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

Analytical Accuracy and Clinical Associations in Systemic Sclerosis

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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

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

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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

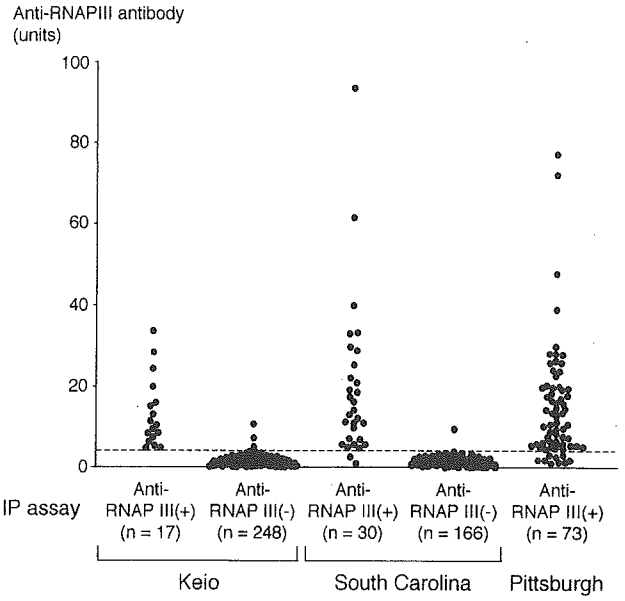

Figure 1. Anti–RNA polymerase III (anti–RNAP III) antibody measured by enzyme-linked immunosorbent assay in a total of 534 sera from systemic sclerosis (SSc) patients at Keio University School of Medicine (Tokyo, Japan), Medical University of South Carolina (Charleston, SC), and University of Pittsburgh 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).

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.

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.

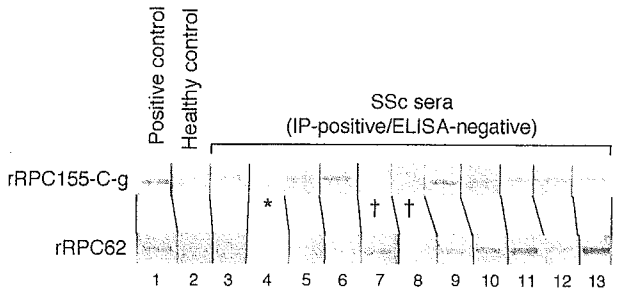

Figure 2. Reactivities to rRPC155C-g and rRPC62 in immunoblots of anti–RNAP III–positive SSc sera that showed a false-negative result in the enzyme-linked immunosorbent assay (ELISA). The bacterial lysates containing recombinant fragments were fractionated on sodium dodecyl sulfate–10% polyacrylamide 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.

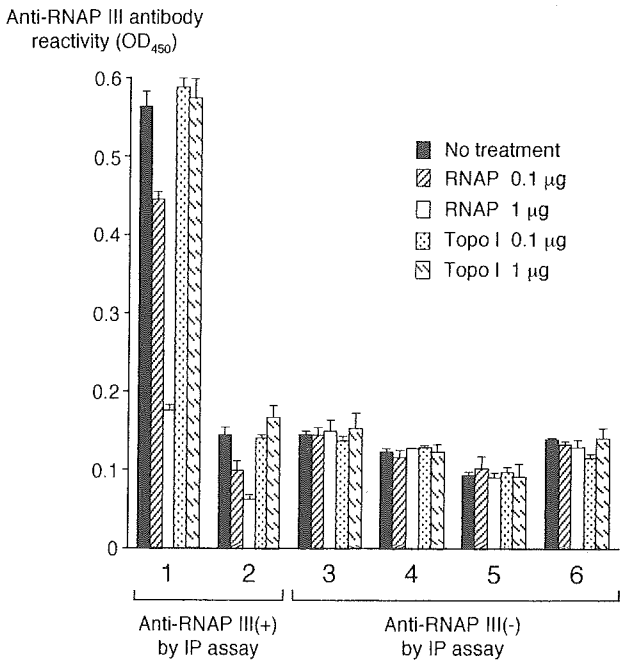

Figure 3. Results of a competitive inhibition assay to examine the specificity of the anti–RNAP III antibody reactivity detected by enzyme-linked immunosorbent assay (ELISA) in 2 SSc sera that were positive for anti–RNAP III antibody by IP assay and 4 SSc sera that were negative for anti–RNAP III antibody 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

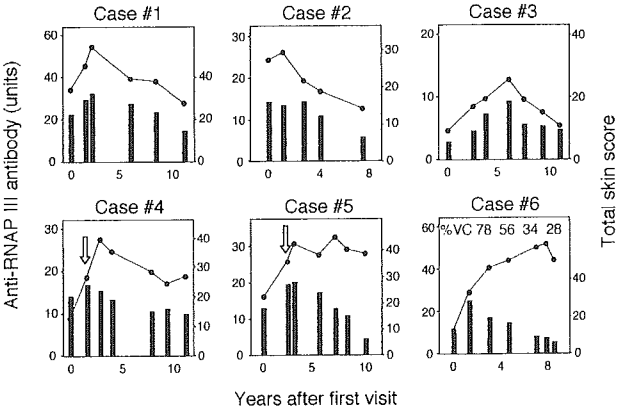

Figure 4. Serial measurements of anti–RNAP III antibody and total skin score in 6 anti–RNAP III antibody–positive SSc patients. Scales for the anti–RNAP III antibody level (circles) and total skin score (bars) are shown at the left and right, respectively. Arrows indicate the onset of renal crisis. Serial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

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Novel Autoantibodies Against 7SL RNA in Patients with Polymyositis/Dermatomyositis

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ABSTRACT. ***Objective.*** Autoantibodies against signal recognition particle (SRP) are detected in patients with polymyositis/dermatomyositis (PM/DM). The SRP consists of 7SL RNA and 6 protein components. We examined autoantibodies against deproteinized 7SL RNA in PM/DM patients with anti-SRP antibodies and evaluated the association of anti-7SL RNA antibodies with PM/DM clinically and serologically.

Methods. Sera from 10 Japanese and 22 North American PM/DM patients with anti-SRP antibodies were tested for the presence of anti-7SL RNA antibodies, using the sera to immunoprecipitate deproteinized RNA extracts derived from HeLa cells.

Results. The immunoprecipitation analysis indicated that 5 Japanese (50%) and one North American (5%) patient with anti-SRP antibodies had novel autoantibodies against deproteinized 7SL RNA. The frequency of anti-7SL RNA antibodies was significantly higher in Japanese than North American patients ($p = 0.006$). The presence of anti-7SL RNA antibodies appeared to be associated with DM (2 patients) and finger swelling (2 PM patients). The seasonal onset of the disease was different ($p = 0.008$) for Japanese PM/DM patients with anti-7SL RNA antibodies, who developed the disease between October and January (mean month November; $p = 0.01$) from that of patients without these antibodies, who developed it between June and August (mean month July; $p = 0.01$).

Conclusion. Novel autoantibodies against 7SL RNA were identified in patients with PM/DM, and the presence of these antibodies was correlated to ethnic background, clinical features, and season of disease onset. These findings indicated that autoantibodies against 7SL RNA are a novel serological marker for a subset of PM/DM cases. (J Rheumatol 2005;32:1727–33)

Key Indexing Terms:
SIGNAL RECOGNITION PARTICLE AUTOANTIBODY POLYMYOSITIS
DERMATOMYOSITIS 7SL RNA ETHNIC BACKGROUND SEASONS

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Polymyositis (PM) and dermatomyositis (DM) are idiopathic inflammatory myopathies characterized by subacute skeletal muscle involvement resulting in muscle weakness and elevated serum levels of muscle enzymes[1]. Patients with PM/DM produce a number of autoantibodies, whose presence indicates a close relationship between the antibodies and the clinical features of these diseases. Target molecules of the autoantibodies that are specific to PM/DM have been identified as a subset of aminoacyl-tRNA synthetases (ARS), transfer RNA (tRNA), the nuclear helicase/ATPase Mi-2, and components of the signal recognition particle (SRP)[2]. The target molecules of anti-ARS antibodies include histidyl-tRNA synthetase (Jo-1), threonyl-tRNA synthetase (PL-7), alanyl-tRNA synthetase (PL-12), glycyl-tRNA synthetase (EJ), isoleucyl-tRNA synthetase (OJ), and asparaginyl-tRNA synthetase (KS). A number of studies have demonstrated that patients with anti-ARS antibodies frequently exhibit certain clinical features, such as interstitial lung disease, arthritis, and myositis[3,4]. Anti-Mi-2 antibodies are specific for DM and are hardly ever found in PM[2].

The SRP is a complex consisting of 7SL RNA and 6 protein components of 9, 14, 19, 54, 68, and 72 kDa[5]. In eukaryotes, the 7SL RNA consists of 2 domains, the Alu and

S domains[6]. The Alu domain is complexed with a heterodimer of the 9 and 14 kDa proteins, and plays a role in elongation arrest[7]. The S domain, which binds to both the signal peptide and a membrane-bound SRP receptor, is complexed with the other 4 proteins[8]. The SRP is assumed to regulate the translocation of protein from the ribosome to the endoplasmic reticulum[9]. Reeves, *et al* first reported the presence of autoantibodies against the SRP in patients with typical PM[10]. Anti-SRP antibodies are also detected in 4%–9% of patients with myositis. Several lines of evidence indicate that these patients have a distinct seasonal onset of the disease and severe myositis resistant to corticosteroid treatment, and almost all of them have PM rather than DM[3,11,12]. Anti-SRP antibodies have also been found in patients with systemic sclerosis (SSc)[13]. Autoantibodies recognizing several components of RNA have been shown to have a close association with the clinical features and disease progression of various connective tissue diseases (CTD)[14]. It was reported that the major antigen for anti-SRP antibodies is the 54 kDa protein, and not a component of the 7SL RNA[11,15]. However, autoantibodies against the 7SL RNA were only looked for in a limited number of serum samples, and further investigation should be performed to evaluate the presence of anti-7SL RNA antibodies in PM/DM patients.

Racial differences in the frequency of autoantibodies have been found for various CTD, suggesting that susceptibility to CTD is influenced by immunogenetic and environmental factors[16-18]. An association of HLA-DR3 with anti-Jo-1 antibodies was found in Caucasian patients with myositis, but this association was not found in Black patients[19]. It was also shown that anti-SRP antibodies are especially frequent in Black female patients with HLA-DR5 and DRw52 in the United states and in Japanese patients with HLA-DR8[3,20].

We investigated the presence of autoantibodies against 7SL RNA in PM/DM patients with anti-SRP antibodies, and evaluated the association between the presence of these antibodies and the race of the patients and their clinical and serological findings.

MATERIALS AND METHODS

Patients. Thirty-two PM/DM patients with anti-SRP antibodies were evaluated. Of these patients, 10 Japanese patients with PM/DM (8 with PM, 2 with DM) were treated at the Division of Rheumatology, Kitasato University Hospital, Kanagawa. They included 8 women and 2 men, with a mean age of 44.5 ± 10.4 years. Twenty-two North American patients (15 Caucasians, 6 Blacks, and a patient of unknown race) with PM were treated at the Division of Rheumatology and Clinical Immunology, University of Pittsburgh. They included 13 women and 9 men, mean age 48.3 ± 15.3 years. In Japanese patients with anti-SRP antibodies, the seasonal onset of disease was determined by the appearance of initial symptom. The presence of anti-SRP antibodies was determined by immunoprecipitation. Serum samples obtained from these patients were stored at –20°C until they were used. Clinical information on all patients was retrospectively obtained from their clinical charts. All selected patients had no other CTD. Diagnoses of PM/DM were made according to the criteria of Bohan and Peter[1].

Preparation of cell lysate and deproteinized RNA. HeLa cells were maintained at 37°C, 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS, USA). HeLa cells were washed twice with phosphate buffered saline and resuspended in NET-2 buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Nonidet P-40, pH 7.4) at 6×10^7 cells/ml. The cells were sonicated and the lysate was spun at 13,000 rpm for 15 min at 4°C. Supernatants were used for the immunoprecipitation analysis.

Deproteinized RNA was recovered from the HeLa cell lysate by 2 phenol extractions, and then dissolved in NET-2 buffer. Deproteinization was also performed using the treatment with proteinase K. HeLa cell lysate was incubated with 0.5% sodium dodecyl sulfate (SDS) and proteinase K (100 μg/ml) for 40 min at 37°C. After incubation, cell lysate was treated with phenol extraction and ethanol precipitation.

Immunoprecipitation of RNA. Immunoprecipitation analysis was performed according to the method described by Forman, *et al*[21]. Two milligrams of protein A sepharose CL-4B (Pharmacia, Piscataway, NJ, USA) were suspended in 500 μl IPP buffer (10 mM Tris-HCl, 500 mM NaCl, 0.1% Nonidet P-40, pH 8.0) and incubated with 10 μl of a serum sample with rotation for 12 h at 4°C. The sepharose beads were washed 3 times with 500 μl IPP buffer, and were then resuspended in 400 μl NET-2 buffer and 100 μl of total cell extract or deproteinized RNA. After incubation for 2 h at 4°C, the beads were washed 5 times with 500 μl of NET-2 buffer and resuspended in 300 μl NET-2 buffer. After incubation, bound RNA was extracted with 30 μl of 3.0 M sodium acetate, 30 μl of 10% SDS, and 300 μl of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. Precipitants were resolved in a 10% polyacrylamide gel containing 7 M urea and detected by silver staining (Bio-Rad, Hercules, CA, USA).

Statistical analysis. Continuous variables are shown as a mean ± standard deviation. Frequencies of clinical, demographic, and serologic findings were tested for statistical significance using Fisher's exact test. The odds ratio with a 95% confidence interval (CI) was calculated for statistically significant differences. The seasonal patterns were evaluated using Rayleigh's test of the length of mean vector. Further, seasonal variation of the seasonal onset was compared by Fisher's exact test analysis of four 3-month clusters, December to February, March to May, June to August, and September to November; this was also performed for two 6-month clusters (January to June and July to December)[12,22]. The significance of differences in the distribution of the 3 groups was assessed by Kruskal-Wallis test. Differences in the continuous variables were examined by nonparametric Mann-Whitney U test between 2 patient groups. Differences were considered significant at a value of $p < 0.05$.

RESULTS

Anti-RNA antibodies in patients with anti-SRP antibodies. Anti-SRP antibodies were screened by immunoprecipitation using total HeLa cell extract, and 10 Japanese and 22 North American patients with PM/DM were positive. Anti-SRP antibodies that were precipitated migrated to the 7S region by electrophoresis. To evaluate anti-RNA antibodies, immunoprecipitation analysis was performed using HeLa cell extracts or purified RNA from the cells. As shown in Figure 1A, serum samples precipitated ribonucleoproteins (RNP) from total cell extracts that were found in the 7SL RNA region (Figure 1A, "+" lanes). When purified RNA was used as the antigen (Figure 1A, "–" lanes), precipitates were also detected in the 7SL RNA region for some samples (Figure 1A, sections A–C), but no positive signal was obtained for others (Figure 1A, D–F). The deproteinization of RNA samples by the procedure we used may have been complete, since the precipitates of other antibodies including anti-SSA/Ro antibodies were not detected (Figure 1A,

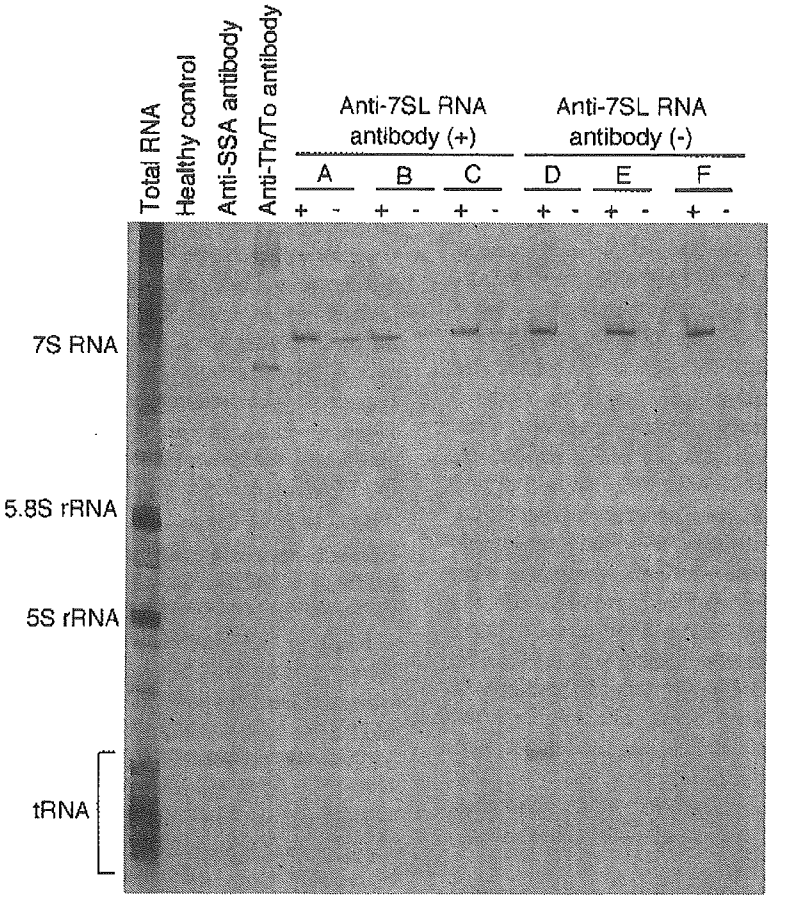

Figure 1A. Analysis of anti-RNA antibodies in serum samples from Japanese patients with PM/DM. Sera (A–F) were screened for anti-SRP antibodies or anti-7SL RNA antibodies by immunoprecipitation using either total (+) or deproteinized (–) cell extracts, respectively. Immunoprecipitates were prepared with sera A, B, D, and E from Japanese patients and sera C and F from North American patients. Positions of prominent small RNA bands are indicated on the left.

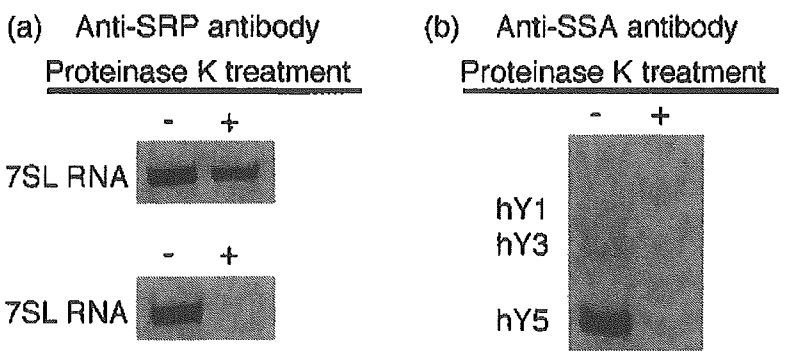

Figure 1B. Detection of anti-RNA antibodies in serum samples from patients with PM/DM. Immunoprecipitation was performed by using proteinase K-untreated (–) or proteinase K-treated (+) antigens to evaluate anti-SRP antibodies (a) and anti-SSA/Ro antibodies (b).

sections A and D). To ensure the antibodies were against the RNA, we performed immunoprecipitation analysis using deproteinized cell extracts obtained by the treatment with proteinase K[23,24]. Anti-7SL RNA antibodies were also detected in this experiment (Figure 1B). Anti-RNA antibodies had completely disappeared at hY RNA regions after the treatment of anti-SSA/Ro antibody-positive serum with proteinase K, indicating the presence of antibody to recognize both protein and RNA (Figure 1B, panel b). In the case of anti-SRP antibodies, anti-7SL RNA antibodies were detected in some serum samples even after the proteinase K treatment, indicating the presence of anti-7SL RNA antibodies (Figure 1B, panel a). Anti-7SL RNA antibodies could not be detected if 2-fold amounts of antigens or serum derived from anti-7SL RNA antibody-negative patients were applied. Among these patients with anti-SRP antibodies, autoantibodies against 7SL RNA were detected in 5 out of the 10 Japanese patients and in one Caucasian among the 22 North American patients. Anti-7SL RNA antibodies were frequently detected in Japanese patients compared with North American patients (50% vs 5%; p = 0.006, OR 21, 95% CI 2–222) (Table 1). Anti-SRP antibodies were found in 10 of the 84 Japanese patients with PM/DM (11.9%). We have reported[13] that the frequency of anti-SRP antibodies was 6.1% in North American patients. The frequency of anti-SRP antibodies was not statistically significant between Japanese and North American patients with PM/DM (11.9% vs 6.1%; p = 0.08). Titration of anti-7SL RNA antibodies and identification of the epitope recognized by this antibody were not performed in this study.

Autoantibodies against other RNP were found in patients with anti-SRP antibodies: anti-SSA/Ro antibodies were found in 5 Japanese and one North American patient, anti-SSB/La in one Japanese patient, and anti-SSA/Ro and anti-U1-RNP antibodies in one North American patient each. Precipitated anti-SSA/Ro, SSB/La, and U1-RNP antibodies migrated to the hY1, hY3 and hY5 region, the hY RNA and 5S rRNA region, and the U1 RNA region, respectively. The frequency of the anti-SSA/Ro antibodies was significantly higher in Japanese patients than North American patients (50% vs 5%; p = 0.006, OR 21, 95% CI 2–222) (Table 1). Finally, anti-U1 RNA antibodies were found in 2 North American patients with anti-SRP antibodies, but not in Japanese patients.

Clinical features of Japanese and North American patients with anti-SRP antibodies. Of the 10 Japanese patients with anti-SRP antibodies, 8 were given a diagnosis of PM and 2 DM; in contrast, all the North American patients with anti-SRP antibodies were given a diagnosis of PM. Although the frequency of DM among the patients with the anti-SRP antibodies appeared to be higher in the Japanese population, the difference was not statistically significant. To evaluate racial differences in anti-SRP antibody-positive patients, their clinical features were investigated. There were no significant differences in the clinical features of age at onset, sex, Raynaud's phenomenon, arthritis, interstitial lung disease, finger swelling, or cardiac involvement between Japanese and North American patients with anti-SRP antibodies. All the Japanese patients with anti-SRP antibodies were treated with corticosteroid.

Clinical features of patients with or without anti-7SL RNA antibodies. The patients were divided into 2 groups according to the presence (5 Japanese patients and one North American patient) or the absence (5 Japanese, 20 North American patients) of anti-7SL RNA antibodies. The clinical characteristics of these 2 groups are shown in Table 2. The frequency of DM was higher in the antibody-positive patients than in the antibody-negative patients (33.3% vs 0%; p = 0.03, OR 28.3, 95% CI 3–272). Finger swelling without clinical features of hyperkeratotic eruption, scarring, hyperpigmentation, and arthritis was found in 2 PM patients with anti-7SL RNA antibodies, but it was not found in the antibody-negative patients (p = 0.03, OR 28.3, 95% CI 3–272). Finger swelling in these patients disappeared after the treatment with corticosteroid. Patients with anti-7SL RNA antibodies did not have other SSc related features, such as esophageal dysfunction, digital pitting scars, and SSc-specific autoantibodies. There were no significant differences in other clinical features between these patient groups (Table 2).

Clinical characteristics of Japanese patients with or without anti-7SL RNA antibodies. Leff, *et al* reported that PM/DM patients with anti-SRP antibodies developed the disease during the season between September and February[12]. Since we

Table 1. Coexistence of autoantibodies in anti-SRP antibody-positive sera from Japanese and North American patients with PM/DM. Values show the number of patients (%).

Autoantibodies	Japanese Patients, n = 10	North American Patients, n = 22	p†
Anti-7SL RNA*	5 (50)	1 (5)	0.006
Anti-SSA(Ro)	5 (50)	1 (5)	0.006
Anti-SSB(La)	1 (10)	0 (0)	NS
Anti-U1 RNP	0 (0)	1 (4)	NS
Anti-U1 RNA*	0 (0)	2 (9)	NS

* Anti-SRP antibody-positive sera were evaluated for the presence of anti-7SL RNA antibodies and anti-U1 RNA antibodies using immunoprecipitation of deproteinized cell extracts. † Fisher's exact test. NS: not significant.

Table 2. Clinical findings of PM/DM patients (Japanese and North American) with or without anti-7SL RNA antibodies.

Clinical Findings	Anti-7SL RNA-Positive, n = 6	Anti-7SL RNA-Negative, n = 25	p*
PM:DM	4:2	25:0	0.03
Sex, male:female	0:6	11:14	NS
Age at onset, yrs	47.5 ± 5.4	47 ± 15.3	NS†
Raynaud's phenomenon, n (%)	3 (50)	7 (28)	NS
Arthritis, n (%)	1 (17)	6 (24)	NS
Interstitial lung disease, n (%)	1 (17)	6 (24)	NS
Finger swelling, n (%)	2 (33)	0 (0)	0.03
Cardiac involvement, n (%)	2 (33)	3 (12)	NS
Anti-SSA/Ro antibody, n (%)	3 (50)	3 (12)	NS

* Fisher's exact test. †p values were calculated by nonparametric Mann-Whitney U test. NS: not significant.

did not have information about disease onset for the North American patients, we evaluated the seasonal pattern of disease onset only for the Japanese patients. Japanese patients with anti-SRP antibodies predominantly developed the disease during June to January. Patients with anti-SRP antibodies were divided into anti-7SL RNA antibody-positive and negative groups, and the clinical features of these 2 groups were evaluated. There were no statistical differences in the age at onset, sex, clinical findings (Raynaud's phenomenon, arthritis, interstitial lung disease, finger swelling, and cardiac involvement), or the presence of other autoantibodies. The patients with anti-7SL RNA antibodies predominantly developed the disease from October to January, with mean month of onset being November (p = 0.01) (2 patients in November, one each in October, December, and January), whereas the patients without antibodies developed the disease from June to August, with mean month of onset being July (p = 0.01) (3 patients in June and 2 in August; Figure 2). Although there was no significant difference when the year was divided into 4 separate periods of 3 months (in accord with the seasons), analysis of 6-month clusters (March to August vs September to February and April to September vs October to March) showed that the peak season of disease onset for the anti-7SL RNA-positive and negative groups was different (p = 0.008). Patients with anti-7SL RNA antibodies did not have any infectious diseases and vaccination at disease onset. These data indicate the possibility that seasonal pattern of disease onset may be different among patients with and those without anti-7SL RNA antibodies.

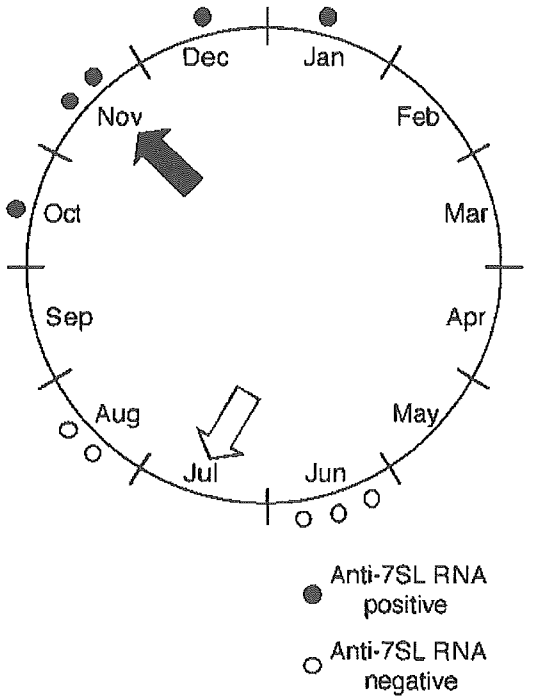

Figure 2. Seasonal deviation of disease onset in Japanese PM/DM patients with anti-SRP antibodies. ●: Patients with anti-7SL RNA antibodies; ○: patients without anti-7SL RNA antibodies. Mean vectors are indicated by arrows (black arrow: anti-7SL RNA antibody-positive patients; white arrow: anti-7SL RNA antibody-negative patients). Rayleigh's test of length of mean vector was performed for anti-7SL RNA antibody-positive patients (p = 0.01, length of mean vector 0.87) and anti-7SL RNA antibody-negative patients (p = 0.01, length of mean vector 0.88). When each year was divided into two 6-month periods, the onset of symptoms for patients with anti-7SL RNA antibodies showed a significant seasonal variation compared to onset of symptoms for patients without these antibodies (p = 0.008, Fisher's exact test).

DISCUSSION

We observed novel antibodies against the deproteinized 7SL RNA in PM/DM patients with autoantibodies against the SRP, and the presence of anti-7SL RNA antibodies was closely associated with ethnic background, clinical features, and seasonal deviation of disease onset.

To evaluate the presence of anti-7SL RNA antibodies in patients with anti-SRP antibodies, we prepared deproteinized purified RNA from HeLa cells by phenol extraction alone (Figure 1A) or proteinase K treatment followed by phenol extraction (Figure 1B). The purity of the isolated RNA was almost 2.0 by the A_{260}/A_{280} ratio, indicating that these results were unlikely to be caused by protein contami-

nants[23]. Immunoprecipitation analysis was performed using these purified RNA as antigens, and the results clearly indicated the presence of autoantibodies directly against 7SL RNA. These results were reproducible even if we used highly purified RNA obtained by repeating the phenol extraction 4 times. Further, we did not detect anti-SSA/Ro and anti-SRP antibodies in some samples when we immunoprecipitated serum samples containing anti-SRP antibodies and anti-SSA/Ro antibodies using deproteinized HeLa cell extracts obtained by phenol extraction (Figure 1A) or proteinase K treatment (Figure 1B). Thus, this technique seems sufficient to detect anti-RNA antibodies.

Although a number of studies have investigated anti-SRP antibodies in PM/DM patients, the anti-7SL RNA antibody has not been described to date. Okada, *et al* evaluated anti-7SL RNA antibodies in a Japanese and an American patient with anti-SRP antibodies; and Targoff, *et al* also measured the antibodies in an American patient with anti-SRP antibodies. However, they failed to detect anti-7SL RNA antibodies in these selected patients[11,15]. The precise reason for the difference between our findings and theirs is not clear. It may be because of the number of serum samples examined and the different patient populations.

It has been reported that patients with connective tissue diseases exhibit various autoantibodies capable of recognizing RNA, including U1 RNA[23], ribosomal RNA[25], hY5 RNA[26], $tRNA^{his}$ [24], and $tRNA^{ala}$ [27]. Several lines of evidence indicate that the epitopes recognized by these antibodies are located at the functional sites of the RNA[25,27,28]. Epitope recognized by anti-7SL RNA antibodies was not identified in our study. We intend to evaluate it in a future investigation. Epitope spreading in autoantibody formation has been postulated. This is based on the idea that autoimmune responses against a self-antigen may expand toward a subsequent intermolecular epitope. This might be the case for anti-$tRNA^{His}$ autoantibodies in PM/DM patients with anti-Jo-1 antibodies and anti-U1 RNA antibodies in systemic lupus erythematosus (SLE) patients with anti-U1-RNP antibodies[26,29]. We also think that the autoimmune response directed toward the protein components might spread to the RNA component of the SRP.

Differences in the distribution of autoantibodies in various racial groups appear to promote differences in the frequencies of CTD. Among patients with the SSc-PM overlap syndrome, an anti-Ku antibody is prevalent in Japanese, whereas an anti-PM-Scl antibody is prevalent in Caucasians[16-18]. The frequency of anti-SRP antibodies in PM/DM patients has been documented to be 4%–9% in Caucasian patients with PM/DM. Anti-SRP antibodies have also been detected in about 6% of Japanese patients with PM/DM[30]. These findings indicate that the frequencies of anti-SRP antibodies are almost the same in several ethnic groups. Several immunogenetic studies have revealed an association between CTD and HLA class II alleles. HLA-DRw52 and DR5 are most frequently associated with the presence of anti-SRP antibodies in American patients, but HLA-DR8 is more frequently observed in Japanese patients with anti-SRP antibodies[3,20]. In contrast, we observed here that the frequency of anti-7SL RNA antibodies in Japanese patients appeared to be higher than in North American patients. However, we could not conclude there was a racial difference in the distribution of anti-7SL RNA antibodies because of the limitation of sample numbers. Further investigation in a multicenter analysis may elucidate the racial differences.

An association between anti-SRP antibodies and PM rather than DM has been documented[3,11,13]. Anti-SRP antibodies were found in 14 PM and 5 DM patients among 379 Europeans[31], and 2 PM patients and one DM patient among 52 Japanese[30], and almost all American patients with anti-SRP antibodies examined have a diagnosis of PM[3,11,12]. We found increased frequencies of DM and finger swelling in patients with anti-7SL RNA antibodies compared with patients without these antibodies. Thus, anti-7SL RNA antibodies may be a novel marker for a subset of PM/DM.

Seasonal variation in onset and disease progression has been reported for various autoimmune diseases, such as rheumatoid arthritis[32], SLE[33], Wegener's granulomatosis[34], and PM/DM[35,36]. Leff, *et al* reported that patients with anti-SRP antibodies predominantly developed PM/DM during the period between September and February, with the average being November[12]. A study by Miller, *et al* showed almost the same results in the pattern of disease onset[37]. Our results also showed the possibility of the same predominant pattern of disease onset. Moreover, we observed that patients with anti-7SL RNA antibodies developed myositis during the winter season, while patients without these antibodies developed the disease from the summer season. The seasonal difference in disease onset in patients with anti-7SL RNA antibodies may indicate preceding infections or the presence of environmental triggers. Several studies have reported that infectious agents may influence the onset of inflammatory myopathies[38,39]. In addition, adrenocortical function is activated during the winter. This variation may influence the cellular function of T and B cells[40]. Because of the limitation of the number of patients with anti-7SL RNA antibodies, it is hard to determine the seasonal difference of disease onset. Multicenter analysis may provide precise evidence of seasonal predominance of disease onset.

We describe the first observation of the presence of autoantibodies directly against 7SL RNA in PM/DM patients with anti-SRP antibodies. The presence of anti-7SL RNA antibodies may be associated with ethnic backgrounds and seasonal patterns of disease onset. Thus, these data indicate that anti-7SL RNA antibodies as well as anti-SRP antibodies may be useful markers for the diagnosis of PM/DM.

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CLINICAL RESEARCH STUDY

Initial laboratory findings useful for predicting the diagnosis of idiopathic thrombocytopenic purpura

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Thrombopoietin

ABSTRACT

PURPOSE: To identify initial laboratory findings useful for the later diagnosis of idiopathic thrombocytopenic purpura (ITP) in adult patients with thrombocytopenia.

SUBJECTS AND METHODS: We studied 62 consecutive adult patients who had thrombocytopenia and whose peripheral blood film was normal except for thrombocytopenia at presentation. Each patient underwent physical examination and routine laboratory tests and was prospectively followed for 22.5 ± 9.8 months (range, 8 to 41 months). The frequency of antiglycoprotein (GP) IIb/IIIa antibody-producing B cells, the presence of platelet-associated and plasma anti-GPIIb/IIIa antibodies, the percentage of reticulated platelets, and the plasma thrombopoietin level were examined at the first visit. The final diagnosis was based on the clinical history, physical examination, complete blood test, bone marrow findings, and the clinical course at last observation.

RESULTS: Forty-six patients were diagnosed as having ITP and 16 as having another disorder, including myelodysplastic syndrome, aplastic anemia, amegakaryocytic thrombocytopenia, and reduced platelet production, with or without other cytopenias, and without dysplasia or evidence for destruction. Six initial laboratory findings discriminated ITP from other diagnoses: the absence of anemia, absence of leukocytopenia, increased frequency of anti-GPIIb/IIIa antibody-producing B cells, increased platelet-associated anti-GPIIb/IIIa antibodies, elevated percentage of reticulated platelets, and a normal or slightly increased plasma thrombopoietin level. Three or more of these ITP-associated findings were found at presentation in 44 patients (96%) with thrombocytopenia later diagnosed as ITP, compared with only 1 patient (6%) whose disorder was non-ITP.

CONCLUSION: Initial laboratory findings can well predict future diagnosis of ITP. Further studies prospectively evaluating these same diagnostic criteria on another, independent set of patients are necessary.

Thrombocytopenia is a common clinical manifestation of many diseases and has various causes, including decreased bone marrow production, increased splenic sequestration, and accelerated destruction of platelets.[1] One of the major causes of accelerated platelet consumption is immune thrombocytopenia, in which platelet destruction is mediated by antiplatelet autoantibodies.[1,2] This condition is seen in patients with various diseases, such as systemic lupus ery-

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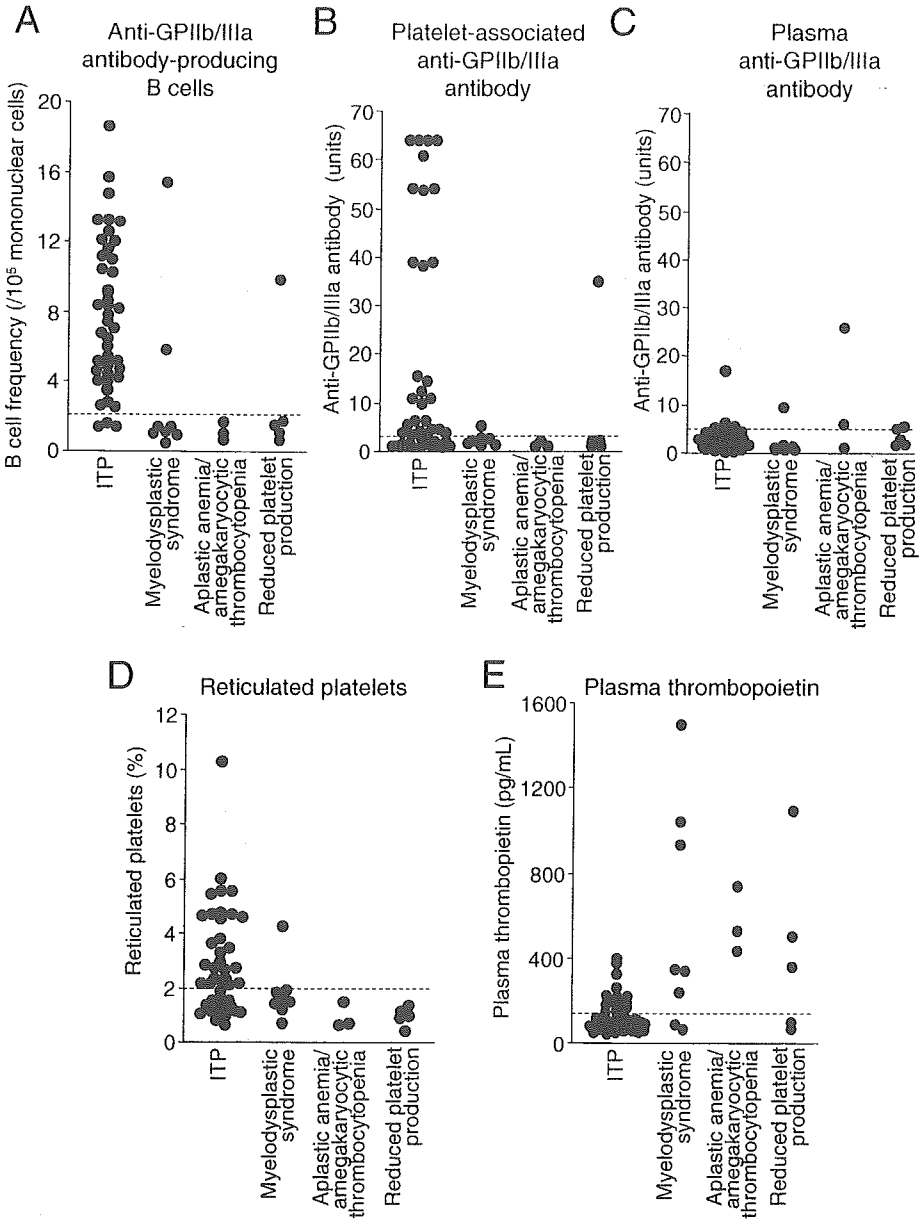

Figure 1 Anti-glycoprotein (GP) IIb/IIIa antibody-producing B cell frequency (A), platelet-associated anti-GP IIb/IIIa antibodies (B), plasma anti-GPIIb/IIIa antibodies (C), percentage of reticulated platelets (D), and plasma thrombopoietin level (E) in 62 patients with thrombocytopenia at first visit, grouped according to the final diagnosis. Forty-six patients received the diagnosis of idiopathic thrombocytopenic purpura (ITP), 8 patients received the diagnosis of myelodysplastic syndrome, 3 patients received the diagnosis of aplastic anemia/amegakaryocytic thrombocytopenia, and 5 patients received the tentative diagnosis of reduced platelet production, with or without other cytopenias, without dysplasia or evidence for destruction. Broken lines in individual panels denote cut-off levels that were based on the results of healthy controls: anti-GPIIb/IIIa antibody-producing B cell frequency, $2.0/10^5$ peripheral blood mononuclear cells; platelet-associated anti-GPIIb/IIIa antibodies, 3.3 units; plasma anti-GPIIb/IIIa antibodies, 5.0 units; percentage of reticulated platelets, 2.0%; and plasma thrombopoietin level, 142 pg/mL.

thematosus and infection with the human immunodeficiency virus, and it can also occur without an underlying disease, in which case it is known as idiopathic thrombocytopenic purpura (ITP).[2] Currently, the diagnosis of ITP is principally based on the exclusion of other possible concurrent causes of thrombocytopenia.[2,3] In the guidelines proposed by the American Society of Hematology,[4] the panel recommended that the diagnosis of ITP be made in patients who have thrombocytopenia and who lack findings that are atypical for ITP or that suggest another diagnosis by history, physical examination, complete blood count, or peripheral blood film. No further laboratory tests are considered necessary. A similar guideline has been reported by the British Committee for Standards in Haematology General Haematology Task Force.[5] However, there are potential problems with a diagnosis of exclusion, as noted by Chong and Keng[6]

Table 1 Demographic and laboratory findings at first visit of 46 patients later diagnosed as having ITP and 16 later diagnosed as having a non-ITP disorder

Demographic and laboratory findings	Number (%), Mean ± SD		
	ITP (n = 46)	Non-ITP (n = 16)	*P* value
Women	33 (72)	8 (50)	0.2
Age at first visit (years)	50.2 ± 18.2	59.0 ± 17.4	0.09
Anemia	11 (24)	14 (88)	<0.001
Leukocyte count (x 10^9/L)	5.3 ± 1.5	4.0 ± 0.9	<0.001
Platelet count (x 10^9/L)	46 ± 24	59 ± 20	0.04
Anti-GPIIb/IIIa antibody-producing B cells (/10^5 peripheral blood mononuclear cells)	7.6 ± 4.2	2.9 ± 4.1	<0.001
Platelet-associated anti-GPIIb/IIIa antibodies (units)	16.3 ± 22.3	4.2 ± 8.3	0.01
Plasma anti-GPIIb/IIIa antibodies (units)	2.9 ± 2.5	4.1 ± 5.9	0.8
Reticulated platelets (%)	2.8 ± 1.9	1.4 ± 0.9	0.001
Plasma thrombopoietin (pg/mL)	126 ± 85	525 ± 429	<0.001

GP = glycoprotein; ITP = idiopathic thrombocytopenic purpura.

and by McMillan et al,[7] who suggested that the guideline's recommendations are not rigorous enough to make an accurate diagnosis of ITP. In addition, this diagnostic process largely relies on the experience of hematologists who specialize in this field, whereas many less experienced physicians see patients with decreased platelet counts. Therefore, other practical criteria are needed for diagnostic accuracy in clinical settings.

The presence of anti-platelet antibodies is a hallmark of the autoimmune nature of ITP.[8] Anti-platelet antibodies in patients with ITP preferentially recognize platelet surface glycoproteins (GP), and the most common target is GPIIb/IIIa.[8] Several antigen-specific assays are reported to be useful in identifying patients with ITP.[7,9,10] We have also reported that an enzyme-linked immunospot assay for the detection of circulating B cells secreting anti-GPIIb/IIIa antibodies is a sensitive, specific, and convenient method for evaluating the presence or absence of autoantibody-mediated thrombocytopenia.[11] In addition, the percentage of reticulated platelets and the circulating thrombopoietin level are reported to be useful in discriminating a state of accelerated platelet destruction from that of decreased platelet production.[12-15] To evaluate the potential usefulness of these laboratory tests for the diagnosis of ITP, we conducted a prospective study of patients who had thrombocytopenia without any other morphologic abnormalities in their peripheral blood film at first visit.

Subjects and methods

Subjects

We prospectively investigated all 62 adult patients who had thrombocytopenia, who first visited the outpatient clinic at Keio University Hospital during a 3-year period (from January 2000 to December 2002), and who met the inclusion criteria: thrombocytopenia $<100 \times 10^9$/L; the absence of any other morphologic abnormalities in the peripheral blood film; exclusion of pseudothrombocytopenia; no clinical or serologic evidence of associated conditions or factors that can cause thrombocytopenia, such as systemic lupus erythematosus, infection with the human immunodeficiency virus, lymphoproliferative disorders, liver cirrhosis, or therapy with drugs such as heparin or quinidine; and no previous treatment with corticosteroids or splenectomy. In our outpatient clinic, antinuclear antibody testing was routinely performed on patients with thrombocytopenia, and serologic tests for human immunodeficiency virus and hepatitis C virus were performed on patients who were judged to be at clinical risk. At the first visit, a detailed history and physical examination and routine laboratory tests, including complete blood count and peripheral blood film, were performed on all patients. At the same time, 20 mL of peripheral blood was obtained for the evaluation of the anti-GPIIb/IIIa antibody response and platelet turnover. A total of 10 demographic and laboratory findings were recorded for each patient at study entry. These included sex, age at first visit, erythrocyte count, leukocyte count, platelet count, anti-GPIIb/IIIa antibody-producing B cell frequency, platelet-associated and plasma anti-GPIIb/IIIa antibodies, percentage of reticulated platelets, and plasma thrombopoietin level. An erythrocyte count $<4.3 \times 10^{12}$/L (men) or $<3.7 \times 10^{12}$/L (women) was regarded as anemia. All blood samples were obtained after the patients gave written informed consent, as approved by the Keio University Institutional Review Board.

All patients underwent a bone marrow examination and were followed by one of the investigators for 22.5 ± 9.8 months (range, 8 to 41 months). The investigator was blinded to the results of the 5 specialized tests for evaluating

Table 2 Sensitivity, specificity, positive predictive value, and negative predictive value of initial laboratory findings associated with a later diagnosis of ITP

Laboratory findings	Number					Percentage (95% confidence interval)			
	ITP (n = 46)		Non-ITP (n = 16)						
	Positive	Negative	Positive	Negative	*P* value	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Erythrocyte count ≥ 4.3 × 10^{12}/L (male) ≥3.7 × 10^{12}/L (female)	35	11	2	14	<0.001	76 (61-87)	88 (62-98)	95 (82-99)	56 (35-76)
Leukocyte count ≥ 4 × 109/L	38	8	5	11	<0.001	83 (69-92)	69 (41-89)	88 (75-96)	58 (33-80)
Anti-GPIIb/IIIa antibody-producing B cells ≥ 2/105 peripheral blood mononuclear cells	43	3	3	13	<0.001	93 (82-99)	81 (54-96)	93 (82-99)	81 (54-96)
Platelet-associated anti-GPIIb/IIIa antibodies ≥ 3.3 units	28	18	2	14	0.002	61 (45-75)	88 (62-98)	93 (78-99)	44 (26-62)
Percentage of reticulated platelets ≥2%	27	19	1	15	<0.001	59 (43-73)	94 (70-100)	96 (82-100)	44 (27-62)
Plasma thrombopoietin <300 pg/mL	43	3	5	11	<0.001	93 (82-99)	69 (41-89)	90 (77-97)	79 (49-95)

GP = glycoprotein; ITP = idiopathic thrombocytopenic purpura.

the anti-GPIIb/IIIa antibody response and platelet turnover, which were done for this study.

Diagnosis

A diagnosis of ITP was made on the basis of clinical history, physical examination, complete blood count, bone marrow findings, and the clinical course for at least 8 months; the diagnosis was not based on the results of any of the 5 specialized studies done as part of this study. All patients with ITP fulfilled the criteria advised by the American Society of Hematology Practice Guidelines[4] and had chronic ITP based on thrombocytopenia persisting longer than 6 months. Diagnoses of myelodysplastic syndrome, aplastic anemia, and amegakaryocytic thrombocytopenia were based principally on bone marrow findings and cytogenetic analysis.[16-18]

Sample preparation

Platelet-rich plasma was prepared from heparinized venous blood by centrifugation at 120 g for 10 minutes, and the remaining cell component was subjected to a Lymphoprep (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation to isolate the peripheral blood mononuclear cells. After 10^6 platelets were used in the assay for reticulated platelets, the remaining platelet-rich plasma was spun to separate the platelets from platelet-poor plasma.

Evaluation of anti-GPIIb/IIIa antibody responses

B cells producing immunoglobulin G (IgG) anti-GPIIb/IIIa antibodies were detected using the enzyme-linked immunospot assay.[11,19] Briefly, affinity-purified human GPIIb/IIIa was coated onto a polyvinylidene difluoride-bottomed 96-well microplate. Peripheral blood mononuclear cells were pipetted into the wells (10^5/well) and cultured at 37°C for 4 hours. During this incubation period, secreted anti-GPIIb/IIIa antibodies were bound to the immobilized GPIIb/IIIa in the immediate vicinity of the secreting cells. After washing away the cells, the membranes were incubated with alkaline phosphatase-conjugated goat anti-human IgG, and anti-GPIIb/IIIa antibodies bound to the membrane were visualized as spots by incubation with a substrate. Blue-purple spots that were round and had a dark center with slightly fuzzy edges were regarded as the sites of anti-GPIIb/IIIa antibody-secreting B cells. Each assay was conducted in 5 independent wells, and the results represent the mean of the 5 values. The frequency of circulating anti-GPIIb/IIIa antibody-producing B cells was calculated as the number per 10^5 peripheral blood mononuclear cells, and an abnormal value was defined as ≥2.0 based on 5 standard deviations above the mean obtained from 52 healthy individuals.[11]

IgG anti-GPIIb/IIIa antibodies in platelet eluates (from 5 × 10^7 platelets) and plasma were measured by enzyme-linked immunosorbent assay using purified human GPIIb/IIIa as the

Table 3 Sensitivity, specificity, positive predictive value, and negative predictive value of 3 simple ITP-associated laboratory tests* in combination with other tests for the diagnosis of ITP

Combinations of ITP-associated laboratory tests	Number of findings required	Number					Percentage (95% confidence interval)			
		ITP (n = 46)		Non-ITP (n = 16)						
		Positive	Negative	Positive	Negative	*P* value	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Three simple ITP-associated laboratory tests*	2 or more	38	8	2	14	<0.001	83 (69-92)	88 (62-98)	95 (83-99)	64 (41-83)
±Anti-GPIIb/IIIa antibody-producing B cells	2 or more	43	3	2	14	<0.001	93 (82-99)	88 (62-98)	96 (85-99)	82 (57-96)
±Platelet-associated anti-GPIIb/IIIa antibodies	2 or more	41	5	2	14	<0.001	89 (76-96)	88 (62-98)	95 (84-99)	74 (49-91)
±Plasma thrombopoietin	2 or more	42	4	3	13	<0.001	91 (79-98)	81 (54-96)	93 (82-99)	77 (50-93)
±Anti-GPIIb/IIIa antibody-producing B cells and platelet-associated anti-GPIIb/IIIa antibodies	2 or more	45	1	5	11	<0.001	98 (88-100)	69 (41-89)	90 (78-97)	92 (62-100)
±Anti-GPIIb/IIIa antibody-producing B cells and plasma thrombopoietin	2 or more	44	2	4	12	<0.001	96 (85-99)	75 (48-93)	92 (80-98)	86 (57-98)
±Platelet-associated anti-GPIIb/IIIa antibodies and plasma thrombopoietin	2 or more	45	1	3	13	<0.001	98 (88-100)	81 (54-96)	94 (83-99)	92 (66-100)
±Anti-GPIIb/IIIa antibody-producing B cells, platelet-associated anti-GPIIb/IIIa antibodies, and plasma thrombopoietin	3 or more	44	2	1	15	<0.001	96 (85-99)	94 (70-100)	98 (88-100)	88 (64-99)

GP = glycoprotein; ITP = idiopathic thrombocytopenic purpura.
*Anemia, leukocyte count, and percentage of reticulated platelets.