The role of autoantibody-producing plasma cells in immune thrombocytopenic purpura refractory to rituximab

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Rituximab is becoming popular as a treatment for immune thrombocytopenic purpura (ITP). We report here a patient with ITP, who initially responded to rituximab, but later became refractory. In this patient, the appearance of plasma cells producing anti-platelet autoantibodies is likely to be one of the mechanisms for rituximab resistance. Am. J. Hematol. 82:846–848, 2007.

Introduction

Immune thrombocytopenic purpura (ITP) is an acquired hemorrhagic condition of accelerated platelet consumption caused by anti-platelet autoantibodies, which mainly target platelet surface glycoprotein IIb/IIIa (GPIIb/IIIa) [1]. The majority of ITP patients maintain a safe platelet count by taking corticosteroids and/or undergoing a splenectomy, but 9–30% require additional treatment after failing to respond to initial treatment [2]. A number of immunosuppressants and other reagents are currently used for refractory ITP, but no gold standard has been established to date. Rituximab is a chimeric monoclonal antibody directed against CD20, which is a transmembrane protein present on the surface of a broad spectrum of B cells, but not on plasma cells [3]. Rituximab has been used extensively in the treatment of B-cell lymphoma, and several studies and case series in adults and children with refractory ITP have reported that approximately half the patients show a significant platelet response [3–6]. Because of its efficacy and relative safety, rituximab is becoming popular as a treatment for refractory ITP. Patients who achieve a complete response tend to maintain it for more than one year, but the relapse rate is approximately 40% [4,6]. We report here a patient with refractory ITP who initially responded to rituximab, but later became refractory. In this patient, the appearance of plasma cells producing anti-GPIIb/IIIa antibodies is likely to be responsible for rituximab resistance.

Results

As shown in Fig. 1A, platelet-associated anti-GPIIb/IIIa antibodies and anti-GPIIb/IIIa antibody-producing cells were markedly reduced during the first rituximab treatment, but these levels did not change during the third one. Flow cytometric analysis of the patient's PBMC revealed that $CD19^+CD3^-$ and $CD20^+CD138^-$ B cells were completely lost after rituximab treatment in both the first and third courses, although the pretreatment proportion of B cells was very low (<1%) for the third rituximab treatment (Fig. 1B). Interestingly, $CD20^-CD138^+$ plasma cells were detected in the circulation before and after the third rituximab course, when the patient had become refractory. To determine whether the $CD20^-CD138^+$ plasma cells in the peripheral blood produced anti-GPIIb/IIIa antibodies, PBMC depleted of $CD19^+$, $CD20^+$, $CD138^+$, or $CD34^+$ cells were used for the ELISPOT assay. As shown in Fig. 2, in samples taken before the first rituximab treatment, cells producing anti-GPIIb/IIIa antibodies were completely lost following the depletion of $CD19^+$ or $CD20^+$ cells, but not following the depletion of $CD138^+$ cells, indicating that the anti-GPIIb/IIIa antibody-producing cells were exclusively $CD19^+CD20^+CD138^-$ B cells. In contrast, before the third rituximab treatment, when the patient had become refractory to rituximab, the depletion of the $CD19^+$ or $CD20^+$ cells had a minimal effect on the number of antibody-producing cells, but $CD138^+$ cell depletion markedly reduced them, indicating that $CD19^-CD20^-CD138^+$ plasma cells predominantly produced the anti-GPIIb/IIIa antibodies. Bone marrow cells obtained three months after the third rituximab treatment also showed plasma cells to be the dominant population responsible for anti-GPIIb/IIIa antibody production. When PBMC from 10 randomly selected ITP patients who never received rituximab were examined using the ELISPOT assay, none of them had $CD138^+$ plasma cells producing anti-GPIIb/IIIa antibodies. HACA were positive and at an intermediate titer (174 ng/mL) at the time of the third rituximab treatment, but were not detectable prior to rituximab use.

Discussion

Our patient with refractory ITP initially responded to rituximab, but after retreatment she became refractory to it. Rituximab resistance is probably not simply due to HACA induced by the repeated use of this chimeric antibody, because the third rituximab treatment still induced complete B-cell depletion, and bone marrow cells responsible for anti-GPIIb/IIIa antibody production obtained three months after the third rituximab treatment showed a plasma cells phenotype. Data on the clinical significance of HACA during rituximab therapy are limited, but HACA have been discussed primarily in association with adverse events, including infusion reactions and serum sickness [7]. Rapid clearance of the drug may be of concern, but successful rituximab retreatment of lymphoma has been reported in a patient with HACA [8]. An alternative explanation, however, was suggested by our patient's having plasma cells producing anti-GPIIb/IIIa antibodies in the circulation as well as in

Contract grant sponsor: The Japanese Ministry of Health, Welfare and Labour.

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Received for publication 17 January 2007; Accepted 26 February 2007

Am. J. Hematol. 82:846–848, 2007.

Published online 15 May 2007 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/ajh.20951

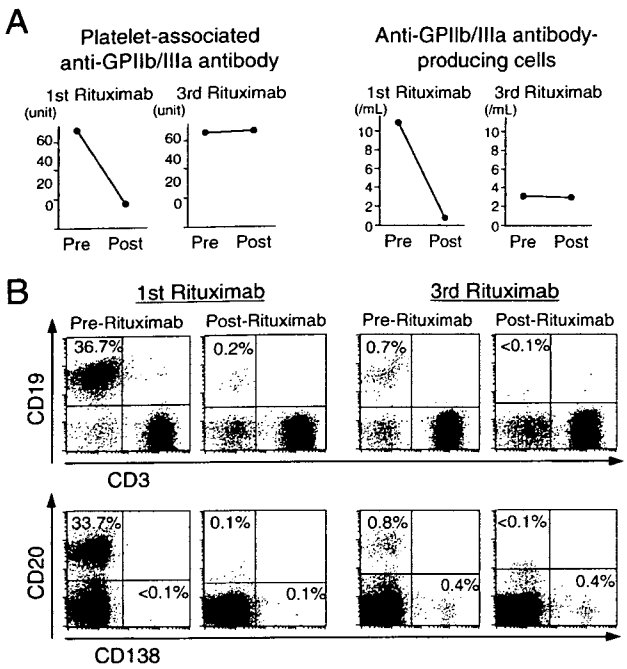

Figure 1. Serial measurements of anti-GPIIb/IIIa antibody responses and circulating B cell subsets before and after the first and third courses of rituximab. A: Platelet-associated IgG anti-GPIIb/IIIa antibody was measured by enzyme-linked immunosorbent assay using purified human GPIIb/IIIa as the antigen, and the antibody units were calculated based on a standard curve obtained from serial concentrations of a standard plasma. IgG anti-GPIIb/IIIa antibody-producing cells were detected using enzyme-linked immunospot assay and are shown in the absolute number per 1 mL of peripheral blood. B: Serial flow cytometric analysis of $CD19^+CD3^-$ B cells, $CD20^+CD138^-$ B cells, and $CD20^-CD138^+$ plasma cells. Relative proportions of the subsets of interest are shown in each panel.

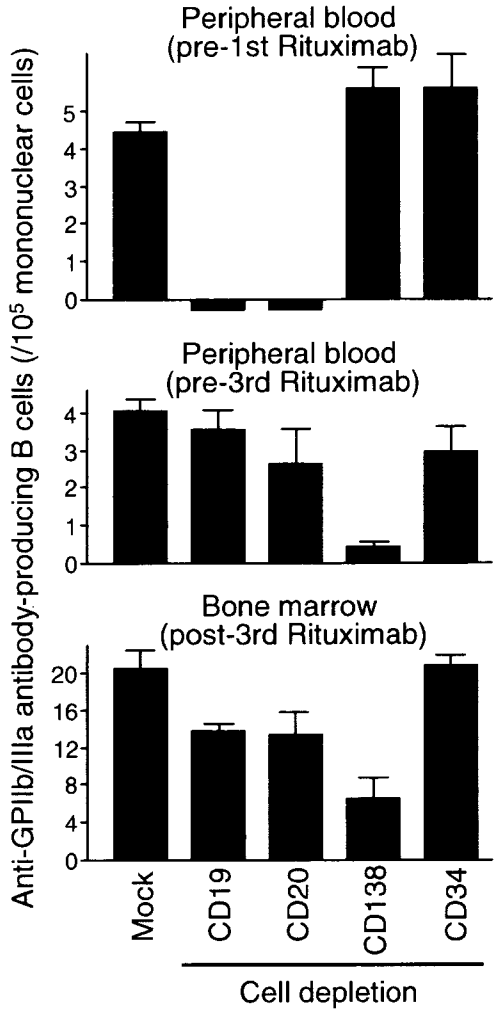

Figure 2. IgG anti-GPIIb/IIIa antibody-producing cells in mononuclear cells that were mock-treated or were depleted of $CD19^+$, $CD20^+$, $CD138^+$, or $CD34^+$ cells. IgG anti-GPIIb/IIIa antibody-producing cells in peripheral blood or bone marrow mononuclear cells were detected using enzyme-linked immunospot assay. The cell depletion was performed using magnetic bead-coupled corresponding monoclonal antibodies, followed by separation on a MACS column. The frequency of circulating anti-GPIIb/IIIa antibody-producing B cells was calculated as the number per 10^5 mononuclear cells. Results shown are the mean and standard deviation obtained from three independent wells.

the bone marrow while she was refractory to rituximab. Since rituximab, which was designed to target B lineage cells, does not eliminate plasma cells, the appearance of plasma cells producing anti-platelet autoantibodies could be one of the mechanisms of rituximab resistance. In this regard, there is increasing evidence that plasma cells can survive as long as memory B cells, and such long-lived plasma cells are thought to be involved in the pathogenesis of various autoimmune diseases through their continuous production of pathogenic autoantibodies [9]. Because anti-GPIIb/IIIa antibody-producing plasma cells were undetectable in our patient before rituximab treatment and in 10 additional ITP patients who never received rituximab, it is possible that repeated doses of rituximab might induce a compensatory shift in the dominance of autoantibody-producing cells from B cells to plasma cells, following the rituximab-induced loss of the B cells. The mechanism involved in this process remains unclear, but it might be mediated through longevity of autoantibody-producing plasma cells, because rituximab blocks differentiation of memory B cells into plasma cells. Currently, there is little information regarding the efficacy of rituximab retreatment in ITP patients who relapse after one cycle of treatment, but we should take this potential mechanism for rituximab resistance into account in developing future therapeutic strategies for refractory ITP.

Materials and Methods

Case report

A 75-year-old woman presented with multiple ecchymoses and nasal bleeding in June 2003. Physical examination revealed petechiae on her palate and scattered diffusely on her arms, legs, and trunk. Her complete blood count showed hemoglobin 8.5 g/dL, leukocyte 4.0×10^9 L^{-1} with normal differential, and platelet 12×10^9 L^{-1}. Coagulation and blood chemistry studies were normal. Antinuclear antibody was negative, but IgG anti-cardiolipin antibody was strongly positive (74 GPL). A bone marrow evaluation showed normocellular marrow without dysplasia or megakaryocytic hypoplasia. The patient was initially treated with prednisolone (1 mg/kg), but there was no clear platelet response, and she required frequent platelet transfusions, because of severe persistent thrombocytopenia (platelets $< 5 \times 10^9$ L^{-1}) with oral mucosal bleeding. The patient was then given intravenous immunoglobulin (IVIG) 0.4 g/kg for 5 days, which was followed by an increased platelet count (96×10^9 L^{-1}). Splenectomy was performed, but her platelet count fell

quickly to $10 \times 10^9\ L^{-1}$. She was treated, successively, with *Helicobacter pylori* eradication, danazol, azathoprine, and cyclosporine, but her platelets remained $<10 \times 10^9\ L^{-1}$, and she required weekly platelet transfusions. In August 2003, we tried rituximab at a dose of 375 mg/m^2 weekly for 4 doses. The patient's platelet count rose to $>100 \times 10^9\ L^{-1}$ within 5 weeks and remained at this level for 9 weeks thereafter. However, she relapsed 13 weeks after the rituximab treatment, and was again transfusion-dependent, despite treatment with high-dose dexamethasone (40 mg for 4 days), prednisolone (1 mg/kg), vincristine, danazol, and azathioprine. She received a second rituximab therapy in December 2003, which resulted in a partial platelet response (platelets $30–50 \times 10^9\ L^{-1}$) that eliminated the need for platelet transfusion. A monthly pulse cyclophosphamide (300–500 mg) was added, and the patient's platelet count remained $>50 \times 10^9\ L^{-1}$ for the next 10 months. In November 2004, the patient developed gastrointestinal bleeding and her platelet count was $<10 \times 10^9\ L^{-1}$, requiring IVIG treatment and platelet transfusion. A third rituximab treatment was given in March 2005, but there was no platelet response. Since then, she has received biweekly IVIG (a single dose of 0.4 g/kg) to maintain a safe platelet count.

Immunologic analyses

Platelet-associated IgG anti-GPIIb/IIIa antibodies and circulating IgG anti-GPIIb/IIIa antibody-producing cells were measured using, respectively, an enzyme-linked immunosorbent assay (ELISA) [10] and an enzyme-linked immunospot (ELISPOT) assay [11]. Human antichimeric antibodies (HACA) were measured using ELISA [12]. Flow cytometric analysis was performed using the following mouse monoclonal antibodies, which were conjugated to fluorescein isothiocyanate, phycoerythrin, or allophycocyanin: anti-CD3, anti-CD138 (Beckman Coulter, Fullerton, CA), anti-CD19 (Sigma, St. Louis, MO), anti-CD20 (BD Biosciences, San Diego, CA), and anti-CD34 (Miltenyi Biotech, Bergisch Gladbach, Germany). Negative controls were cells incubated with an isotype-matched mouse monoclonal antibody to an irrelevant antigen. The cells were analyzed on an FACS® Calibur flow cytometer (BD Biosciences) using CellQuest software. In some experiments, peripheral blood mononuclear cells (PBMC) depleted of $CD19^+$, $CD20^+$, $CD138^+$, or $CD34^+$ cells were used for the ELISPOT assay. The cell depletion was performed using magnetic bead-coupled anti-CD19, anti-CD20, or anti-CD34 monoclonal antibodies (Miltenyi Biotech) or anti-CD138 monoclonal antibody (Beckman Coulter) in combination with magnetic bead-coupled anti-mouse IgG antibodies (Miltenyi Biotech), followed by separation on a MACS column. Flow cytometric analysis revealed that cells positive for corresponding markers were nearly absent in the treated fractions ($<0.5\%$ for $CD19^+$ and $CD20^+$ cells, and $<0.01\%$ for $CD138^+$ and $CD34^+$ cells).

Acknowledgment

We thank Yuka Okazaki for excellent technical assistance.

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Clinical Evaluation of Anti-Aminoacyl tRNA Synthetase Antibodies in Japanese Patients with Dermatomyositis

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ABSTRACT. ***Objective.*** To investigate the distribution of anti-aminoacyl-tRNA synthetase (anti-ARS) antibodies among patients with autoimmune diseases, and to analyze the clinical features of patients with dermatomyositis (DM) with anti-ARS antibodies.

Methods. Serum samples from 315 patients with autoimmune diseases or related disorders who had visited Kanazawa University Hospital or affiliated facilities were assessed for anti-ARS antibodies by immunoprecipitation. In particular, the association between anti-ARS antibodies and clinical features was investigated in detail in patients with DM.

Results. Anti-ARS antibody was positive in 16 (29%) of 55 patients with DM, 2 (22%) of 9 patients with polymyositis, and 7 (25%) of 28 patients with idiopathic pulmonary fibrosis. Although anti-ARS antibody was detected with high frequency (63%, 15/24) in DM patients with interstitital lung disease (ILD), the incidence of anti-ARS antibody was very low (3%, 1/31) in DM patients without ILD. Anti-ARS antibody-positive patients with DM had significantly higher incidences of ILD (94% vs 23%) and fever (64% vs 10%) than the antibody-negative patients. Some immunosuppressive agents, in addition to oral corticosteroids, were required more frequently in the antibody-positive patients with DM than the antibody-negative patients (88% vs 26%). Although 60% of DM patients with ILD simultaneously developed ILD and myositis, ILD preceded myositis in 33% of patients.

Conclusion. Among patients with DM, anti-ARS antibodies are found in a subset with ILD. DM patients with anti-ARS antibodies appear to have a more persistent disease course that requires additional therapy compared to those without anti-ARS antibodies. (First Release Feb 15 2007; J Rheumatol 2007;34:1012–8)

Key Indexing Terms:
ANTI-AMINOACYL tRNA SYNTHETASE ANTIBODY
DERMATOMYOSITIS
ANTISYNTHETASE SYNDROME
INTERSTITIAL LUNG DISEASE

Polymyositis (PM) and dermatomyositis (DM) are idiopathic inflammatory diseases affecting skeletal muscles. A subset of patients with PM has interstitial lung disease (ILD), and there are some subsets of patients with DM who have ILD or internal malignancy[1]. Early studies showed that anti-Jo-1 antibody is positive in 20%–30% of patients with PM and is associated with the involvement of ILD[2]. Six autoantibodies to aminoacyl-tRNAsynthetase (ARS) have been detected to date, including anti-Jo-1 antibody[3]. ARS is an enzyme catalyzing the binding of the corresponding amino acid to the 3'-terminal of transfer RNA to yield aminoacyl-tRNA. Anti-ARS antibodies include anti-Jo-1 (histidyl-tRNA synthetase),

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Accepted for publication January 8, 2007.

anti-PL-7 (threonyl-tRNA synthetase), anti-PL-12 (alanyl-tRNA synthetase), anti-OJ (isoleucyl-tRNA synthetase), anti-EJ (glycyl-tRNA synthetase), and anti-KS (asparaginyl-tRNA synthetase). Recent studies showed that anti-ARS antibodies are detected in patients with DM as well as those with PM[4,5]. Anti-ARS antibody-positive patients present common symptoms such as myositis, ILD, polyarthritis, Raynaud's phenomenon, fever, and mechanic's hand. Further, anti-ARS antibody-positive individuals showing these abnormalities are defined as having antisynthetase syndrome[6-9]. No valid method based on enzyme linked immunosorbent assay (ELISA) has been established, except anti-Jo-1 antibody, to detect anti-ARS antibody. Immunoprecipitation is therefore indispensable and an excellent method in terms of both sensitivity and specificity to detect these antibodies, although the number of facilities capable of carrying out this assay is limited.

ARS can be divided into class I and class II by its molecular structure and the aminoacylation-initiating codon. Other than isoleucyl-tRNA synthetase, all targets of anti-ARS antibodies identified to date correspond to class II. It is estimated that class II ARS is present in a free form in the cytoplasm, and that it is likely to be exposed on the surface to serve as an autoantigen[10]. It has recently been reported that histidyl-tRNA synthetase and asparaginyl-tRNA synthetase have a chemokine-like function, and may induce the migration of T lymphocytes and monocytes through their interaction with chemokine receptors (CCR3 and CCR5) expressed on dendritic cells[11]. Therefore, the presence of ARS in inflamed tissue may stimulate the uptake of ARS by the invading dendritic cells, and the resultant presentation of ARS as an autoantigen may induce the production of autoantibody against ARS[11]. Nonetheless, it remains unknown whether anti-ARS antibodies induce the development of antisynthetase syndrome.

We investigated the clinical features and the prevalence of anti-ARS antibodies in Japanese patients with DM.

MATERIALS AND METHODS

Patients. Serum samples were obtained from 315 Japanese patients with autoimmune diseases or related disorders who had visited Kanazawa University Hospital or collaborating medical centers. These included 55 patients with DM, 9 with PM, 28 with idiopathic pulmonary fibrosis (IPF), and 223 with other diseases [126 with systemic sclerosis (SSc); 27 with systemic lupus erythematosus (SLE); 10 with mixed connective tissue disease (MCTD); 10 with Sjögren's syndrome; 9 with rheumatoid arthritis (RA); 9 with localized scleroderma; 6 with overlap syndrome; 6 with adult-onset Still's disease; 6 with primary antiphospholipid syndrome; 4 with unclassified connective tissue diseases; 4 with cryoglobulinemia; 3 with Behçet's disease; and 3 with eosinophilic fasciitis]. Patients with DM visit our department (specializing in dermatology) more frequently than patients with PM. Therefore, the percentage of patients who visited the dermatology associations was 71% (39/55) in patients with DM, 22% (2/9) in patients with PM, 0% (0/28) in patients with IPF, and 77% (203/223) in patients with other diseases.

All patients with DM and PM fulfilled the Bohan and Peter criteria[12,13]. We distinguished DM from PM by the presence of heliotrope rash or Gottron's lesions (Gottron's papules and/or Gottron's sign). The diagnosis of IPF was based on the presence of unexpected dyspnea, progressive bilateral pulmonary infiltrates, and evidence of pulmonary fibrosis in histological specimens obtained by lung biopsy[14]. Patients with IPF were examined for muscle weakness using a manual muscle test and muscle enzyme levels (creatine phosphokinase and aldolase) during the followup period (44 ± 12 mo), since the onset of ILD might precede the onset of myositis. Some patients were also examined by electromyogram and muscle magnetic resonance imaging, and by pathologic analysis of the muscle.

The criteria proposed by the American College of Rheumatology[15-18] were used to diagnose patients with SSc, SLE, RA, or Sjögren's syndrome. MCTD was diagnosed according to preliminary diagnostic criteria proposed by Kasukawa and Miyawaki[19]. Localized scleroderma was diagnosed when a patient had typical symptoms characterized by fibrosis of skin and subcutaneous tissue[20]. Overlap syndrome was diagnosed by the coexistence of 2 or more connective tissue diseases, such as SLE, DM, PM, SSc. Adult-onset Still's disease was diagnosed according to the criteria proposed by Medsger and Christy[21]. Primary antiphospholipid syndrome was diagnosed according to the Sapporo criteria[22]. Unclassified connective tissue disease was diagnosed by the presence of various connective tissue disease symptoms without meeting the full criteria for any one of them[23]. Cryoglobulinemia was diagnosed by the presence of single or mixed immunoglobulins that undergo reversible precipitation at low temperatures[24]. Behçet's disease was diagnosed according to the criteria of the International Study Group for Behçet's Disease[25]. Eosinophilic fasciitis is diagnosed by symmetrical scleroderma-like induration of the skin over one or more distal extremities, accompanying thickened fascia and eosinophil infiltrations[26].

Immunoprecipitation. The presence of anti-ARS antibody, anti-Mi-2 antibody, anti-signal recognition particle antibody, anti-PM/Scl, anti-Ro, or anti-La was confirmed by the immunoprecipitation assay. The immunoprecipitation assay was performed using extracts of the leukemia cell line K562 as described[10]. A total of 10 μl of patient serum was mixed with 2 mg of protein A-sepharose beads (Amersham Biosciences, Piscataway, NJ, USA) in 500 μl of immunoprecipitation buffer (10 mM Tris HCl, pH 8.0, 500 mM NaCl, 0.1% Nonidet P40) and incubated for 2 h at 4°C, and then washed 5 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 h. After 5 washes, the sepharose beads were resuspended in sodium dodecyl sulfate (SDS) sample buffer and the polypeptides were fractionated by 8.5% SDS-polyacrylamide gels (PAGE). Radiolabeled polypeptide components were analyzed using autoradiography.

For the analysis of RNA, the antigen-bound sepharose beads were incubated with 300 μl of K562 cell extracts (3×10^6 cell equivalents per sample) for 2 h at 4°C. The bound RNA from the sepharose beads was extracted using Isogen (Nippon Gene, Toyama, Japan) in accordance with manufacturer's protocols. After ethanol precipitation, the RNA was resolved using a 7 M urea-10% PAGE, which was subsequently silver stained (Bio-Rad, Hercules, CA, USA).

Indirect immunofluorescence. Indirect immunofluorescence was performed using HEp-2 cells and fluorescein-labeled antihuman immunoglobulin (Medical & Biological Laboratories, Nagoya, Japan) in patients with DM, as described[27].

Clinical studies. Complete medical histories, examinations, and laboratory tests could be obtained from 35 patients among 55 patients with DM analyzed for anti-ARS antibody. Therefore, the associations between anti-ARS antibody and clinical features were assessed in these 35 patients with DM. The patients were diagnosed as having ILD according to chest radiography, chest computed tomography, and pulmonary function testing, which included the percentage-predicted values for forced vital capacity (FVC) and diffusing capacity for carbon monoxide (DLCO). Serum KL-6 levels were determined by ELISA, as described[28]. Internal malignancy was carefully examined using chest and abdominal computed tomography, gastrointestinal fiberscope, gallium scintigraphy, and other procedures according to need. The protocol was approved by the Kanazawa University Graduate School of Medical Science

and Kanazawa University Hospital, and informed consent was obtained from all patients.

Statistical analysis. Statistical analysis was performed using the Mann-Whitney U-test for the comparison of values, and Fisher's exact probability test for the comparison of frequencies. p values less than 0.05 were considered to be statistically significant.

RESULTS

Prevalence of anti-ARS antibody in patients with autoimmune diseases. We investigated the existence of anti-ARS antibodies by immunoprecipitation. Representative immunoprecipitation for RNA or protein with anti-ARS antibody-positive sera is shown in Figure 1. Of the 315 patients with autoimmune diseases or related disorders, 25 patients (8%) were anti-ARS antibody-positive. Among these 25 patients, 18 satisfied the diagnostic criteria for PM/DM, and 7 were diagnosed with IPF without myositis. The anti-ARS antibody-positive rate was 29% (16/55) in patients with DM, 22% (2/9) in patients with PM, and 25% (7/28) in patients with IPF (Table 1). No significant association was detected between each anti-ARS antibody and the disease subset. It was striking that no patients with the other autoimmune diseases were positive for anti-ARS antibody (Table 1).

Association between ILD and anti-ARS antibody. The frequency of patients having ILD was 44% (24/55) in patients with DM, 33% (3/9) in patients with PM. ILD was detected in 54 (52 SSc and 2 MCTD) of the 223 (24%) patients with other diseases (Table 2A). Almost all DM (94%, 15/16) or PM (100%, 2/2) patients with anti-ARS antibody had ILD (Table 2A). Anti-ARS antibody was detected with high frequency in DM (63%, 15/24) or PM patients (67%, 2/3) with ILD (Table 2B). In contrast, the incidence of anti-ARS antibody was very low in DM (3%, 1/31) or PM (0%, 0/6) patients without ILD (Table 2B). These findings indicate that anti-ARS antibody is not myositis-specific, but rather an ILD-specific marker in patients with DM or PM.

Clinical correlation of anti-ARS antibodies in Japanese

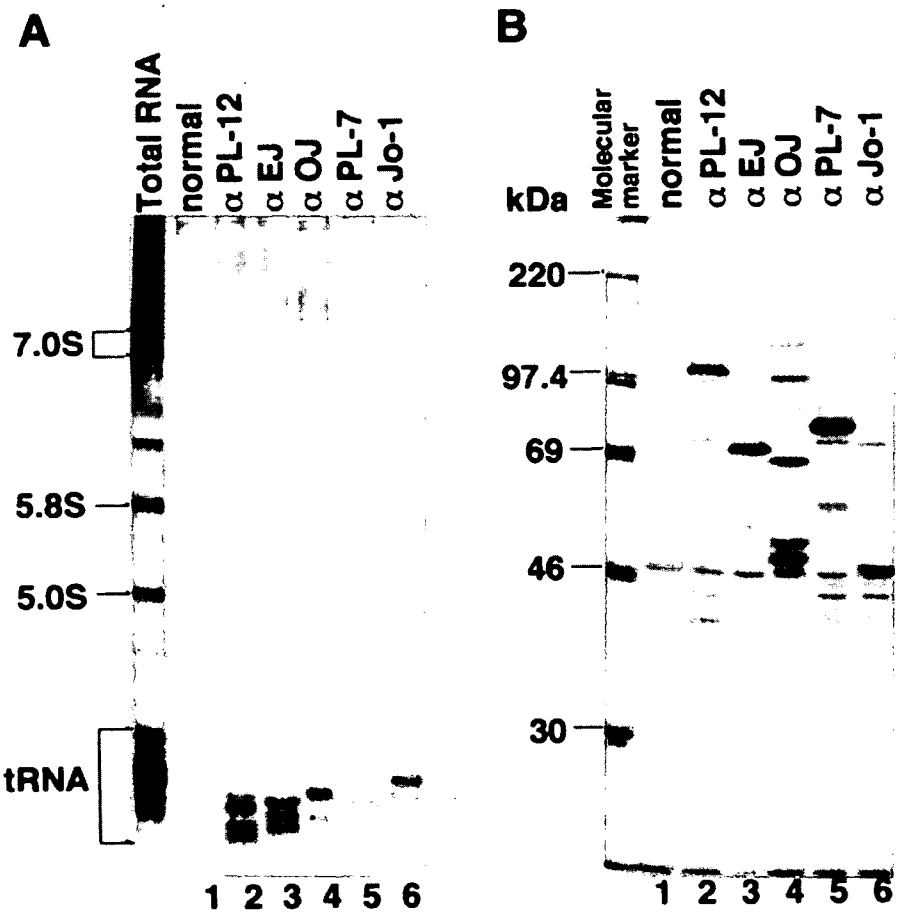

Figure 1. A. Representative immunoprecipitation for RNA with anti-aminoacyl tRNA synthetase (anti-ARS) sera and the normal control. 7 M urea-10% polyacrylamide gel (PAGE) of phenol-extracted immunoprecipitates from K562 cell extract, developed with silver stain. Total RNA lane indicates the 7.0S, 5.8S, and 5.0S small ribosomal RNA and the tRNA region. Sera used for immunoprecipitation: Lane 1: normal control serum indicated; Lanes 2–5: anti-ARS sera indicated, with antibodies to PL-12 (alanyl-tRNA synthetase), EJ (glycyl-tRNA synthetase), OJ (isoleucyl-tRNA synthetase), PL-7 (threonyl-tRNA synthetase), Jo-1 (histidyl-tRNA synthetase). B. Representative immunoprecipitation for proteins with anti-ARS sera and normal control. Autoradiogram of 8.5% SDS-PAGE of immunoprecipitates from ^{35}S-methionine-labeled K562 cell extract.

Table 1. Prevalence of anti-ARS antibodies in autoimmune diseases.

	Clinical Composition, no. (%)			
	DM, n = 55	PM, n = 9	IPF, n = 28	Others, n = 223
Anti-ARS (total)	16 (29)	2 (22)	7 (25)	0 (0)
Anti-Jo-1	3 (5)	1 (11)	1 (4)	0 (0)
Anti-EJ	6 (11)	0 (0)	2 (7)	0 (0)
Anti-PL-7	4 (7)	1 (11)	0 (0)	0 (0)
Anti-PL-12	3 (5)	0 (0)	3 (11)	0 (0)
Anti-OJ	0 (0)	0 (0)	1 (4)	0 (0)
Anti-Mi-2	3 (5)	0 (0)	0 (0)	0 (0)
Anti-SRP	1 (2)	1 (11)	0 (0)	0 (0)
Anti-PM/Scl	0 (0)	0 (0)	0 (0)	0 (0)
Anti-Ro	9 (16)	2 (22)	2 (7)	56 (26)
Anti-La	0 (0)	0 (0)	0 (0)	8 (4)

DM: dermatomyositis; PM: polymyositis; IPF: idiopathic pulmonary fibrosis; SRP: signal recognition particle.

patients with DM. Table 3 shows the association between anti-ARS antibody and clinical features in 35 patients with DM who were examined in detail. Of these 35 patients, 16 were anti-ARS antibody-positive. There was no significant difference in age or sex between the anti-ARS antibody-positive group and the antibody-negative group. However, the disease duration (interval from initial symptom to diagnosis) was significantly longer in patients in the antibody-positive group compared to the antibody-negative group [1.4 ± 1.5 vs 0.4 ± 0.4 yrs (mean ± SD); $p < 0.01$]. The patients with anti-ARS antibody frequently had fever and elevated CRP levels, indicating that the inflammation was remarkable in the antibody-positive patients. The percentage of patients with ILD was dramatically elevated in the antibody-positive group compared to the antibody-negative group (94% vs 23%; $p < 0.005$). In addition, respiratory function, such as %FVC and %DLCO, declined significantly ($p < 0.005$) and the levels of serum KL-6, a serum marker for ILD, were elevated significantly ($p < 0.05$) in the antibody-positive group compared to the antibody-negative group. The frequency of Raynaud's phenomenon and arthritis was higher in the antibody-positive group than in the antibody-negative group, although the difference was not significant. The complication of internal malignancy was not seen in any DM patient with anti-ARS antibodies, while internal malignancy was detected in 16% of DM patients without the antibody. The incidence of DM-specific eruptions, such as heliotrope rash, Gottron's lesions, and flagellate erythema, was similar between the 2 groups. While mechanic's hands were detected in 19% of patients with, but not in patients without the antibody, the difference between the 2 groups was not significant. The antinuclear antibody-positive rate was significantly lower in the anti-ARS antibody-positive group. In contrast, the frequency of anti-cytoplasmic antibody was significantly higher in the anti-ARS antibody-positive group than in the anti-ARS antibody-negative group (75% vs 5%; $p < 0.005$), since ARS is localized at cell cytoplasm. The anti-ARS antibody-positive patients with DM did not have other myositis-specific antibodies, such as anti-Mi-2 antibody and anti-signal recognition particle antibody.

Treatment with other immunosuppressive agents in addition to oral corticosteroid was significantly more frequently needed for anti-ARS antibody-positive patients with DM for the treatment of ILD or myositis compared to the anti-ARS antibody-negative patients (88% vs 26%; $p < 0.005$). Among the immunosuppressive agents, mainly cyclosporine or cyclophosphamide was used as an additional treatment (Table 4).

PM patients with anti-ARS antibody showed clinical features and received therapy similar to patients with DM, although the number of patients was too small to analyze (data not shown). The complication of internal malignancy was not found in any patient with PM, irrespective of anti-ARS antibody status (data not shown). Only one IPF patient with the antibody had developed lung cancer that preceded IPF by 2 years (data not shown).

Onset time of ILD and myositis in DM patients with anti-ARS antibodies. When the onset time of ILD or myositis was analyzed in the 15 anti-ARS antibody-positive cases of DM

Table 2A. Prevalence of ILD in patients with anti-ARS antibodies.

	Clinical Composition, no. (%)				
	DM	PM	IPF	Others	Total
Anti-ARS-positive	15/16 (94)	2/2 (100)	7/7 (100)	0/0 (0)	24/25 (96)
Anti-ARS-negative	9/39 (23)	1/7 (14)	21/21 (100)	54/223 (24)	85/290 (29)
All	24/55 (44)	3/9 (33)	28/28 (100)	54/223 (24)	109/315 (35)

Table 2B. Prevalence of anti-ARS antibodies in patients with ILD.

	Clinical Composition, no. (%)				
	DM	PM	IPF	Others	Total
ILD-positive	15/24 (63)	2/3 (67)	7/28 (25)	0/54 (0)	24/109 (22)
ILD-negative	1/31 (3)	0/6 (0)	0/0 (0)	0/169 (0)	1/206 (0.5)

ILD: interstitial lung disease; DM: dermatomyositis; PM: polymyositis; IPF: idiopathic pulmonary fibrosis.

Table 3. Clinical and laboratory data of DM patients with anti-ARS antibodies.

	Dermatomyositis	
	Anti-ARS-positive, n = 16	Anti-ARS-negative, n = 19
Age at onset, mean ± SD, yrs	57 ± 13	50 ± 20
Sex (female/male)	13/3	13/6
Disease duration, mean ± SD, yrs	1.4 ± 1.5**	0.4 ± 0.4
Clinical features		
Fever	64***	10
Raynaud's phenomenon	31	5
Arthritis	43	21
Interstitial lung disease	94***	23
Internal malignancy	0	16
Skin eruptions		
Heliotrope rash	38	47
Gottron's lesions	69	53
Flagellate erythema	19	16
Periungual erythema	38	47
Nailfold capillary changes	31	42
Mechanic's hands	19	0
Laboratory findings		
Positive antinuclear antibody	6***	58
Positive anti-cytoplasmic antibody	75***	5
Positive anti-Mi-2 antibody	0	16
Positive anti-SRP antibody	0	5
Positive anti-Ro antibody	25	16
CPK, IU/L, mean ± SD	1476 ± 2963	2801 ± 6301
CRP, mg/dl, mean ± SD	2.7 ± 2.4***	0.7 ± 1.0
IgG, mg/ml, mean ± SD	1716 ± 602	1561 ± 542
FVC, %, mean ± SD	73 ± 14***	98 ± 25
DLCO, %, mean ± SD	47 ± 13***	72 ± 23
KL-6 U/ml, mean ± SD	2088 ± 2304*	497 ± 491
Usage of other immunosuppressive agents, in addition to oral steroid	88***	26

Values are percentages unless indicated. SRP: signal recognition particle; CPK: creatine phosphokinase; CRP: C-reactive protein. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ vs DM patients without anti-ARS antibodies.

patients with ILD (Table 5), the onset of ILD preceded the onset of myositis in 5 (33%) patients, myositis preceded ILD in one patient (7%), and myositis and ILD developed simultaneously in 9 patients (60%). These results suggest that patients diagnosed as having IPF need to be checked for anti-ARS antibody and that if antibody is positive, close attention is needed for the development of myositis.

DISCUSSION

In our study, the frequency of anti-ARS antibodies was similar (22%–29%) among patients with DM, PM, and IPF (Table 1). Anti-ARS antibody was negative in all patients with other autoimmune diseases, including SSc, SLE, and MCTD. These findings indicate that anti-ARS antibody is a highly disease-specific autoantibody for a proportion of patients with DM, PM, or IPF. In addition, virtually all DM or PM patients with anti-ARS antibody had ILD (Table 2A). Although anti-ARS antibody was detected with high frequency in DM or PM patients with ILD, the incidence of anti-ARS antibody was very low in DM or PM patients without ILD (Table 2B). Therefore, it is likely that anti-ARS antibody is a marker of ILD but not for myositis in patients with DM or PM.

In our Japanese patients with DM, symptoms of antisynthetase syndrome, such as ILD, arthritis, Raynaud's phenomenon, fever, and mechanic's hand, were frequently detected in anti-ARS antibody-positive patients (Table 3). In general, ILD with anti-ARS antibody-positive patients can be characterized by the chronic course of the disease and elevation of the diaphragm (so-called "shrinking lung")[29]. On the other hand, it has recently been shown that cases of therapy-resistant rapidly progressive ILD, often accompanied by amyopathic DM, are negative for anti-ARS antibody and positive for anti-CADM-140 antibody[30]. In our study, there was no DM patient with anti-CADM-140 antibody. While the characteristics of DM-specific eruptions, such as heliotrope coloration, Gottron's lesions, and flagellate erythema[31], were similar between DM patients with anti-ARS antibody and those without antibody, mechanic's hands were found only in the antibody-positive DM group. Therefore, mechanic's hands may be closely associated with the existence of anti-ARS antibody. Our study showed that the disease duration was significantly longer in patients with the anti-ARS antibody-positive group compared to the antibody-negative group (Table 3) and there was no significant seasonal pattern of disease onset in the anti-ARS antibody-positive group (data not shown). However, prior studies demonstrated a rather more acute onset with a seasonal pattern in DM patients with the anti-ARS antibody[32,33]. The reasons for this discrepancy may be due to the small population or ethnic difference in our study. However, ILD preceded myositis for a long period (20 ± 20 mo) in 33% of the DM patients with ILD (Table 5). By contrast, only one of 15 (7%) DM patients with ILD showed myositis 2 months earlier than ILD. Other patients (60%) simultaneously developed ILD and myositis. These findings indicate that the onset of antisynthetase syndrome is acute, but the development of myositis may lag behind the onset of ILD in patients with DM. Nonetheless, future large studies will be needed to determine this in various ethnic populations.

Although corticosteroids remain the mainstay of therapy in DM patients with anti-ARS antibodies, most patients failed to fully respond to this treatment or manifested recurrent flares when the steroid dosage was tapered (data not shown), consistent with previous reports[34,35]. Most patients with anti-ARS antibody need to be treated with the other immunosuppressive agents such as cyclosporine A and/or cyclophosphamide, in addition to corticosteroids therapy (Table 4). Oddis, *et al* showed that tacrolimus (FK-506), an immunosuppressant pharmacologically similar to cyclosporine, is effective for managing refractory ILD and myositis in patients with anti-ARS antibodies[35,36]. The therapeutic guideline of antisynthetase syndrome should be established in the near future.

Immunological specificity differs among different types of

Table 4. Treatment history in DM patients with anti-ARS antibodies.

Anti-ARS	Age, yrs	Sex	ILD	Treatment
Anti-Jo-1	58	F	+	PSL 40 mg, steroid pulse, CYC
Anti-Jo-1	66	F	+	PSL 60 mg, steroid pulse, CyA
Anti-Jo-1	64	F	+	PSL 60 mg, steroid pulse, CyA
Anti-PL-7	56	F	+	PSL 50 mg, CYC pulse, CyA
Anti-PL-7	56	M	+	PSL 60 mg, CYC pulse
Anti-PL-7	72	F	+	PSL 60 mg, steroid pulse, CYC pulse, CyA
Anti-PL-7	83	F	+	PSL 60 mg, CYC pulse
Anti-PL-12	59	F	+	mPSL 120 mg, CyA, CYC
Anti-PL-12	58	F	+	PSL 30 mg, AZP, CyA
Anti-PL-12	38	F	+	PSL 30 mg, CYC pulse
Anti-EJ	58	M	+	PSL 60 mg, steroid pulse, CyA, MTX
Anti-EJ	20	F	–	PSL 60 mg, steroid pulse, CyA
Anti-EJ	50	F	+	PSL 40 mg, steroid pulse, CyA
Anti-EJ	56	F	+	PSL 55 mg, CyA
Anti-EJ	52	M	+	PSL 55 mg
Anti-EJ	62	F	+	PSL 40 mg

Anti-ARS: anti-aminoacyl-tRNA synthetase; ILD: interstitial lung disease; PSL: prednisolone (initial dose); mPSL: methylprednisolone (initial dose); CYC: cyclophosphamide; CyA: cyclosporine A; MTX: methotrexate; AZP: azathioprine.

Table 5. The onset time of ILD and myositis in DM patients with anti-ARS antibodies.

	ILD Preceded Myositis	Myositis Preceded ILD	ILD and Myositis Developed Simultaneously*
Frequency of patients (%)	5/15 (33)	1/15 (7)	9/15 (60)
Duration, mean ± SD, mo	20 ± 20	2	

* Both ILD and myositis developed within 1 month. Anti-ARS: anti-aminoacyl-tRNA synthetase; ILD: interstitial lung disease.

ARS, and differences in clinical features have also been reported depending on the type of anti-ARS antibody. Anti-Jo-1 antibody has been reported to be specific for PM rather than DM, while anti-EJ antibody is often positive in patients with DM[37]. Anti-PL-12 and anti-KS are detected in ILD patients with myositis that is poor in symptoms[10,38], while anti-PL-7 is related to sclerodactyly[39]. Although the reason is unclear, each anti-ARS antibody is mutually exclusive[40]. In our study, no significant difference in the clinical features was found depending on the type of anti-ARS antibody (data not shown), probably due to the small population. Alternatively, this may be due to the racial difference, since our patients were restricted to those of Japanese ethnicity. Further studies will be needed to determine the association between each anti-ARS antibody and the clinical features in a large population.

Anti-ARS antibodies appear to be associated with 2 subgroups of subjects: those who develop myositis with a high prevalence of ILD, and those who develop ILD without clinical evidence of myositis. Further, DM patients with anti-ARS antibodies appear to require additional therapy compared to those without anti-ARS antibodies.

ACKNOWLEDGMENT

We thank Ms. M. Matsubara and Y. Yamada for their technical assistance.

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Rheumatology 2007;46:842–845
Advance Access publication 25 January 2007

doi:10.1093/rheumatology/kel435

Clinical characteristics of Japanese patients with anti-OJ (anti-isoleucyl-tRNA synthetase) autoantibodies

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Objectives. The clinical and laboratory characteristics of seven patients with anti-aminoacyl-tRNA synthetase (ARS) autoantibodies, specifically anti-OJ (anti-isoleucyl-tRNA synthetase), were examined and compared with previously published findings.
Methods. Serum samples from 1135 Japanese patients with various autoimmune diseases and 48 normal individuals were screened for anti-OJ antibodies using RNA and protein immunoprecipitation assays. The patients whose sera contained anti-OJ antibodies were assessed regarding clinical symptoms, clinical course, laboratory findings, chest radiography and chest computed tomography.
Results. Sera from seven patients were found to contain anti-OJ antibodies. These autoantibodies were associated with interstitial lung disease (ILD) and myositis. The diagnoses of the seven patients were idiopathic interstitial pneumonias (IIPs) in three, polymyositis (PM) in three and PM-rheumatoid arthritis (RA) overlap in the remaining one. All patients had ILD, but muscle weakness and polyarthritis were seen only in four. Raynaud's phenomenon and sclerodactyly were absent in all patients.
Conclusions. These results indicate that the presence of anti-OJ autoantibodies may distinguish a subtype of anti-ARS syndrome that is more closely associated with ILD than myositis or Raynaud's phenomenon.

KEY WORDS: Interstitial lung disease (ILD), Polymyositis/dermatomyositis (PM/DM), Anti-aminoacyl-tRNA synthetase (ARS) antibodies.

Introduction

Anti-aminoacyl-tRNA synthetase (anti-ARS) autoantibodies have been found in patients with polymyositis/dermatomyositis (PM/DM) [1, 2]. Six anti-ARS autoantibodies have been described, as follows: anti-histidyl (anti-Jo-1), anti-threonyl (anti-PL-7), anti-alanyl (anti-PL-12), anti-glycyl (anti-EJ), anti-isoleucyl (anti-OJ), and anti-asparaginyl (anti-KS) tRNA synthetases [1–7]. The most common anti-ARS antibodies, anti-Jo-1, are found in approximately 20–30% of PM/DM patients. Anti-OJ antibodies are also found in PM/DM patients, although the frequency is low [1]. In previous studies, anti-OJ antibodies were found in less than 2% of all patients with PM/DM [8]. These anti-ARS antibodies have been reported to be associated with a similar syndrome characterized by myositis with a high frequency of interstitial lung disease (ILD) and arthritis, as well as increased fever, Raynaud's phenomenon, and mechanic's hands compared to the overall myositis population [9]. Although anti-ARS syndromes have common clinical symptoms, further observations have distinguished certain differences in clinical features associated with each of the different anti-ARS antibodies. It has been reported that anti-Jo-1 antibodies are closely associated with myositis [1, 3], whereas patients with anti-PL-12 and anti-KS antibodies are more likely to have ILD without clinical evidence of myositis [7]. On the other hand, we previously observed that the presence of anti-PL-7 antibodies is closely associated with PM/DM-SSc overlap as well as ILD in Japanese patients [10].

There have been two reports on the clinical significance of anti-OJ antibodies in patients in North America [11, 12], and two case reports of anti-OJ antibodies in Japan [8, 13]. However, the clinical characteristics of the Japanese patients with anti-OJ antibodies have not been examined in detail and their clinical significance remains uncertain. Here, we analyse the clinical and laboratory characteristics of Japanese patients with antibodies against anti-OJ and review published reports from elsewhere.

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Submitted 21 June 2006; revised version accepted 8 December 2006.

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Patients, materials and methods

Patients and sera

Serum samples were obtained from 1135 Japanese patients who had or were suspected of having connective tissue diseases (CTDs) seen at the Keio University Hospital and collaborating centers between 1990 and 2000. These included 120 with PM/DM, 400 with systemic lupus erythematosus, 192 with systemic sclerosis, 58 with rheumatoid arthritis (RA), 101 with overlap syndrome including mixed connective tissue disease, 114 with ILD and 150 patients with arthritis or erythema who were suspected to have CTDs. These included three patients with anti-OJ antibodies previously reported by our study group [8, 13]. We also examined 48 sera from normal individuals. Blood samples were obtained after the patients and normal controls had provided written informed consent approved by the Keio University Institutional Review Board.

Immunoprecipitation (IPP)

The IPP assay with HeLa cell extracts was performed as previously described [6]. For the analysis of RNAs, antibodies bound to protein A-Sepharose CL-4B beads were incubated with extracts of HeLa cells. They were then washed with NET-2 buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40). After ethanol precipitation, RNAs were dissolved in electrophoresis sample buffer composed of 10 M urea, 0.025% bromophenol blue, and 0.025% xylene cyanol-FF in TBE buffer (90 mM Tris-HCl, pH 8.6, 90 mM boric acid and 1 mM EDTA). The RNA samples were resolved in 7 M urea-10% polyacrylamide gels, which were then silver stained (Bio-Rad Laboratories, Hercules, CA, USA). For protein studies, antibody-coated Sepharose beads were mixed with [^{35}S] methionine-labelled HeLa extracts. After washing, the Sepharose beads were resuspended in SDS–sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8, 0.005% bromophenol blue). The proteins were then fractionated by 10% SDS-PAGE gels and dried. Radiolabelled protein components were analysed by autoradiography. With these assays, myositis-specific or -associated autoantibodies such as anti-ARS, anti-signal recognition particle, anti-Mi-2, anti-SSA, anti-SSB, anti-U1-RNP and anti-Ku autoantibodies are distinguishable, compared with corresponding standard sera [2]. The identification of anti-OJ antibodies was accomplished by

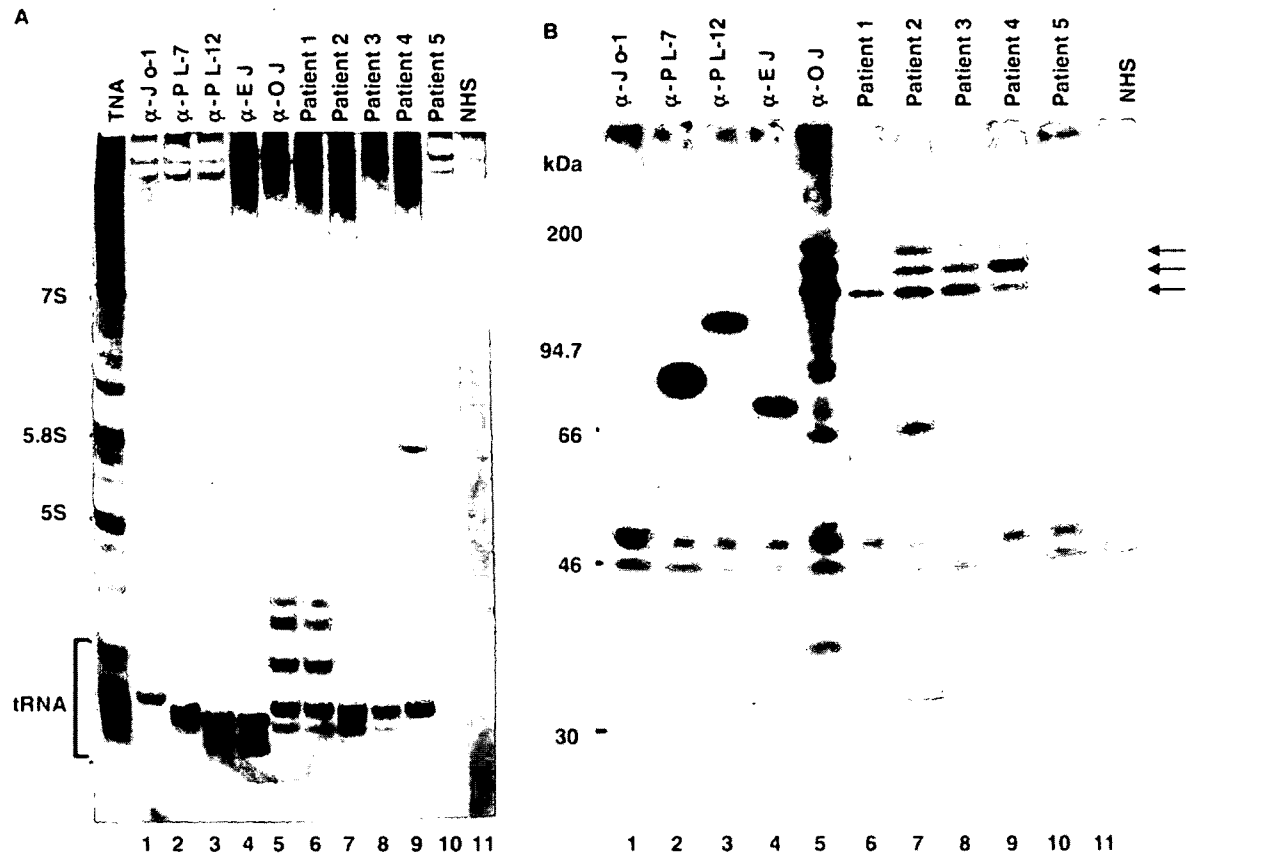

FIG. 1. (A) Immunoprecipitation (IPP) of nucleic acids with representative anti-OJ sera and controls. Urea (7M) and 10% PAGE of phenol-extracted immunoprecipitates from HeLa cell extracts were developed with silver stain. TNA, total nucleic acids, with the 5.8 and 5.0 S small ribosomal RNAs and the tRNA region indicated. Sera used for IPP include: lanes 1-5, anti-synthetase sera indicated, with antibodies to Jo-1 (histidyl-tRNA synthetase), PL-7 (threonyl-tRNA synthetase), PL-12 (alanyl-tRNA synthetase), EJ (glycyl-tRNA synthetase), OJ (isoleucyl-tRNA synthetase); lanes 6-10, anti-OJ sera as indicated; and lane 11, control serum indicated (NHS, normal human serum). The tRNA pattern with anti-OJ sera is easily distinguishable from that of the other anti-synthetases. (B) IPP of proteins with anti-OJ sera and controls. Autoradiogram of 10% SDS-PAGE of immunoprecipitates from [^{35}S] methionine-labelled HeLa cell extracts. Mr, molecular weight markers of the sizes indicated to the left (kDa). The sera used for immunoprecipitation are the same as those in Fig. 1A. The same characteristic pattern of protein bands including at least three that were thought to be glutamine, isoleucine and leucine tRNA synthetases (arrows) was seen with each of the five anti-OJ sera. And the pattern was easily different from the bands immunoprecipitated by sera containing the other anti-synthetases.

comparing IPP patterns for both RNA and protein with standard anti-OJ serum as described previously [13].

Clinical features

The clinical symptoms, clinical course, laboratory findings, the results of chest radiography and chest computed tomography (CT) were retrospectively assessed from medical records in all patients positive for anti-OJ antibodies. Some patients were also assessed for electromyogram (EMG), muscle biopsy, and pathological findings from video-assisted thoracoscopic surgery (VATS) at first evaluation. The assessment of muscle weakness was performed using a manual muscle test (MMT) [14].

The diagnosis of PM/DM was based on criteria proposed by Bohan and Peter [15]. ILD was defined according to the results of chest radiography, chest CT, lung function testing (percentage predicted forced vital capacity: FVC and carbon monoxide diffusing capacity: DLCO) and the diagnosis of IIPs was based on consensus classification of IIPs [16]. The resolution of myositis symptoms was defined as both improved muscle strength on a manual muscle test and normalization of the serum CK value. Pulmonary symptoms were considered improved when shown by both chest CT and pulmonary function testing.

Statistical analysis

All comparisons between the two patient groups were performed using Fisher's 2-tailed exact test or Student's *t*-test.

Results

Identification of anti-OJ antibodies

Of the 1183 sera tested, seven immunoprecipitated a characteristic identical nucleic acid band of tRNA of a size identical to anti-isoleucyl tRNA synthetase (anti-OJ). Representative examples are shown in Fig. 1A. Two bands of RNA in the tRNA size range were immunoprecipitated and are clearly distinguishable from the pattern of tRNAs precipitated by the other anti-ARS antibodies. The same sera also immunoprecipitated several protein bands corresponding to polypeptides precipitated by anti-OJ standard serum. These included at least three protein bands that were more intense than the other seven bands of the OJ complex. These proteins were easily distinguishable from those immunoprecipitated by sera reactive with the other described anti-ARS antibodies (Fig. 1B). Thus, it is concluded that they contained anti-OJ antibodies.

Clinical features in patients with anti-OJ antibodies

In this study, anti-OJ antibodies were detected in four of 120 PM/DM patients (3.3%) and three of 112 ILD patients (2.7%). However, anti-OJ antibodies were not detected in other CTDs or normal human sera.

Clinical features in the seven patients with anti-OJ autoantibodies are summarized in Table 1. Of these seven, four were female. All seven patients had ILD, although none of them

TABLE 1. Clinical features of patients with anti-OJ antibodies

Clinical findings	#1	#2	#3	#4	#5	#6	#7
Age/gender	51/female	62/male	75/female	53/male	69/female	27/male	32/female
Diagnosis	PM/RA	PM	PM	PM	IIPs	IIPs	IIPs
ILD	(+)	(+)	(+)	(+)	(+)	(+)	(+)
%VC (%)	56	88	46	n.a.	70	74	38
DLCO (ml/min[a]tor)	8.5	12.5	n.a.	n.a.	10.4	5.9	4.1
Histopathology in VATS	n.d.	n.d.	n.d.	n.d.	COP	UIP	NSIP
DM rush	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Muscle weakness	(+)	(+)	(+)	(+)	(−)	(−)	(−)
Maximun CK level (IU/l)	3,297	648	672	1,682	125	113	33
EMG findings	Myogenic[a]	n.d.	Myogenic[a]	Myogenic[a]	n.d.	n.d.	n.d.
Muscle biopsy	Myositis[b]	Myositis[b]	n.d.	n.d.	n.d.	n.d.	n.d.
Arthritis	(+)	(+)	(+)	(+)	(−)	(−)	(−)
Raynaud's phenomenon	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Sclerodactyly	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Sjögren's syndrome	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Duration of the disease (month)	57	49	42	28	43	35	63
Treatment	PSL 50 mg	PSL 60 mg, mPSL pulse, AZA 100 mg	PSL 20 mg	(−)	PSL 20 mg	PSL 60 mg	PSL 50 mg
Duration of treatment (month)	57	48	41	0	24	11	15
Effect of treatment	(+)[c]	(+)[c]	(+)[c]		(+)[d]	(−)[d]	(+)[d]

PM: polymyositis, RA: rheumatoid arthritis, DM: dermatomyositis, IIPs: idiopathic interstitial pneumonias, CK: creatine kinase, EMG: electromyogram, ILD: interstitial lung disease, VATS: video-assisted thoracoscopic surgery, COP: cryptogenic organizing pneumonia, UIP: usual interstitial pneumonia, NSIP: non-specific interstitial pneumonia, PSL: prednisolone, mPSL: methylprednisolone, AZA: azathioprine. [a] Low amplitude, resting fibrillation, positive sharpe wave (denervatrion potencials) were present. [b] Atrophy, necrosis with regeneration and infiltration of lymphocytes of muscle fibres were present. [c] Both improvement of muscle weakness on a manual muscle test and the normalization of serum CK value. [d] Improvement of chest CT and/or pulmonary function testing.

TABLE 2. Comparison of clinical features in anti-OJ-positive *vs* anti-Jo-1-positive patients

	Anti-OJ ($n=7$)	Anti-Jo-1 ($n=22$)
Age at onset, mean ± S.D. years	53 ± 18	49 ± 15
No male/no. female	3/4	7/15
Fever (%)	3 (43)	14 (64)
DM rash (%)	0 (0)	6 (27)
Arthritis (%)	4 (57)	20 (91)
Myositis (%)	4 (57)*	22 (100)*
ILD (%)	7 (100)	20 (91)
Raynaud's phenomenon (%)	0 (0)**	15 (68)**
Sclerodactyly (%)	0 (0)	1 (5)

PM: polymyositis, DM: dermatomyositis, ILD: interstitial lung disease, IPF: idiopathic pulmonary fibrosis.
*$P=0.010$. **$P=0.002$.

progressed to severe acute respiratory failure. Four patients had muscle weakness that was graded as 4/5 based on MMT and serum CK elevation. From the results of EMG and muscle biopsy, four patients were diagnosed as definite or probable PM. Two of these manifested symptoms of ILD preceding their myositis symptoms. One of the four PM patients had destructive changes in joint radiography compatible with RA. The other three patients had no muscle or skin manifestations but interstitial changes in the lung at chest high resolution CT and/or histopathlogical change in VATS resulted in a diagnosis of IIPs. VATS was done in all patients with IIPs and the results were compatible with cryptogenic organizing pneumonia (patient #5), usual interstitial pneumonia (patient #6) and non-specific interstitial pneumonia (patient #7). No Raynaud's phenomenon or sclerodactyly was present at any time in any of the seven patients. Treatment of the myositis with prednisolone and/or other immunosupressants resulted in improvement of muscle strength assessed by MMT and reduction of serum CK level in three PM patients. Three patients with IIPs had also received prednisolone that had then been gradually tapered and discontinued; improvement of chest CT and pulmonary function was achieved in two of them.

Comparison with the clinical features of patients with anti-OJ in the literature

The clinical features of patients with anti-OJ antibodies reported in the English literature were previously reviewed [11, 12]. Targoff *et al.* [11] reported on nine patients with anti-OJ and Gelpi *et al.* [12] described one patient with co-existing anti-Jo-1 and anti-OJ antibodies.

Frequencies of several clinical manifestations can be compared between the anti-OJ-positive patients reported by Targoff *et al.* and those in the present study. The frequency of myositis in our Japanese patients with anti-OJ antibodies tended to be lower than in the patients of Targoff *et al.* (57% *vs* 89%), but this difference did not reach statistical significance. The frequencies of ILD and Raynaud's phenomenon in our series were similar compared with previously reported patients [11].

Comparison with the clinical features of patients with anti-OJ and anti-Jo-1 antibodies

In the present study, anti-Jo-1 antibodies, representative of anti-ARS antibodies, were detected in 22 patients with PM/DM (18% of PM/DM in our study). We compared the frequencies of several clinical manifestations between anti-OJ- and anti-Jo-1-positive patients in our series (Table 2). It was found that the frequency of myositis and Raynaud's phenomenon in patients with anti-OJ was significantly lower than in those with anti-Jo-1 ($P=0.010$ and $P=0.002$, respectively), whereas the frequency of ILD was similar.

Discussion

Seven sera from 1135 CTD patients or suspected CTDs were found to contain anti-OJ antibodies. In the present study, these seven patients seemed not to have the typical features characteristic of the anti-ARS syndromes previously described. The most striking differences were that none of them had Raynaud's phenomenon that is common in anti-ARS syndromes in general. In fact, the frequency of Raynaud's phenomenon in our series was significantly lower than in our patients with anti-Jo-1 antibodies that are the representative anti-ARS cases. However, the sample is too small to draw a definitive conclusion in this study, but our results are similar to those in North American patients with anti-OJ antibodies [11]. Thus, the low frequency of Raynaud's phenomenon seems to be a characteristic feature of patients with anti-OJ autoantibodies compared to other anti-ARS syndromes.

Three of the anti-OJ-positive patients had no signs of myositis and were diagnosed as IIPs. This suggested that the presence of anti-OJ is more closely associated with ILD than myositis, as is the case with anti-PL-12 or anti-KS antibodies.

Although three patients received a diagnosis of IIPs at this time, the possibility remains that muscle symptoms may appear in the future, because it is known that pulmonary manifestations can appear before muscle symptoms in PM/DM patients. Another possibility is that existing myositis was underdiagnosed because of the effect of prednisolone treatment; indeed, three patients diagnosed as IIPs had been taking prednisolone for their pulmonary symptoms. Nonetheless, it is unlikely that they had myositis because the duration of prednisolone therapy was relatively short and they never had any clinical symptoms related to myositis throughout their clinical course. However, observation should be continued for identification of any future muscle symptoms.

The mechanisms responsible for these differences in clinical features associated with each of the anti-ARS autoantibodies are unknown and an accumulation of larger numbers of cases will be required to clarify this in the future.

In conclusion, we report seven Japanese patients with anti-OJ antibodies classed as suffering from IIPs or PM accompanied by ILD. These patients lacked any manifestations of Raynaud's phenomenon or sclerodactyly. Anti-OJ autoantibodies are a clinically important marker for a specific subset of anti-ARS syndrome that is more closely associated with ILD than myositis or Raynaud's phenomenon. The measurement of anti-OJ was found to be useful for diagnosis of patients with ILD with or without myositis. Further analysis of these autoantibodies may provide insights into the etiological and pathogenetic mechanisms of ILD and myositis.

Rheumatology key messages

- Anti-OJ autoantibodies may distinguish a subtype of anti-ARS syndrome that is more closely associated with ILD than myositis or Raynaud's phenomenon.
- Anti-OJ autoantibodies are closely associated with ILD.

Acknowledgements

We thank Ms Mutsuko Ishida for assisting with the RNA immunoprecipitation assay.

The authors have declared no conflict of interest.

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Mod Rheumatol (2008) 18:15–22
DOI 10.1007/s10165-007-0015-y

ORIGINAL ARTICLE

Interleukin-17 gene expression in patients with rheumatoid arthritis

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Received: 14 August 2007 / Accepted: 28 September 2007 / Published online: 20 December 2007

Abstract Interleukin-17 is a proinflammatory cytokine. Recent animal studies have shown that IL-17 plays a role in the initiation and progression of arthritis. However, whether IL-17 has a prominent role in human rheumatoid arthritis (RA) or not remains unclear. Here we investigated the role of IL-17 in patients with RA. cDNA was prepared from knee joint synovial tissues of RA ($n = 11$) and osteoarthritic (OA, $n = 10$) patients and PBMC of RA ($n = 52$) and healthy subjects ($n = 34$). IL-17 gene expression level was measured by real-time PCR, and was compared with various clinical parameters. IL-17 gene expression in synovial tissues of RA was similar to that in OA. IL-17 gene expression level in PBMC of RA patients was significantly higher than in the control. The response (changes in DAS) to two-week treatment with anti-TNF-α blockers (infliximab or etanercept) did not correlate with changes in IL-17 gene expression levels. The IL-17/TNF-α gene expression ratio at baseline (before treatment) tended to be lower in responders to the treatment. Expression of IL-17 gene in PBMC may be associated with the inflammatory process of RA. IL-17/TNF-α expression ratio is a potentially suitable marker of response to anti-TNF-α therapy.

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Keywords Etanercept · Infliximab · Interleukin-17 (IL-17) · Rheumatoid arthritis · Tumor necrosis factor-α (TNF-α)

Introduction

Rheumatoid arthritis (RA) is characterized by chronic synovial inflammation, cartilage degradation and bone erosion in multiple joints, which ultimately lead to joint destruction and disability. Inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin-1 (IL-1) and IL-6 are involved in the pathogenesis of RA [1–3], and the beneficial effects of antagonists to these cytokines have been reported [4–6]. However, not all patients respond to these agents, suggesting that other pro-inflammatory cytokines, e.g., IL-17, may be important in the pathogenesis of RA.

IL-17 is a proinflammatory cytokine produced by activated and memory $CD4^+$ $CD45RO^+$ T cells [7–8], and is a potent inducer of other cytokines, such as TNF-α, IL-1β, IL-6, IL-8, and granulocyte colony stimulating factor (G-CSF) in a variety of epithelial, endothelial, and fibroblastic cell types [9]. In experimental arthritis, IL-17 was found to be important in both the early initiation phase and late progression phase, especially in arthritis models driven mainly by T cells, such as the IL-1 $Ra^{-/-}$ mouse model and streptococcal cell wall-induced arthritis [10–15].

Several studies have suggested that IL-17 plays a role in the pathogenesis of RA. Chabaud et al. [16] reported that the proportion of synovial membrane cultures that produced IL-17 was higher in those from RA patients than those from OA patients or healthy controls. In addition, previous studies indicated the presence of elevated IL-17 levels in the synovial fluid of patients with sRA [17, 18].

Although these observations imply that IL-17 is involved in the pathogenesis of human RA, there is no solid information on whether IL-17 actually plays a role in RA pathogenesis.

With regard to TNF-α, previous studies reported that IL-17 contributes to the arthritic process not only by inducing the production of TNF-α, but also acting in synergy with or independently of TNF-α [8, 19–23]. Although it is unknown whether these observations are relevant in human RA, we hypothesized that IL-17 gene expression level in PBMC might be enhanced independent of that of TNF-α in some patients with RA, and that these patients may comprise a subpopulation of RA refractory to anti-TNF-α therapy.

The aims of the present study were to clarify the role of IL-17 in disease activity and progression of human RA and to explore the possibility of IL-17 as a target for the treatment for RA. For this purpose, we measured the gene expression of IL-17 in peripheral blood mononuclear cells (PBMC) and synovial tissues from RA patients. We correlated the findings of PBMC samples with various clinical parameters, TNF-α gene expression and the efficacy of infliximab or etanercept.

Methods

Patients

Peripheral blood was taken from 52 patients who fulfilled the 1987 American Rheumatism Association criteria for the classification of RA [24]. The patient group comprised 19 males and 33 females (age, 25–84 years, mean ± SD: 54.0 ± 13.0 years). The fifty-two RA patients included 25 infliximab- and 11 etanercept-treated patients who attended our unit between 29 September 2003 and 22 August 2006, and, as a control group, 16 RA patients who visited our unit between October 11 and October 25 2006. Clinical data such as white blood cell count (WBC), erythrocyte sediment rate (ESR), c-reactive protein (CRP) and matrix metalloproteinase 3 (MMP-3) was obtained at the time of blood sampling. We also included a group of control healthy donors (13 men and 21 women, age 30.0 ± 5.0 years). Synovial tissues were obtained at the time of total knee replacement performed from April 2001 to June 2002 on 11 RA patients (one male and ten females, age 56.8 ± 8.7 years) and ten osteoarthritis (OA) patients (ten females, age 73.4 ± 2.7 years). No patient underwent both blood and synovial tissue sampling. Patient demographics are listed in Table 1. Written informed consent was obtained from all subjects, and the study was approved by the appropriate ethics committee.

Treatment with infliximab or etanercept and assessment of efficacy

Twenty-five RA patients were treated with 3 mg/kg of infliximab at weeks 0, 2, 6, and 14, and every eight weeks thereafter. Another RA group consisting of 11 patients were treated with etanercept (25 mg injection twice weekly). Drug efficacy was evaluated by comparing the differences in European League Against Rheumatism improvement criteria [Disease Activity Score (DAS)] at week 0 (before treatment) and at 2 weeks.

Synovial and blood samples for cDNA synthesis

Part of each synovial tissue was cut into small pieces and rinsed with phosphate-buffered saline (PBS). Complementary DNA (cDNA) was prepared from synovial tissues

Table 1 Demographics of the subjects included in the study

PBMC	Mean ± SD	Range	Synovium	Mean ± SD	Range
RA patients			RA patients		
Age (years)	54.0 ± 13.0	(25–84)	Age (years)	56.8 ± 8.7	(37–70)
Males	19		Males	1	
Females	33		Females	10	
C-reactive protein (mg/dl)	2.15 ± 1.63	(0.25–8.95)	C-reactive protein (mg/dl)	1.65 ± 2.64	(0.07–7.17)
ESR (mm/h)	48.5 ± 21.1	(12–104)	ESR (mm/h)	33.4 ± 25.1	(10–95)
Rheumatoid factor (IU/ml)	245 ± 231	(5–1790)	Rheumatoid factor (IU/ml)	161 ± 199	(4–715)
Healthy controls			OA patients		
Age (years)	30.0 ± 5.0	(22–50)	Age (years)	73.4 ± 2.7	(69–78)
Males	13		Males	0	
Females	21		Females	10	

ESR erythrocyte sedimentation rate

using Revertaid first strand cDNA synthesis kit (Fermentas, Hanover, MD, USA), following the instructions provided by the manufacturer.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood obtained from normal subjects and RA patients using Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden) following the protocol recommended by the manufacturer. Cells were spun down to pellets and total RNA was extracted from the cell pellets using Isogen (Nippongene, Tokyo, Japan). The cDNAs were synthesized using a Revertaid first strand cDNA synthesis kit (Fermentas), following the instructions provided by the manufacturer. These cDNA samples underwent gene expression analyses.

Quantification of gene expression by real-time polymerase chain reaction

The cDNA samples were amplified with specific primers and fluorescence-labeled probes for the target genes. Specific primers and probes for IL-17, IL-6. TNF-α and glyceraldehyde-2-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems Japan (Tokyo). The amplified product genes were monitored on an ABI 7700 sequence detector (Applied Biosystems Japan). The qPCR master mix was also purchased from Applied Biosystems Japan. The final concentrations of the primers were 200 nM for each of the 5′ and 3′ primers, and the final probe concentration was 100 nM. The thermal cycler conditions used were 50 °C for 2 min, 95 °C for 10 min, then 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Serial dilutions of a standard sample were included in every assay, and standard curves for the genes of interest and GAPDH genes were generated. All measurements were performed in triplicate. The level of gene expression was calculated from the standard curve, and expressed relative to GAPDH gene expression.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). The Mann–Whitney U test was used to compare the expression levels of genes between RA patients and healthy controls. Spearman's correlation coefficient by rank test was calculated to assess the correlations between the expression levels of IL-17 and TNF-α genes, as was the correlation between IL-17/TNF-α gene expression ratio and changes in DAS after two weeks of therapy with etanercept or infliximab relative to baseline (before treatment, i.e., DAS 0–2 weeks). Paired Wilcoxon's rank test was used to compare the gene expression levels between week 0 and week 2. A P value of less than 0.05 was considered significant.

Results

Expression levels of IL-17 genes in synovial tissues

We anticipated that the expression of IL-17 would be enhanced in RA patients at the site of inflammation, namely the synovial tissue. However, the expression of IL-17 gene in synovial tissues of RA patients, as measured by real-time PCR, was not significantly different from that of the OA patients (Fig. 1).

Expression levels of IL-17 genes in PBMC

We next asked whether the expression level of IL-17 gene is upregulated in PBMC of RA patients. Expression of the IL-17 gene in PBMC from RA patients was significantly higher than that of the control (RA: 0.0437 ± 0.1112, control: 0.0134 ± 0.0033, $P = 0.011$, Fig. 2).

Relationship between IL-17 and IL-6 or TNF-α gene expression in RA patients

We wished to determine whether the expression of IL-17 gene in patients with RA is significantly associated with

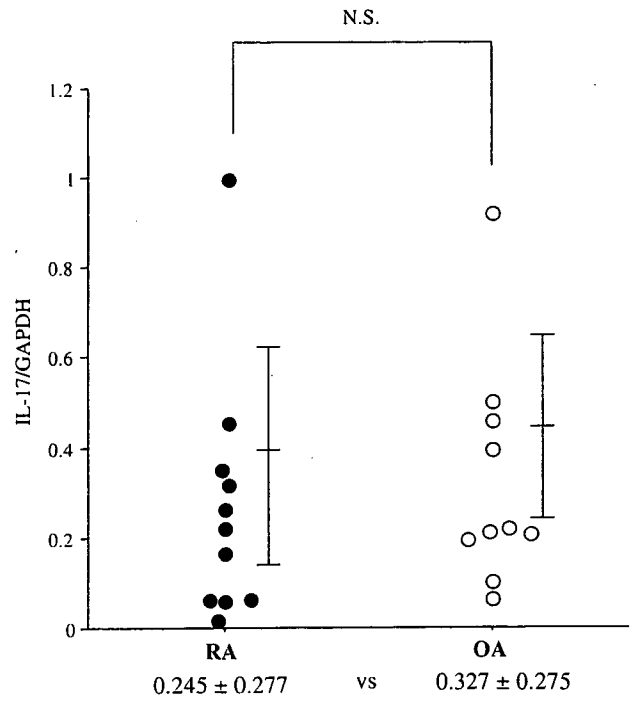

Fig. 1 Expression levels of IL-17 gene in the synovium tissues of patients with RA (*closed circles*, $n = 11$) and osteoarthritis (OA, *open circles*, $n = 10$). Data are mean ± SD. The P value was calculated by the Mann–Whitney U test

expressions of other inflammatory cytokines such as TNF-α and IL-6. No significant relationship between expression of the IL-17 gene and that of IL-6 or TNF-α was observed (Fig. 3a, b). In addition, there was no significant relationship between the expression level of IL-6 and that of TNF-α (Fig. 3c). Furthermore, the expression levels of IL-17 in PBMC of RA patients did not correlate significantly with WBC count, serum CRP, ESR or serum MMP-3 (data not shown).

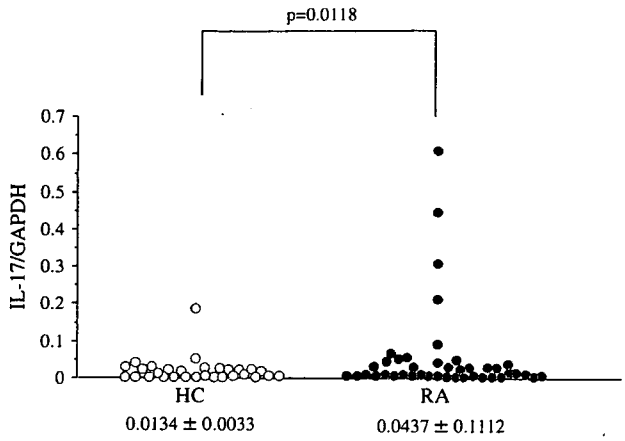

Fig. 2 Expression levels of IL-17 gene in peripheral blood mononuclear cells from patients with RA (*closed circles*, $n = 52$) and from healthy controls (HC, *open circles*, $n = 34$). Data are mean ± SD. The P values were calculated by the Mann–Whitney U test

Effects of infliximab and etanercept therapy on IL-17 gene expression in PBMC

We compared the gene expression levels of IL-17 in PBMC samples at baseline, and two weeks after the first infusion or injection of biologics that target TNF-α. In patients who received infliximab, no significant differences were observed between baseline and week 2 samples (week 0: 0.089 ± 0.157, week 2: 0.044 ± 0.056, Fig. 4a). On the other hand, etanercept significantly increased IL-17 gene expression at two weeks after initiation of therapy (week 0: 0.041 ± 0.132, week 2: 0.134 ± 0.274, $P = 0.028$, Fig. 4 b). There were no relationships between changes in IL-17 gene expression levels and efficacy (changes in DAS) of infliximab and etanercept.

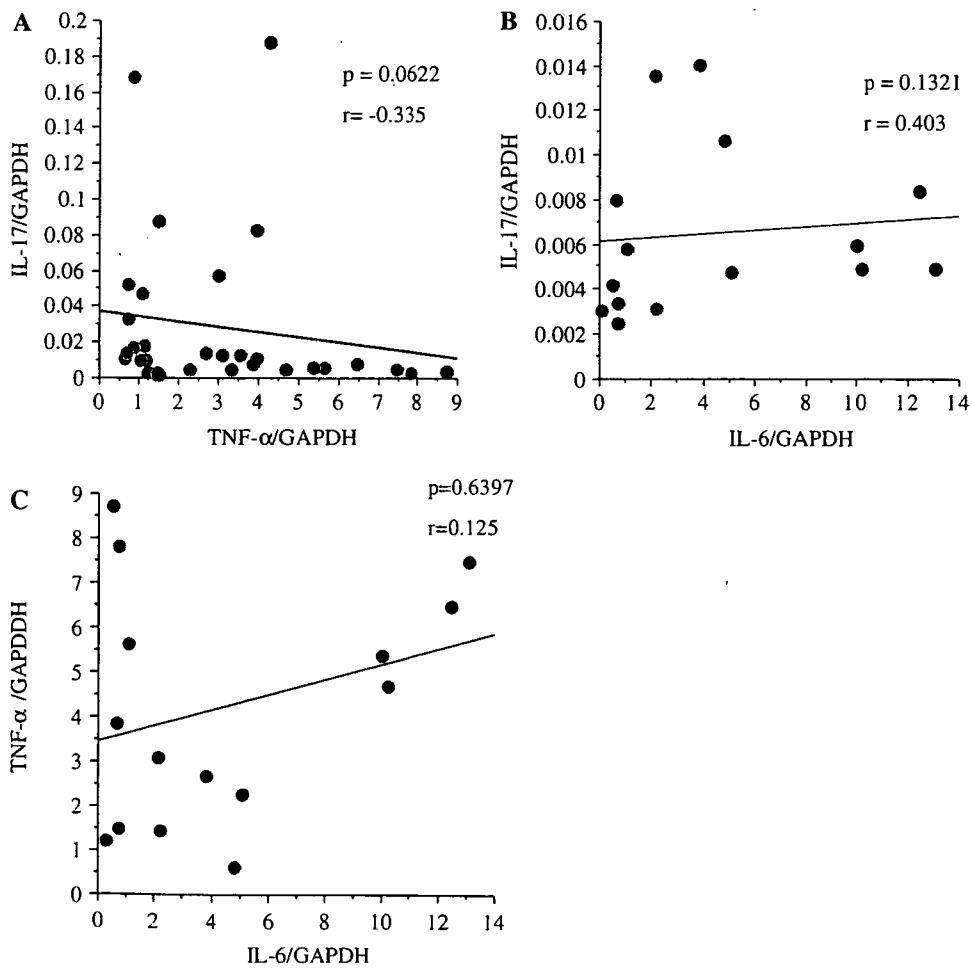

Fig. 3 Relationships between expression levels of IL-17, TNF-α and IL-6 genes in peripheral blood mononuclear cells from rheumatoid arthritis (RA) patients. **a** Relationship between expression levels of IL-17 and TNF-α in PBMC from RA ($n = 31$). **b** Relationship between expression levels of IL-17 and IL-6 in PBMC from RA ($n = 15$). **c** Relationship between expression levels of TNF-α and IL-6 in PBMC from RA ($n = 15$) r = correlation coefficient. The P values were calculated by Spearman's correlation coefficient by rank test

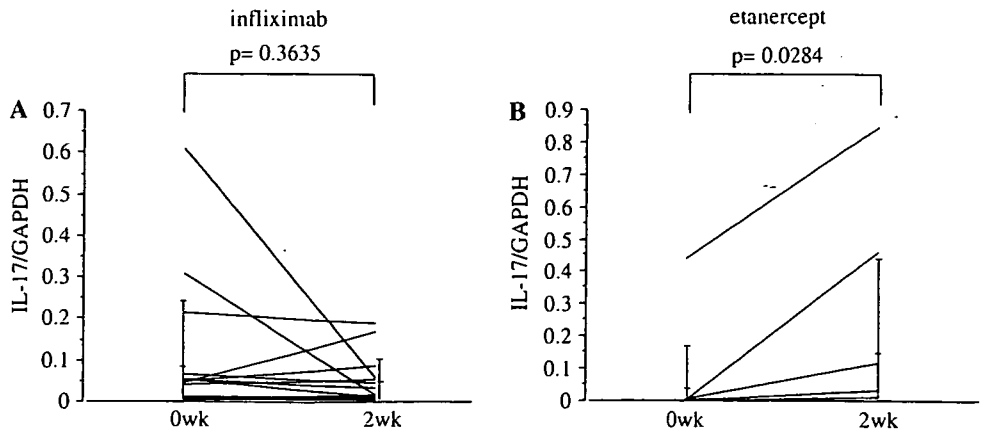

Fig. 4 IL-17 gene expression levels in peripheral blood mononuclear cells at baseline and two weeks after the first infusion of infliximab (n = 17) (**a**), and after the first injection of etanercept (n = 11) (**b**). Data are mean ± SD. The P values were calculated by Wilcoxon's rank sum test

Relationships between IL-17 and TNF-α gene expression and efficacy of anti-TNF-α therapy

We next examined whether patients with a high expression level of IL-17 gene relative to that of the TNF-α gene were more refractory to anti-TNF-α therapy. For this purpose, we calculated the IL-17/TNF-α gene expression ratio in PBMC of RA patients prior to anti-TNF-α therapy and correlated it with changes in DAS from pre- to two-week post-therapy (DAS0-2 week). The results showed that the IL-17/TNF-α gene expression ratio prior to treatment tended to be lower in patients who responded to anti-TNFα therapy, although the relationship was not statistically significant (Fig. 5).

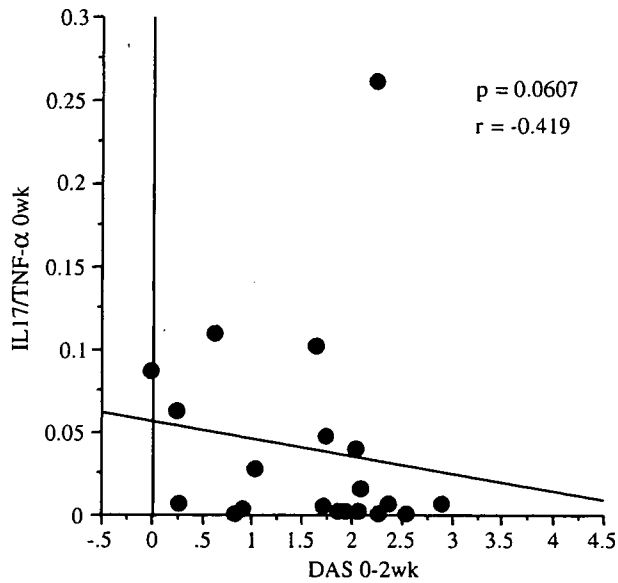

Fig. 5 Relationship between IL-17/TNF-α ratio in peripheral blood mononuclear cells and response to anti-TNF-α therapy. Number of patients = 20, r = correlation coefficient. The P values were calculated by Spearman's correlation coefficient by the rank test

Discussion

The active involvement of IL-17 in both the initiation stage and the progression stage of murine experimental arthritis has been demonstrated and IL-17 is considered a key cytokine in the pathogenesis of arthritis in such experimental models [10–13]. A few studies suggest that IL-17 may have a role in the pathogenesis of human RA as well [16–18].

Based on the above background, we measured IL-17 gene expression in synovial tissues and PBMC of RA patients, and compared them with those in control samples. We first compared the expression of IL-17 gene in synovial tissues from RA patients with that in OA patients. Unexpectedly, the IL-17 gene expression level in synovial tissues of OA patients was comparable to that in RA patients. This result is in contrast to that described by Chabaud et al. [16], where expression of IL-17 gene was higher in RA synovium than in OA synovium. Differences in patient demographics and sample size may partly account for this discrepancy. While OA is not generally considered an inflammatory disorder, it is reported that IL-17 upregulates the release of IL-8 and GRO-α in synovial fibroblasts and chondrocytes isolated from patients from OA, suggesting that IL-17 does play a role in the joint destruction process of OA [25]. In this study, we were unable to obtain synovial tissues from healthy individuals, which would have been a better control for this study. In contrast to our study, Kotake et al. [17] reported the presence of high IL-17 protein levels in synovial fluids of patients with RA, but not those with OA. The reason for this discrepancy is unclear, but it is possible that differences in post-transcription regulation of IL-17 production between RA and OA partly contributes to the difference in protein levels in the synovial fluid.

We next examined whether IL-17 gene expression in PBMC is elevated in patients with RA. As expected, the expression of IL-17 gene was significantly higher in PBMC of RA patients than in those of healthy controls. This result

implies that IL-17 does have a role in the inflammatory process of RA, at least in some patients. We did not measure expression of the IL-17 gene in PBMC from OA patients. Comparison of IL-17 gene expression in synovial tissues and PBMC taken simultaneously in both RA patients and OA patients could be informative. Expression of the IL-17 gene in PMBC of RA patients differed greatly among individuals, suggesting that such expression is important in some patients at some points during the course of the disease. Future studies with serial samples from RA patients would be informative. In this regard, IL-17 protein levels were undetectable in serum samples of 15 RA and 15 healthy controls (<15.6 pg/ml) by enzyme immunoassay (data not shown). Thus, we were unable to determine whether IL-17 gene expression levels in PBMC reflect the levels of serum IL-17 protein.

Interleukin-17 is known to enhance the pro-inflammatory effects of TNF-α in vitro and to act in synergy with TNF-α in the progression of arthritis in experimental arthritis mouse models [8, 19, 20, 21]. IL-17 is reported to depend strongly on TNF-α in induction of arthritis under naïve conditions in another experimental arthritis model [15]. However, it was recently reported that IL-17 could induce arthritis in the absence of TNFα in an experimental arthritis model [22]. Therefore, we considered it possible that (1) the expression of IL-17 gene in PBMC may be related to that of TNF-α, or (2) some RA patients show enhanced IL-17 gene expression level in PBMC regardless of the TNFα gene expression level, and comprise a subpopulation of anti-TNF-α refractory patients. We found a tendency towards a negative relationship between the expression of IL-17 gene and that of TNF-α gene in PBMC of RA patients, although the relationship was not significant. In addition, we found no significant relationship between IL-17 gene expression in PBMC and various parameters of inflammation, including ESR and serum CRP. These results imply that, in RA, which is a multifactorial and heterogeneous disease, gene expressions of TNF-α and IL-17 in PBMC are not directly associated with each other, and the expression of IL-17 in PBMC does not have an overwhelming influence on the inflammatory status.

Interleukin-6 is also a pro-inflammatory cytokine known to play an important role in the pathogenesis of RA [26, 27], and also to regulate the differentiation of Th17 T cells. Previous studies indicated that IL-6$^{-/-}$CD4+ T cells from draining lymph nodes produced less IL-17 than cells from wild-type mice, and that IL-6-deficient SKG mice were completely devoid of IL-17^{+}CD4+ T cells [28–30]. Conversely, IL-17 induced secretion of IL-6 from cultured fibroblasts [7, 8]. Based on this background, we compared the expression of IL-17 and IL-6 genes in PBMC of RA patients. In contrast to the relationship between IL-17 gene expression and TNF-α gene expression, a tendency towards a positive relationship was observed, albeit statistically insignificant.

It has been reported that IL-23 is important in the survival and expansion of IL-17- and IL-6-producing Th17 T cells and the development of collagen-induced arthritis in mice [30–33]. A recent study reported that self-reactive T cells produced by genetic alteration of thymic T cell selection spontaneously differentiate into Th17 T cells, and that these T cells stimulate antigen presenting cells (APC) to secrete IL-6 [29]. APC-derived IL-6 and T-cell derived IL-6 drive naïve T cells to differentiate into arthritogenic Th17 T cells [34]. Our observation may reflect the fact that Th17 T cells have an important role in the pathogenesis of human RA.

The efficacy of anti-TNF-α therapy in RA is well established [35]. However, non-responders hardly show any improvement in symptoms even after continuous injection or infusion of TNF-α antagonists. These observations imply that some cytokines other than TNF-α can act independently of TNF-α in the pathogenesis of RA. If IL-17 is one of these cytokines, IL-17 may become an appropriate target for treatment of RA patients refractory to TNF-α antagonists. To gain an insight into this question, we first addressed how TNF-α blockage affected the expression of IL-17 gene in PBMC of patients with RA by comparing IL-17 gene expression before and two weeks after the first infliximab or etanercept injection. Infliximab did not significantly affect IL-17 expression but etanercept significantly increased the expression in PBMC after two weeks of therapy. The reason for this difference is not clear at present. However, etanercept is known to be a decoy receptor and inhibitor of the action of soluble TNF-α, while infliximab is an antibody against TNF-α and is reported to induce negative signals through membrane TNF-α [36]. This may partly account for the observed differences in the action of these two agents. At present, we could not find any significant relationship between the efficacy of TNF-α blockade therapy and fluctuation of IL-17 gene expression in PBMC. A study in a larger number of patients is warranted to examine this issue.

We also calculated the IL-17/TNF-α ratio in PBMC of RA patients prior to initiation of therapy using TNF-α blocking agents, and examined the changes in the ratio after such treatment. While there was no relationship between DAS 0–2 week and IL-17 or TNF-α gene expression, the IL-17/TNF-α ratio tended to be lower in responders. These results suggest that RA patients with low IL-17 gene expression and high TNF-α gene expression in PBMC before treatment are more likely to respond to anti-TNF-α therapy. Currently there are no tools available to distinguish between responders and non-responders before TNF-α blockade therapy. IL-17/TNF-α expression ratio in