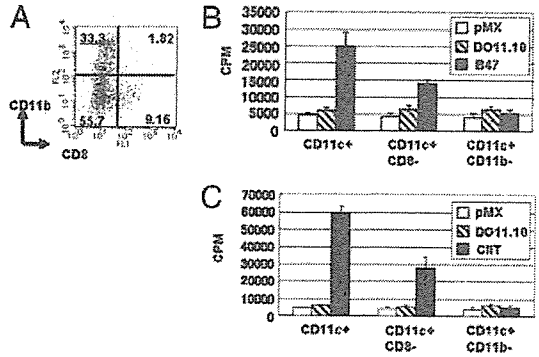

FIGURE 4. A DC subpopulation that stimulated B47- and CII-transduced $CD4^+$ T cells. *A*, ILN cells were stained with CD8-FITC, CD11b-PE, and CD11c-Tricolor and analyzed by FACS. A representative CD11c gated dot plot is shown. *B*, Stimulation of B47 TCR-transduced cells with ILN whole $CD11c^+$ cells, CD8-depleted $CD11c^+$ cells, and CD11b-depleted $CD11c^+$ cells. *C*, Stimulation of CII TCR-transduced cells with ILNl whole $CD11c^+$ cells, CD8-depleted $CD11c^+$ cells, and CD11b-depleted $CD11c^+$ cells.

(Fig. 2*D*). B47-transduced $CD4^+$ cells proliferated in the presence of autologous $CD11c^+$ DCs of the spleen and draining lymph nodes from naive mice (Fig. 2*E*). Though mock- and DO11.10-transduced $CD4^+$ cells were stimulated weakly by $CD11c^+$ DCs from naive and arthritic mice, B47-transduced $CD4^+$ cells proliferated more strongly in the presence of $CD11c^+$ DCs from arthritic mice (Fig. 2*E*). In addition, B47-transduced $CD4^+$ cells did not show increased proliferation in response to mCII and bCII (Fig. 2*F*). Therefore, B47 TCR was found to recognize an autoantigen that is presented more efficiently in arthritic mice.

We next examined the kinetics of B47-transduced $CD4^+$ cells in the arthritic mice. Mock- or B47-transduced $CD4^+$ cells were labeled for CFSE and transferred to arthritic mice via the tail vein. These mice groups were designated as the mock group and the B47 group, respectively. Five days after transfer, the accumulation of $V\alpha2^+V\beta8.1/8.2^+CFSE^+CD4^+$ T cells was similar in the spleen and lung of the mock group and the B47 group (Fig. 3, *A* and *B*). In contrast, the accumulation of $V\alpha2^+V\beta8.1/8.2^+CFSE^+CD4^+$ T cells in the ILN and paws of B47 group was significantly greater than that in mock group (Fig. 3, *A* and *B*). Moreover, $V\alpha2^+V\beta8.1/8.2^+CFSE^+CD4^+$ T cells in the ILN and paws showed lower CFSE fluorescence than those in the spleen of the B47 group and in the ILN and paws of the mock group (Fig. 3*C*). $V\alpha2^+V\beta8.1/8.2^+CFSE^-CD4^+$ T cells in the ILN of the B47 group showed higher expression of IFN-γ than those in the spleen of the B47 group or in the spleen and ILN of the mock group (Fig. 3*D*). This result indicated that transfer of B47 allowed $CD4^+$ T cells to accumulate in the draining lymph nodes and arthritic paws.

In Fig. 3*A*, the $V\alpha2^+V\beta8.1/8.2^-$ population also increased in these mice. In in vitro experiments using GFP-reported TCR α and β expression vectors (pMIG-TCRα and pMIG-TCRβ), the expression of the transduced TCR β-chain was rather unstable compared with that of the transduced TCR α-chain (K. Fujio, unpublished data). We suppose that this phenomenon was related to phenotypic allelic exclusion of the TCR β protein, because internal ribosomal entry site (IRES)-driven GFP expression was sustained despite a decrease of TCR β-chain expression. We think that at least a part of the $V\alpha2^+V\beta8.1/8.2^-$ population may have come from B47-transduced cells that lost TCR β expression.

We next explored the subpopulation of $CD11c^+$ DCs that can present arthritis-associated autoantigens. $CD11c^+$ DCs in ILN can classified into three groups, $CD11c^+CD11b^-CD8^-$ cells, $CD11c^+CD11b^+CD8^-$ cells, and $CD11c^+CD11b^-CD8^+$ cells (Fig. 4*A*). We compared the Ag presentation of total ILN $CD11c^+$ DCs, MACS-depleted $CD8^-CD11c^+$ DCs, and $CD11b^-CD11c^+$ DCs to

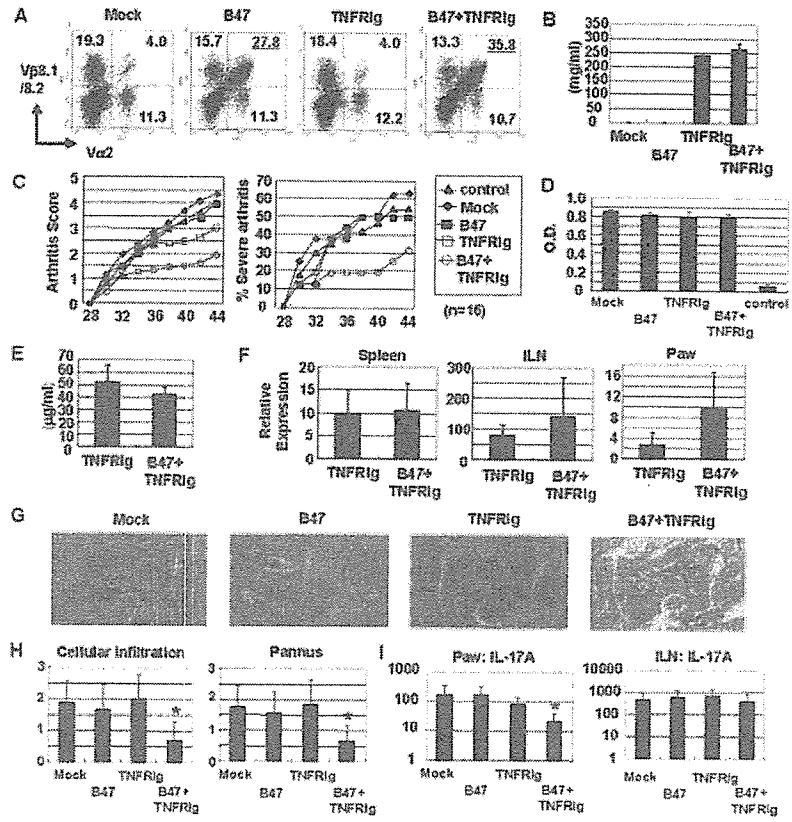

FIGURE 5. B47 and TNFRIg cotransduced $CD4^+$ T cells suppressed CIA progression. *A*, Retroviral gene transfer of B47 and TNFRIg in DBA1 splenocytes. The results shown are representative of three independent experiments. The cells were triple stained for $V\alpha2$, $V\beta8.1/8.2$, and CD4. CD4 gated dot plots are shown. *B*, TNFRIg concentrations in the culture supernatant of each experimental group. *C*, B47+TNFRIg-transduced $CD4^+$ cells containing 0.5–1 $\times$ 10^6 B47 and TNFRIg cotransduced cells were transferred to bCII-immunized mice just before the onset of arthritis (day 28). Data are shown as the mean of the clinical scores (*left panel*) and the incidence of severe arthritis (arthritis score $\geq$4) (*right panel*) at the indicated time points after the bCII priming (n = 16 per each group). ▲, PBS; ◆, Mock; ■, B47; □, TNFRIg; ○, B47+TNFRIg. *D*, Serum concentrations of anti-CII Ab in each experimental group. *E*, Serum concentrations of TNFRIg protein in the TNFRIg and B47+TNFRIg groups. *F*, Accumulation of the TNFRIg gene in arthritic paws. The relative expressions were determined by quantitative PCR. *G*, Histologic examination of each experimental group. *H*, The cellular infiltration and pannus invasion were scored for each experimental group. Data represent the mean values and SEs. * indicates a significant difference ($p < 0.05$) compared with mock group. *I*, The expressions of cytokine mRNAs were determined by quantitative PCR analysis. cDNAs were synthesized from the paws of each experimental group. Cytokine expressions in the paws (*left panel*) and ILN (*right panel*) are shown.

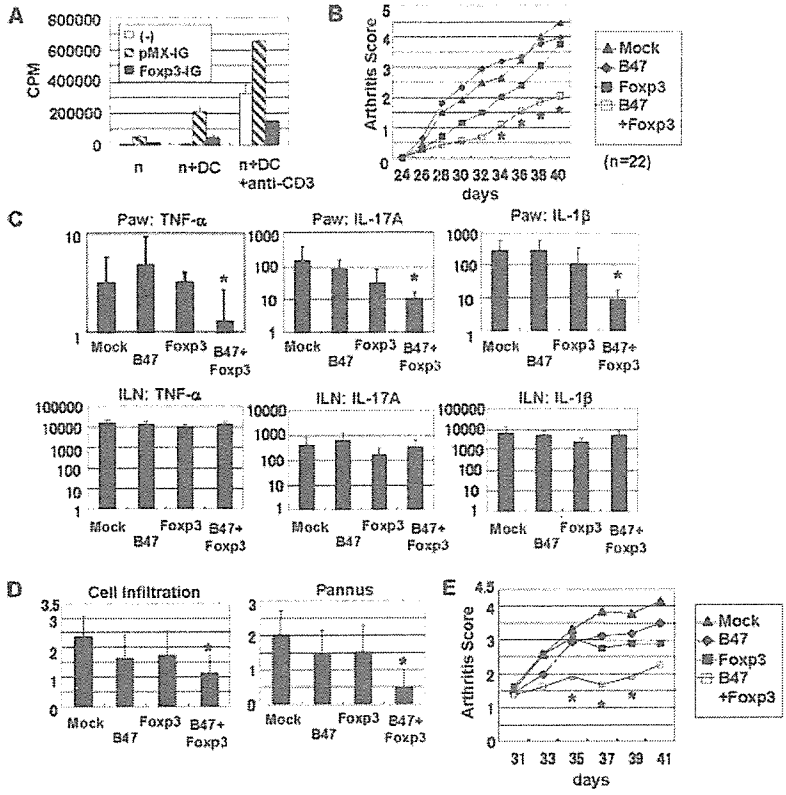

FIGURE 6. B47 and Foxp3 cotransduced T cells suppressed CIA and expression of pathogenic cytokines. *A*, Foxp3-reconstituted cells showed suppressive activity. A total of 1×10^5 naive $CD4^+$ cells (*n*) and 1×10^4 $CD11c^+$ cells (DC) in the presence or absence of anti-CD3 Ab (10 μg/ml) were cultured with no cells, 1×10^5 mock (pMX-IRES-GFP)-transduced $CD4^+$ cells, or Foxp3-transduced $CD4^+$ cells. Representative results of three experiments are shown. *B*, B47 and Foxp3 cotransduced T cells suppressed CIA progression. B47+Foxp3-transduced cells containing $0.5–1 \times 10^6$ of $V\alpha2^+V\beta8^+GFP^+CD4^+$ cells were transferred to bCII-immunized mice before the onset of arthritis (day 23). Equivalent numbers of mock, B47, and Foxp3-transduced cells were transferred as controls. Data are shown as the mean of clinical scores at the indicated time points after the bCII priming (n = 22 per each group). ▲, Mock; ◆, B47; ■, Foxp3; □, B47+Foxp3. *, A significant difference ($p < 0.05$) compared with the mock-transduced cells. *C*, The expressions of cytokine mRNAs were determined by quantitative PCR analysis. cDNAs were synthesized from the paws of each experimental group. Cytokine expressions in the paws (*upper panel*) and ILN (*lower panel*) are shown. *D*, The cellular infiltration and pannus invasion were scored for each experimental group. Data represent the mean values and SEs. *, A significant difference ($p < 0.05$) compared with the mock group. *E*, Reconstituted regulatory T cells were transferred to bCII-immunized mice just after the arthritis score reached approximately two points. Data are shown as the mean of clinical scores at the indicated time points after the bCII priming (n = 12 per each group). ▲, Mock; ◆, B47; ■, Foxp3; □, B47+Foxp3. *, A significant difference ($p < 0.05$) compared with the mock group.

B47-transduced $CD4^+$ cells. As shown in Fig. 4*B*, CD11b-depleted $CD11c^+$ DCs lost their autoantigen presentation to B47-transduced $CD4^+$ cells. We next examined the autoantigen presentation to CIIT-transduced $CD4^+$ cells. CD11b-depleted $CD11c^+$ DCs from ILN cells of CIA mice also lost their autoantigen presentation to CII-specific T cells (Fig. 4*C*). These results indicated that $CD11b^+CD11c^+$ DCs are important APCs in arthritis.

B47 plus TNFRIg-transduced cells suppressed CIA

We next attempted to use paw-directed B47-transduced $CD4^+$ cells as a vehicle for therapeutic molecules. We constructed a TNFRIg-expressing vector by fusing the murine p75 TNFR and Fc domain of IgG2a. TNFRIg-producing paw-directed cells were generated by triple gene transfer of B47 TCR and TNFRIg. We prepared three groups receiving controlled gene transfer of either mock vector, B47 alone, or TNFRIg alone. The clonotypic transduction efficiency was ~30% on average (Fig. 5*A*). Though we could not directly detect the transduction efficiency of TNFRIg, the TNFRIg protein concentrations in the culture supernatant of B47 plus TNFRIg-transduced $CD4^+$ cells were equivalent to those of TNFRIg (Fig. 5*B*). Therefore, the transduction efficiency of the *TNFRIg* gene was considered to be almost equal in these two groups.

These mock, B47, TNFRIg, or B47 plus TNFRIg transduced cells were i.v. transferred to CII-immunized mice via the tail vein just before the onset of arthritis at day 28. These mice groups were designated as mock group, B47 group, TNFRIg group, and B47 plus TNFRIg group, respectively. The arthritic score of B47 plus TNFRIg group was evidently suppressed compared with those of the mock and B47 groups (Fig. 5*C*). The arthritis score of the TNFRIg group was slightly suppressed. In terms of the incidence of severe arthritis, the B47 plus TNFRIg group clearly showed the lowest rate.

Accumulation of TNFRIg transcript in the paws was important for arthritis suppression

We next examined the kinetics of the transduced *TNFRIg* gene. Because the titers of anti-CII IgG at day 38 were equivalent in all experimental groups, TNFRIg did not directly affect the humoral immune response (Fig. 5*D*). The serum concentrations of TNFRIg protein in the B47 plus TNFRIg group were equivalent to those in the TNFRIg group at day 38 (Fig. 5*E*). This result indicated that the serum concentration of TNFRIg was not the main determinant of arthritis suppression in the B47 plus TNFRIg group.

We then checked the accumulation of TNFRIg transcript in the lymphoid organs and paws. The amount of TNFRIg transcript was

determined by real-time PCR of cDNAs from tissues of day 46 (Fig. 5*F*). The amount of TNFRIg transcript was equivalent between these two groups in the spleen and ILN. This result was consistent with the equality of the serum concentration of TNFRIg. In contrast, the amount of TNFRIg in the paws of the B47 plus TNFRIg group was significantly higher than that in the paws of the TNFRIg group. Therefore, local accumulation of the TNFRIg transcript suppressed arthritis in the B47 plus TNFRIg group.

On histologic examination, although the control groups showed severe inflammation, the B47 plus TNFRIg group showed only marginal inflammation (Fig. 5*G*). We graded mononuclear cell infiltration and cartilage/bone destruction by histopathological determination of the pannus invasion. Mononuclear cell infiltration and pannus formation were significantly suppressed in the B47 plus TNFRIg group (Fig. 5*H*).

We next evaluated the gene expression profiles of the paws and ILN by quantitative PCR. Among the cytokines important for arthritis progression, the expression of IL-17A was significantly suppressed in the paws, but not in ILN (Fig. 5*I*). In contrast, the expressions of TNF-α and IL-1β were not significantly suppressed in either the paws or ILN.

B47 plus Foxp3-transduced T cells suppressed CIA

We next tried to generate paw-directed regulatory T cells by cotransfer of B47 and Foxp3. It has previously been shown that retroviral transduction of Foxp3 confers a regulatory function onto $CD4^-$ T cells (5) (Fig. 6*A*). We generated B47 plus Foxp3-transduced cells (B47 plus pMX-Foxp3-IRES-GFP); three groups received controlled gene transfer of either the mock vector (pMX plus pMX-IRES-GFP), B47 alone (B47 plus pMX-IRES-GFP) or Foxp3 alone (pMX plus pMX-Foxp3-IRES-GFP). These gene-transduced cells were i.v. transferred to bCII-immunized mice before the onset of arthritis (day 23). These mice groups were designated as mock group, B47 group, Foxp3 group, and B47 plus Foxp3 group, respectively. B47 plus Foxp3 group showed a significant suppression in the development of arthritis (Fig. 6*B*). Foxp3 group showed only a marginal suppression of arthritis.

The titers of anti-CII Abs did not differ among these experimental groups (data not shown). When we evaluated the gene expression profiles of the paws and ILN by quantitative PCR, TNF-α, IL-17A, and IL-1β were found to be significantly suppressed (Fig. 6*C*). A suppressive cytokine, IL-10, was not up-regulated in the B47 plus Foxp3 group (data not shown). In ILN, the expression of TNF-α, IL-17A, and IL-1β was not suppressed in the B47 plus Foxp3 group (data not shown).

On histologic examination, although the control groups showed severe inflammation, the B47 plus Foxp3 group showed only marginal inflammation (data not shown). Mononuclear cell infiltration and pannus formation were suppressed in the B47 plus Foxp3 group (Fig. 6*D*). These results suggest that regulatory T cells at arthritic sites suppress bone destruction as well as inflammation. In contrast, Foxp3-transduced T cells without Ag specificity were not sufficient for arthritis suppression. Reconstituted regulatory cells also showed effective suppression when transferred after the onset of arthritis, at which time the average arthritic score reached around two points (Fig. 6*E*).

Discussion

We demonstrated the therapeutic efficacy of T cells transduced with an arthritis-associated TCR and a soluble and intracellular molecule. To obtain these paw-homing T cells, we cloned TCR from T cells expanded in the arthritic paws using a combination of single-cell sorting and TCR-SSCP. This method enabled us to identify the TCRs expanded in the inflamed tissues.

In response to the treatment with TNFRIg, T cells coexpressing B47 and TNFRIg exhibited suppressive activity associated with local accumulation. This result suggested that the main determinant of therapeutic efficacy in anti-TNF therapy is local accumulation, not serum concentration. Therefore, the conventional systemic administration of an anti-TNF drug that depends on serum concentration may not be a reasonable therapy. An elevated serum concentration is associated with systemic immunosuppression and high cost of treatment. Local injection of an anti-TNF drug is another approach to avoid a systemic suppressive effect (23, 24). However, this approach is not ideal due to the polyarthritic nature of RA. In contrast, T cells that produce TNFRIg and accumulate in the paws at the arthritic sites can reach multiple paws with reduced systemic effect.

The TCR transfer was also effective in the treatment with intracellular Foxp3 expression. Though suppression of murine arthritis with polyclonal regulatory T cells have been reported (25), the importance of T cell specificity has not been addressed. In the Foxp3 transfer experiment, Foxp3-expressing T cells with arthritis-associated TCR were effective. Once activated, regulatory T cells exhibit suppression in an Ag nonspecific manner (26). However, Ag specificity is important in the migration and expansion of regulatory T cells (27, 28). Indeed, Ag-specific regulatory T cells are efficient in suppressing various autoimmune diseases. The problem is how to obtain a sufficient amount of organ-Ag-specific regulatory T cells for therapeutic transfer. TCR and Foxp3 gene transfer is one possible approach to overcome this problem. Many mice spleens may be required to obtain 0.5–1.0 $\times$ 10^6 of $CD4^+CD25^+$ regulatory T cells, which is required to treat one mouse in the prior CIA treatment (25). In contrast, in vitro-expanded cells derived from a quarter of a spleen were sufficient to treat one mouse in our experiment.

Several groups have reported that regulatory T cells are accumulated in the joints of arthritis patients (29, 30). These joint-accumulating $CD4^+CD25^+$ T cells display a greater ability to suppress arthritis than blood $CD4^+CD25^+$ T cells. However, the precise role that these accumulating regulatory T cells play in the pathology of arthritis has not been clarified. Our experiments suggest that regulatory T cells in arthritic joints have the capacity to suppress pathogenic cytokine expression and bone destruction. Moreover, it is noteworthy that reconstituted regulatory T cells suppressed ongoing arthritis (Fig. 6*E*). There are several evidences that blocking of a specific inflammatory cascade ameliorates CIA after the onset. IL-10 and anti-IL-17A have been reported to inhibit ongoing CIA (31, 32). Our results suggested that regulatory T cells suppress arthritis by blocking the continuous inflammatory process. Therefore, regulatory T cells or Foxp3 therapy may be a feasible approach for established RA patients.

In therapeutic experiments for autoimmune diseases, use of a TCR without specificity to the disease-priming Ag can be an advantage. In our experiment, transfer of B47-transduced cells did not exacerbate arthritis. If CII-specific TCR is used for treatment, there is a possibility that the arthritis will be exacerbated due to enhancement of anti-CII immunity. This potential risk is important for the priming Ag-specific T cell-based treatment of other autoimmune diseases or human diseases that last for a significantly longer period than the diseases in mouse models. Indeed, it is necessary to clarify the specificity of these TCRs associated with arthritis or other autoimmune disorders before clinical application. Despite epitope screening with synthetic combinatorial peptide libraries in a positional scanning format (PS-SCL) (33), the precise autoantigen for B47 has not been determined.

We confirmed the clonal expansion of autoreactive $CD4^+$ T cells that were not specific to the priming Ag in the arthritic paws

of this mouse model. This result may have important implications for the treatment of autoimmune inflammation. Because $CD11c^+CD11b^+$DCs present both CII and an Ag recognized by B47, this DC population may be associated with copriming of B47 upon CII immunization.

In summary, we identified a TCR that is expanded in arthritic paws by a combination of TCR-SSCP and single-cell sorting. This arthritis-associated TCR that was not specific to the disease-priming Ag was used as a highly effective therapeutic vehicle for both soluble and intracellular molecules.

Acknowledgments

We thank Kazumi Abe and Yayoi Tsukahara for their excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

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Autoimmunity, May 2006; 39(3): 233–241

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Anti-aminoacyl-tRNA synthetase antibodies in clinical course prediction of interstitial lung disease complicated with idiopathic inflammatory myopathies

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Abstract

In the treatment of polymyositis and dermatomyositis (PM/DM), the complication of interstitial lung disease (ILD) is an important prognostic factor. It has been reported that autoantibodies against aminoacyl-tRNA synthetases (ARS) are strongly associated with ILD. The aim of this study is to examine the correlation between anti-ARS and the clinical course of ILD. We investigated 41 cases of PM/DM with ILD. The response of ILD to corticosteroids (CS) was determined according to the change in respiratory symptoms, image findings, and pulmonary function between, before and 2 months after the treatment. Anti-ARS (anti-Jo-1, PL-7, PL-12, EJ, OJ and KS) antibodies were screened with the RNA immunoprecipitation assay. In the stratification into ILD-preceding, simultaneous and myopathy-preceding types, anti-ARS antibodies were significantly frequent in the ILD-preceding type ($p < 0.05$). In the stratification into anti-ARS-positive and negative groups, the response of ILD to CS was significantly better in the positive group ($p < 0.05$). However, recurrence of ILD was significantly more frequent in the positive group ($p < 0.01$), and 2 year prognoses of pulmonary function (%VC and $\%DL_{CO}$) were not different between the two groups. In conclusion, screening of anti-ARS may be useful to predict late-onset myopathy in ILD-preceding patients and to predict the clinical course of ILD in PM/DM patients.

Keywords: *Polymyositis, dermatomyositis, autoantibody, anti-synthetase syndrome, corticosteroid, prognosis*

Abbreviations: *$A\text{–}aD_{O2}$, alveolar–arterial oxygen tension difference; ADM, amyopathic dermatomyositis; ARS, aminoacyl-tRNA synthetases; CK, creatine kinase; CS, corticosteroids; DL_{CO}, diffusing capacity of the lung for carbon monoxide; DM, dermatomyositis; IBM, inclusion body myositis; IIM, idiopathic inflammatory myopathies; ILD, interstitial lung disease; MAA, myositis-associated antibodies; MSA, myositis-specific antibodies; PM, polymyositis; PSL, prednisolone; SAILD, seropositive amyopathic interstitial lung disease; SRP, signal recognition particle; VC, vital capacity*

Introduction

Idiopathic inflammatory myopathies (IIM) including polymyositis and dermatomyositis (PM/DM) are recognized as a heterogenous category of various disease subsets, among which clinical properties, responses to treatment, and prognoses are different [1,2]. Six types of autoantibodies against aminoacyl-tRNA synthetases (ARS) have been identified in patients with PM/DM and/or interstitial lung disease (ILD): anti-Jo-1, PL-7, PL-12, EJ, OJ and KS antibodies [3]. Anti-ARS-positive patients develop common characteristic symptoms known as anti-synthetase syndrome, such as myopathy, ILD, Raynaud's phenomenon, polyarthritis, high fever,

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ISSN 0891-6934 print/ISSN 1607-842X online © 2006 Taylor & Francis
DOI: 10.1080/08916930600622884

and mechanic's hand [2]. Since an association between anti-ARS and specific MHC alleles has been also reported [2,4], anti-ARS-positive patients are considered to form a homogeneous disease subset.

The diagnosis and management of ILD is important in the treatment of IIM, since IIM patients with ILD have poorer prognoses than those without ILD [5,6]. It has been reported that anti-ARS antibodies are strongly associated with ILD [7,8]. Love et al. [2] reported that the recurrence of myopathy was frequent in anti-ARS-positive PM/DM patients. If anti-ARS-positive patients have similar clinical properties of ILD, information on anti-ARS may be useful in the treatment of ILD complicated with IIM. In the present study, we examined whether these antibodies are predictive of the clinical course of ILD in IIM patients.

Patients and methods

Diagnostic criteria

We examined 140 patients who were suspected of having IIM or related diseases and consulted our Department in Kyoto University Hospital from May 2001 to September 2004. Patients satisfying at least three of the five Peter–Bohan's criteria were diagnosed as having PM/DM [1], while obvious secondary myopathies were excluded. Amyopathic dermatomyositis (ADM) was diagnosed, when a patient fulfilled both of the following items: (1) heliotrope rash and/or Gottron's sign and (2) no muscle weakness with normal serum muscle enzyme levels for the entire observation period. Inclusion body myositis (IBM) was diagnosed based on Calabrese–Mitsumoto–Chou's criteria [9]. "Seropositive amyopathic interstitial lung disease (SAILD)" was diagnosed using our original criteria, when a patient fulfilled all of the following items: (1) positive anti-ARS, (2) ILD, (3) no muscle weakness with normal serum muscle enzyme levels for the entire observation period and (4) no specific skin symptoms during the observation period. Serum muscle enzyme levels were considered elevated if creatine kinase (CK) levels were 200 IU/l or above at least twice consecutively, while elevations caused by other factors were excluded. ILD was diagnosed by radiologists according to X-ray photos and/or CT images.

Evaluation of clinical course

Data were collected from medical records, retrospectively. The standard protocol for high dose corticosteroids (CS) was defined as an initial dosage of at least 30 mg/day of prednisolone (PSL) for 2–4 weeks and tapering by less than 10% of the former dosage every 1–2 weeks. In the analysis of the response to CS, patients treated with less than 30 mg/day of PSL or the equivalent dosage of other CS were excluded. If a patient had received plural courses of high dose CS, the episode with the maximum dosage of CS was adopted. To analyze the response of ILD to CS, we compared respiratory symptoms, image findings, and pulmonary function between, before and 2 months after the initiation of high dose CS. The changes in the three items were graded according to five ranks as indicated in Table I. In the evaluation of pulmonary function, the item most changed among %VC, $\%DL_{CO}$ and $A\text{–}aD_{O2}$ was chosen. An ILD patient was defined as a responder to CS, if at least one of the three items were remarkably improved or at least two of the three were slightly improved. Otherwise the patient was defined as a non-responder. A patient who had died of ILD within 8 weeks was defined as a non-responder. In the analysis of the response of myopathy to CS, patients whose serum CK levels were less than 200 IU/l at the initiation of high dose CS were excluded. A patient with myopathy was defined as a responder to CS, if serum CK levels decreased below 200 IU/l by the eighth week from the

Table I. Evaluation criteria for response of ILD to treatment.

Grade	Respiratory symptoms	Image findings	Pulmonary function
Remarkably improved	Improved in Fletcher–Hugh–Jones' criteria	Obviously improved X-ray photos	$\Delta\%VC \geq +20\%$ $\Delta\%DL_{CO} \geq +20\%$ $\Delta A\text{–}aD_{O2} \leq -20$ Torr
Slightly improved	Improved cough and/or dyspnea	Improved CT images	$\Delta\%VC \geq +10\%$ $\Delta\%DL_{CO} \geq +10\%$ $\Delta A\text{–}aD_{O2} \leq -10$ Torr
No change	No change	No change	$\lvert\Delta\%VC\rvert < 10\%$ $\lvert\Delta\%DL_{CO}\rvert < 10\%$ $\lvert\Delta A\text{–}aD_{O2}\rvert < 10$ Torr
Slightly worsened	Worsened cough and/or dyspnea	Worsened CT images	$\Delta\%VC \leq -10\%$ $\Delta\%DL_{CO} \leq -10\%$ $\Delta A\text{–}aD_{O2} \geq +10$ Torr
Remarkably worsened	Worsened in Fletcher–Hugh–Jones' criteria	Obviously worsened X-ray photos	$\Delta\%VC \leq -20\%$ $\Delta\%DL_{CO} \leq -20\%$ $\Delta A\text{–}aD_{O2} \geq +20$ Torr

ILD, interstitial lung disease; VC, vital capacity; DL_{CO}, diffusing capacity of the lung for carbon monoxide; $A\text{–}aD_{O2}$, alveolar–arterial oxygen tension difference.

initiation of high dose CS. Otherwise the patient was defined as a non-responder. Effects of methylprednisolone pulse therapy were not analyzed in this study.

The patients were classified into four types according to the course of ILD: (1) single rush then static or very slow, (2) recurrent, (3) static or very slow and (4) rush and fatal. "Rush" means that the patients needed high dose CS. "Recurrent" means that the patients needed plural courses of high dose CS or additional immunosuppressants to treat the flare-up of ILD. The patients were also classified into four types according to the course of myopathy: (1) monocyclic, (2) recurrent, (3) continuous and (4) hypoenzymatic. Remission was defined as continuously low serum CK levels (less than 200 IU/l) for at least 3 months. Monocyclic type means that a remission was observed within 12 months after the first high dose CS, and the serum CK levels did not increase again. Recurrent type means that the serum CK levels increased again to 200 IU/l or above after a remission. Continuous type means that a remission was not observed within the first 12 months. Hypoenzymatic type means that the serum CK levels stayed below 200 IU/l for the entire observation period, although, other items of Peter–Bohan's criteria were compatible and the patient was diagnosed as having PM/DM. The hypoenzymatic-type patients and patients with short observation periods (less than 6 months) were excluded from the analysis.

RNA immunoprecipitation assay

Autoantibodies were screened with the RNA immunoprecipitation assay established by Lerner and Steitz [10] and modified by Forman et al. [11]. Briefly, HeLa cells were sonicated, and centrifuged supernatants were incubated with protein A-Sepharose beads coated with the patient's IgG. Then, nucleic acids were extracted from the Sepharose particles, separated by electrophoresis in 8 M urea–10% polyacrylamide gels, and visualized with silver stain. According to the specific band patterns, anti-Jo-1, PL-7, PL-12, EJ, OJ, KS, SRP and U1-RNP were identified. Anti-Ku can be screened with this assay, since it gives high molecular weight smear patterns (Figure 1) [12]. Anti-Ku was further confirmed with the protein immunoprecipitation assay using ^{35}S-methionine-labeled HeLa cells [10,12].

Statistical analysis

A matrix analysis was performed using the χ^2 test. Onset intervals, %VC and $\%DL_{CO}$ were compared with the Student-*t* test. *p* values less than 0.05 were considered significant.

Results

Profiles of the patients

We selected 88 out of 140 patients who were suspected of having IIM or related diseases and consulted our department (Table II). Seventy-four probable or definite PM/DM patients were entered into the study according to Peter–Bohan's criteria [1], while five patients satisfying at least three of the five criteria were considered secondary myopathy and excluded: two with hypothyroidism, two with graft vs. host disease-associated myopathy, and one with drug-induced myopathy. Six ADM patients were selected as described in patients and methods. To define "amyopathic" status, Euwer–Sontheimer's criteria required only a lack of muscle weakness and normal serum muscle enzyme levels, and allowed for minor findings of myopathy in the electromyogram or muscle biopsy [13]. Accordingly, five of the six ADM patients showed myogenic patterns in the present study, and three of the four biopsied patients showed some degenerative muscle fibers, however, without cellular infiltration. There was a biopsy-confirmed IBM case. We also selected seven anti-ARS-positive ILD patients

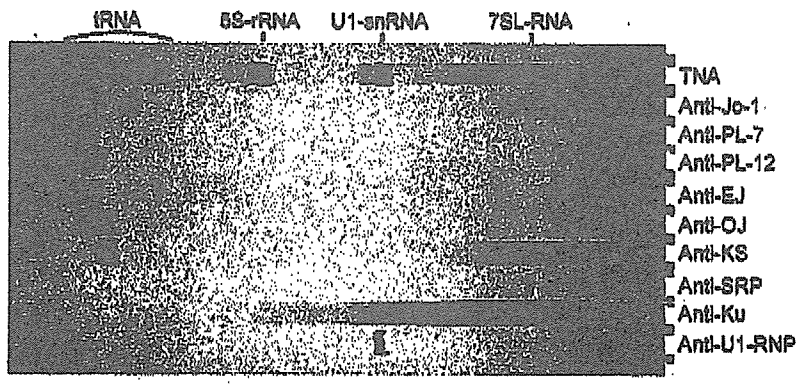

Figure 1. RNA immunoprecipitation assay. Each of the anti-ARS antibodies precipitates plural bands, mainly because there are several base-pair lengths of tRNA corresponding to an amino acid. Smear bands seen in the anti-Jo-1 and anti-KS lanes are artifacts, which appear when an overdose of cell lysate has been used. TNA, total nucleic acids.

Table II. Profiles of 86 IIM patients.

Subgroup	*N*	Age (Median)	M:F	Peter-Bohan's criteria	Max CK (IU/l)	ILD	*N*	Max KL-6 (U/ml)
PM/DM	74	25–83 (55)	16:58	3–5	3361 ± 4104	(+)	41	2052 ± 1997
						(−)	33	342 ± 155
ADM	6	31–66 (48)	1:5	1–2	98 ± 52	(+)	4	2478 ± 1495
						(−)	2	Not tested
IBM	1	67	1:0	3	1268	(−)	1	351
SAILD	7	47–70 (64)	3:4	0	105 ± 43	(+)	7	2725 ± 1479
Total	88	25–83 (55)	21:67	0–5	2832 ± 3928	(+)	52	2186 ± 1882
						(−)	36	342 ± 152

PM/DM, polymyositis/dermatomyositis; ADM, amyopathic dermatomyositis; IBM, inclusion body myositis; SAILD, seropositive amyopathic interstitial lung disease; ILD, interstitial lung disease; CK, creatine kinase. Laboratory data are means ± SD.

without muscle lesions, as having "seropositive amyopathic interstitial lung disease (SAILD)".

Myositis-specific antibodies (MSA) and myositis-associated antibodies (MAA) were screened with the RNA immunoprecipitation assay (Figure 1). The prevalence of MSA/MAA in each subgroup of IIM is shown in Figure 2. Anti-ARS antibodies were detected in 28% of PM/DM cases, among which anti-Jo-1 and anti-EJ predominated. In the PM/DM patients with ILD, the frequency of anti-ARS was as high as 49%, reflecting the strong association between anti-ARS and ILD. One patient was positive for both anti-Jo-1 and anti-PL-7. Such a case is very rare, although, there has been a report of a patient positive for anti-Jo-1 and anti-OJ [14]. All of the ADM and IBM patients were negative for MSA/MAA.

To analyze the clinical properties of anti-ARS-positive patients, we stratified the 74 PM/DM patients into anti-ARS-positive and negative groups (Figure 3). The prevalence of ILD was as high as 95% in the anti-ARS-positive PM/DM patients, consistent with previous reports [2,15]. ILD and mechanic's hand occurred significantly more frequently in the anti-ARS-positive group than in the negative group. Raynaud's phenomenon, polyarthritis, and high fever were also frequent in the positive group, although they were not rare in the negative group and a significant difference was not detected. When the patients were stratified into a group positive for either anti-ARS or anti-U1-RNP, or a group negative for both, a significant difference was detected for Raynaud's phenomenon ($p = 0.002$, data not shown).

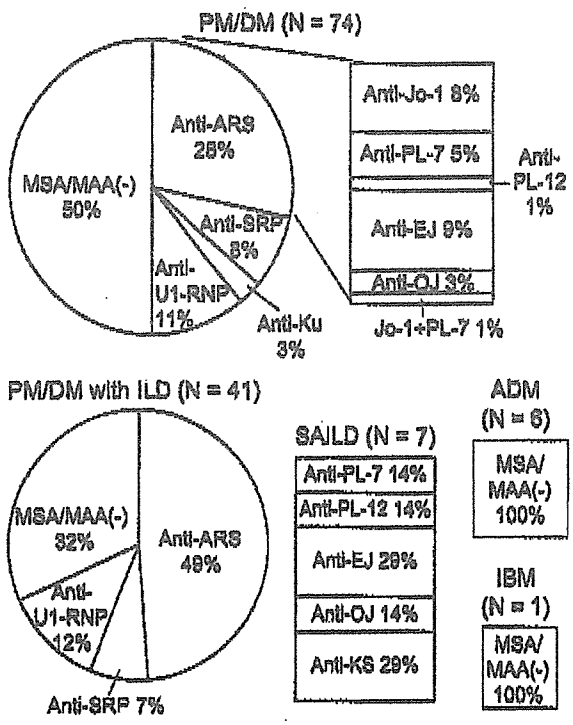

Figure 2. Prevalence of MSA/MAA in each subgroup of IIM.

Pattern of onset

In some PM/DM-ILD patients, the onset of ILD precedes that of myopathy. We stratified the 41 PM/DM-ILD patients into three types according to the pattern of onset (Table III) and examined the frequency of anti-ARS antibodies in each type (Figure 4(A)). Anti-ARS antibodies were frequent in type I (ILD-preceding)

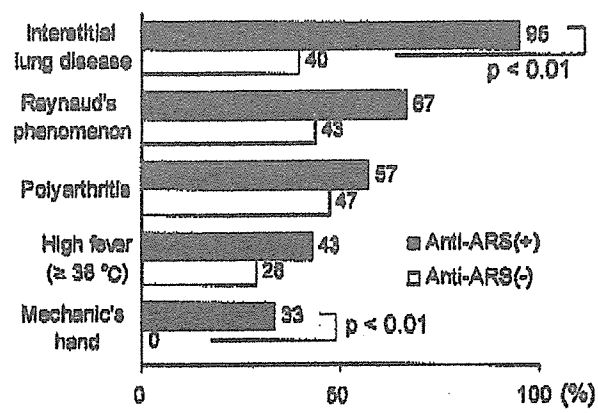

Figure 3. Prevalence of characteristic symptoms of anti-synthetase syndrome. The 74 PM/DM patients were stratified into anti-ARS-positive ($n = 21$) and negative ($n = 53$) groups, and the incidence of each symptom was compared between the two groups. $p = 0.001$, 0.071, 0.439, 0.228 and 0.001 for ILD, Raynaud's phenomenon, polyarthritis, high fever and mechanic's hand, respectively, with the χ^2 test.

Table III. Stratification of 41 PM/DM-ILD patients according to the pattern of onset.

	N (%)	Onset intervals (median)
Type I (ILD-preceding)	13 (32)	ILD → myopathy: 132–4 months (11)
Type II (Simultaneous)	18 (44)	Defined as "within 1 month"
Type III (Myopathy-preceding)	10 (24)	Myopathy → ILD: 2–137 months (24.5)
Total (PM/DM with ILD)	41 (100)	−132– + 137 months (0)

PM/DM, polymyositis/dermatomyositis; ILD: interstitial lung disease.

and rare in type III (myositis-preceding) with statistical significance. We also analyzed the distribution of the onset intervals between ILD and myopathy, stratifying the patients into anti-ARS-positive and negative groups (Figure 4(B)). ILD preceded myopathy by 14 months on average in the anti-ARS-positive group, while myopathy preceded ILD by 11 months in the negative group, with a significant difference.

Clinical course of ILD

To evaluate the response of ILD to CS, we prepared criteria described in patients and methods. The response to CS was significantly better in the anti-ARS-positive group than in the negative group (Figure 5(A)). In the stratification according to the pattern of onset, type I, II and III patients tended to show better, intermediate and worse responses, respectively, although without statistical significance (Figure 5(A)). To analyze the course of ILD, we classified the patients into four types as described in

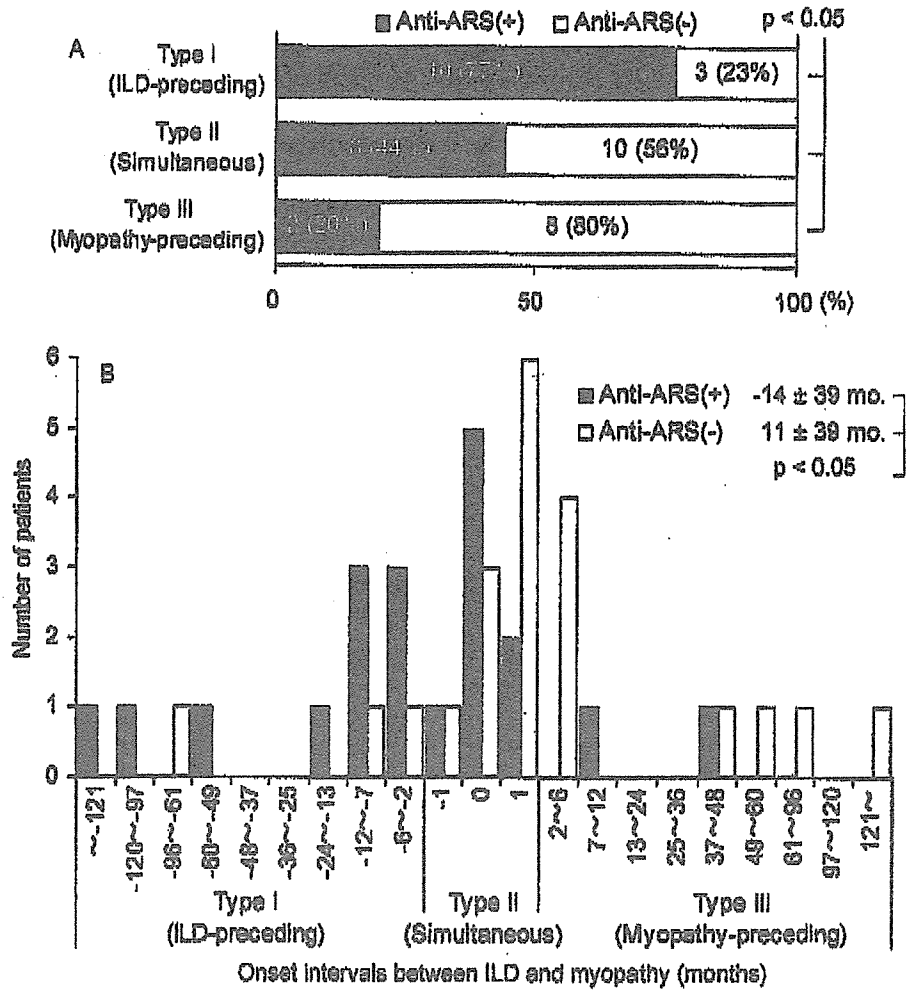

Figure 4. Relationship between anti-ARS antibodies and pattern of disease onset in 41 PM/DM-ILD patients. (A) Frequency of anti-ARS in each type. $p = 0.023$ with the χ^2 test using a 3 × 2 matrix. (B) Onset intervals between ILD and myopathy in anti-ARS-positive and negative patients. Values in the upper-right corner are means ± SD of the onset intervals. $p = 0.046$ between the two groups with the Student-*t* test.

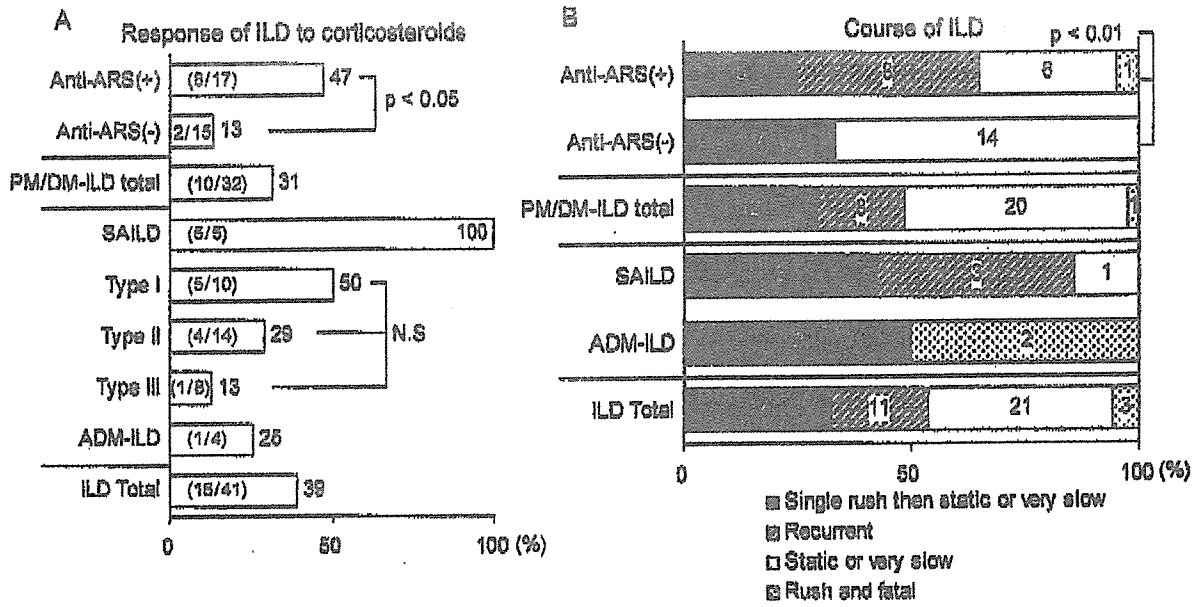

Figure 5. Clinical course of ILD and response to CS. (A) Percentages of responders to responders + non-responders are indicated. Some patients were excluded from the analysis: patients treated with less than 30 mg/day of prednisolone and patients with unknown details. $p = 0.040$ between the anti-ARS-positive and negative groups with the χ^2 test. $p = 0.224$ among the types I, II and III with the χ^2 test using a 3×2 matrix. (B) According to the course of ILD, patients were classified into four types. $p = 0.006$ between the anti-ARS-positive and negative groups with the χ^2 test using a 2×4 matrix.

patients and methods. Recurrent type was remarkably frequent in the anti-ARS-positive group, while not observed in the negative group (Figure 5(B)). Static type predominated in the negative group. There was a significant difference in the course of ILD between the anti-ARS-positive and negative groups. The tapering speeds of the dose of CS were not significantly different between the two groups (data not shown).

We examined the prognosis of pulmonary function in PM/DM-ILD patients. In the anti-ARS-positive group, %VC and $\%DL_{CO}$ 2 years from the onset of ILD were 82 ± 17 and $52 \pm 20\%$ (means ± SD), respectively, and were not significantly different from values, 93 ± 26 and $62 \pm 18\%$, in the negative group ($p = 0.238$ and 0.738). In the assessment of the prognosis of life, there was no significant difference in the 2 year survival rate between the anti-ARS-positive (95%) and negative groups (88%).

Clinical course of myopathy

The response of myopathy to CS was evaluated according to the change in serum CK levels. Seventy-four PM/DM patients were stratified into anti-ARS-positive and negative groups. The anti-ARS-positive group showed a better response than the negative group, although a significant difference was not detected (Figure 6(A)). To analyze the course of myopathy, we classified the patients into monocyclic, recurrent and continuous types as described in patients and methods. Recurrent type predominated in the anti-ARS-positive group, while monocyclic type predominated in the negative group, although without a significant difference (Figure 6(B)). IBM and anti-SRP-positive PM/DM patients are known to develop refractory myopathy [9,16]. The anti-SRP-positive patients showed a significantly poorer response to CS than the anti-SRP-negative patients (Figure 6(A)), and continuous-type myopathy was significantly more frequent in the anti-SRP-positive group (Figure 6(B)). The IBM patient also showed a poor response and continuous-type myopathy.

Discussion

The prevalence of ILD in PM/DM was as high as 53% in the present study (Table II), although it has been about 30% in European studies [6,17]. Since it has reached 50% in other Japanese studies [15,18], the discrepancy can be explained by ethnic difference.

Non-radioactive RNA immunoprecipitation assays can be quickly carried out with minimal loss of sensitivity and specificity to detect autoantibodies against ribonucleoproteins [11], and have been applied to anti-ARS and anti-SRP [15,19–21]. This method is not available for anti-Mi-2 and anti-PM/Scl, which are also myositis-specific or associated [22,23], so that some patients positive for anti-Mi-2 or anti-PM/Scl might be classified into the MSA/MAA-negative group in the present study. However, that might not have affected the results, since anti-Mi-2-positive patients do not tend to develop ILD [24] and anti-PM/Scl antibodies are very rare in Japanese patients [15].

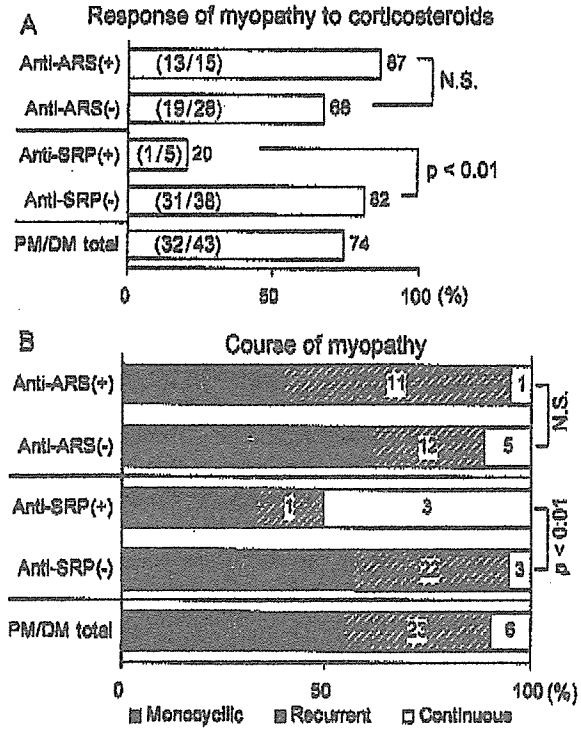

Figure 6. Clinical course of myopathy. (A) The response of myopathy to CS was evaluated as described in patients and methods. Percentages of responders to responders + non-responders are indicated. Some patients were excluded from the analysis: patients treated with less than 30 mg/day of prednisolone, patients whose serum CK levels were less than 200 IU/l at the initiation of high dose CS, and patients with unknown details. $p = 0.178$ between the anti-ARS-positive and negative groups, $p = 0.009$ between the anti-SRP-positive and negative groups, with the χ^2 test. (B) According to the course of myopathy, patients were classified into three types. Four hypoenzymatic-type patients and six patients with short observation periods were excluded. $p = 0.096$ between the anti-ARS-positive and negative groups, $p = 0.002$ between the anti-SRP-positive and negative groups, with the χ^2 test.

When we stratified the PM/DM-ILD patients into three types according to the pattern of onset (Table III), 32% of PM/DM-ILD patients were type I (ILD-preceding). Douglas et al. [25] have also reported that 30% of PM/DM-ILD patients were type I, although the classification criteria were unclear. We demonstrated that anti-ARS antibodies were significantly associated with type I (Figure 4(A)). An important concern is when the anti-ARS antibodies have become positive in type I patients, or in other words, whether there is a patient who has shifted from SAILD to PM/DM-ILD. Since MSA/MAA are usually tested after the onset of myopathy, it is rare that anti-ARS antibodies are checked during the period of ILD in type I patients. In the present study, one of the type I patients had been proven anti-Jo-1-positive at the initial manifestation of ILD, and 8 months later, developed definite PM. Two similar cases were reported in 1984 [8]. Apparently, anti-ARS antibodies can be a predictive factor of late-onset myopathy in type I cases. Another concern is that type I patients tend to be misdiagnosed as having rheumatoid arthritis due to the high frequency of polyarthritis (57%, Figure 3) and positive rheumatoid factors (30%, data not shown) in anti-ARS-positive patients. Indeed, three of our 13 type I patients were initially diagnosed as having rheumatoid arthritis (with ILD) and treated with anti-rheumatic drugs. The early screening of anti-ARS is important in such cases.

Takizawa et al. [26] evaluated the response of ILD to treatment in DM patients according to the changes in respiratory symptoms, X-ray findings, and pulmonary function. Marie et al. [6] used similar criteria. Since those criteria were not precisely designed, we prepared original criteria (Table I). Marie et al. [6] reported a better response of ILD to treatment in anti-Jo-1-positive PM/DM patients than in negative patients, however, without a significant difference. This might be because other anti-ARS antibodies had not been examined in their study, since we observed a significantly better response of ILD to CS in patients with any anti-ARS antibodies than in patients negative for anti-ARS (Figure 5(A)). The reason why the response to CS was better in the type I patients than in the type III patients (Figure 5(A)) is explained by the association between type I and anti-ARS (Figure 4(A)). However, as to the course of ILD, recurrence was remarkably frequent in the anti-ARS-positive group, while not observed in the negative group (Figure 5(B)). Moreover, the pulmonary function 2 years from the onset of ILD was almost the same in both groups. Consequently, although the anti-ARS-positive patients showed a good response to CS, they tended to suffer from recurrent ILD, and the prognosis did not differ from that of the anti-ARS-negative patients.

In the study of Love et al. [2], frequent recurrence and a moderate response of myopathy to CS was reported in anti-ARS-positive PM/DM patients, although the definition of the response to treatment was unclear. In the present study, frequent recurrence of myopathy was confirmed, whereas the response to CS was relatively good in the anti-ARS-positive group (Figure 6). We also found that anti-ARS-positive patients with a good response of ILD to CS tended to show a good response of myopathy ($p = 0.121$) and that anti-ARS-positive patients with recurrent ILD developed recurrent myopathy ($p = 0.003$, data not shown).

Ten SAILD cases were first reported by Friedman et al. [27], and seven cases were analyzed in the present study. Although this subset can be recognized as type I (ILD-preceding) "before late-onset myopathy", some patients stay free from myopathy for a long period: 6.8 years in our study and 13.5 years in the former study. In the present study, characteristic symptoms of anti-synthetase syndrome were observed in the SAILD patients (data not shown), and the

clinical properties such as a response to CS and a course of ILD were similar to the anti-ARS-positive patients with myopathy (Figure 5). Friedman et al. also reported a good response of ILD to CS, distinct from other subsets of idiopathic ILD. After Friedman's report, Hirakata et al. [21] discovered anti-KS antibodies in three SAILD cases. Consistently, anti-KS antibodies were detected only in SAILD patients in the present study (Figure 2). Although anti-KS are not "myopathy"-specific antibodies, anti-KS-positive patients manifested characteristic symptoms of anti-synthetase syndrome in both Hirakata's and our studies. These clinical properties support that anti-KS should also be included in the anti-ARS group.

Considering the clinical significance of anti-ARS in PM/DM and ILD shown in the present study, the relationship among disease categories can be shown as Figure 7. We speculate that anti-ARS antibodies may be primarily related with ILD, and the relationship with myopathy comes next, followed by the other characteristic symptoms. The incidence of SAILD in idiopathic ILD may be more than 6%, since the frequencies of anti-Jo-1 and anti-KS in idiopathic ILD have been reported as 3%, respectively [8,21]. A large-scale prospective study is required.

There is increasing interest in ADM, since a proportion of ADM patients develop acute and fatal ILD [28,29]. Sontheimer [30] stated that such patients are rare but disproportionately frequent in Japan. In the present study, two of the four ADM-ILD patients developed treatment-resistant ILD and died within 3 months (Figure 5). However, Euwer–Sontheimer's criteria cannot cover such catastrophic cases, since they require a 2-year observation considering the possibility of late-onset myopathy. Therefore, we diagnosed ADM if a patient showed no sign of myopathy "for the entire observation period" instead of "for 2 years". It has been postulated that anti-Jo-1 is not associated with ADM [31], and all of our six ADM patients were negative for not only anti-Jo-1 but also other MSA/MAA (Figure 2). Such information is also useful to understand the disease subset.

In conclusion, screening of anti-ARS antibodies may be useful to predict late-onset myopathy in ILD-preceding patients and to predict the clinical course of muscle and lung diseases in IIM patients. Although anti-ARS-positive patients show a good response to CS, the recurrence of disease is frequent. Slow and careful tapering of the dose of CS is recommended, and early indications of additional immunosuppressants should also be considered.

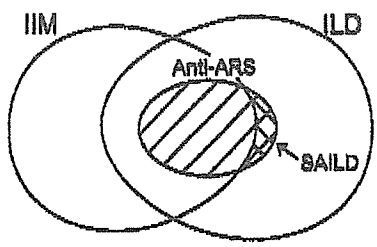

Figure 7. Relationship among disease categories.

Acknowledgements

This work was supported by a grant for studies on the prophylaxis and treatment of immunologic and allergic diseases from the Ministry of Health, Labour and Welfare of Japan.

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doi: 10.1111/j.1365-3083.2006.01747.x

Anti-p97/VCP Antibodies: An Autoantibody Marker for a Subset of Primary Biliary Cirrhosis Patients with Milder Disease?

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Received 27 September 2005; Accepted in revised form 16 February 2006

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Abstract

We previously reported that 12.5% of primary biliary cirrhosis (PBC) sera reacted with a 95 kDa cytosol protein (p95c) that was subsequently identified as a p97/valosin-containing protein (VCP). The clinical features and course of the six anti-p97/VCP-positive PBC patients with Scheuer's stage 1 and 2 liver biopsies were monitored for an average of 15 years. This group was compared with 50 PBC patients that did not have detectable anti-VCP. Autoantibodies to a full-length recombinant p97/VCP were assayed by immunoprecipitation. All six PBC patients with anti-VCP had antibodies to the mitochondrial pyruvate dehydrogenase complex-E2 antigen as measured by an addressable laser bead immunoassay. The first was a male with no evidence of liver failure that died of cerebral infarction at the age of 85. The second was a 73-year-old female with Hashimoto's thyroiditis who has remained clinically stable without ursodeoxycolic acid (UDCA) treatment. Although the third had no HCV antibodies, he developed hepatocellular carcinoma at the age of 76 and died of renal failure at 78. The fourth was a 50-year-old female who remained clinically stable during follow-up and the fifth with Hashimoto's thyroiditis and stable liver function following UCDA treatment. The sixth was a male patient presenting a mild clinical course. The clinical course of these patients was in contrast to the 50 comparison group PBC patients who did not have anti-p97/VCP. As the six PBC patients with anti-p97/VCP antibodies had slowly progressive liver disease and no mortality related to autoimmune liver disease, our observations suggest that this autoantibody might be an indicator of a favourable prognosis.

Introduction

Patients with autoimmune diseases produce a variety of autoantibodies [1]. Antimitochondrial antibodies (AMA), are a serological hallmark and are found in 85% of the primary biliary cirrhosis (PBC) patients, but they are not considered to be pathogenic [2, 3]. In addition to AMA, other autoantibodies that are detected in PBC patients include anticentromere [4], anti-SP100 [5], antibodies to nuclear envelope components gp210, p62 complex and Tpr [6–8] and the high mobility group (HMG) proteins HMG-1 and -2 [9]. Except for anti-HMG-1 and -2, the prevalence of these antibodies in PBC sera is less than 30%. Although there is no evidence that these antibodies are responsible for pathogenesis, they are considered helpful in the diagnosis of PBC in the subset AMA-negative PBC patients [7]. Of interest in the context of the present study, antibodies to the nuclear pore complex proteins gp210 and/or p62 are reported to be a marker for progressive PBC and a relatively poor prognosis [10, 11].

In 1990, Klein and Berg [12] reported that the anti-M9 antibodies were a marker for mild and slowly progressive PBC. In that study, the anti-M9 autoantigen was estimated to have a molecular mass of 97 kDa by immunoblot and was reported to be identical to glycogen phosphorylase. Subsequently, we studied a PBC serum and immunoprecipitated a cytosolic 95 kDa autoantigen (p95c) from HeLa cell lysates [13]. As the p95c autoantigen was not detected by immunoblot, we concluded that p95c was not identical to M9 and that anti-p95c antibodies reacted with a conformational epitope of the cognate cytosolic antigen [14].

More recently, we became aware that p97/VCP (valosin-containing protein), a member of the extended AAA ATPase family of proteins, plays an important role in homotypic assembly of endoplasmic reticulum (ER), the Golgi complex and the nuclear membrane [15, 16]. In addition, it has been shown that p97 also participates in endoplasmic reticulum-associated degradation (ERAD) of aberrant proteins in the secretory pathway [17]. Based on observations that human anti-p95c antibodies inhibited *in vitro* nuclear assembly in a *Xenopus* oocyte assay and immunoprecipitated human recombinant p97/VCP, we concluded that p95c was identical to p97/VCP [18]. In the present study, we report on the clinical significance of anti-p97/VCP found in PBC patients that were followed for an average of 15 years. Our study suggests that anti-p97/VCP is a marker for a more favourable clinical course in PBC patients.

Materials and methods

Patients. In an earlier study, 30 PBC patients were identified with anti-p95c/p97/VCP autoantibodies, but only 13 were initially available for detailed analysis [18], of which, six patients had liver biopsies. However, the detailed histopathological report could not be retrieved in one patient, and she was excluded from the analysis. The remaining five patients were followed for a longer period (9–21 years, mean 14 years), and adequate sera and detailed clinical and laboratory data were available. In addition, one patient (no. 14) [18] became available for this study after the patient's consent was received from the attending physician. One patient with a Scheuer stage 1 biopsy was symptomatic. As a comparison group, we included a random cohort of 50 PBC patients from a speciality hepatology clinic who had been followed for a minimum of 1 year. All patients were evaluated routinely as dictated by standards of clinical practice and had documented clinical features and outcomes.

Immunoassays: immunodiffusion, immunoprecipitation and addressable laser bead immunoassay. The Ouchterlony double immunodiffusion method was used to screen for anti-p97/VCP antibodies [13, 14, 18]. Briefly, the prototype serum and 20 mg of rabbit thymus extract or rat liver supernatant were used as antigen source to detect autoantibodies as previously described [13, 14, 18].

HeLa cells were cultured in monolayers in methionine-free medium, radiolabelled for 20 h with 370 kBq/ml of [^{35}S]-methionine (ICN Radiochemicals, Irvine, CA, USA) and reactive proteins were immunoprecipitated from the cell lysates and identified as previously published [19, 20].

The cDNA representing the full-length VCP (p97/VCP: accession number *CAA78412*; a gift from Dr Graham Warren, Yale University, New Haven, CT, USA) was used as a template for the *in vitro* transcription and translation reactions (TnT, Promega, Madison, WI, USA) in the presence of [^{35}S]-methionine as previously described [21, 22]. TnT incubations were conducted at 30 °C for 1.5–2 h, and the presence of the translation products was confirmed by subjecting 2–5 µl samples to SDS-PAGE and analysis by autoradiography. The *in vitro* translated products were then used as antigen source. Immunoprecipitation (IP) reactions were prepared by combining 100 µl 10% protein A-Sepharose beads (Sigma, St Louis, MO, USA, catalogue #p-3391), 10 µl human serum, 500 µl NET2 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet p-40, 0.5% deoxycholic acid, 0.1% SDS, 0.02% sodium azide) and 5–10 µl of labelled recombinant protein obtained from the TnT reaction. After 1 h of incubation at 4–8 °C, the beads were washed five times in NET2, and the proteins eluted in 10 µl of SDS-PAGE sample buffer. The reactive proteins were then analysed by 10 or 12.5% SDS-PAGE.

An addressable laser bead immunoassay (ALBIA, developed by INOVA, San Diego, CA, USA) was used to test for antibodies to pyruvate dehydrogenase (M2), soluble liver antigen (SLA), chromatin and liver kidney microsome (LKM) antigen.

Nuclar assembly assay. Demembranated sperm chromatin was prepared [23] and stored at − 80 °C at a concentration of 40,000/µl. *Xenopus laevis* eggs were collected, dejellied and lysed to prepare an interphase extract [24]. Nuclear envelope assembly assays were then performed essentially as described by Smythe and Newport [25] and published in our previous report [18].

Results

Immunoprecipitation

The heterogeneity and specificity of autoantibodies found in patients with PBC were first identified by IP using [^{35}S]-methionine-labelled HeLa lysates (Fig. 1). All six sera immunoprecipitated an approximately 95 kDa protein, which was previously shown to be identical to p97/VCP [18] and three sera (lanes 3–6) also immunoprecipitated a 74 kDa protein that comigrated with markers for pyruvate dehydrogenase complex (PDC)-E2. One serum (lane 4) bound an approximately 40 kDa protein, which is presumed to be PDC-E1α.

IP of the full-length radiolabelled recombinant p97/VCP protein was used to confirm the presence of anti-p97/VCP in the six sera (Fig. 2). Serum from the index PBC patient [18] was used as positive control (lane 7), and a normal human serum was included as a negative control (lane 8). Five sera immunoprecipitated the recombinant p97/VCP protein (lanes 1–4, 6) and although the fifth serum (lane 5) exhibited a precipitin line of identity with the index serum in the immunodiffusion assay and immunoprecipitated p95c from HeLa cell lysates (Fig. 1, lane 5), it did not immunoprecipitate the recombinant protein.

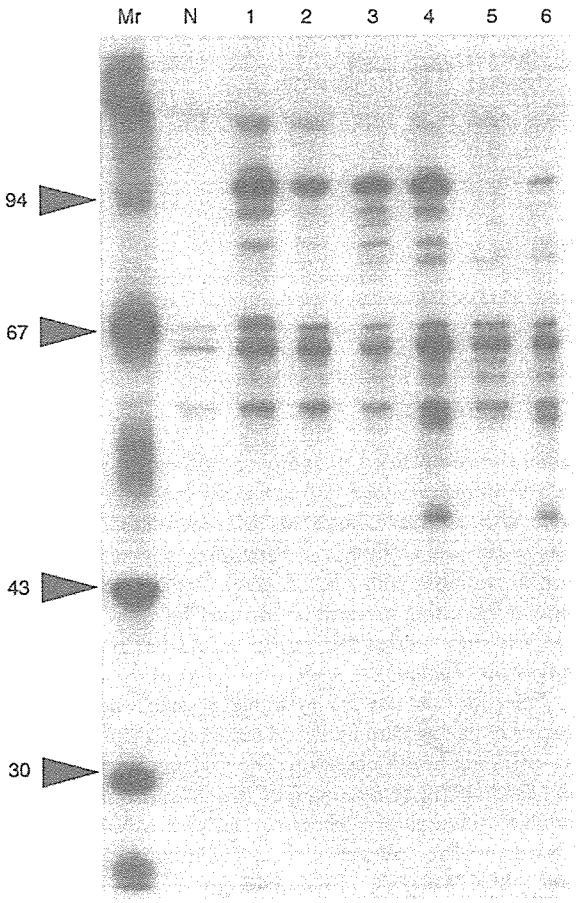

Figure 1 Immunoprecipitation of [^{35}S]-methionine-labelled HeLa cell lysate by sera from PBC patients with anti-p95c antibodies. PBC sera (lanes 1–6) immunoprecipitate an approximately 95 kDa protein but normal human serum (lane N) does not. The 74 kDa protein comigrated with an antibody to PDC-E2, and the 40 kDa protein is assumed to be PDC-E1α. A ~45 kDa protein is also immunoprecipitated by all sera and is regarded as a nonspecific reaction. Molecular weight markers (Mr) are shown on the left.

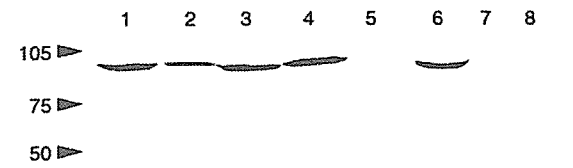

Figure 2 Immunoprecipitation of p97/VCP recombinant protein with human anti-p95 sera. The p97/VCP protein was expressed as a [^{35}S]-labelled *in vitro* transcription and translation (TnT) product and then immunoprecipitated with human sera. Four sera from PBC patients (lanes 1–4, 7) and the index anti-p95c sera (lane 6) immunoprecipitated the approximately 97 kDa recombinant protein, whereas normal human serum (lane 8) and a PBC patient (lane 5) did not. The serum in lane 5 had immunoprecipitins by immunodiffusion but demonstrated weak reactivity in immunoprecipitation of HeLa cell lysates (see Fig. 1). Molecular weight markers are indicated on the left.

Clinical and serological features of 6 patients with anti-p97/VCP antibodies (Tables 1 and 2)

Among the six patients with anti-p97/VCP antibodies, there were three females and three males who had an age range of 58–84 years (mean 72 years). Coincident morbidity included two patients with Hashimoto thyroiditis and two with Sjögren's syndrome. All the six patients had elevated levels of antibodies to pyruvate dehydrogenase (M2, range 840–4000 mean fluorescence units) but did not have antibodies to SLA, LKM or chromatin as measured by ALBIA. All patients had liver histopathology findings consistent with a diagnosis of PBC. The liver biopsy specimens obtained from cases 1 and 2 showed bile duct destruction compatible with PBC Scheuer stage 2. Another specimen revealed chronic nonsuppurative destructive cholangitis (CNSDC), compatible with Scheuer stage 1. The titre of serum anti-p97/VCP from case 5 as determined by double immunodiffusion was relatively low and may explain why it did not immunoprecipitate recombinant p97/VCP. The remaining four sera all demonstrated moderate (++) to strong (+++) IP reactions. Sera from the five patients demonstrated varying degrees of inhibition of nuclear envelope assembly (19–82%) in the *in vitro* assay, which was variably correlated with the titre of anti-p97/VCP antibodies (Table 1). The lack of complete correlation between these assays must take into account the different nature of the two assays (IP versus functional inhibition) and the possibility that other related antibodies may also participate in the INA reaction.

Longitudinal laboratory findings of six patients with anti-p97/VCP antibody (Fig. 3, Table 1)

Case 1 was a 69-year-old male previously diagnosed with obstructive liver disease in 1978. AMA was detected by indirect immunofluorescence at a dilution of 1:160, but could not be detected by double immunodiffusion using a rat liver mitochondrial fraction. This serum did exhibit a strong immunoprecipitin reaction when tested against rat liver supernatant. Abnormal liver functions as determined in 1983 included GOT 60 IU/l, GPT 55 IU/l, ALP 1766 IU/l, γ-GTP 614 IU/l and IgM 915 mg/dl. His liver biopsy specimen obtained in 1985 revealed typical chronic NSDC with histiocytes, including a multinucleated one around a damaged bile duct that demonstrated focal condensation of collagen at sites where the duct was probably present. The limiting plate showed segmental erosion and early short radiating septum, suggesting PBC at Scheuer stage 2 (Fig. 4). Administration of ursodeoxycolic acid (UDCA) (300–600 mg/day) was effective in maintaining liver function, but at the age of 84, he died of cerebral thrombosis (Table 1).

Case 2 was a 59-year-old female who visited the hospital in 1990 for evaluation and had liver function tests

Table 1 Clinical and serological features of five patients with anti-p97/VCP

Number	Sex	Primary diagnosis*	Secondary diagnosis	AMA M2 ALBIA†	Anti-p97/VCP†	Anti-VCP†	INA (%)
1	M	PBC2	SjS	1256	128	+++	82
2	F	PBC2	Hashimoto	1191	64	++	45
3	M	PBC1	None	840	64	+++	44
4	F	PBC1	SjS	4000	16	+++	70
5	F	PBC1	Hashimoto	1136	1	–	19
6	M	PBC1	Cholelithiasis	1404	4	++	43

ALBIA, addressable laser bead immunoassay; AMA M2, antimitochondrial antibodies; ELISA, enzyme-linked immunosorbent assay; INA, inhibition of nuclear assembly; IP, immunoprecipitation; PBC, primary biliary cirrhosis; SjS, Sjögren's syndrome; VCP, valosin-containing protein.
*Arabic numeral indicates Scheuer stage.
†AMA M2 (pyruvate dehydrogenase complex) antibodies detected by ALBIA expressed as median fluorescence units (normal = <124); anti-p97/VCP detected by immunodiffusion using liver cytosol extracts as antigen source; anti-VCP detected by IP of recombinant VCP.

with results as follows: GOT 49 IU/l, GPT 30 IU/l, ALP 798 IU/l, γ-GTP 180 IU/l and IgM 1360 mg/ml. A liver biopsy performed in April 1990 showed Scheuer stage 1–2 CNSDC and the follicles were judged to be normal. Although this patient was not treated with UDCA, her health and liver status has remained stable (Table 1).

Case 3, a 57-year-old male, initially visited the hospital in 1984 complaining of symptoms of liver dysfunction and laboratory evaluation revealed GOT 42 IU/l, GPT 44 IU/l, ALP 563 IU/l, γ-GTP 194 IU/l and IgM 997 mg/ml. A liver biopsy was performed in February 1986, and histology revealed marked portal inflammation with numerous foci of piecemeal necrosis. There were also a few foci of destructive duct lesions, compatible with florid Scheuer stage 1 duct lesion. The patient was treated with UDCA (150 mg/day) from 1989 to 1998 when the dosage was increased to 600 mg/day. HCV antibodies and HBsAg have been absent since 1990 onwards but hepatocellular carcinoma was detected in July 2003 (Table 1).

The fourth patient initially visited the hospital for treatment of hypertension in 1982 at the age of 54. At that time, she complained of morning stiffness of her fingers and xeropthalmia. She had a positive AMA but did not have any clinical features of liver dysfunction. A liver biopsy revealed CNSDC, and attending physicians elected not to treat her with UDCA until October 1996, when laboratory testing revealed an elevated IgM of 733 mg/dl. She has since moved her residence and has been lost to follow-up (Table 1).

The fifth case was a 53-year-old woman who was suspected of having chronic liver disease since 1983 following an employee health check-up. She visited a medical clinic in March 1998, complaining of struma and pruritus, and exhibited clinical and laboratory features consistent with obstructive liver disease: γ-GTP 250 IU/l, ALP 454 IU/l and IgM 671 mg/ml. Her liver biopsy revealed mild fatty deposition in liver cells, the limiting plate was not destroyed and there was marked mononuclear cell infiltration into one of the Glisson's areas, where CNSDC was noted. Following administration of UDCA (300 mg/day) in July 1998, the liver function tests returned to within the normal range (γ-GTP 29 IU/l, ALP 243 IU/l, IgM 108 mg/ml). The patient currently maintains a stable clinical condition (Table 1).

The sixth case was a man who presented with epigastric pain and icterus at the age of 53 years in 1984. He was suspected of having cholelithiasis and the presence of AMA suggested a diagnosis of PBC. Following surgical removal of a common bile duct stone, obstructive liver impairment continued. His liver biopsy done at 55 years revealed an early stage of PBC. Although recent IgM levels performed at the present age of 72 years had increased from 569 mg/dl on the first visit to 881 mg/dl, the AST (33 IU/l), ALT (46 IU/l), ALP (261 IU/l) and γ-GTP (99 IU/l) were not significantly changed.

Table 2 Comparison of clinical features of six PBC patients with anti-VCP and a cohort of 50 PBC patients without anti-VCP antibodies

Patient group	Anti-VCP(+) ($n=6$)	Anti-VCP(–) ($n=50$)
Male/Female	3/3	7/43
Age range/median	58–84/72	42–77/60
Number of symptomatic/ number of asymptomatic	1/5	10/40
Follow-up year/mean year	9–21/15	1–20/7
Number of deaths because of hepatic failure number (%)	0	8(16)
Number of deaths because of other cause (%)	2(33)	5(10)
Number of AMA positive (%)	6(100)	39(78)
Number of ACA positive (%)	1(16)	16(32)

ACA, anticentromere antibodies detected by indirect immunofluorescence; AMA, antimitochondrial antibodies detected by indirect immunofluorescence; VCP, valosin-containing protein.

Comparison with 50 patients without anti-VCP

The background of 50 PBC patients without anti-VCP is summarized and compared with the five study patients in Table 2. Forty-three were females and seven were males

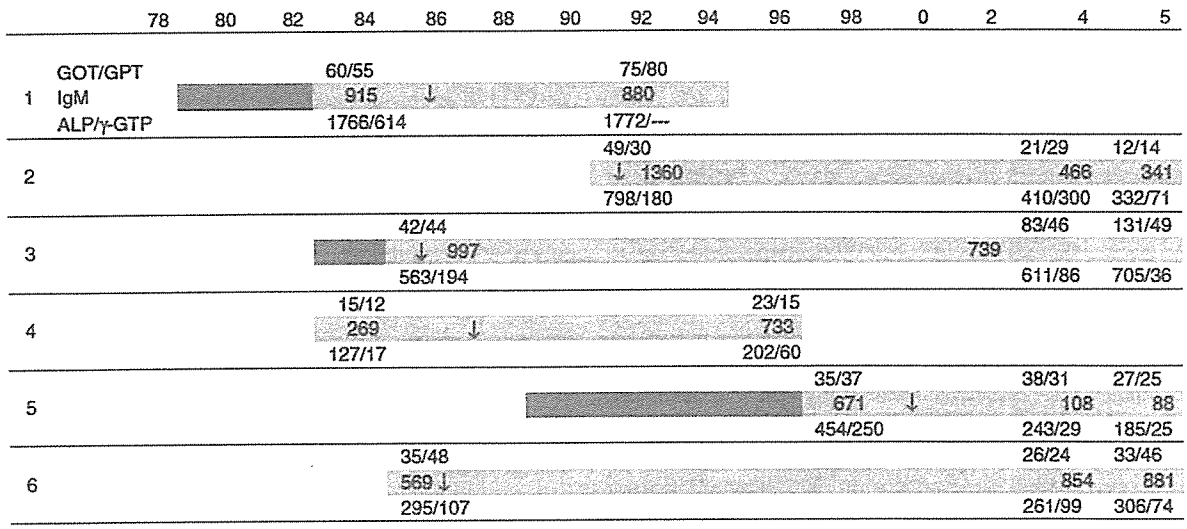

Figure 3 Longitudinal follow-up and laboratory findings of six patients with anti-p97/VCP antibodies. Normal ranges for laboratory tests include GOT (AST) 10–40 IU/L; GPT (ALT) 5–45 IU/l, ALP 110–360 IU/l, γ-GTP <75 IU/L and IgM 35–220 mg/dl indicates the year liver biopsy was performed. The grey area indicates the period that the patient was observed by a hepatologist. The purple area shows the period over which liver dysfunction was known.

with an age range of 42–77 years (median 60 years). The ratio of symptomatic to asymptomatic patients (1:5) was nearly identical to that of the anti-VCP group. Of note, 8/50 (16%) patients without anti-VCP died because of hepatic failure compared with none with anti-VCP. This is remarkable considering that the follow-up period of the PBC patients without anti-VCP was shorter than the anti-VCP group (mean 7 versus 15 years). Thirty-three percent of death due to other causes in the p97/VCP-positive patients was higher than 5% in the p97/VCP-negative patients. However, this was because the average age of the p97/VCP patients (72 years) was remarkably higher than that of the PBC patients (60 years) without anti-p97/VCP. In this comparison group, 39/50 (78%) patients had AMA and 16/50 (32%) had anticentromere antibodies.

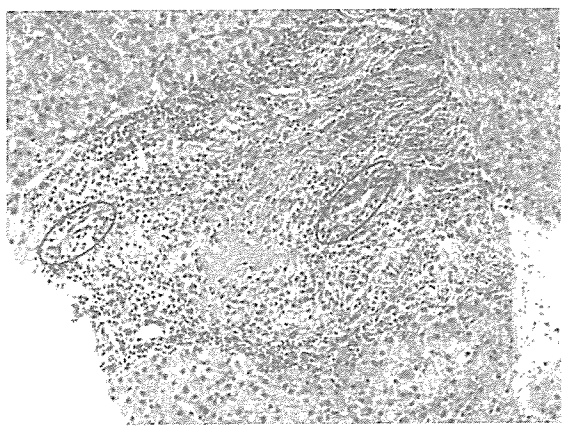

Figure 4 Liver biopsy specimen showing marked mononuclear cell infiltration associated with focal replacing fibrosis in Glisson's area and CNSDC (oval right side). Mononuclear cell infiltration into small interlobular bile duct with vacuolated degenerating epithelial cells was observed. The limiting plate showed segmental erosion. A small arteriole is identified by the oval on the left side of the figure (magnification ×200).

Discussion

Previously, we reported a novel antibody in autoimmune liver disease sera that reacted with a conformational epitope of a cytosolic 95 kDa protein, which we tentatively named p95c [14]. These antibodies were found in 12.5% of PBC and 9.8% of autoimmune hepatitis sera. Recently, we have provided evidence that the p95c antigen is identical to p97/VCP based on *in vitro* experiments which showed anti-p95c antibody inhibiting nuclear assembly, the major function of p97/VCP, and reacting with the 97 kDa radiolabelled recombinant protein [18]. The clinical features and outcome of patients became of interest when it was noted that they might have a more favourable clinical course. We originally gathered 30 sera over the past 10 years containing anti-p97/VCP. However, most sera were sent from the small hospital and private clinics and detailed clinical information could not be obtained. A few sera were gathered from large hospitals, but the patients' consent could not be obtained and as a result of which, only sera of six patients were available for inclusion in this study.

The p97/VCP protein was first associated with the AAA (ATPase associated with diverse cellular activities) family of proteins and accounts for as much as 1% of the cellular

protein [26]. The AAA family of proteins contain ~230 amino acid residues and common ATPase domains (i.e. Walker motif) and a characteristic second region of homology [27, 28]. The crystal structure of p97/VCP has been reported [16] and was shown to play a key role in postmitotic assembly of the ER, Golgi apparatus and nuclear membrane [29]. Furthermore, as an unfoldlase, it is associated with degradation of misfolded proteins in the ER that are released into the cytoplasm (ERAD) [17]. Another important function of p97/VCP has been found to be augmentation of signals to nuclear factor kappa B (NF-κB) in carcinoma cells such as gastric cancer and hepatocellular carcinoma [30, 31].

Using conventional diagnostic assays, AMA are found in at least 85% of patients with PBC and is generally regarded as a diagnostic hallmark for this disease [2, 3]. It has been noted, however, that the clinical manifestations of PBC patients with AMA are almost the same as those of PBC patients without AMA. In 1990, Klein and Berg [12] proposed that anti-M4 was a marker for a poor prognosis of PBC and that anti-M9 indicated a good prognosis. However, these conclusions have not been widely validated.

In general, asymptomatic primary biliary cirrhosis (a-PBC) patients have a longer life expectancy than those who are symptomatic and have pruritus or/and scleral icterus. For example, in one cohort of 250 patients followed for 24 years, the median survival for a-PBC and symptomatic primary biliary cirrhosis (s-PBC) was 16 and 7.5 years, respectively [32]. In Japan, the 5-year survival rates following diagnosis with s-PBC and only pruritus, and s-PBC with both pruritus and icterus, were calculated as 88 and 53%, respectively [33].

In this study, we have reported that six PBC patients with anti-p97/VCP antibodies had an overall survival rate of 67% over a 15-year period. Notably, the first and third patients who died during this period did not succumb to autoimmune liver failure. In contrast, the survival rate of the 50 comparison PBC patients was 74%, with 16% dying of liver failure. It is not clear why the PBC patients with anti-p97/VCP appeared to have a milder clinical course but because our number of patients is admittedly small, multicentre studies will be required to validate these impressions. Unfortunately, liver biopsies were performed only at clinical presentation in each of our six patients, which makes it difficult to confirm the histopathological status of relevant tissues later in their clinical course. It was noted that ALP, γ-GTP and IgM levels were more stable and the clinical course was thus considered to be less aggressive than patients without anti-p97/VCP.

In light of our observation that patients with anti-p97/VCP have a stable clinical course, it is intriguing to postulate that anti-p97/VCP antibodies have a protective role Most autoantibodies are not able to bind the cognate intracellular antigen of living cells. However, the anti-p97/VCP antibody could be unique in another way by gaining entry into certain cells through receptor-mediated endocytosis and a clathrin-binding protein such as Vsp25/SKD [34]. If this occurs in some mononuclear cells that infiltrate and surround the small bile ducts, obstructive liver impairment may be diminished or delayed via mechanisms as previously described [35, 36]. Relevant to these considerations is our observation that anti-p97/VCP antibodies were detected at presentation and at an early stage of PBC. Currently, we are trying to explore this hypothesis in cell culture assays.

Acknowledgments

We thank Hiroshi Chiba for technical assistance at Health Sciences Research Institute. This research was supported in part by a grant from the Canadian Institutes for Health Research (#10884) to M. J. F.

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