantibodies to RNA-polymerases in Italian patients with systemic sclerosis

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Abstract

Objective

T*o assess the frequency and clinical correlates of the systemic sclerosis-related autoantibodies to RNA polymerases in Italian patients.*

Methods

Sera from 115 patients with systemic sclerosis (SSc) and 10 patients with systemic sclerosis-overlap syndromes recruited from a single center in northern Italy were investigated for antibodies to RNA polymerase I, II, and III by means of immunoprecipitation using ^{35}S-labeled HeLa cell antigen extract. Twenty-five normal volunteers and 91 patients with different connective tissue diseases were studied as a control group.

Results

Antibodies to RNA-polymerases were found in 14/115 SSc patients (12.1%). None of the normal controls and none of the patients with other connective tissue diseases, including overlap syndromes, were positive. Antibodies reacting with RNA-polymerase I and III (± RNA-polymerase II) were found in 9/115 patients (7.8%) and were mutually exclusive with respect to other scleroderma-related autoantibodies. Isolated anti-RNA polymerase II reactivity was found in 5 patients and was associated with anti-topoisomerase I antibodies in 4 cases. Anti-RNA-polymerase I and III antibodies were associated with diffuse cutaneous involvement and male gender. Only two patients from our series had scleroderma renal crisis, and one of them had anti-RNA polymerase antibodies.

Conclusions

Anti-RNA-polymerase antibodies appear to be less frequent in Italian patients than in Caucasian patients from the United Kingdom or USA. This might be associated with the lower frequency of scleroderma renal crisis.

Key words

Systemic sclerosis, connective tissue disease, autoantibodies, RNA-polymerases, scleroderma renal crisis.

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Introduction

Autoantibodies directed towards RNA polymerases (RNAP) were first detected 20 years ago (1) and have been extensively studied in systemic sclerosis (SSc) thereafter (2-11). There are three different RNAP in eukaryotic cells (RNAP I, II and III), and three main groups of autoantibodies to RNAP (anti-RNAP) in SSc patients. A first group recognizes all 3 RNAP, a second group recognizes only RNAP I and III, and a third group is directed only to RNAP II, in particular to its phosphorilated form IIo (11). The latter specificity may be found in SSc along with other autoantibodies such as anti-topoisomerase I (topo-I) (7,11) and it has been reported in some patients with systemic lupus erythematosus (SLE) and mixed connective tissue disease as well (12). On the other hand, the autoantibodies directed to RNAP I, III were almost exclusively found in patients with SSc and can identify a definite clinical and serologic subset. Anti-RNAP I, III antibodies were reported to be mutually exclusive with respect to either anti-topo-I and anticentromere antibodies (ACA) and associated with an increased frequency of renal involvement, including scleroderma renal crisis (4,5,8,11,13). A significant association with heart involvement and poor survival rate was also found in one study (4).

The relative frequency and the prognostic meaning of the different serologic subsets in SSc may vary according to racial or geographic factors (14-16); in particular a higher frequency of anti-RNAP I, III was found in Caucasian patients from USA than in American blacks and Japanese patients (17). At present, no data are available about the frequency of occurrence and clinical significance of anti-RNAP in patients from Mediterranean countries. This may be of interest since several recent surveys from Italy, Spain and Greece have shown that SSc may have a lower rate of renal involvement with respect to Northern Europe and America (18-20).

Patients and methods

Patients

We studied 115 patients with SSc recruited from the Division of Rheumatology of the Pavia University Hospital in northern Italy. All these patients were consecutively recruited from 1995 and 1999, and followed-up for at least two years. 104 patients (90.4%) fulfilled the American College of Rheumatology preliminary criteria for classification as definite SSc (21); the remaining 11 patients were suffering from Raynaud's phenomenon with a positive test for ACA and a scleroderma pattern at nail-fold capillaroscopy, thus fulfilling the recently proposed criteria for limited SSc (21). In most patients blood samples were obtained at the first visit and in all cases before any treatment with immunosuppressive agents. 22 patients had been previously treated with steroids. Sera from patients with scleroderma renal crisis were evaluated both at the first visit and when the renal crisis was diagnosed.

We also studied 10 patients with SSc-overlap syndrome. All of these patients fulfilled the criteria for both SSc and another connective tissue disease, including polymyositis or dermatomyositis (23) in 5 cases, Sjögren's syndrome (24) in 3 cases, and SLE (25) and rheumatoid arthritis (26) in one case, each.

Twenty-five age- and sex-matched normal volunteers recruited from blood donors and laboratory staff, and 91 age- and sex-matched patients with other autoimmune rheumatic diseases (30 SLE,27 rheumatoid arthritis, 21 primary Sjögren's syndrome, 13 polymyositis/dermatomyositis) were evaluated as a control group.

Clinical evaluation

In order to compare our findings with those reported in previous studies, SSc patients were classified as having either limited cutaneous disease (lcSSc) or diffuse cutaneous disease (dcSSc). Patients with lcSSc had skin sclerosis restricted to the extremities and face, while in patients with dcSSc skin sclerosis was proximal to the elbows or knees and could extend toward the trunk (5). Patients with limited SSc without cutaneous sclerosis were grouped along with lcSSc.

The onset of the disease was taken as the appearance of the first definite SSc

sign or symptom other than Raynaud's phenomenon. Organ involvement was defined as at least grade one severity within each organ system according to the disease severity scale for SSc (27) with few additional items. In particular, respiratory involvement was determined in all cases by carbon monoxide transfer (TLCO), forced vital capacity (FVC), chest radiograph, and high resolution computed tomography (HRCT). Interstitial lung disease was defined as a > 20% reduction from the expected TLCO or FVC values, and/or significant fibrosis or ground glass lesions at HRCT scored according to Kazerooni *et al.* (28). Isolated pulmonary hypertension was suggested by a reduction of TLCO with normal FVC, no evidence of interstitial lung disease by HRCT, and an estimated mean pulmonary arterial pressure >30 mm Hg by Doppler echocardiography.

Cardiac involvement was recorded when one or more of the following features were present: left ventricular ejection fraction < 50% on echocardiography, conduction defects on EKG, arrhythmia requiring specific treatment, and evidence of pericarditis or pericardial effusion on EKG and/or echocardiography.

Renal involvement was defined as a persistent and otherwise inexplicable renal impairment (serum creatinine > 1.3 mg/dl) or proteinuria (> 0.5 g/day). Renal crisis was defined as acute or subacute development of renal insufficiency often associated with accelerated arterial hypertension or microangiopathic haemolytic anemia or both (5).

Anticentromere and anti-topoisomerase I antibodies

Antinuclear antibodies (ANA) were detected by indirect immunofluorescence as described (29), on HEp-2 cells (Diamedix Co, Miami Florida, USA) with serum diluted 1:80. ACA were identified from the ANA pattern.

Antibodies to extractable nuclear antigens were evaluated in all sera by counterimmunoelectrophoresis using soluble extracts from human spleen and rabbit thymus (Pelfreez Biologicals, Rogers Akansas, USA) followed by comparison with sera of known specificity according to standard technical procedures previously described (30). Positive sera were confirmed by ELISA using a commercially available kit (Diamedix Co, Miami Florida, USA). All anti-topoI positive sera by CIE gave positive results by ELISA.

Anti-RNA-polymerase antibodies

Anti-RNAP were evaluated by immunoprecipitation from ^{35}S methionine-labelled HeLa cell extract using sera from all patients and controls.

HeLa cells were maintained at 37°C and 5% CO_2 in RPMI 1640 medium supplemented with 5% heat inactivated fetal bovine serum (Biowhittaker Europe, Verviers, Belgium), 60 µg/ml penicillin, and 100 µg/ml streptomycin. For the preparation of radiolabeled cell extracts, 1×10^7 HeLa cells were cultured in methionine- and cysteine-free RPMI 1640 with 6 µCi/ml (at 2×10^5 cell/ml) [^{35}S] methionine-cysteine (ICN Radiochemicals, Costa Mesa, CA, USA) supplemented with 2.5% PBS dialyzed fetal calf serum for 14h (4). The cells were harvested and washed with cold tris-buffered saline (TBS) (140 mM NaCl, 40 mM Tris-HCl, pH 7.4) and resuspended in buffer containing 500 mM NaCl, 10 mMTris –HCl, pH 8.0, 0.1% Igepal CA 630 (Sigma, St.Louis, Missouri USA), supplemented with 2 mM phenyl-methyl-sulfonyl-fluoride to minimize proteolytic degradation. The cells were then sonicated on ice three times for 40 sec each and centrifuged at 14,000 g for 15 min. The supernatant was used as the source of antigen.

Immunoprecipitation from radiolabeled cellular extract was performed at 4°C as described by Kuwana *et al.* (4). Briefly, 20 µl of undiluted sera were incubated with 0.5 ml of 4 mg/ml protein A-Sepharose CL 4B (Pharmacia Amersham Biosciences, Uppsala, Sweden) in IPP buffer and rotated end-over-end overnight. The antibody-coated protein A beads were then washed 5 times with IPP buffer, centrifuged at 9000 rpm for 1 minute and resuspended in 400 µl di IPP buffer. Fifteen µl of radiolabeled cell extract were added to each sample and the samples were rotated end-over-end for 2 hr at 4°C; after 10 washes with IPP buffer, the

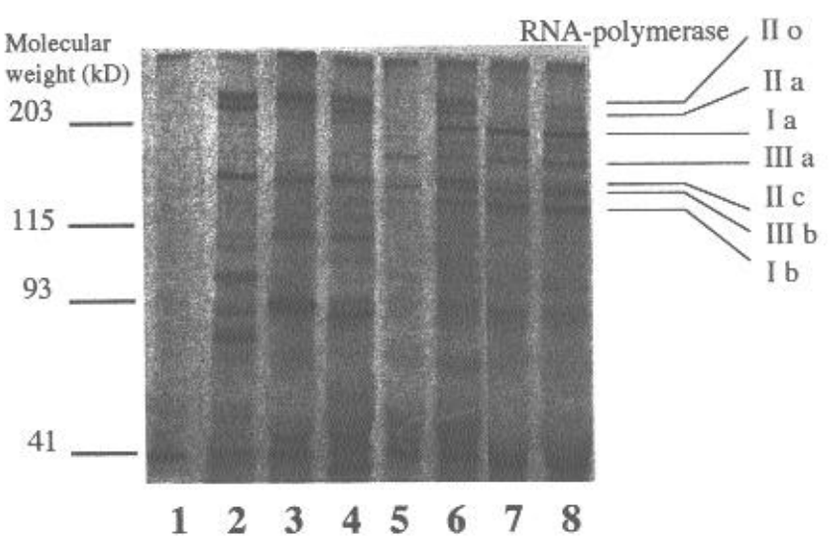

Fig. 1. Autoradiograph of 7.5% polyacrylamide gel showing the precipitated proteins of [^{35}S] methionine-labeled HeLa cell extract using reference samples or patient sera. **Lane 1**: normal control serum. **Lane 2**: anti-RNA polymerase II positive control reacting to the phosphorylated form IIo (240 kD) as well as to the subunits IIa (220 kD) and IIc (145 kD). **Lane 3**: SSc serum reacting to RNAP IIo and IIc. **Lane 4**: SSc serum reacting to RNA polymerase IIo,IIa and IIc. **Lane 5**: anti-RNA polymerase III positive control serum reacting to the subunits IIIa (155 kD) and IIIb (138 kD). **Lane 6**: SSc serum reacting to all three RNA polymerases. **Lane 7**: anti-RNA polymerase I positive serum reacting to RNA polymerase I subunits Ia (190 kD) and Ib (120 kD) as well as to RNA polymerase III. **Lane 8**: SSc serum reacting to RNA polymerase I and III.

samples were centrifuged at 9000 rpm for 1 minute at 4°C. The immunoprecipitated proteins were denaturated by boiling for 5 min in 25 µl sample buffer and fractionated on 7.5% polyacrylamide-SDS gels. Gels were then treated with EN^{3}HANCE (DuPont NEN, Bruxelles Belgium) and radiolabeled proteins were visualized by autoradiography.

Anti-RNAP reactivity was assessed by comparison with reference samples (Fig. 1). Anti-RNAP I and III reference sera were a kind gift from Dr. Chris Bunn (Department of Clinical Immunology, Royal Free Hospital, London, UK). Anti-RNAP II reference standard was a IgG2a mouse monoclonal antibody directed towards RNAP II (Clone 8WG16; BabCO, Richmond California, USA) used at a 1:50 working dilution.

Statistical analysis

Continuous variables were reported as mean and standard deviation, or median and quartiles if skewed. Frequencies and percentages were calculated for categorical variables. SSc subsets were compared by means of Student's t-test (or Mann Whitney U test if appropriate) and Fisher exact test for continuous and categorical variables, respectively. Stata 7 (StataCorp, USA) was used for computation. A 2-sided p-value < 0.05 was retained for statistical significance.

Results

Frequency of autoantibodies

ANA were present 111 out of 115 SSc patients (96.5%) and ACA were identified in 42 patients (36.5%). Anti-topo I were detected in 27 patients (23.5%) while 11 patients had other defined specificities; anti-SSA/Ro (5 patients), anti-SSA/Ro and SSB/La (2 patients), anti-U1RNP (2 patients), anti-U3RNP and anti-PM-Scl.

By immunoprecipitation, banding patterns characteristic of RNAP I, II and III, or RNAP I and III, or RNAP II were detected (Fig. 1) in 14 out of 115 patients with SSc (12.1%). Three patients showed antibodies directed to all three RNAPs, 6 patients had antibodies directed to both RNAP I and RNAP III, and 5 patients had antibodies directed only to RNAP II. Thus, anti-RNAP I, III reactivity was found in 9 patients (7.8%) and isolated anti-RNAP II in 5 (4.3%).

None of the normal controls and none of the other CTDs, including SSc-overlap syndromes, showed anti-RNAP reactivity.

Table I shows the association between anti-RNAP and other defined serologic markers in SSc patients. Anti-RNAP I, III were never associated with other systemic sclerosis-related autoantibodies, while isolated anti-RNAP II were frequently found along with anti-topo I. All patients with anti-RNAP I, III had a fine speckled ANA fluorescence pattern and 3 of them also showed a nucleolar pattern. Patients with anti-RNP II and anti-topo I had a diffuse grainy pattern staining as usually found in anti-topo I positive cases. The only one patient with isolated anti-RNP II reactivity who was negative for anti-topo I exhibited a diffuse fine speckled pattern.

Clinical features associated with antibodies to RNAP

The median follow-up of our patients was 4.87 (interquartile range = 2.8-5.9) years, and the median duration of the disease at the end of follow-up 8.9 (3.7 - 15.5) years. Table II shows the main clinical features in patients with anti-RNAP I, III as compared with the other serological subsets. A statistically significant difference with respect to the other subsets ($p = 0.02$) was sex distribution with a male to female ratio of 4:5. Furthermore, anti-RNAP I, III reactivity was associated with dcSSc, showing a frequency similar to that of anti-topo I positive patients and significantly higher than that found in the other serological subsets ($p < 0.01$).

No differences were found with regard to involvement of the peripheral vascular system, joints and tendons, muscles, or gastrointestinal tract between the patients with anti-RNAP I, III and the remaining group. As for anti-RNAP II positive patients, 3 had dcSSc, and 3 had moderate to severe interstitial lung

Table I. Frequency of anti-RNA polymerase (RNAP) antibodies in SSc patients according to the presence of other autoantibody specificities (ACA: anticentromere antibodies; anti-topoI: anti-topoisomerase I antibodies).

Serologic subset	Anti-RNAP I, II, III	Anti-RNAP I, III	Anti-RNAP II
All SSc (115 pts)	3	6	5
ACA positive (42 pts)	0	0	0
Anti-topo I positive (27 pts)	0	0	4
Other defined specificities (11 pts)	0	0	0

Table II. Main clinical characteristics of 115 patients with SSc according to the presence of anti-topoisomerase I antibodies (topo-I), anti-centromere antibodies (ACA) or anti-RNA polymerase I-III antibodies (RNAP I, III).

Characteristic	Topo-I positive (27 patients)	ACA positive (42 patients)	RNAP I,III positive (9 patients)	RNAP I,III negative (106 patients)
Male gender	4 (15%)	2 (5%)	4 (44%)§	12 (11%)
Age at onset	55.9±11	56.8±11	52.2±12	55.7±12
Limited SSc	8 (30%)	41 (98%)	3 (33%)	71 (67%)
Diffuse cutaneous SSc	19 (70%)	1 (2%)	6 (67%)	35 (33%)
Lung involvement	20 (74%)	12 (29%)	4 (44%)	46 (43%)
Isolated pulmonary hypertension	0	6 (14%)	1 (11%)	9 (8%)
Kidney involvement	3 (11%)	0	2 (22%)	5 (5%)
Heart involvement	7 (26%)	5 (12%)	3 (33%)	14 (13%)
Deceased patients	3 (11%)	1 (2%)	1 (11%)	6 (6%)

§ $p < 0.05$ vs anti-RNAP negative patients.

disease, as usually found in anti-topo I positive patients.

Only 2 of our patients developed a full-blown picture of scleroderma renal crisis. One had anti-topo I, and one anti-RNAP I, III antibodies. The former patient had dcSSc and was negative for anti-RNAP antibodies both at the first visit and at the time of renal crisis; the latter patient had lcSSC and was also suffering from isolated pulmonary hypertension.

Discussion

The present study shows that antibodies to RNAP I,III were present in 9 sera from 115 patients with any form of SSc (7.8%) and in 6 sera from 41 patients with dcSSc (14.6%). These figures, obtained in unselected Caucasian patients from Italy, are distinctly lower than those reported in Caucasian patients from USA and UK (Table III).

Our results are in keeping with previous studies showing that anti-RNAP reactivity is highly specific for SSc (4, 5,8,11). Also, the subgroups of anti-RNAP and their association with the different serologic subsets in our patients was similar to those reported in previous studies (8, 11). In fact, no serum with isolated reactivity to RNAP I or RNAP III was found in our series, while 6 sera recognised both RNAP I and RNAP III, 3 sera recognized all three RNAPs and 5 additional sera recognized only RNAP II. Isolated anti-RNAP II was closely associated with anti-topo I reactivity, while anti-RNAP I, III reactivity was mutually exclusive with respect to either ACA or anti-topo-I.

From a clinical point of view, in the present study anti-RNAP I, III antibodies were more frequently found in dcSSc and in male patients. The former association was present in all of the reported studies. On the other hand, it is quite interesting to note that the association with male gender was previously reported only in Japanese subjects, who had a low frequency of anti-RNAP similar to that found in our patients (4). In previous studies SSc patients with anti-RNAP I, III were shown to have a higher incidence of kidney involvement while lung disease was less frequent than in anti-topo I positive patients (5, 8, 11). Our results are in general agreement with these findings; however, the analysis of the clinical data was limited by the low frequency of both anti-RNAP I, III and renal involvement in our series. Thus, the lack of significant differences between the anti-RNAP positive patients and those belonging to other serologic subsets might be due to a type-2 error.

Anti-RNAP I, III were reported to be strictly associated with scleroderma renal crisis in many countries (Table III) and were used in the diagnosis of SSc in patients presenting with renal crisis without other clinical features of the disease (31, 32). An interesting question is whether the same association is valid in Italian patients, as suggested by the lower rate of scleroderma renal crisis (17, 33). The present study confirms that scleroderma renal crisis is rare in our country. It was found only in 2 cases among 115 consecutive SSc, including 41 patients with dcSSc who are known to be at higher risk of developing this disorder early in the course of the disease. One of our patients with scleroderma renal crisis had anti-RNAP I, III along with lcSSc and isolated pulmonary hypertension. This clinical picture is intriguing since the occurrence of scleroderma renal crisis in lcSSc is uncommon, and an association with anti-RNAP I, III has been reported previously (34). On the other hand, the only patient in our study with 'classic' scleroderma renal crisis did not show anti-RNAP I, III in her serum at any time. Thus, the association between anti-RNAP I, III and scleroderma renal crisis in our country deserves further investigation.

In conclusion, our data show that SSc patients from Italy have a lower frequency of anti-RNAP I, III with respect to patients from the UK and America and this might be associated with the lower rate of scleroderma renal crisis. However, because of the limited number of patients with scleroderma renal crisis in our series, studies on larger multicenter series are needed to properly address this issue.

Table III. Frequency of anti-RNA polymerase I, III antibodies in patients with systemic sclerosis (SSc) from different countries.

Study (reference)	Country	Anti-RNApolymerase I,III positive cases				
		SSc			SSc-overlap syndromes	Scleroderma renal crisis
		Total	Limited	Diffuse		
Kuwana *et al.* (4)	Japan	14/183 (8%)	1/114 (0.9%)	13/71 (18%)	0/92	6/7 (85%)
Okano *et al* (5)	USA	57/225 (25.3%)	7/114 (6%)	50/111 (45%)	0/27	12/15 (80%)
Kuwana *et al* (14)	USA (Blaks)	n.d.	n.d.	1/12 (8%)	0/5	n.d.
Chang *et al* (9)	USA	9/89 (10%)	1/53 (2%)	8/36 (22%)	n.d.	2/2 (100%)
Bunn *et al* (8)	UK	≥ 86/735 (≥ 11.7%)	19 (n.d.)	62 (n.d.)	n.d.	n.d.
Harvey *et al* (11)	UK	19/155 (12.2%)	7/101 (7%)	10/21 (47.6%)	n.d.	n.d.
Present study	Italy	9/115 (7.8%)	3/74 (4%)	6/41 (14.6%)	0/10	1/2 (50%)

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