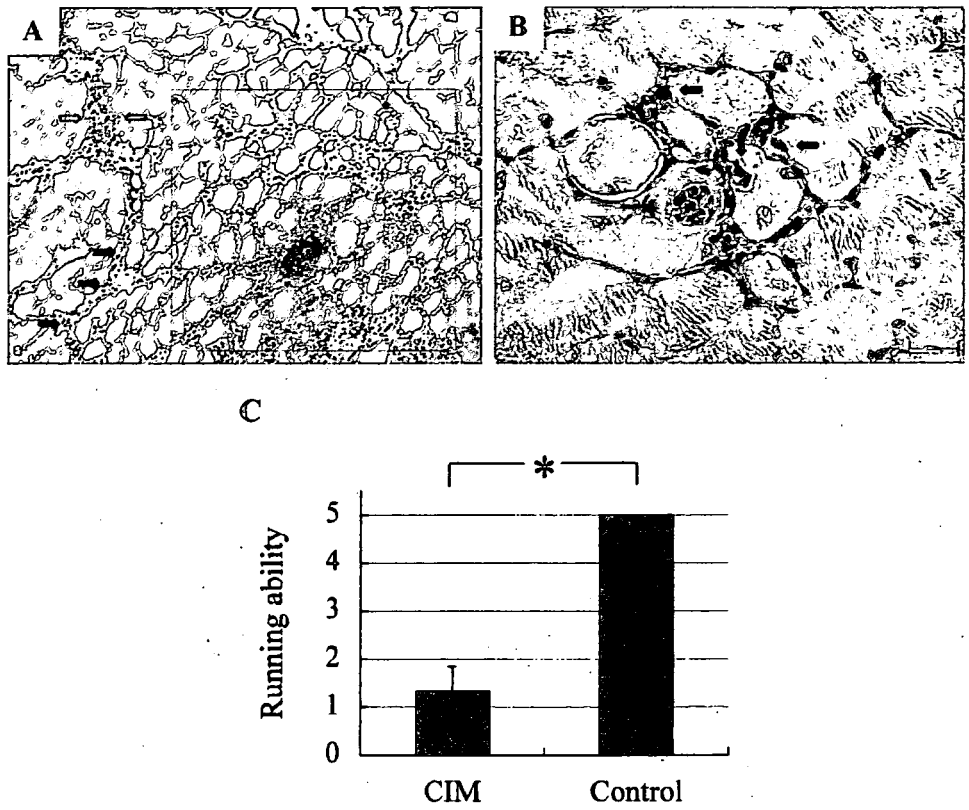

Figure 1. Muscle inflammation in C protein–induced myositis (CIM). C57BL/6 mice were immunized once with recombinant human skeletal C protein fragment 2 to induce CIM. **A** and **B,** Mononuclear cell infiltration was found predominantly in the endomysial site (boxed area) but also in the perimysial site (**solid arrows**) and perivascular site (**open arrows**) (**A**). Many cells invaded non-necrotic muscle fibers (**arrows**), while necrotic fibers were also present (**B**). Bar in **A** = 100 μm; bar in **B** = 25 μm. **C,** Muscle function was evaluated with a rotarod test 21 days after immunization. Six mice with CIM and 5 control mice treated with adjuvant alone were examined. Running ability was scored as described in Materials and Methods. Values are the mean and SD score. * = $P < 0.01$.

fragment, and myositis was studied histologically 21 days after the immunizaton.

None of the mice treated with adjuvant alone or immunized without PT developed myositis. We found that a single immunization of fragments 2 or 4 induced myositis consistently, and the mean ± SD histologic scores were 2.8 ± 0.2 and 1.0 ± 0.3, respectively. Fragments 1 and 3 induced milder myositis at a lower incidence, and the mean ± SD histologic scores were 0.2 ± 0.3 and 0.1 ± 0.2, respectively. Because fragment 2 induced the most severe myositis, it was used as an immunogen in subsequent experiments.

Histologic analysis of the muscles affected by CIM showed that mononuclear cells infiltrated predominantly the endomysial site, but also the perimysial and perivascular sites of the muscle tissue (Figure 1A). Many mononuclear cells invaded non-necrotic muscle fibers (Figure 1B). No abnormality in cardiac muscle and other tissues, including lung and joint tissues, was observed. Muscle function was assessed clinically with a rotarod device on day 21. Consistent with the histologic findings in the muscle tissue, mice with CIM ran for a shorter time than did control mice, indicating a reduction in motor function (Figure 1C).

Inflammation is acute and regresses spontaneously in most animal models of autoimmune diseases. To study the course of CIM in mice, muscle sections from 4 or 5 mice were examined at various time points after the immunization. A small number of mononuclear cells appeared in 50% of the muscle samples on day 7

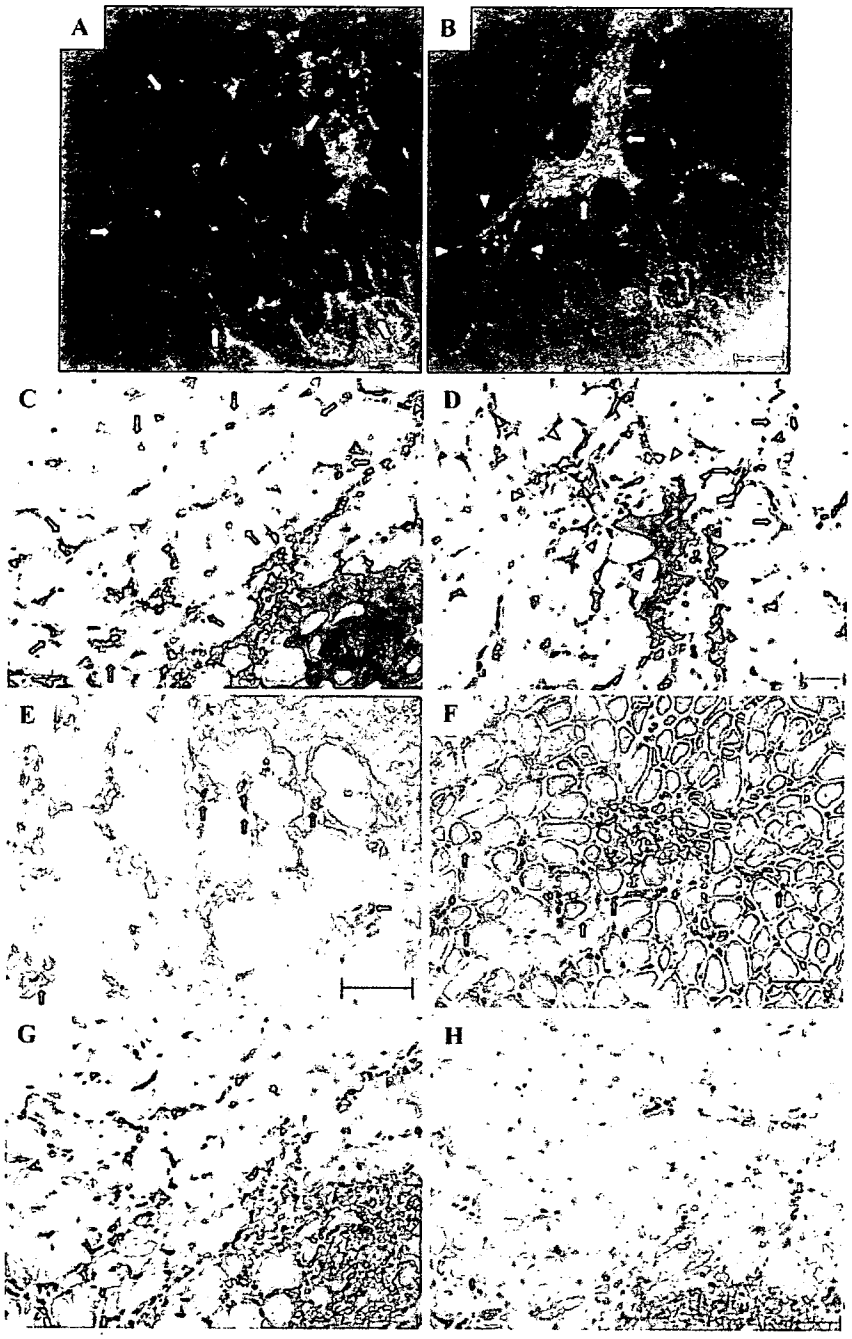

Figure 2. Immunohistochemical findings in muscles of mice with C protein–induced myositis. **A** and **B**, Expression of CD4 (green, fluorescein isothiocyanate) and CD8 (red, Alexa Fluor 647) was examined. Infiltrating cells in the endomysial site (**arrows**) (**A**) and in the perimysial site (**arrows**) and perivascular site (**arrowheads**) (**B**) were individually enumerated for calculating CD4:CD8 ratios. **C–H**, Expression of CD11b (**C** and **D**), perforin (**E**), class I major histocompatibility complex (**F**), interleukin-1α (IL-1α) (**G**), and tumor necrosis factor α (TNFα) (**H**) was examined with immunoperoxidase staining. CD11b-positive cells diffusely infiltrated the endomysial site (**arrows**) (**C**) and both the perimysial (**arrows**) and perivascular sites (**arrowheads**) (**D**). Perforin-positive cells infiltrated around non-necrotic muscle fibers at the endomysial sites (**arrows**) (**E**). Muscle fibers reacted to anti-H2K^b monoclonal antibodies (**arrows**) (**F**). IL-1α and TNFα were expressed on infiltrating mononuclear cells in muscles (**G** and **H**). Bars = 50 μm.

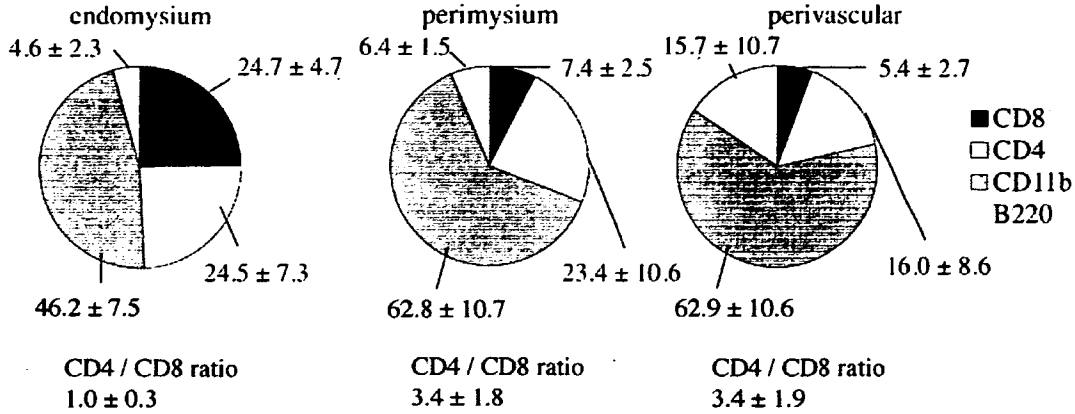

Figure 3. Quantitative immunohistochemical analysis of mononuclear cells in muscles of mice with C protein–induced myositis. Frequencies of CD8, CD4, CD11b, and B220 cells in the infiltrating mononuclear cells at endomysial, perimysial, and perivascular sites are shown. Values are the mean ± SD percentage of each cell subset and mean ± SD CD4:CD8 ratio at 5 inflammatory mononuclear cell foci. The total mononuclear cell counts in the endomysial, perimysial, and perivascular sites were 1,385, 506, and 543, respectively.

(incidence 50%, mean ± SD histologic score 0.6 ± 0.7). Inflammation of the muscle tissue peaked on days 14 and 21 after the immunization (incidence 100%, histologic scores 2.6 ± 0.3 and 2.8 ± 0.2, respectively) and started to resolve after day 28 (incidence 100%, histologic score 1.4 ± 0.6). On day 49, mononuclear cell infiltration was absent from most skeletal muscles. On days 35, 49, and 63, the histologic scores were 0.5 ± 0.6, 0.1 ± 0.2, and 0.0 ± 0.0, respectively, and the incidences of CIM were 38%, 10%, and 0%, respectively.

Other strains of mice were immunized with fragment 2 of the C protein in the same manner as in B6 mice, and the muscles were examined histologically. Studies of 4 or 5 mice per strain showed that myositis developed in NZB and SJL/J mice with an incidence similar to that in B6 mice. However, the mononuclear cell infiltration was less intense. The mean ± SD histologic scores were 1.8 ± 0.6 and 1.3 ± 0.3 in NZB and SJL/J mice, respectively. No inflammation was observed in the muscles from BALB/c, DBA/1, C3H/He, or MRL/Mp+/+ mice immunized with C protein fragments.

Immunohistochemical findings in muscles of mice with CIM. Localization of CD4 and CD8 T cells was studied with double immnunofluorescence labeling or immunohistochemical staining of muscle sections. CD4 cells diffusely infiltrated the endomysial, perimysial, and perivascular sites. In contrast, CD8 cells infiltrated preferentially the endomysial site (Figures 2A and B), which was reflected equally well in the CD4:CD8 cell ratios. Double immnunofluorescence staining of CD4 and CD8 cells showed that the mean ± SD CD4:CD8 ratios in the endomysium, perimysium, and perivascular site were 1.0 ± 0.1, 3.3 ± 0.3, and 3.5 ± 0.4, respectively, which was consistent with the ratios derived from immunohistochemical staining (Figure 3). The frequency of CD8-positive cells in endomysial sites was higher than that in perimysial and perivascular sites, whereas the frequencies of CD4-positive T cells were similar among the 3 sites (Figure 3).

B cells and macrophages were identified by staining with B220 and CD11b antibodies, respectively. CD11b-positive cells were most abundant among the infiltrating cells in all 3 sites (Figures 2C and D and Figure 3). Although natural killer cells are also CD11b positive, our analysis of CD68 and CD11b expression in serial sections showed that 93.4 ± 4.3% (mean ± SD) of CD11b-positive cells were CD68-positive cells, indicating that the majority of CD11b-positive cells were macrophages.

B cells were sparse in the muscle tissue, especially in the endomysial and perimysial sites (Figure 3). Perforin-positive cells were present mostly (82%) around non-necrotic muscle fibers at the endomysial site (Figure 2E) and were sparse in the perimysial and perivascular sites (results not shown). Thus, the distribution of perforin-positive cells corresponded well to that of CD8-positive cells. When muscle fibers were stained with anti-$H2K^b$ (class I MHC) and anti-$I\text{-}A^b$ (class II MHC) mAb, they reacted to the anti–class I

MHC mAb (Figure 2F) but not to the anti–class II MHC mAb (results not shown).

Pathologic role of CD8 and CD4 T cells in CIM. Histologic studies have demonstrated that CD8 T cells function as effector cells in injury of muscle fibers. To establish the pathologic role of CD8 T cells, B6 mice were pretreated by removal of circulating CD8 T cells using specific mAb. Ten days after injection of the antibodies for 3 consecutive days, CD8 T cells in the spleens were depleted to fewer than 2%. The mice were then immunized with C protein and treated with the same antibodies every other day for 14 days. The muscles were examined histologically 14 days after the immunization, when the frequency of CD8 T cells in the spleens and lymph nodes was still less than 2%. The number of CD4 T cells in the spleens and lymph nodes was maintained in the CD8-depleted mice.

Significantly fewer CD8-depleted mice developed myositis compared with control mice, with a 33% incidence of disease compared with 100% in controls (Table 1). The histologic scores of the treated mice were significantly lower than that of the controls. It is known that CD4 T cells help CD8 T cells develop into mature cytotoxic T lymphocytes. They also have the potential to injure muscle fibers. Therefore, CD4 T cells were removed with specific mAb in the same manner as described above for CD8 T cells. The pretreated mice exhibited fewer than 2% of circulating CD4 T cells, and were then immunized for CIM induction. They also developed a milder myositis compared with control mice (Table 1).

Investigation of essential immunologic mediators in mutant mice. Mice with CIM developed serum antibodies directed to C protein. They also developed low-titer autoantibodies with a cytoplasmic pattern or homogeneous and speckled nuclear patterns on Hep-2 staining, which proved to be nonreactive to PM-associated autoantigens (results not shown). However, the contribution of these autoantibodies to myositis was unclear.

The susceptibility of B6 mice to CIM allowed us to study the contribution of different immune mediators to myositis using genetic mutant mice. Igμ-null mutant mice developed CIM with features and a frequency comparable with those in control wild-type (WT) mice (Table 1). These findings indicate that the functions of B cells and immunoglobulins are not necessary for the development of CIM.

Inflammatory cytokines such as IL-1 and TNFα are known to be expressed in mononuclear cells infiltrating the muscles of mice with PM (25,26). Our immunohistochemical analyses showed that these inflammatory cytokines were also found in mice with CIM (Figures 2G and H). We then studied whether the presence of IL-1 and TNFα is required for the development of CIM, using IL-1α/β double-null mutant and TNFα-null mutant B6 mice. Most IL-1α/β–null mutant mice did not develop myositis, and those that did have myositis developed a mild form (Table 1). The rotarod score of 7 for the IL-1α/β–null mutant mice was significantly higher than that for the 6 WT mice (mean ± SD score 4.7 ± 0.5 versus 1.3 ± 0.5; $P < 0.01$). In contrast, TNFα-null mutant mice were as susceptible to CIM as the control mice, and the TNFα-null mice had a similar incidence and severity of myositis (Table 1). These results indicate the differential requirements for the roles of IL-1 and TNFα in the development of CIM.

Table 1. Studies of the pathologic features of C protein–induced polymyositis (CIM) and effects of treatment with intravenous immunoglobulin (IVIG)*

Experiment, mouse group	Incidence of CIM, %	Muscle blocks involved, %†	Histologic score, mean ± SD
CD8 depletion			
CD8-depleted	33	25	0.6 ± 1.0‡
Rat IgG–injected	100	100	1.9 ± 0.6
CD4 depletion			
CD4-depleted	60	50	0.7 ± 0.7‡
Rat IgG–injected	100	100	2.0 ± 0.4
Igμ-null			
Ig$\mu^{-/-}$	100	95	1.7 ± 0.6
WT	100	100	2.1 ± 0.7
IL-1-null			
IL-1$\alpha/\beta^{-/-}$	14	7	0.1 ± 0.2§
WT	100	100	2.1 ± 0.5
TNFα-null			
TNF$\alpha^{-/-}$	80	80	1.9 ± 1.0
WT	100	90	2.3 ± 0.6
IVIG			
IVIG-injected	43	36	0.6 ± 1.1‡
Saline-injected	100	100	2.3 ± 1.0

* Mice were immunized with 200 μg of the recombinant C protein fragments and then examined histologically 14 or 21 days after immunization. For in vivo depletion of CD8 or CD4 T cells, 5 or 6 B6 mice were treated intraperitoneally with anti-CD8 or anti-CD4 monoclonal antibodies, while 5 or 6 control mice were treated with purified rat IgG. To demonstrate the requirement for humoral immunity, the presence of interleukin-1 (IL-1), and the presence of tumor necrosis factor α (TNFα) for the development of CIM, 5 Igμ-null mutant mice, 7 IL-1α/β–null mutant mice, 5 TNFα-null mutant mice, and 5 wild-type (WT) B6 mice were studied. IVIG was administered at a dosage of 400 mg/kg/day intravenously into the tail vein for 5 consecutive days, from day 3 after immunization. Seven mice were treated with IVIG and 6 with control saline.

† Calculated as the number of muscle blocks showing myositis divided by the total number of blocks.

‡ $P < 0.05$ versus control group.

§ $P < 0.01$ versus control group.

Effects of high-dose IVIG treatment. Infusion of high-dose IVIG is effective treatment of inflammatory myopathies that are refractory to conventional immunosuppressants (27–29). Although several mechanisms of action for IVIG have been proposed, they have not been fully characterized. One possibility is that IVIG acts via suppression of pathogenic immunoglobulins and B cells. Thus, whether this treatment improves CIM, which does not depend on humoral immunity for tissue injury, is of special interest. When mice with CIM were treated with high-dose IVIG (400 mg/kg) for 5 consecutive days, beginning 3 days after immunization, the incidence and histologic severity of CIM were suppressed significantly compared with that in control, saline-injected mice (Table 1).

DISCUSSION

CIM was established as a simple murine model of PM. A single injection of mice with recombinant human muscle protein induced severe and clinically significant inflammation of the skeletal muscles. CD8 T cells were enriched in the endomysial site (the site of muscle injury) as compared with their distribution in other sites of the mouse muscle. CD8 cells expressed perforins preferentially at the endomysial site. Class I MHC expression was up-regulated on the muscle fibers. Removal of CD8 T cells significantly suppressed myositis. Thus, muscle injury in CIM appears to be driven, primarily, by cytotoxic CD8 T cells, as is assumed in human PM. In this regard, the new model provides a clear contrast to the previous EAM model, which appears to be driven by CD4 T cells. Induction of EAM requires repeated immunization with a specific mouse strain having a dysferlin gene mutation that induces spontaneous muscular degeneration and inflammation. Sensitivity of B6 mice to CIM prompted the initiation of genetic studies of the pathologic mechanisms of autoimmune myositis, which would provide information for the development of new treatments.

Muscle tissues from mice with CIM contained more CD4 T cells and macrophages than are found in patients with PM, which may reflect the acute disease course of CIM. Although we observed critical participation by CD8 T cells, CD4 T cells were also important in the development of CIM. In this regard, it has been shown that the actions of CD4 T cells are essential for full differentiation of cytotoxic CD8 T cells (30,31). Alternatively, CD4 T cells may also injure muscle tissues directly. This issue should be addressed further in future experiments.

Severe inflammation was found consistently in the proximal muscles of the lower extremities, but not in other sites. Although injection of CFA alone did not induce myositis, we assume that activation of local innate immunity by injection of the foot pad with CFA would contribute to induction of severe myositis. Unlike inflammation in human PM, inflammation in other myositis models, such as EAM and cardiac myosin–induced myocarditis, is transient (32,33). Because lipopolysaccharide injection in the recovery phase of experimental myocarditis can induce a relapse of inflammation (33), unknown factors might perpetuate the chronic disease in humans.

An elevation in the levels of creatine kinase (CK) was found in the mice with CIM. However, since some mice, including healthy animals, have unexpectedly high levels of CK, this elevation could be attributed to uncontrollable hemolysis. Lung involvement in some patients is characteristic of PM and also of dermatomyositis (DM). However, no abnormality in the lungs was observed in the mice with CIM. Considering the frequency of lung disease in PM (34), we may have to undertake extensive studies of this issue using a large number of animals.

Recombinant C protein was used to confirm its immunogenicity, as suggested in a rat myositis model induced by biochemically purified C protein (19). Large-scale production of recombinant C protein fragments in the prokaryotic expression system facilitated multiple experiments to optimize an immunization protocol and to analyze the pathologic features of myositis. Since at least 200 μg of the immunogen had to be injected to induce CIM consistently, we needed to use the back and foot pads of the mice for immunization.

The rotarod test was useful to assess muscle function in the mice with CIM at a single time point. Analysis by Spearman's rank correlation coefficient showed that the rotarod score correlated well with the histologic score ($P < 0.001$). However, this test was less useful in evaluating the disease course in these mice, because the mice could learn how to run for a longer period of time when the test was repeated. We believe that the device should be improved so that muscle function or weakness can be evaluated in an easier way.

Unlike in the EAM model, many strains of mice, including SJL/J mice, were susceptible to CIM. This fact confirms the immunogenicity of the C protein and suggests that mouse strains may have their own hierarchy of susceptibility to myositis.

Our observations of CIM induced in the B6 mice with mutations in inflammatory cytokine genes demon-

strated the importance of IL-1, but not TNFα, in the development of myositis. Previous histologic studies of PM muscle tissue showed that IL-1 and other proinflammatory cytokines, including TNFα, IL-6, and interferon-γ, are expressed by mononuclear cells in the affected muscle tissue (25). IL-1 expression by mononuclear cells accompanies expression of class I MHC molecules on the muscle fibers and expression of adhesion molecules, such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, on endothelial cells in muscle (35). Thus, we assume that IL-1 expression of activated macrophages up-regulates expression of the class I MHC molecules, as well as that of adhesion molecules and chemokines, on muscle fibers, endothelial cells, and inflammatory cells. All of these molecules can trigger CD8-mediated muscle damage. Other studies have shown that antigen-specific T cell responses are impaired in IL-1α/β double-null mutant mice (36,37). In the autoimmune myocarditis model, IL-1 is important for efficient activation of dendritic cells (DCs) and priming of CD4 T cells (38). IL-1 may also contribute to activation of DCs and interacting T lymphocytes.

Similar to IL-1, TNFα induces expression of class I MHC molecules on muscle fibers and expression of adhesion molecules on endothelial cells (39,40). The results of one report suggested that TNFα released from infiltrating CD8 T cells in PM muscles was responsible for the muscle damage (26). However, we found that CD8 T cells can induce typical myositis without TNFα. In this regard, experimental myocarditis is suppressed in both IL-1 receptor– and TNFα receptor–null mutant mice (38,41), and this model is mediated by pathogenic CD4 T cells (33,38). Thus, it is important to note the differences between the 2 murine myositis models.

Our results do not necessarily refute the idea that TNFα is a therapeutic target in PM. Clinical findings from sporadic reports suggest that some patients with PM respond to systemic delivery of anti-TNFα mAb (42). This fact and our results are similar to the findings in collagen-induced arthritis (CIA), which is an animal model of RA. TNFα-null mutant mice are susceptible to CIA (43), but inhibition of TNFα can improve the disease (9). Development of arthritis is suppressed in IL-1α/β double-null mutant mice (37). Studies are in progress to investigate the therapeutic effects of IL-1 or TNFα blockade in CIM.

A high dose of IVIGs, pooled from the plasma of healthy donors, is commonly administered as treatment in patients with autoimmune disorders (44). After an initial study showing that a patient with refractory PM was successfully treated with IVIG (29), the efficacy has been confirmed by a number of studies of patients with PM and DM (27,28). Currently, IVIG therapy is the only treatment that does not induce general immune suppression. The same treatment exerted a minor therapeutic effect in SJL/J mice in the myositis model (45). Although immunoglobulins are derived from human sera, the effect was not due to nonspecific immunomodulation by a xenogenic protein.

We found that IVIG treatment was markedly effective in CIM, suggesting its relevance as a model for human PM. When the treatment was started 8 days after immunization of the mice, the therapeutic effect appeared milder (results not shown). The mode of action of IVIGs could vary, and all of the mechanisms have not been fully characterized (46–48). Modulation of crystallizable fragment Fcγ receptor IIb on phagocytes appears to be the primary mechanism for an increase in platelet counts in patients with immune thrombocytopenia (49). Theoretically, the treatment could also down-regulate activating Fcγ receptors, increase IgG catabolism, neutralize autoantibodies and inflammatory cytokines, attenuate complement-mediated tissue damage, and modulate cytokine production by B cells and monocytes. Because development of CIM does not depend on B cells or antibodies, the efficacy of IVIG treatment for this model suggests that down-regulation of B cells or autoantibody-mediated processes are not a prerequisite to achieve improvement of PM. Further evaluation should lead to identification of key molecules for the effect of IVIG and development of new treatments that target defined molecules.

Our new model mimics human PM and provides a useful tool to investigate its pathologic mechanisms. It holds promise for identification of specific targets that will lead to the development of new therapeutic approaches in the treatment of PM, and will also be useful for confirming the efficacy of these treatments.

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AUTHOR CONTRIBUTIONS

Dr. Kohsaka had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Sugihara, Kohsaka.

Acquisition of data. Sugihara, Sekine, Nakae, Kohyama.

Analysis and interpretation of data. Sugihara, Harigai, Iwakura, Matsumoto, Miyasaka, Kohsaka.
Manuscript preparation. Sugihara, Kohsaka.
Statistical analysis. Sugihara, Kohsaka.

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膠原病に伴う肺障害
pulmonary disorders in collagen vascular disease

諏訪　昭　東海大学准教授・内科学系リウマチ内科学

病態と診断

膠原病における肺障害は高頻度で，肺胞・間質性病変，気道病変，血管病変，胸膜病変など多彩である．各疾患における肺障害の頻度や臨床像は異なり，原因も原病，感染症，薬剤など多岐にわたる．肺障害の診断と活動性評価は，臨床所見，血液検査（SP-A，SP-D，KL-6），画像（胸部X線，CT，Gaシンチ），肺機能検査，気管支肺胞洗浄液検査，肺生検（経気管支/胸腔鏡下/開胸）による．

治療方針

A. 間質性肺炎

臨床像より慢性型，亜急性-急性型，急速進行型に分けられ，組織学的にはDAD（diffuse alveolar damage），COP（cryptogenic organizing pneumonia），UIP（usual interstitial pneumonia），LIP（lymphocytic interstitial pneumonia），NSIP（non-specific interstitial pneumonia）などに分類され，治療反応性や予後が異なる．特発性IPに比べ臨床像・病理組織像も多様である．活動性症例にステロイドを用い，重症例や治療抵抗例ではステロイドパルス療法や免疫抑制薬を併用する．

1. 軽症，慢性例　自然軽快例や，強皮症（SSc），多発性筋炎/皮膚筋炎（PM/DM），関節リウマチ（RA）には治療を要しない慢性例もある．

2. 亜急性-急性例　RA，全身性エリテマトーデス（SLE），PM/DM，重複症候群/MCTDなどの活動性症例でステロイド療法を行う．COPやNSIPの反応性は高い．

℞処方例

プレドニゾロン錠（5 mg）　6-12錠　分3・食後

3. 急速進行性間質性肺炎　筋症状の乏しいamyopathic DM，RA，SLEにみられる予後不良な病態であり，ステロイドパルス療法または免疫抑制薬併用を行う．

℞処方例　1）を用い，効果不十分の場合には下記2）または3）を併用する．

1）ソル・メドロール注　1回1,000 mg　1日1回を5%ブドウ糖注射液500 mLに混和し1時間以上かけて点滴静注　3日間　(保外)．後療法としてプレドニゾロン錠1 mg/kg/日を投与する

2）エンドキサン注　1回0.5-1.0 g/m²　1日1回をソリタ-T3号注500 mLに混和し2時間以上かけて点滴静注（2-4週間ごと）　(保外)．十分な飲水または補液により出血性膀胱炎を予防する

3）ネオーラルカプセル（25 mg）　2-6カプセル　分2-3　食後　(保外)．血中濃度トラフ値100-150 ng/mL程度とする

B. 肺胞出血

SLEや血管炎（特にMPO-ANCA陽性例）にみられる予後不良な病態である．急性呼吸不全，進行性貧血，胸部X線上両側びまん性スリガラス様陰影を呈する．ステロイドパルス療法，シクロホスファミドパルス療法，血漿交換療法を行う．

C. 肺高血圧症

重複症候群/MCTD，強皮症，SLEなどで認める難治性病態である．労作時呼吸困難，第II肺動脈音亢進，胸部X線/CT検査で肺動脈本幹部拡大，左第2弓突出，心電図検査で右室負荷・肥大，右房負荷所見を認め，肺機能検査，ドプラ心エコー検査，右心カテーテル検査，肺換気シンチにより診断する．PGI_2経口薬，Ca拮抗薬，抗凝固療法，抗血小板薬を用い，NYHA分類III度以上ではエンドセリン受容体拮抗薬やPGI_2（エポプロステノール）持続静注療法を考慮する．早期例には免疫抑制療法が有効な場合がある．

℞処方例　下記のいずれか，または適宜組み合わせて用いる．

1）ドルナー錠（20 μg）　6-9錠　分3・食後　(保外)

2）カルスロット錠（20 mg）　1錠　分1　食後　(保外)

3）ワーファリン錠　1-3 mg　分1-2　食後　(保外)

4）プレタール錠（100 mg）　2錠　分2　食後　(保外)

5）トラクリア錠（62.5 mg）　2-4錠　分2　食後

D. 薬剤性肺障害

抗リウマチ薬〔メトトレキサート（MTX），レフルノミド，金製剤，ブシラミン，サラゾスルファピリジン〕は肺障害を惹起する．MTX肺炎は用量非依存的で，高齢，糖尿病，IP，薬剤過敏症はリスクとなる．MTX中止後ステロイド大量療法（パルス療法）を行う．レフルノミド肺炎ではクエストラン投与を行う．

E. 胸膜炎

中等量ステロイドを投与する．

■患者説明のポイント

- 原病・治療薬により免疫能が低下するため，感染に注意するよう指導する．
- 急激な咳嗽，呼吸困難を認めたら，直ちに受診させる．
- ステロイドや免疫抑制薬の効果，副作用〔感染症（日和見感染），胃腸障害，糖尿病，高血圧，高脂血症，骨粗鬆症，精神症状，眼症状，腎障害など〕についてよく説明する．
- 服薬方法を遵守し，自己判断で服薬を変更・中止しないよう指導する．

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Clinical and Immunogenetic Features of Patients With Autoantibodies to Asparaginyl–Transfer RNA Synthetase

Michito Hirakata,[1] Akira Suwa,[2] Tetsuya Takada,[1] Shinji Sato,[1] Sonoko Nagai,[3] Ekkehard Genth,[4] Yeong W. Song,[5] Tsuneyo Mimori,[3] and Ira N. Targoff[6]

Objective. **We have previously described anti-KS autoantibodies and provided evidence that they are directed against asparaginyl–transfer RNA (tRNA) synthetase (AsnRS). The aim of the present study was to identify patients with anti-AsnRS autoantibodies and elucidate the clinical significance of this sixth antisynthetase antibody. In particular, we studied whether it was associated with the syndrome of myositis (polymyositis or dermatomyositis [DM]), interstitial lung disease (ILD), arthritis, and other features that had been previously associated with the 5 other anti–aminoacyl–tRNA synthetase autoantibodies.**

Methods. **More than 2,500 sera from patients with connective tissue disease (including myositis and ILD) and controls were examined for anti-AsnRS autoantibodies by immunoprecipitation (IP). Positive and control sera were tested for the ability to inhibit AsnRS by preincubation of the enzyme source with the serum. The HLA class II (DRB1, DQA1, DQB1, DPB1) alleles were identified from restriction fragment length polymorphism of polymerase chain reaction–amplified genomic DNA.**

Results. **Anti-AsnRS antibodies were identified in the sera of 8 patients (5 Japanese, 1 American, 1 German, and 1 Korean) by IP of the same distinctive set of tRNA and protein that differed from those precipitated by the other 5 antisynthetases, and these antibodies showed specific inhibition of AsnRS activity. Two of these patients had DM, but 7 of 8 (88%) had ILD. Four patients (50%) had arthritis, and 1 had Raynaud's phenomenon. This antisynthetase was very rare among myositis patients (present in 0% of Japanese myositis patients), but it was found in 3% of Japanese ILD patients. Thus, most patients with anti-AsnRS had chronic ILD with or without features of connective tissue disease. Interestingly, all 4 Japanese patients tested had DR2 (DRB1*1501/1502), compared with 33% of healthy controls.**

Conclusion. **These results indicate that anti-AsnRS autoantibodies, like anti–alanyl-tRNA synthetase autoantibodies, have a stronger association with ILD than with myositis and may be associated with the DR2 phenotype.**

The aminoacyl–transfer RNA (aminoacyl-tRNA) synthetases are a family of cytoplasmic enzymes that catalyze the formation of aminoacyl-tRNA from a specific amino acid and its cognate tRNA and play a crucial role in protein synthesis. Autoantibodies to certain of these synthetases (histidyl–, threonyl–, alanyl–, isoleucyl–, and glycyl–tRNA synthetases) have been identified in patients with inflammatory myopathies (1–6). Among these "antisynthetase autoantibodies," the most common is anti–Jo-1 (anti–histidyl–tRNA synthetase [anti-HisRS]), found in 20% of patients with polymyositis/dermatomyositis (PM/DM) (7–11). Anti–PL-7 (anti–threonyl–tRNA synthetase [anti-ThrRS])

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[1]Michito Hirakata, MD, Tetsuya Takada, MD, Shinji Sato, MD: Keio University School of Medicine, Tokyo, Japan; [2]Akira Suwa, MD: Tokai University School of Medicine, Isehara, Japan; [3]Sonoko Nagai, MD, Tsuneyo Mimori, MD: Kyoto University Graduate School of Medicine, Kyoto, Japan; [4]Ekkehard Genth, MD: Clinic and Research Institute for Rheumatic Diseases Aachen, Aachen, Germany; [5]Yeong W. Song, MD: Seoul National University Hospital, Seoul, Korea; [6]Ira N. Targoff, MD: Veterans Affairs Medical Center, University of Oklahoma Health Sciences Center, and Oklahoma Medical Research Foundation, Oklahoma City.

Dr. Targoff serves as a technical consultant to the Oklahoma Medical Research Foundation Clinical Immunology Laboratory.

Address correspondence and reprint requests to Michito Hirakata, MD, Section of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: mhirakat@sc.itc.keio.ac.jp.

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and anti–PL-12 (anti–alanyl–tRNA synthetase [anti-AlaRS]) autoantibodies are less common, found in 3–4% of all patients with PM/DM (4,5,11–13), while anti-OJ (anti–isoleucyl–tRNA synthetase [anti-IleRS]) and anti-EJ (anti–glycyl–tRNA synthetase [anti-GlyRS]) autoantibodies are the least common, occurring in <2% (6,14,15), although the frequency may vary in different populations (16).

Characteristic clinical features have been found in patients with anti-HisRS and other antisynthetase autoantibodies (1,9,10). These features include myositis, interstitial lung disease (ILD), arthritis, Raynaud's phenomenon, fever with exacerbations, and the skin lesion of the fingers referred to as mechanic's hands, and they appear to form a distinct syndrome referred to as the "antisynthetase syndrome" (8–11). Although the similarity of the clinical features associated with different antisynthetases is impressive (17,18), certain differences have been noted, which must be considered preliminary due to the small reported number of patients with non-HisRS antisynthetases (1,9,19). Patients with anti-AlaRS appear to be more likely than those with anti-HisRS to have ILD and/or arthritis either without myositis or with little evidence of muscle disease. Absence of significant myositis over the full disease course in patients with anti-HisRS is rare (<5%), although it may occur. Clinically significant myositis was seen in 60% of US patients with anti-AlaRS (13), whereas none of 6 Japanese patients with anti-AlaRS autoantibodies fulfilled criteria for myositis (20). Among patients with anti-IleRS, 2 of 10 had ILD without evidence of myositis, and 1 had ILD with subclinical myositis (14). In addition, antisynthetases may occur in either PM or DM, but PM is usually more common with anti-HisRS (10,16,21), and DM is usually more common with other antisynthetases, especially anti-GlyRS (15,22).

We recently described anti-KS autoantibodies and provided evidence that the KS antigen is asparaginyl–tRNA synthetase (AsnRS) (23). This sixth antisynthetase was found in sera from 3 patients with ILD and/or inflammatory arthritis without evidence of myositis. It immunoprecipitated a 65-kd protein and a unique tRNA that was distinct from that precipitated by any previously described antisynthetase or other reported tRNA-related antibody. Each of the 3 sera and their IgG fractions showed significant inhibition of AsnRS activity, but did not inhibit any of the other 19 aminoacyl–tRNA synthetase activities.

In this report, we describe the clinical and immunogenetic features of 5 additional patients with anti-AsnRS autoantibodies, most of whom had the syndrome of ILD with arthritis and/or myositis. Immunoprecipitation (IP) and aminoacylation inhibition studies with sera from these patients provide additional evidence that anti-KS (anti-AsnRS) reacts with asparaginyl–tRNA synthetase.

PATIENTS AND METHODS

Sera. Serum samples from a collection of sera from ~800 patients seen at the current or previous collaborating centers of the authors (Keio University, Tokyo, Japan; Kyoto University, Kyoto, Japan; Seoul National University, Seoul, Korea; Clinic and Research Institute for Rheumatic Diseases Aachen, Aachen, Germany; University of Oklahoma Health Sciences Center, Oklahoma City; National Institutes of Health, Bethesda, MD) or sera referred there for testing were stored at −20°C and were tested for the presence of anti-AsnRS autoantibodies. Sera from the following patients were included: 1) patients with PM or DM according to the criteria described by Bohan and Peter (24,25); 2) patients with a condition suggesting the clinical diagnosis of myositis; 3) patients with ILD who had no evidence of myositis and did not meet criteria for other connective tissue diseases; and 4) patients with serum anticytoplasmic antibodies, regardless of diagnosis. Approximately 1,700 other sera have also been tested, including sera from patients with other conditions including systemic lupus erythematosus, systemic sclerosis, and rheumatoid arthritis, as well as sera from normal subjects. Many of the sera were tested in studies of other autoantibodies. All samples were obtained after the patients gave their informed consent, as approved by the corresponding institutional review boards. Stored sera known to contain autoantibodies against synthetases for histidine, threonine, alanine, glycine, and isoleucine were used as controls.

ILD was considered to be present if an interstitial infiltrate was observed on chest radiography. DM was considered to be present if a heliotrope rash and/or Gottron's papules were observed.

IP. IP from HeLa cell extracts was performed as previously described (6,10). Ten microliters of patient sera was mixed with 2 mg of protein A–Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) in 500 μl of IP buffer (10 m*M* Tris HCl at pH 7.5, 500 m*M* NaCl, 0.1% Nonidet P40 [NP40]) and incubated with end-over-end rotation (Labquake shaker; Lab Industries, Berkeley, CA) for 2 hours at 4°C. The IgG-coated Sepharose was washed 4 times in 500 μl of IP buffer using 10-second spins in a microfuge tube, and resuspended in 400 μl of NET-2 buffer (50 m*M* Tris HCl at pH 7.5, 150 m*M* NaCl, 0.05% NP40).

For analysis of RNAs, this suspension was incubated with 100 μl of extracts, derived from 6×10^6 cells, on the rotator for 2 hours at 4°C. The antigen-bound Sepharose was then collected with a 10-second centrifugation in the microfuge, washed 4 times with NET-2 buffer, and resuspended in 300 μl of NET-2 buffer. To extract bound RNAs, 30 μl of 3.0*M* sodium acetate, 30 μl of 10% sodium dodecyl sulfate (SDS), and 300 μl of phenol/chloroform/isoamyl alcohol (50:50:1; containing 0.1% 8-hydroxyquinoline) were added to the Sepharose beads. After agitation in a Vortex mixer and

spinning for 1 minute, RNAs were recovered in the aqueous phase after ethanol precipitation and dissolved in 20 μl of electrophoresis sample buffer, composed of 10*M* urea, 0.025% bromphenol blue, and 0.025% xylene cyanol FF (Bio-Rad, Hercules, CA) in Tris–borate–EDTA buffer (90 m*M* Tris HCl at pH 8.6, 90 m*M* boric acid, and 1 m*M* EDTA). The RNA samples were denatured at 65°C for 5 minutes and then resolved by 7*M* urea–10% polyacrylamide gel electrophoresis (PAGE), with silver staining (Bio-Rad).

For protein studies, antibody-coated Sepharose was mixed with 400 μl of ^{35}S-methionine–labeled HeLa extract derived from 2×10^5 cells and rotated at 4°C for 2 hours. After 4 washes with IP buffer, the Sepharose was resuspended in SDS sample buffer (2% SDS, 10% glycerol, 62.5 m*M* Tris HCl at pH 6.8, 0.005% bromphenol blue). After heating at 90°C for 5 minutes, the proteins were fractionated by 10% SDS-PAGE, enhanced with 0.5*M* sodium salicylate, and dried. Labeled proteins were analyzed by autoradiography.

Aminoacylation. Aminoacylation inhibition reactions were performed as described previously, with minor modification (6,26). Six microliters of HeLa cell extract diluted 1:10 in Tris buffered saline was incubated with 3 μl of a 1:10 dilution of serum for 2 hours at 4°C. This was combined with 17 μl of reaction solution (50 m*M* Tris HCl at pH 7.5, 0.02*M* NaCl, 0.01*M* $MgSO_4$, 1 m*M* dithiothreitol) containing 8 units of yeast tRNA, 3 μl of ^{14}C-asparagine or other ^{3}H-labeled amino acid, and 1 μl of 200 m*M* cold amino acid. Ten-microliter aliquots were tested at 10 minutes and 20 minutes, spotted onto filter paper treated with 5% trichloroacetic acid (TCA), washed 5 times with 5% TCA, then with ethanol, then dried for counting. Results of inhibition testing were expressed as the percent inhibition of the average activity seen with the normal serum included in that experiment, as follows: % inhibition = [(average counts per minute with normal serum) − (cpm with test serum)] × 100/(average cpm with normal serum). Inhibition of >50% compared with the activity with normal serum was considered significant. In previous studies, although nonspecific effects on aminoacylation reactions by serum were common, nonspecific inhibition was usually <25%, and inhibition >50% reliably reflected specific antibody effects (6,7,12,13,26).

DNA typing of the HLA class II (DRB1, DQA1, DQB1, DPB1) alleles by polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP). Genomic DNA was isolated by phenol extraction of SDS-lysed and proteinase K–treated peripheral blood leukocytes, and then amplified by the PCR procedure using an automated PCR thermal cycler (PerkinElmer Cetus, Norwalk, CT). The primers used for specific amplification of the polymorphic exon 2 domains of the DRB1, DQA1, DQB1, and DPB1 genes were previously described (27). Amplified DNA was digested by all-specific restriction endonucleases and subjected to electrophoresis using a 12% polyacrylamide gel. Digested fragments were detected by staining with ethidium bromide, and HLA genotypes were determined on the basis of the RFLP patterns generated as previously described (27).

Other. Ouchterlony double immunodiffusion was performed as described previously, using HeLa cell extract as antigen (10).

Cases. *Patient 1.* The patient, a 61-year-old Japanese woman, noticed chest pain, followed 3 months later by dyspnea on mild exertion. Chest radiography and computed tomography (CT) scanning showed bilateral basilar infiltrates. The patient had hypoxemia, with a restrictive pattern on pulmonary function tests. No muscle weakness was observed, and the creatine kinase (CK) level was normal (67 IU/liter). A lung biopsy specimen obtained by video-assisted thoracic surgery showed mild interstitial chronic inflammation and interstitial fibrosis lacking a temporal heterogeneity pattern, and a diagnosis of fibrotic nonspecific interstitial pneumonia was made.

Patient 2. The patient, a 51-year-old German woman, developed a nonproductive cough and dyspnea on exertion. Chest radiography showed bibasilar interstitial fibrosis, and pulmonary function tests showed a restrictive pattern with decreased diffusing capacity for carbon monoxide (DLco). A diagnosis of ILD was made, and the patient's pulmonary function remained stable throughout her disease course. She had polyarthralgia and developed erythema and keratosis of the palms and fingers consistent with mechanic's hands, but no cutaneous scleroderma, Raynaud's phenomenon, or DM rash (Gottron's papules or heliotrope rash) was observed. No muscle weakness was found, and the CK level was normal (56 IU/liter at the first visit) each time it was measured. When the patient was age 58 years, ovarian carcinoma was found, and surgery with subsequent irradiation was performed. She died of metastatic ovarian carcinoma at age 63 years.

Patient 3. The patient, a 72-year-old American woman, developed an itchy red eczematous rash that was thought to be due to a medication for hypertension. The rash was soon accompanied by progressive weakness, myalgias, mild dyspnea, and difficulty swallowing. She was started on prednisone and methotrexate, and 6 months after the rash had first appeared, she was referred to the Arthritis and Rheumatism Branch of the National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health. There was a widespread maculopapular rash of the trunk, extremities, and head, and Gottron's papules were observed. Proximal muscle weakness was present, and her CK level was 358 IU/liter. Magnetic resonance imaging of the thighs showed both atrophy and probable inflammation on the STIR images. A biopsy of the deltoid muscle showed changes of an active inflammatory myopathy. No malignancy was identified. She was treated with pulse methylprednisolone. However, her muscle weakness and rash were not significantly improved, and infectious complications limited the therapeutic options. Her disease course was subsequently complicated by herpes zoster and the Ramsay-Hunt syndrome as well as by skin infections and cellulitis, mastoiditis, heart failure, and a cerebrovascular accident.

Patient 4. The patient, a 53-year-old Korean woman with intermittent episodes of productive cough due to bronchiectasis, noticed easy fatigability and myalgia in 1994 and later developed muscle weakness and was admitted to Seoul National University Hospital in February 1995. Proximal muscle weakness in her extremities and a dark pigmentation over the extensor surface of both knees were observed. The CK level was elevated at 3,808 IU/liter. The findings on electromyogram and muscle biopsy were consistent with inflammatory myopathy. A diagnosis of DM associated with ILD was made, and she was treated with prednisolone (60 mg/day). Her muscle enzyme levels gradually normalized, and her muscle weakness improved. Her chest radiograph and high-resolution

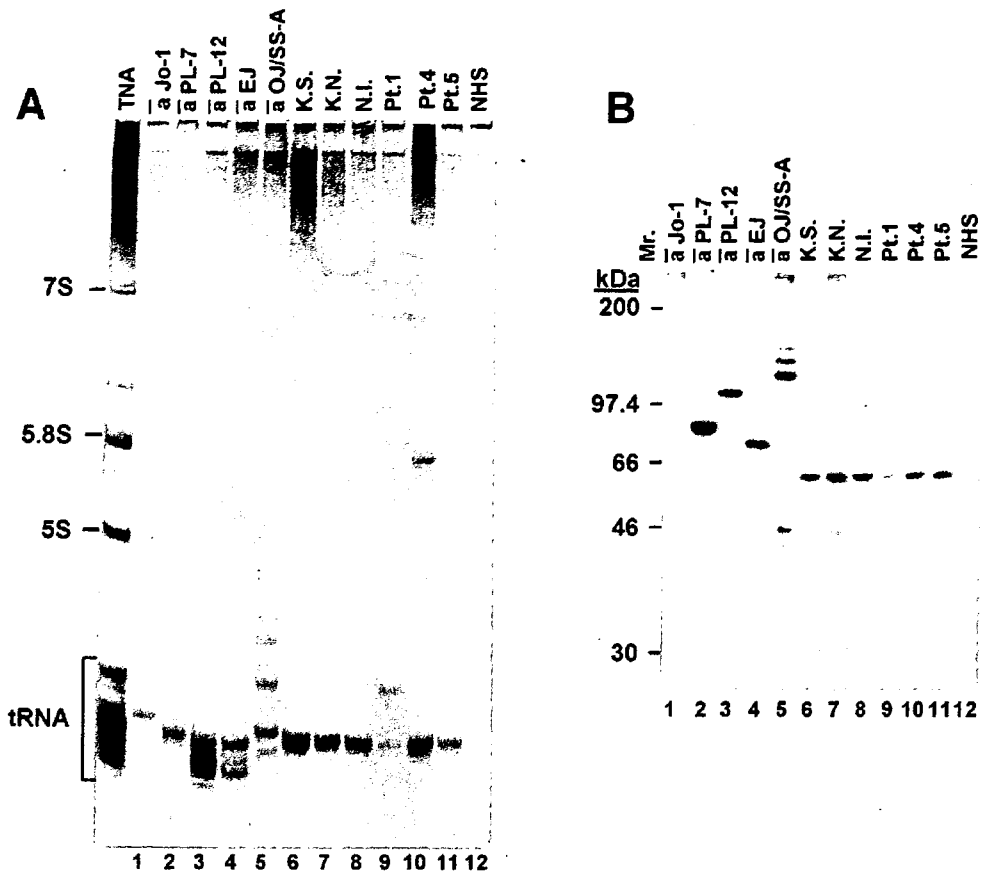

Figure 1. **A,** Immunoprecipitation (IP) for nucleic acids with anti-KS and control sera. Shown are patterns of transfer RNA (tRNA) resulting from 7*M* urea–10% polyacrylamide gel electrophoresis (PAGE) of phenol-extracted immunoprecipitates from HeLa cell extract, developed with silver stain. TNA = total nucleic acids, with the 5.8S and 5S small ribosomal RNAs and the tRNA region indicated. Antisynthetase sera used for IP are indicated. Lane 1, Anti–histidyl–tRNA synthetase (a Jo-1); lane 2, anti–threonyl–tRNA synthetase (a PL-7); lane 3, anti–alanyl–tRNA synthetase (a PL-12); lane 4, anti–glycyl–tRNA synthetase (a EJ); lane 5, anti–isoleucyl–tRNA synthetase (a OJ/SS-A); lanes 6–11, anti-KS sera from patients KS, KN, and NI in the previous study (23) and from patients 1, 4, and 5 in the present study; lane 12, normal human serum (NHS) control. The tRNA pattern with anti-KS sera is easily distinguishable from that of other antisynthetases. **B,** IP for proteins with anti-KS and control sera. Autoradiogram of 10% sodium dodecyl sulfate–PAGE of immunoprecipitates from ^{35}S-methionine–labeled HeLa cell extract. Mr. = molecular weight markers. Antisynthetase sera used for IP are indicated as in A. Anti-KS sera immunoprecipitated a very strong protein band from ^{35}S-methionine–labeled HeLa cell extracts (lanes 6–11), migrating at 65 kd, that was clearly different from the bands immunoprecipitated by sera with the described antisynthetases.

CT scan showed bilateral basilar interstitial fibrosis, and pulmonary function tests showed a restrictive pattern with decreased DLco. Her muscle weakness gradually improved, and the CK level normalized in January 1996. Prednisolone was tapered and discontinued in March 1996.

Patient 5. The patient, a 64-year-old Japanese man with a previous history of prostatic carcinoma, was admitted to the hospital due to bilateral infiltrates on chest radiography. He did not notice cough or dyspnea at that time, but a chest CT scan revealed bibasilar interstitial fibrosis. A transbronchial lung biopsy was performed, with histology showing usual interstitial pneumonia. He was started on prednisolone (40 mg/day), resulting in slight improvement seen on his chest radiograph. Prednisolone was tapered and discontinued in April 1998. He then developed polyarthritis and was treated with a nonsteroidal antiinflammatory drug. No muscle weakness was found, and the CK level was normal (50 IU/liter at the first visit) throughout his disease course.

RESULTS

Identification of anti-KS (anti-AsnRS) antibodies. Sera from all 8 patients (the 3 patients with ILD and/or inflammatory arthritis without evidence of myositis in our previous study [patients KS, KN, and NI; see

Table 1. Clinical features of 8 patients with anti-KS antibodies*

	Patient							
	KS	KN	NI	1	2	3	4	5.
Age at onset, years/sex	36/F	44/F	61/F	60/F	51/F	72/F	53/F	65/M
Ethnic background	Japanese	Japanese	Japanese	Japanese	German	US	Korean	Japanese
ILD	Yes	Yes	Yes	Yes.	Yes	No	Yes	Yes
Myositis	No	No	No	No	No	Yes	Yes	No
DM rash	No	No	No	No	No	Yes	Yes	No
Arthritis	Yes	No	No	No	Yes	Yes	No	Yes
Malignancy	No	No	No	No	Ovarian cancer	No	No	Prostate cancer
Raynaud's phenomenon	No	Yes	No	No	No	No	No	No
Other autoantibodies	No	No	No	Anti-SSA/Ro	No	No	No	No
Diagnosis	ILD with arthritis	Idiopathic ILD	Idiopathic ILD	Idiopathic ILD	Idiopathic ILD	DM	DM	ILD with arthritis

* ILD = interstitial lung disease; DM = dermatomyositis.

ref. 23] and the 5 additional patients described above) were shown to immunoprecipitate a characteristic, identical pattern of tRNA, with a strong predominant nucleic acid band of tRNA size, accompanied by a faster faint band (Figure 1A). This gel pattern of tRNA was clearly distinguishable from the pattern of tRNA precipitated by the 5 other described antisynthetases (Figure 1A) and was identical in mobility and appearance to that of serum KS, the originally reported anti-KS serum (23) (Figure 1A).

A very strong band from ^{35}S-methionine–labeled HeLa cell extracts (Figure 1B), migrating at 65 kd, that was also identical in mobility to that of serum KS, was found by IP for all 8 sera, with 5 representative sera shown in Figure 1B. This was clearly different from the characteristic bands immunoprecipitated by sera with the other described antisynthetases (Figure 1B).

Five of the newly recognized anti-KS antibody–positive sera were tested for their ability to inhibit the in vitro enzymatic function of AsnRS (aminoacylation of $tRNA^{Asn}$). Four of the 5 new anti-KS sera significantly inhibited (by >50% at 10 minutes) AsnRS activity compared with normal serum or other controls (serum from patient KS by 87%, serum from patient KN by 99%, serum from patient NI by 91%, serum from patient 1 by 82%, serum from patient 2 by 100%, serum from patient 3 by 18%, serum from patient 4 by 87%, and serum from patient 5 by 91%). This inhibition was strong and comparable with that seen with serum KS, for 4 of the 5 new anti-KS sera. Purified IgG from the third new serum (from patient 3) showed significant, but not strong, inhibition (52%) that increased at 20 minutes (to 84%).

There was no significant inhibition of other synthetases. Normal control serum and anti-KS–negative myositis serum did not show significant inhibition of AsnRS, although sera with other antisynthetases inhibited the expected enzymes. These results indicated that sera with anti-KS by IP showed specific inhibition of AsnRS, further supporting previous data indicating that anti-KS reacted with AsnRS.

Clinical findings. The clinical features of the 5 newly identified patients (patients 1–5) and the 3 patients with anti-AsnRS reported previously (patients KS, KN, and NI) (23) are summarized in Table 1. All patients with anti-AsnRS antibodies were middle-aged or elderly, and 7 of them were women. Five patients were Japanese, 1 was from the US, 1 was German, and 1 was Korean. Seven of these 8 patients (88%) had ILD, documented in each case by both chest radiography and pulmonary function tests. In addition, 2 patients had myositis and a diagnosis of DM. Their clinical courses of ILD were classified as the chronic type. Four patients (50%) had nonerosive arthritis or arthralgia. Raynaud's phenomenon was seen in only 1 patient. None of the patients had sclerodactyly or overlap syndromes with other connective tissue diseases. Malignant diseases (ovarian carcinoma and prostatic carcinoma) were observed in 2 patients. Regarding other autoantibodies, anti-SSA/Ro antibodies were detected in only 1 patient.

Anti-AsnRS was found in 0% of Japanese patients with myositis, but was found in 3% of Japanese patients with "idiopathic" ILD. Thus, most patients with anti-AsnRS antibodies had chronic ILD with or without features of PM/DM or other connective tissue disease.

Immunogenetic features. The HLA class II gene was determined in 4 Japanese patients (Table 2). All 4 patients had DR2 (DRB1*1501 or DRB1*1502) compared with 33% of healthy local controls. It should be noted that all patients with anti-AsnRS antibodies had DR2, but the frequency of DR2 did not reach statistical significance ($P > 0.05$).

Table 2. HLA class II genes in Japanese patients with anti-KS autoantibodies

	Patient			
	KS	KN	NI	1
DR	2/5	2/1	2/2	2/4
DRB1*	1502/1101	1501/0101	1502/1502	1501/0405
DQA1*	0103/0501	0102/0101	0103/0103	0102/0303
DQB1*	0601/0301	0602/0501	0601/0601	0602/0401
DPB1*	0901/1401	0201/0501	0901/0901	0201/0402

DISCUSSION

We have identified anti-KS (anti-AsnRS) autoantibodies in 8 patients with ILD and DM, by IP of the same distinctive set of tRNA and protein that differed from those precipitated by the other 5 antisynthetases. Most of the anti-KS sera showed specific inhibition of the enzyme target, AsnRS, without inhibiting other synthetases.

Several interesting characteristics of the previously studied antisynthetases have been described: 1) they are associated with a distinctive clinical syndrome referred to as the antisynthetase syndrome, 2) they are directed at functionally related enzymes (performing the same function for different amino acids), 3) they do not cross-react with other synthetases, and 4) they tend to be mutually exclusive. Anti-AsnRS antibodies seem to have the same features. No serum with any other antisynthetase has had antibodies to AsnRS, and none of the 8 anti-AsnRS sera reported here showed signs of reaction with other synthetases. The mechanism of this phenomenon remains unknown.

Multiple tRNA bands immunoprecipitated by anti-AsnRS were found on urea-PAGE. The patterns of tRNA for each of the 8 patients were very similar, highly restricted compared with total tRNA, and distinctive compared with the pattern of other anti–aminoacyl tRNA synthetase autoantibodies. These bands are likely to represent different forms of tRNA for asparagine, which can include tRNA with different asparagine anticodons (uracil-uracil-adenine, uracil-uracil-guanine) or tRNA with the same anticodon but differences in other parts of the sequence. Most sera with anti-HisRS, anti-ThrRS, anti-GlyRS, and anti-IleRS had not been described to react directly with tRNA, suggesting indirect precipitation of tRNA. However, approximately one-third of anti-HisRS–positive sera were reported to contain autoantibodies recognizing $tRNA^{His}$ (28). Most anti-AlaRS sera react directly with the sets of $tRNA^{Ala}$ with the inosine-guanine-cytosine anticodon (29). We previously found that the 3 original anti-KS (anti-AsnRS) sera did not immunoprecipitate any RNA from deproteinized HeLa extracts (23). This suggests that anti-AsnRS antibodies can precipitate $tRNA^{Asn}$ indirectly, through its affinity for AsnRS, although the possibility of conformational epitopes on the tRNA has not been excluded (28). Further analysis will be necessary to determine the sequence and specificity of tRNA immunoprecipitated by anti-AsnRS.

The specific inhibition of AsnRS function by most of the sera found to have anti-KS is consistent with findings observed for other antisynthetases. It should be noted that our anti-KS sera also demonstrated inhibition of enzymatically active recombinant AsnRS (30). Most sera with any of the 5 reported antisynthetases specifically inhibit the aminoacylation of the respective tRNA, indicating inhibition of the enzymatic function of the synthetase (3,5–7,12). This functional inhibition may indicate that the autoantibodies are recognizing the active sites of the synthetases. In contrast, it has been reported that animal antisera raised against synthetases do not consistently show such inhibition, suggesting that active sites tend not to be immunogenic for animals (31). Hypothetically, this could relate to relative conservation of the active site. However, there might be an alternative mechanism for inhibition. For example, binding of antibodies outside the active site may alter the structure of the enzyme or interfere with enzyme activity sterically. Further studies of the precise epitope on the aminoacyl–tRNA synthetase might help to explain the development of these autoantibodies.

Each of the 5 previous antisynthetases was first identified in patients with myositis and then found to be associated with ILD. In previous studies, these autoantibodies were associated with myositis with a high frequency of ILD (50–80%) and arthritis (50–90%) (1,2,17,18), as well as an increase in Raynaud's phenomenon (60%), fever with exacerbations (80%), and the skin lesion of the fingers referred to as mechanic's hands (70%) when compared with the overall population of patients with myositis (9–11). The similarities between patients with different antisynthetases have been noted, whereas certain differences have been found, which must be considered preliminary due to the small reported number of patients with non-HisRS antisynthetases. Absence of significant myositis over the full disease course in patients with anti-HisRS is rare (<5%) (32), whereas patients with anti-AlaRS are more likely than patients with anti-HisRS to have ILD and/or arthritis without clinical evidence of myositis (19). Anti-ThrRS

resembles anti-HisRS more than anti-AlaRS in Japanese patients (33).

In the present study, 7 of 8 patients (88%) with anti-AsnRS autoantibodies had ILD, some with other associated features of connective tissue disease including arthritis and Raynaud's phenomenon. In this respect, anti-AsnRS appears to resemble anti-AlaRS more than anti-HisRS. It is noteworthy that the 2 patients with both anti-AsnRS and myositis were among the 3 patients from outside Japan, while none of 5 patients from Japan had myositis. Thus, as with patients with anti-AlaRS, for patients with anti-AsnRS, the frequency of ILD without myositis may be higher in Japanese patients. However, most of the group of patients with ILD without myositis who were tested in this study were from Japan.

The features of these 8 patients with anti-KS appeared to reside within the spectrum of the antisynthetase syndrome that has been associated with other antisynthetases. ILD is one of the most important features of the antisynthetase syndrome, and Raynaud's phenomenon and arthritis, as seen in some patients with anti-AsnRS, are also likely to be part of the syndrome. The syndrome associated with anti-AsnRS may be one end of the spectrum of patients with antisynthetase. This highlights the clinical importance of looking for such antibodies in patients with ILD even if there are no signs of myositis or connective tissue diseases.

The typical cutaneous features of DM were observed in 2 patients with anti-AsnRS antibodies. PM has been reported to be much more common (60–80% or more) than DM in patients with anti-HisRS in most studies, whereas DM was most frequent with anti-GlyRS (15) and was also found to be common among patients with anti-AlaRS (13). Like anti-GlyRS and anti-AlaRS antibodies, anti-AsnRS antibodies were more associated with DM in the small number of patients available.

Malignancy has been reported to be unusual in patients with antisynthetases. In our studies, 2 patients were found to have malignancy during their disease course. However, malignancy in these patients may not be related to the DM or ILD, since these malignancies occurred separated in time from each other.

Immunogenetic studies of connective tissue disease have been performed, but HLA associations produced conflicting results. However, a strong correlation of HLA class II antigens with some autoantibodies has been reported (34). With regard to antisynthetase antibodies, HLA–DR3 (DRB1*0301), DQA1*0501, or DQA1*0401 was found to be significantly increased in myositis patients with antisynthetases (9,21). In Japanese patients, we have reported that 7 of 9 patients (78%) with anti-HisRS tested had the HLA class II DRB1*0405;DQA1*0302;DQB1*0401 haplotype, compared with 22% of healthy controls (odds ratio [OR] 13, $P = 0.002$), while 4 of 7 patients (57%) with anti-AlaRS had the DRB1*1501;DQA1*0102;DQB1*0602 haplotype, compared with 9% of healthy controls (OR 14, $P = 0.006$) (35). Interestingly, all 4 Japanese patients tested had DR2 (DRB1*1501/1502), compared with 33% of healthy controls, although a definite statistical association could not be established. These results suggest that the stronger association of anti-AlaRS and anti-KS with ILD may be related to the DR2 phenotype. However, it has been noted that different ethnic groups exhibit different immunogenetic profiles that link with specific autoantibodies (36). Therefore, further studies including analysis of more patients with anti-KS antibodies in different ethnic groups and major histocompatibility complex–restricted T cell responses could provide important clues for understanding the possible mechanisms for the development of antisynthetase antibodies.

The mechanism for the association of antisynthetases with ILD is unknown, but it seems to be related to etiologic factors (37). Recently, a new association of anti-HisRS–positive PM and ILD was reported in a patient with hepatitis C virus infection (38). It was hypothesized that viruses might interact with the synthetases and induce autoantibodies by molecular mimicry or antiidiotype mechanisms in the anti-HisRS–positive patient with myositis associated with ILD (3,39). Another mechanism for generating autoantigenic epitopes of synthetase by granzyme B cleavage in apoptosis was also described recently (40,41). However, these proposed mechanisms remain speculative, and further studies could provide important clues for understanding the possible mechanisms for the development of these antibodies. Studies of these antibodies may provide insight into the etiologic and pathogenetic mechanisms of ILD and myositis.

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AUTHOR CONTRIBUTIONS

Dr. Hirakata had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Hirakata.
Acquisition of data. Hirakata, Nagai, Genth, Song, Targoff.
Analysis and interpretation of data. Hirakata, Suwa, Takada, Sato, Mimori.
Manuscript preparation. Hirakata, Takada, Targoff.
Statistical analysis. Hirakata, Suwa, Targoff.

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Longterm Effect of Intermittent Cyclical Etidronate Therapy on Corticosteroid-Induced Osteoporosis in Japanese Patients with Connective Tissue Disease: 7-Year Followup

SHINJI SATO, TETSUYA TAKADA, YUMIKO KATSUKI, NORIKO KIMURA, YUKO KANEKO, AKIRA SUWA, MICHITO HIRAKATA, and MASATAKA KUWANA

ABSTRACT. ***Objective.*** To determine the efficacy and safety of intermittent cyclical etidronate therapy of up to 7 years for corticosteroid-induced osteoporosis.

Methods. One hundred two Japanese patients who originally participated in a 3-year prospective randomized study were enrolled into an open-label followup study. All patients had received > 7.5 mg of prednisolone daily for at least 90 days before entry into the original study and were randomly assigned to 2 treatment arms: E, those receiving etidronate disodium (200 mg per day) for 2 weeks together with 3.0 g of calcium lactate and 0.75 μg of alphacalcidol daily; and C, controls receiving only the latter. Endpoints included changes from baseline in bone mineral density (BMD) of the lumbar spine and the rate of new vertebral fractures.

Results. The mean (± SD) lumbar spine BMD had increased by 5.9% ± 8.8% (p = 0.00007) and 2.2% ± 5.8% (p = 0.013) from baseline after 7 years in groups E and C, respectively. This improvement in BMD in group E was significantly better than in group C (p = 0.02). The frequency of new vertebral fractures was lower in group E, resulting in reduction of the risk of such new fractures by 67% at year 7 (odds ratio 3.000; 95% confidence interval, 0.604–14.90; p = 0.18). There were no severe adverse events in group E during our study.

Conclusion. Our results indicate that longterm (up to 7 years) intermittent cyclical etidronate therapy is safe and effective for prevention and treatment of corticosteroid-induced osteoporosis in patients with connective tissue diseases. (First Release Nov 15 2007; J Rheumatol 2008;35:142–6)

Key Indexing Terms:
CORTICOSTEROID-INDUCED OSTEOPOROSIS
BONE MINERAL DENSITY
BISPHOSPHONATE
CONNECTIVE TISSUE DISEASES

Longterm corticosteroid treatment in patients with connective tissue disease (CTD) causes osteoporosis as the major adverse event. Bisphosphonate therapy has proven to be effective in both prevention and treatment of corticosteroid-induced osteoporosis (CIOP)[1-4]. Guidelines for treating patients with CIOP recommend the use of bisphosphonates as a first-line drug[5]. Nitrogen-containing bisphosphonates, such as alendronate or risedronate, have proven efficacy for both prevention and treatment of CIOP. However, use of these bisphosphonates is associated with gastrointestinal adverse events[6]. We previously conducted a 3-year prospective randomized study to determine the efficacy and safety of etidronate (the first available nitrogen-free bisphosphonate) for treating CIOP[7]. Although longterm followup (7 yrs) of intermittent cyclical etidronate therapy in patients with postmenopausal osteoporosis has been reported[8], few studies are available on the longterm effects of etidronate in patients with CIOP[9]. Further, there are no reports on the continued effectiveness and safety of etidronate for CIOP in patients with CTD. For this reason, we have followed up the original 3-year prospective study for an additional 4 years to determine the longterm efficacy of intermittent cyclical etidronate for treating CIOP in patients with CTD.

MATERIALS AND METHODS

Patients. In the original 3-year study, 102 patients with different CTD were enrolled (56 with systemic lupus erythematosus; 12 rheumatoid arthritis; 10 polymyositis/dermatomyositis; 9 vasculitis syndrome; 8 adult-onset Still disease; 5 polymyalgia rheumatica; 1 systemic sclerosis; and 1 Sjögren's syndrome). Patients' ages ranged from 21 to 73 years and they had been taking

From the Department of Internal Medicine, Keio University, School of Medicine, Tokyo; and the Division of Rheumatology, Department of Internal Medicine, Tokai University School of Medicine, Kanagawa, Japan.

S. Sato, MD; T. Takada, MD; Y. Katsuki, MD; N. Kimura, MD; Y. Kaneko, MD; M. Hirakata, MD; M. Kuwana, MD, Department of Internal Medicine, Keio University; A. Suwa, MD, Division of Rheumatology, Department of Internal Medicine, Tokai University School of Medicine.

Address reprint requests to Dr. S. Sato, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: shins@sc.itc.keio.ac.jp

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> 7.5 mg of prednisolone (PSL) daily for at least 90 days. The results of this study have been reported elsewhere[7].

Study design. In the original 3-year prospective randomized study, all patients were randomly assigned to one of 2 investigational groups. Patients in the etidronate group (E) received 200 mg/day etidronate disodium (Didronel, Sumitomo Pharmaceuticals, Osaka, Japan) for 2 weeks, together with 3.0 g of calcium lactate and 0.75 μg of alphacalcidol (Alfarol, Chugai Pharmaceuticals, Tokyo, Japan) daily for 90 days. This cycle was repeated 28 times during the 7-year observation period. Patients were instructed to take their medication with water at bedtime. The control group (C) received only 3.0 g of calcium lactate and 0.75 μg of alphacalcidol daily for 90 days. In the followup study, the patients were to continue taking the same treatments to which they had been assigned earlier. However, a change of treatment was allowed if their doctor decided that it was necessary for treatment. All patients had provided written informed consent.

Bone mineral density (BMD) and radiological measurements. Lateral and anteroposterior lumbar and thoracic spine radiographs were taken and evaluated at Keio University Hospital at baseline and every year for 7 years. All lumbar and thoracic spine images were evaluated by experienced physicians who were blinded to treatment assignments. The diagnosis of vertebral fracture and osteoporosis was based on the criteria defined by the Japanese Society for Bone and Mineral Research in 1996[10]. A vertebral fracture was defined as: (1) The ratio of the central height (C) to the anterior height (A) of the vertebra was less than 0.8, or the ratio of C to the posterior height (P) was less than 0.8. (2) The ratio of A to P was less than 0.75. (3) A crushed vertebra was recorded when its height was reduced by more than 20% in either A, C, or P compared with the adjacent vertebrae.

Classification of BMD was based on the following criteria: Normal BMD: > 80% of the young adult mean (YAM). Osteopenia: between 70% and 80% of YAM. Osteoporosis: < 70% of YAM.

This definition of osteoporosis (i.e., < 70% of YAM) also corresponds to the osteoporosis criteria recommended by the World Health Organization [less than –2.5 standard deviation (SD) of YAM]. All BMD measurements were made by dual-energy x-ray absorptiometry using an XR-36 (Norland Medical Systems, Fort Atkinson, WI, USA). Because we had already documented changes in bone formation and bone absorption markers in the original 3-year prospective randomized study, we did not monitor biochemical markers of bone turnover in this followup study.

Statistical analysis. The baseline characteristics and homogeneity of the patients' background in an intent-to-treat population was compared between the 2 investigational groups by chi-square test, Student's t-test, and Mann-Whitney U-test, as appropriate. Regardless of whether patients were still receiving the assigned medication, all available BMD data were used to perform an intent-to-treat analysis. If the measurement of the lumbar spine BMD at 7 years was not available, the measurement obtained at the time closest to this was used in the analysis. The patients whose BMD data could not be evaluated correctly because of previous compression fractures are excluded from the analysis. The primary efficacy analysis was based on the differences between the 2 investigational groups in the percentage change of lumbar spine BMD (L2-L4) from baseline to last measurement. The percentage change of BMD from the baseline was compared by an analysis of variance model (SPSS version 14.0). The comparison of percentage change of BMD between the 2 groups was calculated by Student's t-test. Odds ratios adjusted by menopausal status stratum as a factor were calculated for differences of the incidence of vertebral fractures at 7 years between the 2 treatment groups. Significance level was set at 5% and all results expressed as mean ± SD.

RESULTS

At the beginning of this followup study, 7 patients in group E and 6 patients in group C could not be included because of death or loss to followup. There were no significant differences between groups in baseline characteristics in that subset of patients whose data could be used for an intent-to-treat analysis (43 and 45 in groups E and C whose BMD data were available, respectively; Table 1). During 7 years, the average daily dose of PSL in each year was not significantly different between the 2 groups. The number of patients taking steroid in groups E and C was also not significantly different (Table 2). During the followup study, there were no adverse events in either group. However, 2 patients in group E and one in group C died due to progression of their underlying CTD or infection during this study. Ten patients in group C began to receive bisphosphonate as well as alphacalcidol and calcium lactate because their rheumatologist decided that they would benefit from such treatment. On the other hand, only 1 patient in group E was changed from etidronate to alendronate.

After 7 years of treatment, the mean (± SD) percentage change in BMD of the lumbar spine in group E (5.9% ± 8.8%) was significant compared to baseline ($p = 0.00007$). This was also the case, albeit to a lesser extent (2.2% ± 5.8%), in group C ($p = 0.013$; Figure 1). This improvement of BMD was significantly greater in group E than in group C ($p = 0.02$).

In a separate analysis of premenopausal and postmenopausal women, both of these subgroups of group E showed an increase in the mean percentage change in BMD during their treatment course. In premenopausal women, both groups E and C had significant increases of lumbar spine BMD from baseline at 7 years ($p = 0.001$ and $p = 0.02$, respectively). These increases were significantly higher in group E than C in a subgroup of premenopausal women (6.7% ± 9.1% vs 2.3% ± 4.5%; $p = 0.04$). Although the postmenopausal subgroup of group E showed an increase in BMD of the lumbar spine, this failed to achieve significance (2.8% ± 8.0%; $p = 0.23$). BMD of this subgroup in group C remained at baseline (–0.03% ± 7.60%; $p = 0.99$). There were also no significant differences between the 2 groups in this respect.

Analysis of the subgroups based on the baseline BMD revealed that the osteoporosis + osteopenia subgroup in group E showed a significant increase of the lumbar spine BMD from baseline at 7 years ($p = 0.0009$). This increase was significantly greater in group E than in group C at 7 years (7.8% ± 9.5% vs 2.0% ± 6.3%; $p = 0.04$). Both E and C groups of the normal BMD subgroup showed significant increases in lumbar spine BMD at year 7 (3.9% ± 7.7%, $p = 0.03$ and 2.4% ± 5.6%, $p = 0.03$, respectively). Again, there were no significant differences between the 2 groups ($p = 0.40$).

The mean percentage change in lumbar spine BMD in group E improved from baseline by approximately 5% over the 7 years (Figure 2). Although group C showed no decrease of BMD from baseline, the increase in group E was significantly greater than in group C at 7 years ($p = 0.02$).

Six patients in group C had a total of 11 new vertebral fractures during the followup period (Table 3), whereas only 2 patients in group E had a total of 3 new fractures. At year 7, cyclic etidronate therapy had reduced the risk of new vertebral fracture by 67% [odds ratio (OR) 3.00; 95% confidence inter-