Enzyme-linked immunosorbent assay for detection of Anti-RNA polymerase III antibody: Analytical accuracy and clinical associations in systemic sclerosis

ARTICLE *in* ARTHRITIS & RHEUMATOLOGY · AUGUST 2005

Impact Factor: 7.76 · DOI: 10.1002/art.21232 · Source: PubMed

6 AUTHORS, INCLUDING:

Masataka Kuwana
Nippon Medical School
360 PUBLICATIONS **7,557** CITATIONS
SEE PROFILE

Yutaka Okano
Kawasaki Medical University
43 PUBLICATIONS **1,481** CITATIONS
SEE PROFILE

Noreen Fertig
University of Pittsburgh
50 PUBLICATIONS **2,034** CITATIONS
SEE PROFILE

Thomas A Medsger, Jr.
University of Pittsburgh
115 PUBLICATIONS **6,650** CITATIONS
SEE PROFILE

ARTHRITIS & RHEUMATISM
Vol. 52, No. 8, August 2005, pp 2425–2432
DOI 10.1002/art.21232

Enzyme-Linked Immunosorbent Assay for Detection of Anti–RNA Polymerase III Antibody

Analytical Accuracy and Clinical Associations in Systemic Sclerosis

Masataka Kuwana,[1] Yutaka Okano,[2] Janardan P. Pandey,[3] Richard M. Silver,[3] Noreen Fertig,[4] and Thomas A. Medsger, Jr.[4]

Objective. **We have recently developed an enzyme-linked immunosorbent assay (ELISA) for detection of anti–RNA polymerase III (anti–RNAP III) antibody, using a recombinant fragment containing the immunodominant epitope as the antigen source. This study was conducted to assess the analytical accuracy and clinical associations of the anti–RNAP III ELISA in patients with systemic sclerosis (SSc).**

Methods. **To evaluate analytical sensitivity and specificity of the ELISA, both immunoprecipitation tests and ELISA were used to detect anti–RNAP III antibody in 534 SSc sera from patients at 3 medical centers. Sera from 522 SSc patients and 516 controls, including patients with other connective tissue diseases and blood bank donors, were also evaluated to assess the clinical sensitivity and specificity of the ELISA. Clinical findings in anti–RNAP III antibody–positive SSc patients were compared between patient groups stratified according to anti–RNAP III antibody levels determined by the ELISA.**

Results. **In SSc patients, our ELISA showed analytical sensitivity of 91% and analytical specificity of 99% compared with the immunoprecipitation assay (a gold standard for detection of anti–RNAP III antibody). The additional analysis using a large series of SSc and control sera showed that clinical sensitivity and specificity of the ELISA with respect to the diagnosis of SSc were 17% and 98%, respectively. A high level of anti–RNAP III antibody was associated with diffuse cutaneous SSc, higher maximum total skin score, and increased frequency of tendon friction rubs.**

Conclusion. **The anti–RNAP III ELISA is analytically accurate and clinically specific. With this assay, testing for anti–RNAP III antibody can be made routinely available.**

Systemic sclerosis (SSc) is a multisystem connective tissue disorder characterized by fibrosis of the skin and microvascular injury (1). A prominent immunologic feature in SSc is the presence of circulating autoantibodies directed against various nuclear antigens, such as topoisomerase I (topo I) and centromere/kinetochore (2). These SSc-associated antibodies are useful in the diagnosis of SSc and classification of patients, and thus are widely used in clinical settings (1,2). In addition, sera from a subset of SSc patients recognize 1 or more subtypes of RNA polymerase (RNAP) (2–4). Autoantibodies to RNAP I and RNAP III always coexist, and this pattern of antibody response is highly specific for SSc (2–4). Some SSc sera that are positive for antibodies to RNAP I and RNAP III contain anti–RNAP II antibody as well. Antibody to RNAP II alone is also detected in SSc patients with anti–topo I antibody and in a small number of patients with systemic lupus erythematosus (SLE) or overlap syndrome (5). Thus, anti–RNAP II

Supported by the Japanese Ministry of Health, Labour and Welfare, US Department of Energy cooperative agreement grant DE-FC09-02CH11109, the Shoemaker Fund, and the Arthritis Foundation, Western Pennsylvania Chapter.

[1]Masataka Kuwana, MD, PhD: Keio University School of Medicine, Tokyo, Japan; [2]Yutaka Okano, MD: Okano Clinic, Abiko, Japan; [3]Janardan P. Pandey, PhD, Richard M. Silver, MD: Medical University of South Carolina, Charleston; [4]Noreen Fertig, Thomas A. Medsger, Jr., MD: University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

Dr. Kuwana holds a patent from Japan on the assay described in this report.

Address correspondence and reprint requests to Masataka Kuwana, MD, PhD, Institute for Advanced Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: kuwanam@sc.itc.keio.ac.jp.

Submitted for publication November 30, 2004; accepted in revised form May 16, 2005.

antibody is not a specific marker for SSc. In North American white patients with SSc, the frequency of serum anti–RNAP III antibody positivity is similar to that of positivity for anti–topo I and anticentromere antibodies (6). Anti–RNAP III antibody is associated with diffuse cutaneous involvement and renal crisis (3,4). Therefore, it is a useful marker for the diagnosis, prognosis, and subclassification of SSc.

Anti–RNAP III antibody studies are not used in routine clinical practice because there is no convenient assay available for detection. Anti–RNAP III antibody was originally identified by immunoprecipitation (IP) assay using radiolabeled cultured cell extracts, requiring the use of radioisotopes and complicated procedures (2–4). We have recently identified an antigenic epitope on amino acid residues 891–1020 on RPC155, the largest subunit of RNAP III, recognized by nearly all SSc sera that are positive for anti–RNAP III antibody (7). Using a recombinant fragment encoding the major epitope region on RPC155 as an antigen source, we have successfully developed an enzyme-linked immunosorbent assay (ELISA) for the detection of anti–RNAP III antibody. This ELISA system appeared highly sensitive and specific based on complete concordance with an IP assay (a gold standard for the presence of anti–RNAP III antibody), but the number of patient sera examined was relatively small and the specimens were obtained from a single medical center (7). In the present study, analytical accuracy and clinical associations of this anti–RNAP III ELISA were examined using a large series of sera collected from 3 medical centers in Japan and the US.

PATIENTS AND METHODS

Patients and controls. Three groups of SSc patients were included in this study: 265 patients from Keio University School of Medicine (all Japanese), 196 patients from the Medical University of South Carolina (134 white, 55 African American, 5 Hispanic, and 2 Asian), and 73 patients from the University of Pittsburgh School of Medicine (65 white and 8 African American). All patients had disease that met the American College of Rheumatology (formerly, the American Rheumatism Association) preliminary classification criteria for SSc (8). SSc patients from Tokyo and South Carolina were randomly selected from computer databanks, while patients from Pittsburgh were selected based on the presence of anti–RNAP III antibody by IP assay. Sera from the Japanese patients included 20 that were tested for a previously described study (7). Serum samples from all patients were obtained within 1 year of first presentation with SSc; serial samples were available from some patients.

To assess clinical sensitivity and specificity, we additionally collected control serum samples from subjects in Tokyo and Pittsburgh. Sera from subjects in Japan were from 82 patients with systemic lupus erythematosus (SLE), 24 patients with polymyositis/dermatomyositis (PM/DM), 54 patients with rheumatoid arthritis (RA), 32 patients with primary Sjögren's syndrome (SS), and 48 blood bank controls; sera from subjects in Pittsburgh were from 53 patients with SLE, 26 patients with PM/DM, 30 patients with RA, and 167 blood bank controls. All patients with SLE, PM/DM, RA, and primary SS met the corresponding disease classification or diagnostic criteria (9–12). For this analysis, 257 SSc sera were newly selected from the serum bank of the University of Pittsburgh. All samples were obtained after the patients and control subjects gave written informed consent, and the study was approved by the respective institutional review boards.

Clinical features. Clinical and laboratory findings were recorded for the majority of SSc patients from Tokyo and Pittsburgh. Complete medical histories, physical examinations, and laboratory analyses were performed on all patients at the first visit, and limited evaluations were completed during followup visits. These patients had been observed regularly by clinical staff for a minimum of 5 years. SSc patients were classified as having diffuse or limited cutaneous disease. Diffuse cutaneous disease was considered present if, at any time during the course, skin thickening proximal to the elbows or knees (e.g., upper arms, thighs, anterior chest, or abdomen) was present. The modified Rodnan total skin score (13) was serially recorded, and the maximum score recorded during the disease course was used for analysis. The definitions used to determine organ involvement, including involvement of the peripheral vasculature (digital ulcers and/or gangrene), joint, esophagus, small intestine, lung (pulmonary interstitial fibrosis and isolated pulmonary arterial hypertension), heart, kidney (renal crisis), and muscle, have been described previously (4,14). End-stage lung disease was defined as forced vital capacity $<50\%$ of predicted and/or lung-related death (15).

IP assay. Anti–RNAP III antibody was detected by IP assay using ^{35}S-labeled HeLa cell extracts (3,16). Identification of anti–RNAP III antibody was based on immunoprecipitation of large subunits for RNAP III, i.e., 155-kd (RPC155) and 138-kd proteins, in comparison with the standard sera.

Expression and purification of recombinant RNAP III fragments. Recombinant fragments encoding RPC155 and RPC62, subunit components of RNAP III, were used in this study. Specifically, amino acid residues 891–1020 of human RPC155 and the entire open-reading frame of human RPC62 were expressed as recombinant maltose-binding protein fusion proteins (rRPC155C-g and rRPC62, respectively) in a bacterial expression system (7). Individual recombinant proteins were purified from soluble bacterial lysates using amylose-resin affinity chromatography according to the protocol suggested by the manufacturer (New England Biolabs, Beverly, MA).

Anti–RNAP III ELISA. We used an ELISA system for detection of anti–RNAP III antibody as described previously (7). Briefly, polyvinyl 96-well plates (Sumilon multiwell plate H type; Sumitomo Bakelite, Tokyo, Japan) were coated with 0.5 μg/ml purified rRPC155C-g diluted in phosphate buffered saline (PBS) containing 0.05% 2-mercaptoethanol, for 12 hours at 4°C. The remaining free binding sites were blocked with 3% bovine serum albumin in PBS for 1 hour at room temperature. Patient sera were diluted at 1:250 in ELISA buffer (PBS containing 0.1% bovine serum albumin and 0.1%

Tween 20) and preincubated with bacterial lysates containing maltose-binding protein. Wells were incubated with the pretreated serum samples at room temperature for 2 hours and subsequently for 1 hour with peroxidase-conjugated goat anti-human IgG (ICN/Cappel, Aurora, OH) diluted 1:5,000 in ELISA buffer. Antibody binding was visualized by incubation with tetramethylbenzidine (1 mg/ml) in phosphate–citrate buffer containing dimethyl sulfoxide. After the reaction was stopped by the addition of 1*M* sulfuric acid, optical density at 450 nm (OD_{450}) was read with an automatic plate reader (Bio-Rad, Hercules, CA). All incubations were followed by 3 washes with ELISA buffer. Samples were tested in duplicate, and antibody units were calculated from the OD_{450} results, using a standard curve obtained with serial concentrations of an anti–RNAP III antibody–positive SSc serum. The cutoff for positivity was set at 4.2 units (5 SD above the mean obtained in 61 healthy controls) (7).

In some experiments, the specificity of anti–RNAP III antibody reactivity in the ELISA was confirmed by competitive inhibition assay (17). Briefly, SSc sera that were positive for anti–RNAP III antibody by ELISA were preincubated with competitors (0.1 μg/ml or 1 μg/ml) at room temperature for 1 hour before their addition to antigen-coated wells. Competitors included topo I and the mixture of RNAP I, RNAP II, and RNAP III, which were affinity purified from HeLa cells (16,18).

Immunoblotting. Antibody reactivities to recombinant proteins were examined by immunoblotting as described previously (16). Briefly, bacterial lysates containing recombinant proteins were fractionated on sodium dodecyl sulfate–10% polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were incubated with a 1:250 dilution of patient sera that had been pretreated with bacterial lysates to remove antibodies reactive with bacterial proteins. The membranes were subsequently incubated with alkaline phosphatase–conjugated goat anti-human IgG (ICN/Cappel). The immunoreactive bands were visualized by development with nitroblue tetrazolium chloride/BCIP.

Statistical analysis. The statistical significance of differences between groups was tested using chi-square analysis or Fisher's 2-tailed exact test, when applicable. All statistical procedures were performed with StatView software (SAS Institute, Cary, NC).

RESULTS

Analytical sensitivity and specificity of the anti–RNAP III ELISA. Using the IP assay, serum anti–RNAP III antibody was detected in 17 patients from Tokyo (6%) and in 30 from South Carolina (15%). The frequency of positivity for anti–RNAP III antibody was significantly different between these 2 patient groups ($P = 0.003$), confirming our previous finding that anti–RNAP III antibody is more prevalent in North American than in Japanese SSc patients (6). All SSc sera from Tokyo and South Carolina were examined using the anti–RNAP III ELISA (Figure 1). Anti–RNAP III antibody was positive in all 17 of the sera from patients in Japan and in 28 (93%) of the 30 sera from patients in South Carolina that immunoprecipitated RNAP III. In contrast, 3 (1%) of 248 sera from the Japanese cohort and 1 (0.6%) of 166 from the South Carolina cohort that were negative for anti–RNAP III antibody by IP assay showed a positive result in the ELISA. Because we considered the number of sera that were positive for anti–RNAP III by IP (n = 47) insufficient for evaluating analytical concordance with the IP results, we additionally screened 73 SSc patient sera from Pittsburgh that had been confirmed to be positive for anti–RNAP III antibody by the IP assay. Sixty-four (88%) of these sera were positive for anti–RNAP III antibody by the ELISA.

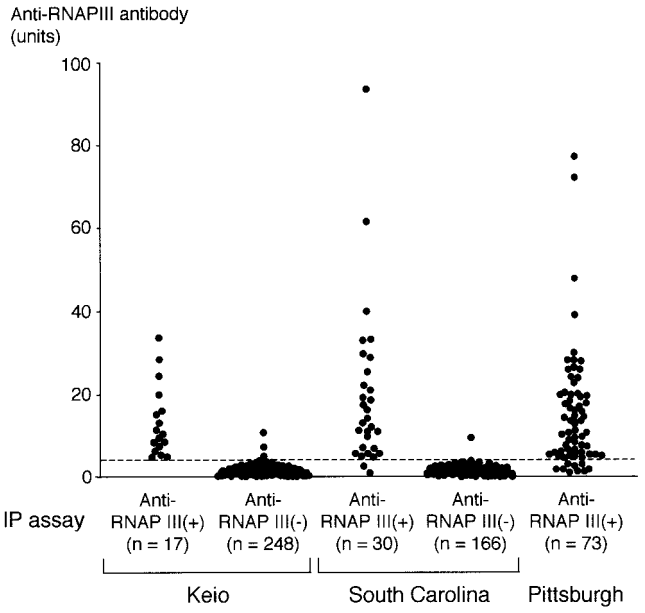

Figure 1. Anti–RNA polymerase III (anti–RNAP III) antibody measured by enzyme-linked immunosorbent assay in a total of 534 sera from systemic sclerosis (SSc) patients at Keio University School of Medicine (Tokyo, Japan), Medical University of South Carolina (Charleston, SC), and University of Pittsburgh School of Medicine (Pittsburgh, PA). SSc patients were divided into those who were positive and those who were negative for anti–RNAP III antibody determined by immunoprecipitation (IP) assay. Broken line indicates the cutoff level for positivity (4.2 units).

Table 1 summarizes the analytical sensitivity, analytical specificity, positive predictive value, and negative predictive value of the anti–RNAP III ELISA in comparison with the IP assay. When 534 SSc sera from 3 medical centers were combined, analytical sensitivity was somewhat low (91%), but other values were high (analytical specificity 99%, positive predictive value 96%, negative predictive value 97%).

Table 1. Analytical sensitivity, analytical specificity, positive predictive value, and negative predictive value of the anti–RNAP III enzyme-linked immunosorbent assay in systemic sclerosis patients from 3 medical centers

Medical center	No. of serum samples	Analytical sensitivity, %	Analytical specificity, %	Positive predictive value, %	Negative predictive value, %
Keio University (Tokyo, Japan)	265	100	99	85	100
Medical University of South Carolina (Charleston, SC)	196	93	99	97	99
University of Pittsburgh (Pittsburgh, PA)	73*	88	–	–	–
Total	534	91	99	96	97

* All sera from the University of Pittsburgh were positive for anti–RNA polymerase III (anti–RNAP III) antibody by immunoprecipitation assay.

Clinical sensitivity and specificity of the anti–RNAP III ELISA. To further assess clinical sensitivity and specificity of the ELISA, control sera obtained from Tokyo and Pittsburgh were subjected to the anti–RNAP III ELISA (Table 2). Of 240 control sera from Tokyo, 2 from patients with RA and 1 from a patient with primary SS showed weakly positive results (<5.0 units), but the percent positivity among the control sera was significantly lower than among the 265 SSc sera (1% versus 6%; $P = 0.002$). Of 276 control sera from Pittsburgh, 2 from SLE patients and 4 from blood bank donors were weakly positive for anti–RNAP III antibody by the ELISA. The frequency of positivity in the control sera was again significantly lower than the frequency in the 257 SSc sera, which were newly selected from the serum bank (2% versus 28%; $P < 0.0001$). When a total of 1,038 sera from the 2 medical centers were combined, clinical sensitivity and specificity with respect to the diagnosis of SSc were 17% and 98%, respectively.

All sera that showed a positive result in the ELISA were subjected to IP assay to assess whether they were true-positive or false-positive. Seven of 9 control sera with a positive result in the ELISA were found to be false-positive, but 1 SLE serum and 1 blood bank control serum did immunoprecipitate RNAP III. A false-positive result was detected in 7 of 72 SSc sera from Pittsburgh, but in none of 17 SSc sera from Tokyo; this difference did not reach statistical significance.

Antigenic specificity of false-negative or false-positive sera. We further evaluated the antigenic specificity of the 15 SSc sera that showed discordant results between IP and ELISA (11 positive by IP but negative by ELISA [false-negative] and 4 negative by IP but positive by ELISA [false-positive]).

Table 2. Clinical sensitivity and specificity of the anti–RNAP III ELISA*

Group	Tokyo			Pittsburgh		
	No. of sera	No. (%) positive by ELISA	No. false-positive	No. of sera	No. (%) positive by ELISA	No. false-positive
SSc†	265	17 (6)	0	257	72 (28)	7
Controls						
SLE	82	0	–	53	2 (4)	1
PM/DM	24	0	–	26	0	–
RA	54	2 (4)	2	30	0	–
Primary SS	32	1 (3)	1	–	–	–
Blood bank controls	48	0	–	167	4 (2)	3
All combined	240	3 (1)	3	276	6 (2)	4

* All sera that were positive by enzyme-linked immunosorbent assay (ELISA) were further analyzed by immunoprecipitation assay to determine whether the ELISA result was true-positive or false-positive. Anti–RNAP III = anti–RNA polymerase III; SLE = systemic lupus erythematosus; PM/DM = polymyositis/dermatomyositis; RA = rheumatoid arthritis; SS = Sjögren's syndrome.
† Systemic sclerosis (SSc) sera from Tokyo were those used for the evaluation of analytical sensitivity and specificity, whereas SSc sera from Pittsburgh were newly selected from the serum bank.

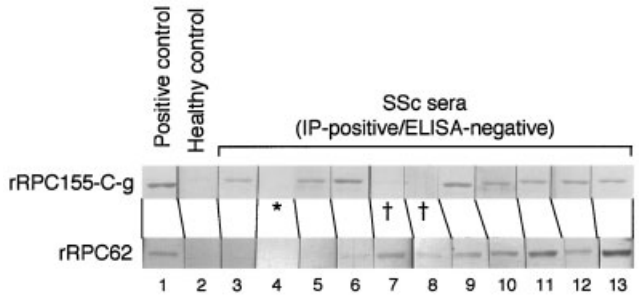

Figure 2. Reactivities to rRPC155C-g and rRPC62 in immunoblots of anti–RNAP III–positive SSc sera that showed a false-negative result in the enzyme-linked immunosorbent assay (ELISA). The bacterial lysates containing recombinant fragments were fractionated on sodium dodecyl sulfate–10% polyacrylamide gels, transferred onto nitrocellulose membranes, and then probed with anti–RNAP III–positive SSc serum with reactivity to both rRPC155C-g and rRPC62 (lane 1), healthy control serum (lane 2), and SSc sera that were positive for anti–RNAP III antibody by IP assay but negative by ELISA (lanes 3–13). Asterisk denotes a serum that did not react with rRPC155C-g or rRPC62; crosses denote sera that reacted with rRPC62 but not with rRPC155C-g. See Figure 1 for other definitions.

The 11 false-negative sera were subjected to immunoblotting to examine antibody reactivity to rRPC155C-g, which was the antigen used in the ELISA (Figure 2). Unexpectedly, 8 sera that did not bind rRPC155C-g in the ELISA reacted with rRPC155C-g by immunoblotting. There was no difference in the intensity of the immunoreactive bands between sera that were positive and those that were negative for anti–RNAP III antibody by the ELISA, suggesting that many false-negative sera recognize the epitope expressed on rRPC155C-g that has been denatured in the presence of sodium dodecyl sulfate and 2-mercaptoethanol, but not on immobilized rRPC155C-g on the ELISA plates. As a result, 117 (98%) of 120 SSc sera that were positive for anti–RNAP III antibody by IP assay recognized rRPC155C-g by either ELISA or immunoblotting, indicating again that amino acids 891–1020 of RPC155 constitute an immunodominant epitope shared by many ethnic groups. On the other hand, 8 of 11 false-negative sera reacted with rRPC62, another RNAP III subunit frequently recognized by anti–RNAP III–positive SSc sera (7,16). As a result, 119 of 120 SSc sera that were positive for anti–RNAP III antibody by the IP assay recognized rRPC155C-g or rRPC62. It should be noted that 2 of the 3 sera that failed to react with rRPC155C-g in both ELISA and immunoblots did recognize rRPC62.

In contrast, the 4 false-positive SSc sera did not bind rRPC155C-g in immunoblots (results not shown). To further examine whether the reactivity of these sera in the ELISA was due to antibody binding to rRPC155C-g itself or to contaminating bacterial components, we conducted a competitive inhibition assay in which serum samples were preincubated with affinity-purified RNAP or topo I antigen (Figure 3). Anti–RNAP III antibody reactivity was inhibited, in a dose-dependent manner, by preincubation of IP-confirmed anti–RNAP III–positive SSc sera with the RNAP antigen, but not by preincubation with topo I. In contrast, anti–RNAP III antibody reactivity was not suppressed by preincubation of the 4 false-positive sera with the RNAP antigen. Similarly, the ELISA reactivity was not inhibited by the RNAP antigen in 3 control sera (2 from patients with RA and 1 from a patient with primary SS) that showed false-positive results. Taken together, these findings suggest that false-negative sera do not recognize rRPC155C-g and that the reactivity in the ELISA was likely due to antibody binding to bacterial components contaminating the rRPC155C-g preparation.

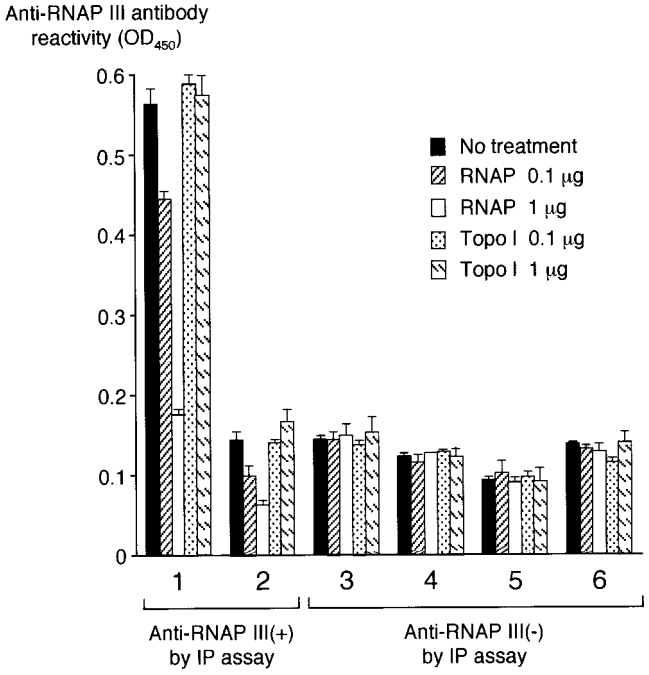

Figure 3. Results of a competitive inhibition assay to examine the specificity of the anti–RNAP III antibody reactivity detected by enzyme-linked immunosorbent assay (ELISA) in 2 SSc sera that were positive for anti–RNAP III antibody by IP assay and 4 SSc sera that were negative for anti–RNAP III antibody by IP assay but showed a false-positive result in the ELISA. Affinity-purified RNAP and topoisomerase I (topo I) antigens were used as competitors. Values are the mean and SD optical density at 450 nm (OD_{450}). See Figure 1 for other definitions.

Table 3. Clinical features in anti–RNAP III antibody–positive SSc patients stratified according to antibody levels determined by ELISA*

Feature	High-level anti–RNAP III (n = 32)	Low-level anti–RNAP III (n = 58)	*P*
Sex, % female	56	78	0.03
Ethnicity, %			NS
White	78	71	
African American	6	9	
Japanese	16	21	
Disease subset, % diffuse	97	81	0.049
Maximum total skin score, mean ± SD	23.8 ± 8.3	18.2 ± 9.3	0.002
Tendon friction rubs, %	88	55	0.002
Organ involvement, %			
Peripheral vascular	44	48	NS
Joint	47	59	NS
Skeletal muscle	0	0	NS
Esophagus	53	52	NS
Small intestine	9	2	NS
Lung (pulmonary interstitial fibrosis)	28	26	NS
Lung (isolated pulmonary arterial hypertension)	6	3	NS
End-stage lung disease	0	7	NS
Heart	13	3	NS
Kidney	28	21	NS

* High and low anti–RNAP III antibody levels were defined as ≥15.2 units and <15.2 units, respectively. NS = not significant (see Table 2 for other definitions).

Clinical findings according to anti–RNAP III antibody levels. Detailed clinical information was available on 90 anti–RNAP III antibody–positive SSc patients (17 from Tokyo and 73 from Pittsburgh). To evaluate clinical correlations with anti–RNAP III antibody levels determined by ELISA, anti–RNAP III–positive SSc patients were divided into 2 groups: 32 with a high level of anti–RNAP III antibody (≥15.2 units), and 58 with a low level of anti–RNAP III antibody (<15.2 units). The cutoff value used to define high versus low antibody levels was determined based on the mean anti–RNAP III antibody levels in all 120 SSc patients who were positive for anti–RNAP III antibody by the IP assay. As shown in Table 3, all but 1 patient with a high level of anti–RNAP III antibody had diffuse cutaneous SSc; this frequency was significantly higher than the frequency in patients with low levels of antibody. The maximum total skin score and frequency of tendon friction rubs were significantly increased in the high-level antibody group compared with the low-level group. Previously reported features of SSc in patients positive for anti–RNAP III antibody included an increased frequency of renal crisis, milder interstitial lung disease, and a lower frequency of inflammatory muscle disease (1–4). However, frequencies of involvement of all internal organs were not different between groups with high levels and those with low levels of the antibody.

When the same comparisons were performed including only the 78 anti–RNAP III–positive patients with diffuse cutaneous SSc, the maximum total skin

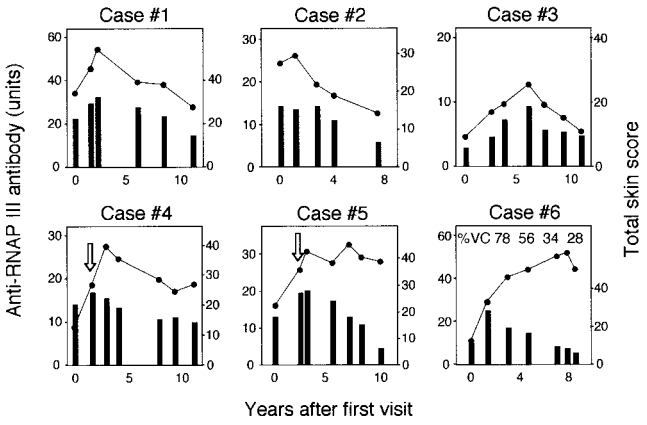

Figure 4. Serial measurements of anti–RNAP III antibody and total skin score in 6 anti–RNAP III antibody–positive SSc patients. Scales for the anti–RNAP III antibody level (circles) and total skin score (bars) are shown at the left and right, respectively. Arrows indicate the onset of renal crisis. Serial measurements of vital capacity (VC) (% predicted) are shown for patient 6. See Figure 1 for other definitions.

score was again significantly increased in the high-level antibody group compared with the low-level antibody group (mean ± SD 24.2 ± 8.1 versus 20.7 ± 7.8; $P = 0.03$), but the difference in the frequency of palpable tendon friction rubs between the high- and low-level antibody groups did not reach statistical significance (87% versus 68%; $P = 0.06$). There was no difference in the frequency of internal organ involvement between the 2 groups when only patients with diffuse cutaneous SSc were included.

Serial anti–RNAP III antibody levels. Anti–RNAP III antibody levels were serially evaluated for >8 years in 6 anti–RNAP III–positive SSc patients. Figure 4 illustrates changes in the anti–RNAP III antibody level together with the total skin score in these patients. In 4 patients, the anti–RNAP III antibody level increased early in the disease course and then decreased, correlating closely with the total skin score. Patient 6 showed a sustained increase in anti–RNAP III antibody levels despite a decrease in the total skin score. This patient had severe interstitial lung disease and died of respiratory failure. Patients 4 and 5 developed renal crisis following rapid increases in the anti–RNAP III antibody level.

DISCUSSION

We have evaluated the analytical accuracy of an anti–RNAP III ELISA, using a large series of sera collected from medical centers in Japan and the US. Among >500 SSc sera screened, our ELISA showed high analytical concordance with the IP assay, the gold standard for detection of anti–RNAP III antibody (specificity 99%), but analytical sensitivity was somewhat low (91%). Both positive and negative predictive values were high (≥96%), indicating that this assay is reliable for the detection of anti–RNAP III antibody. In addition, our ELISA was shown to be clinically specific for SSc in studies using a large series of serum samples from SSc patients, controls with other connective tissue diseases, and blood bank controls. Since ELISAs can be performed easily and quickly and are particularly suitable for screening large numbers of sera, this assay system can substitute for an IP assay for detection of anti–RNAP III antibody in clinical laboratories. However, it should be remembered that a negative result in the ELISA does not necessarily indicate the absence of anti–RNAP III antibody. Thus, our anti–RNAP III ELISA is analytically accurate and clinically specific. Furthermore, this assay affords routine availability of testing for anti–RNAP III antibody. This is important in clinical settings, since anti–RNAP III antibody is known to be useful in the diagnosis, disease classification, and prediction of organ involvement in SSc patients (1–4).

Because a false-positive or false-negative result in the anti–RNAP III ELISA is obtained with some SSc sera, this assay system needs further improvement. Its major weakness is relatively low sensitivity. Based on our detailed assessment of false-negative sera, an increase in sensitivity could potentially be achieved by at least 2 different methods. First, since the majority of sera with false-negative results in the ELISA still recognized rRPC155C-g in immunoblots, the ELISA sensitivity could be improved by enhancing antigenicity of the antigen. It has been shown that autoantibodies to RPC155 in patient sera preferentially recognize a discontinuous or conformational determinant included in the region of rRPC155C-g (7). In fact, structural modification of rRPC155C-g in the presence of 2-mercaptoethanol was needed for sufficient enhancement of the antigenicity in the ELISA (7). Potential approaches for this purpose include expression of a recombinant fragment in different expression systems, such as insect and mammalian systems, and/or structural modification of the antigenic fragment by chemical treatment. An alternative approach would be the addition of other antigenic RNAP I or RNAP III subunits that are frequently recognized by anti–RNAP III–positive SSc sera as a second antigen. RPC62 is one such candidate subunit, because more than half of anti–RNAP III–positive sera have been shown to recognize this subunit (7,16); 2 of 3 anti–RNAP III–positive SSc sera lacking reactivity to rRPC155C-g recognized rRPC62 in the present study.

The majority of sera showing a false-positive result in our ELISA appeared to react with bacterial components contaminating the antigen preparation. Thus, specificity can be further increased by using a more highly purified antigen preparation. Since rRPC155C-g was prepared by 1-step affinity-purification, contaminating bacterial proteins could be removed by additional purification steps, such as ion-exchange chromatography. It is known that the frequency of anti–RNAP III antibody varies in different ethnic groups (6), and our results suggest that the frequency of an analytical false-positive result in SSc patients may also depend on nationality or ethnicity. It is possible that the ELISA can detect antibodies to the RPC155 epitope, which was not expressed on a native RNAP III complex.

One of the advantages of ELISAs is the ability to provide quantitative results, which may be useful in evaluating disease activity. In this regard, serum levels of

anti–topo I antibody have been shown to correlate with disease severity and disease activity in SSc patients (19). In this study, we found that diffuse cutaneous SSc was more prevalent in patients with a high level of anti–RNAP III antibody compared with those with a low level of the antibody. The increased total skin score and high frequency of tendon friction rubs observed in the high-level antibody group are potentially explainable by the increased frequency of diffuse cutaneous SSc in this group. However, the same trends were also observed in a subanalysis including only patients with diffuse cutaneous SSc, suggesting that SSc patients with a higher level of anti–RNAP III antibody may have more extensive skin and tendon involvement independent of the disease subset. Results of our preliminary analysis of serial anti–RNAP III antibody levels should encourage investigators to undertake future prospective studies examining the potential usefulness of serial measurement of anti–RNAP III antibody in predicting disease progression and onset of major organ involvement such as renal crisis.

ACKNOWLEDGMENTS

We thank Yoko Tanaka and Kyoko Kimura for technical assistance, and Hiroko Tanaka for excellent secretarial work.

REFERENCES

1. Medsger TA Jr. Systemic sclerosis (scleroderma): clinical aspects. In: Koopman WJ, editor. Arthritis and allied conditions. 13th ed. Baltimore: Williams & Wilkins; 1997. p. 1433–64.
2. Okano Y. Antinuclear antibody in systemic sclerosis (scleroderma). Rheum Dis Clin North Am 1996;22:709–35.
3. Kuwana M, Kaburaki J, Mimori T, Tojo T, Homma M. Autoantibody reactive with three classes of RNA polymerases in sera from patients with systemic sclerosis. J Clin Invest 1993;91:1399–404.
4. Okano Y, Steen VD, Medsger TA Jr. Autoantibody reactive with RNA polymerase III in systemic sclerosis. Ann Intern Med 1993;119:1005–13.
5. Satoh M, Ajmani AK, Ogasawara T, Langdon JJ, Hirakata M, Wang J, et al. Autoantibodies to RNA polymerase II are common in systemic lupus erythematosus and overlap syndrome. J Clin Invest 1994;94:1981–9.
6. Kuwana M, Okano Y, Kaburaki J, Tojo T, Medsger TA Jr. Racial differences in the distribution of systemic sclerosis–related serum antinuclear antibodies. Arthritis Rheum 1994;37:902–6.
7. Kuwana M, Kimura K, Kawakami Y. Identification of an immunodominant epitope on RNA polymerase III recognized by systemic sclerosis sera: application to enzyme-linked immunosorbent assay. Arthritis Rheum 2002;46:2742–7.
8. 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.
9. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.
10. Bohan A, Peter JB. Polymyositis and dermatomyositis (second of two parts). N Engl J Med 1975;292:403–7.
11. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
12. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002;61:554–8.
13. Clements PJ, Lachenbruch PA, Seibold JR, White B, Weiner S, Martin RW, et al. Inter and intraobserver variability of total skin thickness score (modified Rodnan TSS) in systemic sclerosis. J Rheumatol 1995;22:1281–5.
14. Kuwana M, Kaburaki J, Okano Y, Tojo T, Homma M. Clinical and prognostic associations based on serum antinuclear antibodies in Japanese patients with systemic sclerosis. Arthritis Rheum 1994;37:75–83.
15. Kuwana M, Kaburaki J, Arnett FC, Howard RF, Medsger TA Jr, Wright TM. Influence of ethnic background on clinical and serologic features in patients with systemic sclerosis and anti–DNA topoisomerase I antibody. Arthritis Rheum 1999;42:465–74.
16. Kuwana M, Okano Y, Kaburaki J, Medsger TA Jr, Wright TM. Autoantibodies to RNA polymerases recognize multiple subunits and demonstrate cross-reactivity with RNA polymerase complexes. Arthritis Rheum 1999;42:275–84.
17. Kuwana M, Okazaki Y, Kajihara M, Kaburaki J, Miyazaki H, Kawakami Y, et al. Autoantibody to c-Mpl (thrombopoietin receptor) in systemic lupus erythematosus: relationship to thrombocytopenia with megakaryocytic hypoplasia. Arthritis Rheum 2002;46:2148–59.
18. Kuwana M, Medsger TA Jr, Wright TM. Detection of anti-DNA topoisomerase I antibody by an enzyme-linked immunosorbent assay using overlapping recombinant polypeptides. Clin Immunol Immunopathol 1995;76:266–78.
19. Hu PQ, Fertig N, Medsger TA Jr, Wright TM. Correlation of serum anti-DNA topoisomerase I antibody levels with disease severity and activity in systemic sclerosis. Arthritis Rheum 2003;48:1363–73.