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CLINICAL RESEARCH STUDY

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

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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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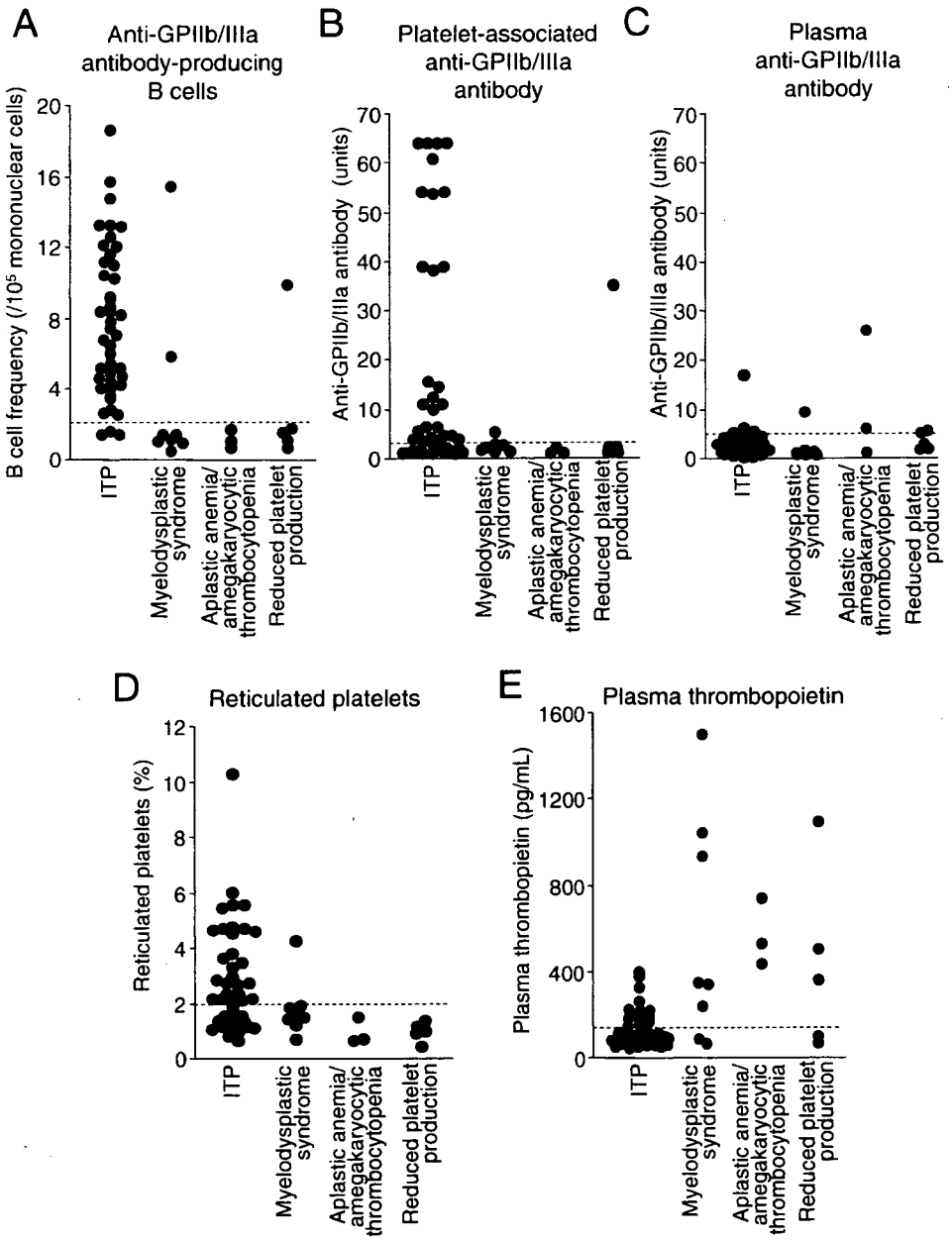

Figure 1 Anti-glycoprotein (GP) IIb/IIIa antibody-producing B cell frequency (A), platelet-associated anti-GP IIb/IIIa antibodies (B), plasma anti-GPIIb/IIIa antibodies (C), percentage of reticulated platelets (D), and plasma thrombopoietin level (E) in 62 patients with thrombocytopenia at first visit, 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				*P* value	Percentage (95% confidence interval)			
	ITP (n = 46)		Non-ITP (n = 16)			Sensitivity	Specificity	Positive predictive value	Negative predictive value
	Positive	Negative	Positive	Negative					
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.

antigen.[19,20] Antibody units were calculated from the OD_{450} results, based on a standard curve obtained from serial concentrations of pooled plasma with a high titer of IgG anti-GPIIb/IIIa antibodies at dilutions ranging from 1:50 to 1:6400. One unit of anti-GPIIb/IIIa antibody was defined as the amount present in the pooled plasma diluted 1:3200. All samples were examined in duplicate, and the results were calculated as the mean of two values. An abnormal value for platelet-associated antibody was defined as ≥ 3.3 units and an abnormal value for plasma anti-GPIIb-IIIa antibody was defined as ≥ 5.0 units, based on 5 standard deviations above the mean obtained from 40 healthy persons.

Evaluation of platelet turnover

Reticulated platelets were detected by staining platelets with thiazole orange (Retic-COUNT, Becton Dickinson, San Jose, Calif) followed by the flow cytometric analysis described previously.[21,22] The fluorescence histogram was analyzed using a linear gate set to capture 1% of the reticulated platelet counts. This standard gate was used to analyze data from all samples and measure the percentage of thiazole orange-positive platelets in this gate. When a single such marker was used to analyze a series of 40 healthy control samples to determine the variability of the technique, the average percentage of reticulated platelets was 0.85% ± 0.23% (range, 0.2% to 1.5%). An abnormal value for the percentage of reticulated platelets was defined as greater than the mean plus 5 standard deviations (2.0%) in our assay system. Possible day-to-day variability was assessed in 2 healthy control subjects; the inter-assay coefficient of variation was 9.6%, and the intra-assay coefficient of variation was 5.4%. The plasma thrombopoietin level was measured using a commercially available enzyme-linked immunosorbent assay kit (Quantikine; R&D Systems, Minneapolis, Minn) according to the manufacturer's protocol. The mean plasma thrombopoietin level in 30 healthy persons was 83.9 ± 11.7 pg/mL (range, 50 to 126 pg/mL). A thrombopoietin level of ≥ 142 pg/mL was defined as abnormal based on a normal mean plus 5 standard deviations.

Statistical analysis

Confidence intervals were calculated for each sensitivity, specificity, positive predictive value, and negative predictive value. Comparisons between 2 patient groups were tested for statistical significance using the nonparametric Mann-Whitney test or the chi-squared test, as appropriate.

Results

Clinical diagnosis of patients with thrombocytopenia

For the 62 patients, the clinical diagnosis was ITP in 46 patients (74%), myelodysplastic syndrome in 8 (13%), aplastic anemia in 2 (3%), and amegakaryocytic thrombocytopenia in 1 (2%). Five patients (8%) did not have a definitive final diagnosis at the last observation (observation period ranged from 11 to 23 months), because they continued to have reduced megakaryocytes in the bone marrow, alone or in combination with other lineages, without morphologic evidence for dysplasia. These patients were tentatively diagnosed as having reduced platelet production, with or without other cytopenias, and without dysplasia or evidence for platelet destruction. Among the 46 patients with a diagnosis of ITP, 32 received corticosteroids and showed a subsequent partial or complete response. Three patients who were refractory to corticosteroid therapy underwent splenectomy, which successfully increased the platelet count. All 6 patients who were treated with intravenous immunoglobulin experienced an increase in platelet count. Five patients with ITP were treated for *Helicobacter pylori*, and 3 showed a significant increase in platelet count after the successful eradication.

Clinical and laboratory findings in ITP versus non-ITP

The initial findings for evaluation of anti-GPIIb/IIIa antibody responses and platelet turnover were compared according to final clinical diagnosis (Figure). High levels of anti-GPIIb/IIIa antibody-producing B cell frequency, platelet-associated anti-GPIIb/IIIa antibodies, and the percentage of reticulated platelets were detected almost exclusively in patients who later received a final diagnosis of ITP. The plasma thrombopoietin level was normal or slightly increased in patients with ITP, but more than half the patients with myelodysplastic syndrome, aplastic anemia, amegakaryocytic thrombocytopenia, or reduced platelet production, with or without other cytopenias, without dysplasia or evidence for platelet destruction, had an extremely high plasma thrombopoietin level. An increased level of plasma anti-GPIIb/IIIa antibodies was found in only a small number of patients, irrespective of the final diagnosis.

The 46 patients with a final diagnosis of ITP tended to be younger than the 16 patients with other diagnoses (Table 1). Six laboratory findings, including the presence or absence of anemia, the leukocyte count, the frequency of anti-GPIIb/IIIa antibody-producing B cells, the platelet-associated anti-GPIIb/IIIa antibody level, the percentage of reticulated platelets, and the plasma thrombopoietin level, were different at presentation in patients later diagnosed as having ITP than in other patients. The initial platelet count was marginally lower in ITP compared with other diagnoses. The sensitivity, specificity, positive predictive value, and negative predictive value for each of these laboratory findings (Table 2) showed that no single test was sufficient to diagnose ITP in patients with thrombocytopenia. The combination of 3 simple and inexpensive tests (anemia, leukocyte count, and reticulated platelets) also had a low sensitivity and negative predictive value, but the addition of any one of

the substantially more costly tests improved sensitivity and negative predictive value (Table 3). When the presence of 3 or more of the 6 initial ITP-associated laboratory findings was regarded as possible ITP, a positive result was detected in 44 of 46 patients ultimately diagnosed as ITP (96%) but in only 1 (6%) of 16 patients without ITP, indicating a sensitivity of 96%, specificity of 94%, positive predictive value of 98%, and negative predictive value of 88%.

Discussion

This prospective study demonstrates that 6 laboratory tests (erythrocyte count, leukocyte count, anti-GPIIb/IIIa antibody-producing B cells, platelet-associated anti-GPIIb/IIIa antibodies, reticulated platelets, and plasma thrombopoietin level) performed at the first visit are useful to predict a future diagnosis of ITP. These laboratory findings associated with the diagnosis of ITP are categorized into 3 groups that individually reflect different aspects of the disease: a lack of abnormality in myeloid and erythroid lineages, the presence of autoantibody response to platelet-specific GPIIb/IIIa, and preserved platelet production. Three simple and inexpensive tests plus at least 1 of the other more costly ITP-associated laboratory tests measured together at first visit were a relatively sensitive and specific way to discriminate between the future diagnosis of ITP as compared with other conditions. However, it was difficult to determine which combination was most reasonable, probably because the number of patients examined in this study was relatively small. Further multicenter studies involving larger patient cohorts are necessary to make a decision regarding the least expensive and more efficient combination of laboratory tests to detect ITP.

To evaluate the autoimmune nature of the disease, the autoantibody response to GPIIb/IIIa, a major target of antiplatelet autoantibodies, was examined using 2 different methods that have complementary advantages and disadvantages. The enzyme-linked immunospot assay is very sensitive, but this assay system detects B cells that produce antibodies reacting with immobilized GPIIb/IIIa irrespective of their capacity to bind platelet surfaces. This assay would therefore give a false-positive result in patients who have antibodies that react with GPIIb/IIIa but fail to bind platelet surfaces, ie, antibodies that recognize a cytoplasmic domain of GPIIIa.[23] This result may have occurred in 1 of the patients with myelodysplastic syndrome, who showed highly positive results with anti-GPIIb/IIIa antibody-producing B cells and plasma anti-GPIIb/IIIa antibodies but who did not have platelet-associated anti-GPIIb/IIIa antibodies. Conversely, immunologic assays for platelet-associated anti-GPIIb/IIIa antibodies specifically detect antibodies capable of binding platelet surfaces, although the sensitivity is somewhat low.[7,9,10,24] The plasma anti-GPIIb/IIIa antibody level was not useful in the diagnosis of ITP, as reported previously,[20,24] because the majority of pathogenic anti-GPIIb/IIIa antibodies capable of binding platelets are present on platelet surfaces, rather than in the circulation. An increase in sensitivity might be achieved by simultaneously measuring the autoantibody response to other platelet-specific GP, such as GPIb/IX.[8]

Reticulated platelets are young platelets that have been recently released into the circulation and contain elevated levels of nucleic acid components.[25] The proportion of reticulated platelets among circulating platelets reflects platelet turnover.[21,22] Thrombopoietin is a principal regulator of megakaryo-thrombopoiesis, and its circulating level is inversely correlated with the absolute number of bone-marrow megakaryocytes and platelets.[26] These parameters are rapid, noninvasive tests that provide information about activity of the megakaryocytes in the bone marrow and platelet life span. Our prospective study showed that these convenient tests are useful for discriminating between ITP and hypoplastic thrombocytopenia, consistent with previous reports.[12-15]

In summary, initial laboratory findings that predicted future diagnosis of ITP identified in this study could potentially be included in future diagnostic criteria for adult ITP. Because the various combinations of diagnostic tests were retrospectively classified in this study, prospective evaluation of the same diagnostic criteria on another, independent set of patients is necessary.

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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

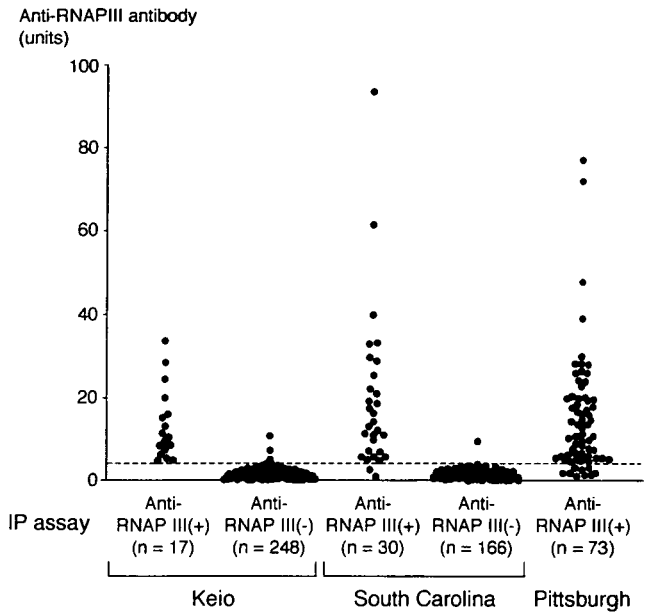

Figure 1. Anti–RNA polymerase III (anti–RNAP III) antibody measured by enzyme-linked immunosorbent assay in a total of 534 sera from systemic sclerosis (SSc) patients at Keio University School of Medicine (Tokyo, Japan), Medical University of South Carolina (Charleston, SC), and University of Pittsburgh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

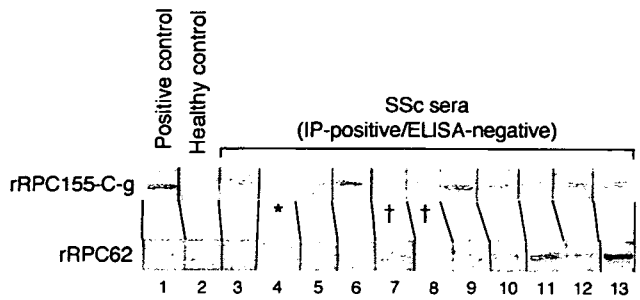

Figure 2. Reactivities to rRPC155C-g and rRPC62 in immunoblots of anti-RNAP III–positive SSc sera that showed a false-negative result in the enzyme-linked immunosorbent assay (ELISA). The bacterial lysates containing recombinant fragments were fractionated on sodium dodecyl sulfate–10% polyacrylamide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

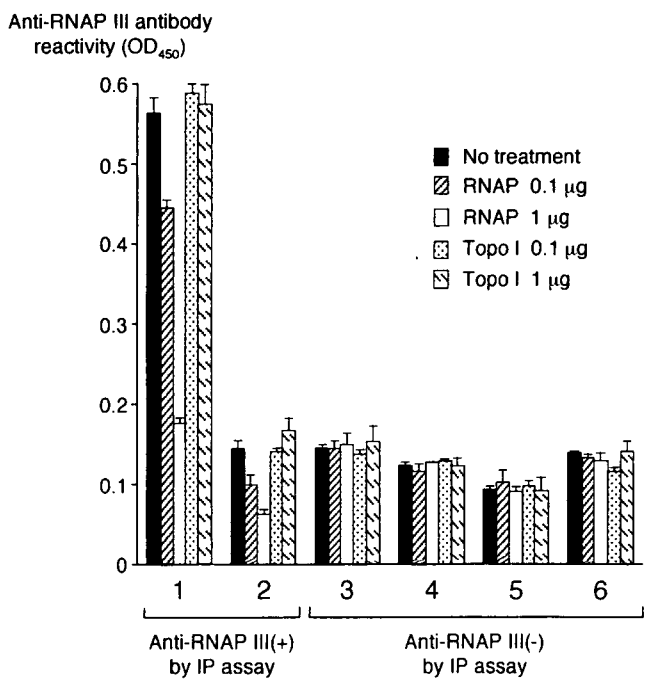

Figure 3. Results of a competitive inhibition assay to examine the specificity of the anti-RNAP III antibody reactivity detected by enzyme-linked immunosorbent assay (ELISA) in 2 SSc sera that were positive for anti-RNAP III antibody by IP assay and 4 SSc sera that were negative for anti-RNAP III antibody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

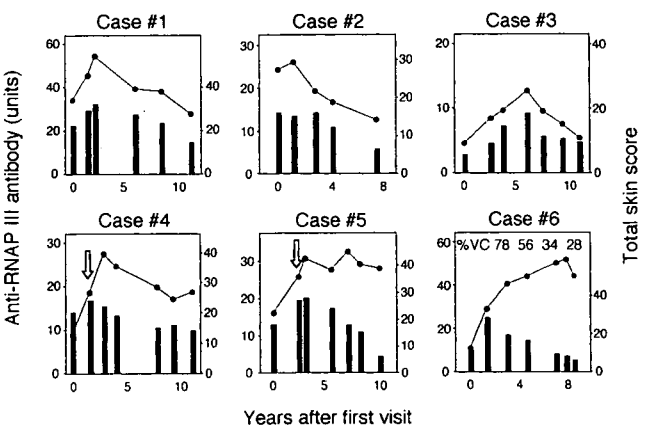

Figure 4. Serial measurements of anti-RNAP III antibody and total skin score in 6 anti-RNAP III antibody-positive SSc patients. Scales for the anti-RNAP III antibody level (circles) and total skin score (bars) are shown at the left and right, respectively. Arrows indicate the onset of renal crisis. Serial 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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Autoantibodies to a 140-kd Polypeptide, CADM-140, in Japanese Patients With Clinically Amyopathic Dermatomyositis

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Objective. **To identify novel autoantibodies specific for dermatomyositis (DM), especially those specific for clinically amyopathic DM (C-ADM).**

Methods. **Autoantibodies were analyzed by immunoprecipitation in 298 serum samples from patients with various connective tissue diseases (CTDs) or idiopathic pulmonary fibrosis (IPF). Antigen specificity of the sera was further examined by immunoblotting and indirect immunofluorescence (IF). The disease specificity and clinical features associated with the antibody of interest were determined.**

Results. **Eight sera recognized a polypeptide of ~140 kd (CADM-140 autoantigen) by immunoprecipitation and immunoblotting. Immunoreactivity was detected in the cytoplasm, and indirect IF revealed a granular or reticular pattern. Anti–CADM-140 antibodies were detected in 8 of 42 patients with DM, but not in patients with other CTDs or IPF. Interestingly, all 8 patients with anti–CADM-140 antibodies had C-ADM. Among 42 patients with DM, those with anti–CADM-140 autoantibodies had significantly more rapidly progressive interstitial lung disease (ILD) when compared with patients without anti–CADM-140 autoantibodies (50% versus 6%; $P = 0.008$).**

Conclusion. **These results indicate that the presence of anti–CADM-140 autoantibodies may be a novel marker for C-ADM. Further attention should be directed to the detection of rapidly progressive ILD in those patients with anti–CADM-140 autoantibodies.**

Polymyositis (PM)/dermatomyositis (DM) is a chronic inflammatory disorder that culminates in injury to the skin and muscle and, sometimes, is associated with interstitial lung disease (ILD) and/or neoplasia (1,2). A number of autoantibodies are associated with myositis, including those specific for aminoacyl–transfer RNA synthetase (anti-ARS) (3), signal recognition particle (anti-SRP) (4), and Mi-2 (5). These autoantibodies have proven to be clinically useful in the diagnosis and classification of these diseases and are predictive of responses to treatment.

It has been known for some time that certain patients may have the typical skin manifestations of DM but no evidence of myositis, a condition known as amyopathic DM. Recently, Sontheimer proposed the existence of a unique subgroup of patients with DM who have the clinical cutaneous features of DM but no evidence of clinical myositis symptoms for at least 2 years after the onset of skin manifestations (referred to as clinically amyopathic DM [C-ADM]) (6). In other words, C-ADM includes patients with amyopathic DM and patients with hypomyopathic DM (patients with subclinical signs of myositis and DM skin manifestations). Some patients with C-ADM, especially those in Japan (7), have been noted to develop rapidly progressive ILD. This condition in many of these patients is resistant to treatment, and fatal outcomes have been observed.

Because of the severity of ILD accompanying C-ADM, a marker autoantibody would be useful for early diagnosis and treatment monitoring. Potential marker autoantibodies have been described by Targoff et al, who, in a preliminary study, described specificity for a 95-kd Sc protein, as well as an unidentified 155-kd protein (8). However, a full survey of the autoantibodies

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associated with C-ADM has not been performed. In the present study, we examined the sera from 15 Japanese patients with C-ADM to identify additional autoantibodies associated with this disease.

PATIENTS AND METHODS

Patients and sera. Serum samples were obtained from 255 randomly selected Japanese adult patients with connective tissue diseases (CTDs) who were being followed up in clinics at Keio University in Tokyo and collaborating medical centers. These sera were obtained, prior to therapy, from a cohort of 61 patients with PM, 42 with DM (including 15 with C-ADM), 50 with rheumatoid arthritis, 46 with systemic lupus erythematosus, 27 with mixed CTD/overlap syndrome, 22 with systemic sclerosis, and 7 with Sjögren's syndrome. Sera from 43 patients with idiopathic pulmonary fibrosis (IPF) and 16 normal human sera were used as control sera. The diagnosis of C-ADM was based on diagnostic criteria proposed by Sontheimer (6), i.e., DM patients with no clinical muscle symptoms for more than 2 years after the onset of skin manifestations.

The patients were diagnosed as having ILD according to the results of chest radiography, chest computed tomography (CT), and pulmonary function testing, which included the percent predicted values for forced vital capacity and diffusing capacity for carbon monoxide. A subset of patients with rapidly progressive ILD was defined as those presenting with progressive dyspnea and progressive hypoxemia, and a worsening of interstitial change on the chest radiograph within 1 month from the onset of respiratory symptoms.

Immunoprecipitation. The immunoprecipitation assay was performed using extracts of the leukemia cell line, K562, as previously described (9). A total of 10 μl of patient serum was mixed with 2 mg of polypeptide A–Sepharose CL-4B (Pharmacia Biotech AB, Uppsala, Sweden) in 500 μl of immunoprecipitation buffer (10 m*M* Tris HCl, pH 8.0, 500 m*M* NaCl, 0.1% Nonidet P40) and incubated for 2 hours at 4°C, and then washed 3 times with immunoprecipitation buffer.

For polypeptide studies, antibody-coated Sepharose beads were mixed with 100 μl of ^{35}S-methionine–labeled K562 cell extracts derived from 2×10^5 cells, and rotated at 4°C for 2 hours. After 6 washes, the Sepharose beads were resuspended in sodium dodecyl sulfate (SDS) sample buffer and the polypeptides were fractionated by 6% SDS–polyacrylamide electrophoresis gels. Radiolabeled polypeptide components were analyzed by autoradiography.

For analysis of RNA, the antigen-bound Sepharose beads were incubated with 100 μl of K562 cell extracts (6×10^6 cell equivalents per sample) for 2 hours at 4°C. To extract bound RNA, 30 μl of 3.0*M* sodium acetate, 30 μl of 10% SDS, 2 μl of carrier yeast transfer RNA (10 mg/ml; Sigma, St. Louis, MO), and 300 μl of phenol:chloroform:isoamyl alcohol (50:50:1, containing 0.1% 8-hydroxyquinoline) were added. After ethanol precipitation, the RNA was resolved using a 7*M* urea–10% polyacrylamide gel, which was subsequently silver-stained (Bio-Rad, Hercules, CA).

Immunoblotting. Immunoblotting analysis was performed using K562 cell extracts in a modification of the procedure described by Towbin et al (10).

Immunodepletion. A 10-μl aliquot of the prototype serum of autoantibodies to the 140-kd polypeptide was mixed with 2 mg of Sepharose beads and incubated for 2 hours at 4°C, followed by 3 washes with immunoprecipitation buffer. Another serum that recognized the 140-kd polypeptide was added in a dose-dependent manner (0 μl, 10 μl, 25 μl, and 50 μl) and then incubated. After 3 washes, immunoprecipitation for polypeptide analysis was performed as described above.

Indirect immunofluorescence (IF). Indirect IF was performed using HEp-2 cells and fluorescein-labeled anti-human immunoglobulin (Inova Diagnostics, San Diego, CA).

Clinical studies. The patients whose sera immunoprecipitated a 140-kd polypeptide were examined for their clinical symptoms, clinical course, muscle enzyme levels (creatine kinase [CK] and aldolase), results on chest radiographic and CT scans, and findings of skin pathology. An assessment of muscle weakness was performed using a manual muscle test (11). Some patients were also examined by electromyogram and muscle magnetic resonance imaging (MRI), and by pathologic analysis of the muscle.

Statistical analysis. The 2 groups of DM patients with or without autoantibodies to the 140-kd polypeptide were compared. The results of comparisons between groups were analyzed using the chi-square test, with Yates' correction where appropriate.

RESULTS

Detection of anti–140-kd polypeptide antibodies in patients with C-ADM. We screened 298 patient sera and 16 normal human sera by immunoprecipitation. Sera from 8 (19%) of 42 patients with DM immunoprecipitated a polypeptide of ~140 kd from ^{35}S-methionine–labeled K562 cell extracts (Figure 1A, lanes 1–8). All 8 patients were diagnosed as having C-ADM, a subtype of DM. In the analysis of RNA specificity, these sera did not immunoprecipitate any nucleic acid band, except for 1 patient's serum, which precipitated hYRNA of SSA/Ro components.

The C-ADM sera that immunoprecipitated the 140-kd polypeptide were also used to immunoblot K562 cell extracts. These sera from C-ADM patients displayed a similar reaction on immunoblot, with a polypeptide band of similar molecular weight (results not shown).

For identification of novel autoantibodies recognizing the 140-kd molecule, the polypeptide immunoprecipitated by the prototype serum was compared with antigens of similar molecular weight recognized by other known autoantibodies (Figure 1B). The protein recognized by the prototype serum migrated slightly faster than the 140-kd protein recognized by anti-MJ antibody (Figure 1B, lane 2) and faster than that recognized by anti–RNA helicase A antibody (Figure 1B, lane 3), but more slowly than the 120-kd protein precipitated by

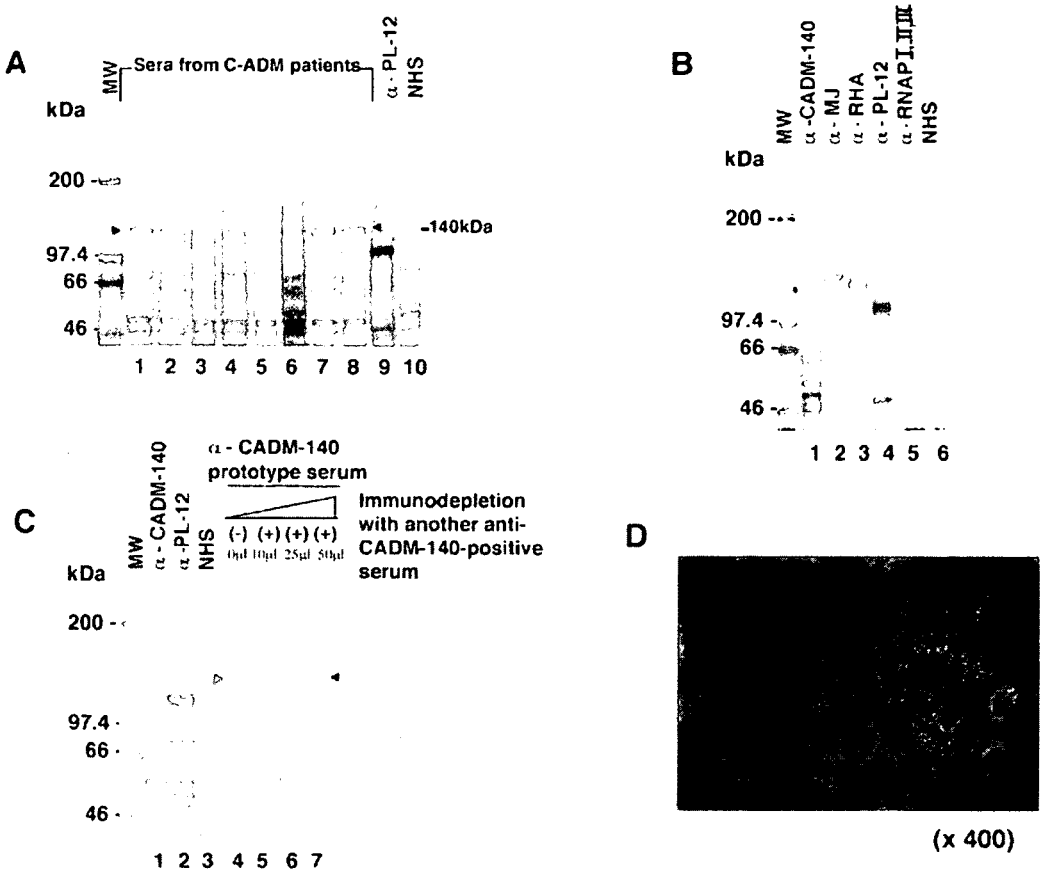

Figure 1. A, Immunoprecipitation of polypeptides with sera from patients with clinically amyopathic dermatomyositis (C-ADM), using ^{35}S-methionine-labeled K562 cell extracts. Lanes 1–8, Sera from C-ADM patients; lane 9, anti-PL-12 serum; lane 10, control normal human serum (NHS). A 140-kd protein was recognized by 8 sera from C-ADM patients (lanes 1–8). **B**, Immunoprecipitation of polypeptides by the prototype serum and by other known autoantibodies. Lane 1, The prototype anti-CADM-140 serum; lane 2, anti-MJ serum; lane 3, anti-RNA helicase A (RHA) serum; lane 4, anti-PL-12 (alanyl-transfer RNA synthetase) serum; lane 5, anti-RNA polymerase I, II, and III (RNAP I, II, and III) serum; lane 6, control NHS. Anti-CADM-140 serum immunoprecipitated an ~140-kd polypeptide that was easily distinguished from that of other known antibodies. **C**, Immunodepletion studies. Sera used for immunoprecipitation were as follows: lane 1, anti-CADM-140; lane 2, anti-PL-12; lane 3, control NHS; lanes 4–7, immunoprecipitation with anti-CADM-140 serum after absorption by another anti-CADM-140–positive serum in a dose-dependent manner. **Arrows** in **A** and **C** denote the 140-kd polypeptide. The sizes of the molecular weight markers are indicated to the left in **A–C**. **D**, Immunofluorescence pattern of HEp-2 cells stained with anti-CADM-140 serum. A granular or reticular cytoplasmic staining pattern on HEp-2 cells was observed. (Original magnification × 400.)

anti–PL-12 antibody (Figure 1B, lane 4). These results clearly indicate that the 140-kd polypeptide immunoprecipitated by the prototype serum was different from the proteins immunoprecipitated by these other known antibodies. We designated this new autoantibody specificity as anti–CADM-140.

The prototype serum depleted extracts of the 140-kd polypeptide in a dose-dependent manner (Figure 1C, lanes 4–7), and the polypeptide recognized by the prototype serum was no longer immunoprecipitated in these extracts (Figure 1C, lane 7). In contrast, the depletion of radiolabeled K562 cell extracts with the use of autoantibodies of different immunologic specificities did not affect the levels of the anti–CADM-140–specific antigen (results not shown). When sera positive for anti–CADM-140 antibodies were assessed in indirect IF studies, a granular or reticular cytoplasmic staining pattern was observed (Figure 1D).