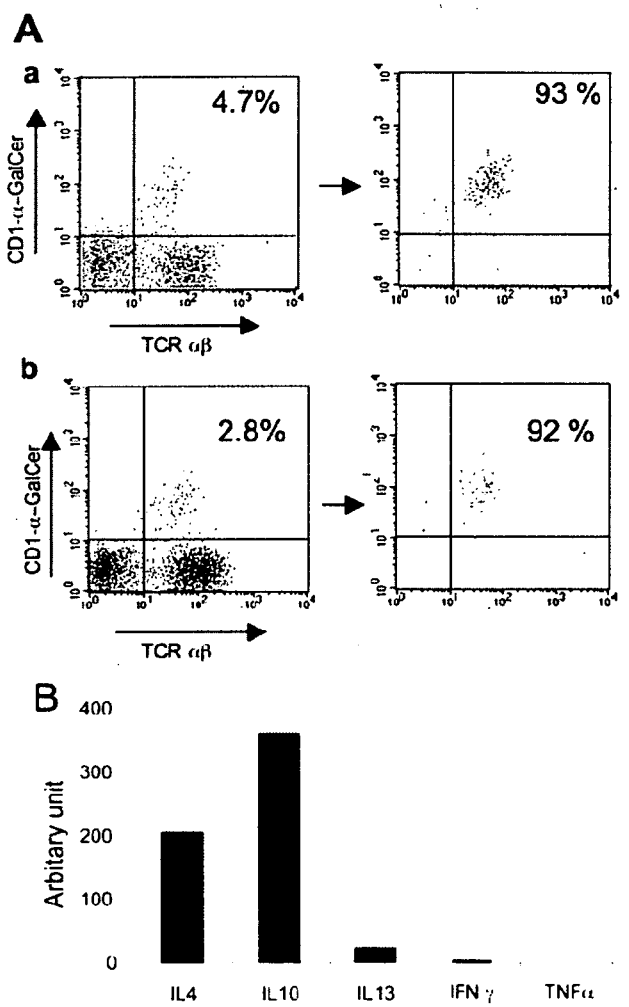

FIGURE 4. NKT cells produce Th2 cytokine after α-GalCer administration and *T. gondii* infection. *A*, NKT cell populations were isolated from the lamina propria of mice treated (*a*) or not treated (*b*; two mice per group) with α-GalCer on day 8 after infection. The cells were then purified on the basis of CD1d/α-GalCer tetramer staining using anti-allophycocyanin magnetic beads. The purity of sorted cells was confirmed by FACS analysis. This experiment was repeated twice. *B*, Purified NKT cells were analyzed for mRNA expression of Th1 and Th2 cytokines by real-time RT-PCR. Results are expressed as the relative increase in the cytokines in NKT cells from treated mice compared with the untreated NKT cells after normalization with the housekeeping gene. Results are representative of two independent experiments.

>90% in both α-GalCer-treated (Fig. 4*A*, *a*) and untreated (Fig. 4*A*, *b*) animals. The mRNA production of different cytokines by the purified NKT cell population was measured by RT-PCR. The results are expressed as the relative increase or decrease in mRNA expression for different cytokines in NKT cells isolated from α-GalCer-treated mice compared with control infected, but untreated, mice. Compared with controls, IL-10, IL-4, and IL-13 mRNA expressions were increased in the NKT cell population isolated on day 8 from mice treated with α-GalCer and infected (Fig. 4*B*). These data indicate that treatment with α-GalCer shifts the NKT cell cytokine pattern to a Th2-like profile.

The production of IL-10 and IL-4 by NKT cells stimulated with α-GalCer was increased in the intestines of treated mice. In contrast, IL-13 production by NKT cells after treatment with α-GalCer did not lead to an increase in this cytokine in the whole intestine throughout the serial time points after infection.

Role of IL-4 in protection against T. gondii*-induced death*

The contribution of IL-4 production associated with α-GalCer treatment to interference with the induction of *T. gondii*-induced death was evaluated by a series of experiments using blocking Ab. Blocking of IL-4 the day before α-GalCer treatment partially reversed its beneficial effect, as shown by a 50% survival rate compared with 100% survival of mice in the α-GalCer alone-treated group (Fig. 5*A*). These observations suggest a partial role for IL-4 in the protection induced by α-GalCer in this model.

Critical role of IL-10 in protection against T. gondii*-induced ileitis*

The contribution of IL-10 production associated with α-GalCer treatment in interfering with the induction of *T. gondii*-induced death was evaluated using genetically deficient and chimeric mice. Strikingly α-GalCer treatment had no beneficial effect on protection in IL-$10^{-/-}$ mice (Fig. 5*B*). These observations suggest a pivotal role for IL-10.

To determine whether IL-10 produced by NKT cells was sufficient to suppress lethal intestinal inflammatory lesions, double-chimeric mice were generated. B6 mice were irradiated and reconstituted by a 50/50% mix of bone marrow cells from Jα281$^{-/-}$ (NKT cell-deficient) and IL-$10^{-/-}$ mice. After reconstitution, the double-chimeric mice expressed a normal immunological phenotype, except for the NKT cells that were IL-$10^{-/-}$ (NKT IL-$10^{-/-}$). These NKT IL-$10^{-/-}$ chimeric mice and their appropriate controls (B6 mice, Jα281$^{-/-}$ and IL-$10^{-/-}$ mice) were treated with α-GalCer the day before infection. NKT IL-$10^{-/-}$ chimeric mice treated with α-GalCer rapidly lost more weight than α-GalCer-treated B6 mice (Fig. 5*C*), indicating that the lack of IL-10 production by the NKT cells alone conferred greater susceptibility to the infection.

However, in contrast to what was expected, the decreased protective effect of α-GalCer treatment in NKT IL-$10^{-/-}$ chimeric mice did not lead to a significant increase in the mortality rate (80% survival; Fig. 5*D*). These results, demonstrating the complete lack of effect of α-GalCer treatment in IL-$10^{-/-}$ mice (Fig. 5*B*) and a reduced effect of this treatment in NKT IL-$10^{-/-}$ chimeric mice (Fig. 5, *C* and *D*), suggested that other cell types might be the source of the IL-10 that is critical for protection. T regulatory cells ($CD4^+CD25^+$) that express the transcription factor FoxP3 and are known as important IL-10 producers were assessed after treatment with α-GalCer and infection. Interestingly, the number of $CD4^+CD25^+$ cells from intestines and MLNs were increased on days 6 and 9, respectively (data not shown), after infection, and this correlates with an increased expression of FoxP3 in the intestine on day 6 and in MLNs on day 9 from B6 mice, but not from Jα281$^{-/-}$ mice (Fig. 6*A*). The sorted $CD4^+CD25^+$ cell subpopulation exhibited IL-10 mRNA expression (data not shown). Whatever the time after infection and the treatment with or without α-GalCer, the sorted NKT cell population failed to express either FoxP3 or CD25. To better characterize the implication of these T regulatory cell subpopulations to the protective process induced by α-GalCer, the effect of this treatment in mice also treated with blocking anti-CD25 Abs was studied. Treatment with anti-CD25 abrogated the protection (Fig. 6*B*), indicating the crucial role of these cells in the anti-inflammatory process induced by treatment with α-GalCer.

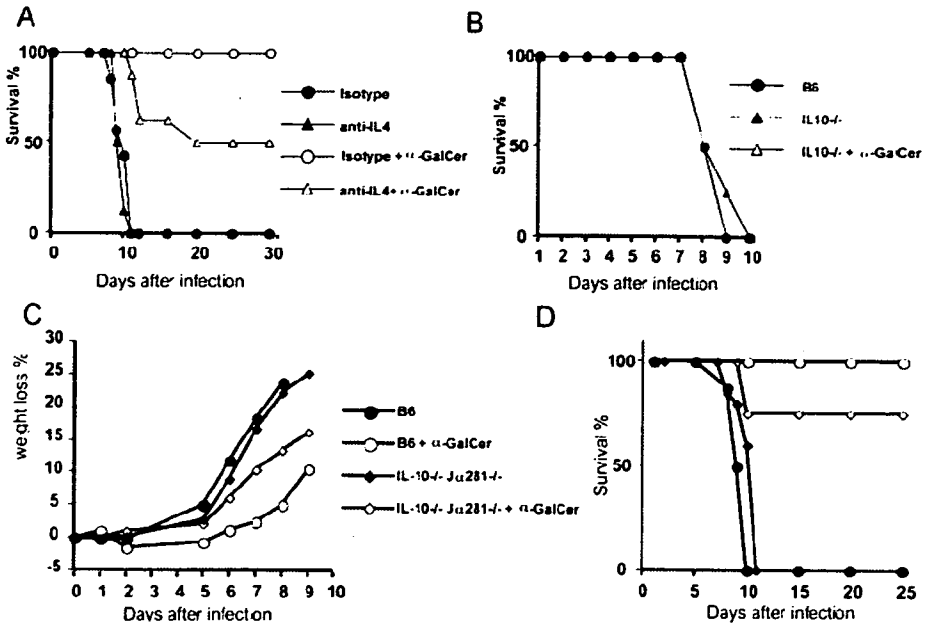

FIGURE 5. Roles of IL-4 and IL-10 in the protective process. *A*, Neutralization of IL-4 the day before α-GalCer treatment partially altered its protective effect. B6 mice were treated with 1 mg of anti-IL-4 Abs 24 h before α-GalCer treatment and 48 h before infection with *T. gondii*. Similarly infected α-GalCer-treated B6 mice and B6 mice treated with rat IgG were used as controls (eight mice per group). The survival rate of these mice was determined. Similar results were obtained in another separate experiment. *B*, α-GalCer treatment fails to protect IL-$10^{-/-}$ mice. IL-$10^{-/-}$ mice were treated (eight mice per group) with 5 μg of α-GalCer i.p. 24 h before infection. As a control, IL-$10^{-/-}$ mice and B6 mice sham-treated with DMSO alone were infected, and the survival rates of all mice were determined. *C* and *D*, IL-10 produced by NKT is partially responsible for the protective effect of α-GalCer. To assess the role of IL-10 produced by NKT cells, chimeric mice were generated. B6 mice were irradiated and then received i.v. 1×10^7 bone marrow cells recovered from femurs and tibias of donor mice. To generate mice in which only NKT cells were devoid of the IL-10 gene, a mixture (50/50%) of bone marrow cells from Jα281$^{-/-}$ mice and IL-$10^{-/-}$ mice was used for reconstitution (12 mice/group). Control mice were reconstituted with bone marrow from B6, Jα281$^{-/-}$, or IL-$10^{-/-}$ mice (six mice per group). Six weeks later, the efficiency of the reconstitution was determined. Chimeric mice were then infected, and weight loss (*C*) and survival rate (*D*) were recorded. This experiment has been performed twice with similar results.

Discussion

In contrast to B6 mice that develop acute lethal ileitis after oral infection with *T. gondii*, mice deficient in NKT cells, although permissive to parasite replication, are more resistant to this severe immunopathological manifestation, suggesting a critical role of these cells in the intestinal inflammation. NKT cells, present in the intestine at early stages after infection, can secrete IFN-γ that will initiate a Th1-like immune response mediating the lethal ileitis. The critical role of IFN-γ was confirmed by studies showing that mice deficient in IFN-γ production do not develop ileitis (27).

Results from this study show that the harmful effect of NKT cells can be neutralized by treatment with a single injection of α-GalCer. When intestinal NKT cells were stimulated by α-GalCer the day before infection, minor intestinal lesions developed, and the mice survived the infection. The beneficial effect of α-GalCer was accompanied by a shift in cytokine production by the intestinal NKT cells toward a Th2 profile (IL-4 and IL-10) and a dramatic increase in $CD4^+CD25^+Foxp3^+$ cells in MLNs. Depletion of regulatory T cells abrogated the protective effect of treatment with α-GalCer before the infection. This observation indicates that activation of NKT cells by α-GalCer triggers a regulatory T cell response that helps control the inflammatory intestinal disease observed after *T. gondii* infection.

We showed for the first time that conventional CD1d-restricted NKT cells are present in the small intestine of *T. gondii*-infected mice; more precisely, they are located within the lamina propria compartment. They are not associated with IELs in this model, contrary to what was described in previous studies that have identified NK-like T cells within the intraepithelial compartment of the mouse small intestine (35). The presence of unconventional NKT cells, non-CD1d-restricted cells, was also described in the large intestine (36). In this study it was observed that the purified NKT cells were mainly of the $CD4^+$ phenotype, with double-negative $CD4^-8^-$ cells making up the difference.

Upon polyclonal or Ag-specific stimulation through the TCR, CD1d-restricted NKT cells have the capacity to produce IL-4 and IFN-γ (11). In this model of pathogen-driven ileitis, we observed that intestinal CD1d-restricted NKT cells promote an IFN-γ response, as reflected by the marked reduction of IFN-γ mRNA expression at serial time points after infection in Jα281$^{-/-}$ mice devoid of NKT cells compared with wild-type control mice. This early IFN-γ production by intestinal NKT cells may influence the Th1/Th2 balance and thus favor the switch toward a local inflammatory Th1 immune response. Secretion of IFN-γ by intestinal NKT cells may induce DC to secrete IL-12, resulting in an increased production of IFN-γ and TNF-α by lamina propria $CD4^+$ T cells that are important effector cells in the hyperinflammatory process associated with oral *T. gondii* infection. IFN-γ produced by NKT may activate other cell types, such as macrophages and neutrophils (37), that will act on NK cells and CD8 T cells to enhance their IFN-γ production. Our data confirmed the findings of previous studies in which $NK1.1^+$ cells were identified as a source of IFN-γ that is essential to limit parasite replication (32, 46) and also point out their role in triggering an exacerbated IFN-γ response leading to immunopathology.

NKT cells are certainly not the only source of IFN-γ. In Jα281$^{-/-}$ mice, characterized by the absence of NKT cells, a limited amount of IFN-γ was secreted after infection, followed by a

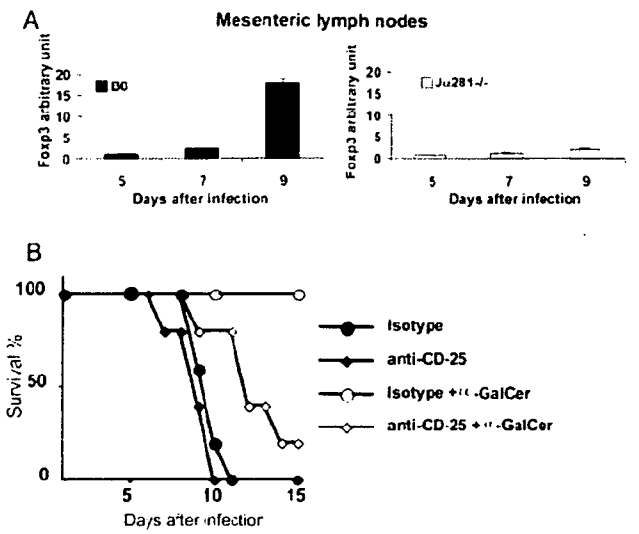

FIGURE 6. Implication of regulatory T cells after α-GalCer treatment. *A*, An increased number of Foxp3 regulatory T cells in MLNs from α-GalCer-treated mice was observed. cDNAs obtained from total MLNs of infected B6 and Jα281$^{-/-}$ mice treated, or not, with α-GalCer were submitted to quantitative real-time PCR with specific primers and probed for Foxp3 and actin (five mice per group). After normalization to actin expression, results were expressed as an increase in Foxp3 expression in α-GalCer-infected B6 or Jα281$^{-/-}$ mice compared with infected B6 or Jα281$^{-/-}$ mice. This experiment was repeated twice with similar results. *B*, B6 mice were depleted of CD25^{+} cells with anti-CD25 mAb i.p. 3 days before α-GalCer treatment. Twenty-four hours later, all mice were infected. Similarly infected α-GalCer-treated B6 mice, anti-CD25-treated B6 mice, and B6 mice treated with isotype Abs were used as controls (five mice per group). Results are representative of two independent experiments.

significant increase in cytokine production with time (day 8). This late IFN-γ production indicates that other cells within the responding immune population (e.g., CD4^{+} T cells from the lamina propria) are specifically activated and probably are responsible for the death of 75% of the Jα281$^{-/-}$ mice and the mild inflammation observed in the intestines of surviving mice.

NKT cells can be activated through different pathways. Activation through TCR ligation by CD1d-associated glycolipid is one possibility. Alternatively, IL-12 might activate NKT cells directly, in the absence of TCR engagement (38, 39), or might synergize its effect to that of TCR engagement (40). The activation pathway responsible for NKT cells activation after *T. gondii* infection remains unclear. It is indeed unknown whether TCR engagement by *Toxoplasma* Ag or through recognition of self Ag is required. Recently, Brigl et al. (40) have described a model in which NKT cells in the presence of IL-12 were activated after recognition of self Ags presented by CD1d. IL-12 was first made by DCs in response to microbial products, and this cytokine, in turn, activated NKT cells to up-regulate CD69 expression and IFN-γ production. One of the potential *Toxoplasma* Ag responsible directly or indirectly for NKT activation is the surface Ag-1 (SAG1) protein, the major surface protein of the parasite. The SAG1 molecule induces the dominant Ab response during infection (41) and a strong Th1 immune response characterized by high levels of IFN-γ production by CD4 T cell from the lamina propria and CD8 T lymphocytes (42, 43). SAG1 is a GPI-anchored protein and could be a potential ligand for CD1d molecule.

The hypothesis of the activation of NKT cells through TCR recognition of CD1d-presented Ag is attractive in our model. However, after oral infection with *T. gondii*, CD1$^{-/-}$ (B6 background) mice developed an acute and lethal ileitis within 7 days despite the absence of NKT cells. This suggests that CD1d may act via several alternative pathways. Besides its activity on NKT cell activation, CD1d is important for the activation of IELs (33) that down-regulate the intestinal inflammation after *T. gondii* infection. Indeed, upon Ag activation these IEL secrete copious amounts of TGF-β that participate in the maintenance of gut homeostasis (28). The lack of CD1 expression leads to the absence of protective IELs, and the absence of regulatory mechanisms overcome the absence of inflammatory NKT cells. In addition, the CD1d molecule is expressed on both the apical and the basolateral membranes of intestinal epithelial cells (44), and its ligation induces IL-10 secretion by these cells (45). Thus, the regulation of CD1 expression and its recognition by the TCR could play important roles in the regulation of intestinal inflammatory processes.

In this model of pathogen-driven inflammatory disease, NKT cells are important for the initiation of the robust Th1 inflammatory immune response in the intestine after oral parasite infection. Alternatively, α-GalCer and related glycolipids can modulate NKT cell responses toward a Th2-like profile (11, 12, 46). Our observations demonstrate that α-GalCer treatment has an impact on the intestinal immune response by shifting the cytokine profile production by NKT cells toward a Th2 phenotype, resulting in orientation of the lamina propria CD4 response. A single dose of α-GalCer prevented the development of lethal ileitis after infection with *T. gondii*. This treatment resulted in a Th2 immune response characterized by the production of IL-4, IL-10, and IL-13 by intestinal NKT cells. The major cytokine implicated in this protection is IL-10, because the beneficial effect of α-GalCer treatment was completely abrogated in IL-10-deficient mice.

Our data are in full agreement with previous work reporting the high susceptibility of IL-10-deficient mice to the development of lethal ileitis after oral *T. gondii* infection (47). This susceptibility is associated with the defect of T cells to produce IL-10, because mice with an inactivation of the IL-10 gene restricted to T cells generated by Cre/loxP-mediated targeting of the IL-10 gene succumb to severe immunopathology upon infection with *T. gondii* (48).

IL-10 secreted by NKT cells also participated in the protective effect of α-GalCer treatment, because double-chimeric mice in which NKT cells alone were impaired in IL-10 secretion were more susceptible to the development of ileitis than controls after α-GalCer injection. However, other IL-10-producing cells are also implicated, because treatment with α-GalCer reduced the mortality of these double-chimeric mice. Regulatory CD25^{+} T cells are the likely candidates, because they are present in the intestine, and the anti-CD25 treatment blocked the protective effect of α-GalCer injection.

IL-10 produced by NKT cells has been shown to exert an important regulatory function in experimental models of different pathologies, such as diabetes (49) and allergic encephalomyelitis (50). The link between the shift in the cytokine profile produced by NKT cells toward a Th2 profile and the activation of regulatory CD4^{+} T cells is as yet unknown. IL-10-producing CD4^{+} NKT cells are involved in the generation of regulatory CD8^{+} T cells after Ag exposure in the anterior chamber of the eye (51). Several reports indicate that NKT cells may contribute to immunoregulation via DC maturation (52). DC maturation in the presence of IL-10 may equally induce T regulatory 1 or Th3 regulatory T cells (53). Secretion of IL-4 and IL-10 by intestinal NKT cells after α-GalCer treatment may act directly on local DCs during induction of the polarization of the immune response and promote a Th2 profile. There is evidence that DCs that mature in the presence of NKT cells produce greater amounts of IL-10

and lose the ability to secrete IL-12, a phenotype consistent with a tolerogenic function (17).

The participation of IL-4 in this process cannot be ruled out. The role of IL-4 seems to be complex in toxoplasmosis. Our data indicate that neutralization of IL-4 cannot render α-GalCer-treated mice as susceptible as wild-type, infected, untreated mice, indicating the participation of other cytokine, such as IL-10. In addition, these experiments might indicate, as suggested by Nickdel et al. (54), that IL4-deficient mice are more resistant than wild-type mice to the development of ileitis. However, our data for IL-4 corroborate previous findings reporting that treatment with α-GalCer or OCH (a synthetic glycolipid that has shorter hydrophobic chain) improves mucosal Th1/Th2 cytokine balance by increasing IL-10 and IL-4 production and prevents experimental colitis in mice (55).

The important role played by NKT cells in the regulation of the intestinal immune response has also been previously suggested in a colitis model induce by chemical agents such as dextran sodium sulfate (56) or oxazolone (57). The pathogenic pathway leading to tissue injury in dextran sodium sulfate-induced colitis and, by extension, in Crohn's disease was attributed to production of Th1 cytokines such as IFN-γ and to the presence of $NK1.1^+$ T cells (56). However, the pathogenic pathway leading to tissue injury in oxazolone colitis was also associated with NKT cells secreting IL-13 (57).

The presence of IL-10-secreting T regulatory lymphocytes has been associated with regulation of intestinal inflammation (33), and in our model these cells may be ultimately responsible for the protective effect seen after treatment with α-GalCer. These data illustrate the dual potential of NKT cells in orienting distinct (i.e., Th1 or Th2) immune responses depending on the stimuli used.

After activation with *T. gondii*, NKT cells are important mediators of the immune response via a robust IFN-γ-mediated effect that limits parasite replication and allows for parasite clearance. However, this early and influential response is not without drawbacks and can be detrimental to the host. This response, when uncontrolled, leads to the development of an acute inflammatory process and death within 7 days of infection in this experimental model of pathogen-driven ileitis. Our data highlight the crucial role of NKT cells derived from the gut in the modulation of intestinal homeostasis.

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Disclosures

The authors have no financial conflict of interest.

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EXTENDED REPORT

Efficacy of rituximab (anti-CD20) for refractory systemic lupus erythematosus involving the central nervous system

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Aim: Neuropsychiatric systemic lupus erythematosus (NPSLE) is a serious treatment-resistant phenotype of systemic lupus erythematosus. A standard treatment for NPSLE is not available. This report describes the clinical and laboratory tests of 10 patients with NPSLE before and after rituximab treatment, including changes in lymphocyte phenotypes.

Methods: Rituximab was administered at different doses in 10 patients with refractory NPSLE, despite intensive treatment.

Results: Treatment with rituximab resulted in rapid improvement of central nervous system-related manifestations, particularly acute confusional state. Rituximab also improved cognitive dysfunction, psychosis and seizure, and reduced the SLE Disease Activity Index Score at day 28 in all 10 patients. These effects lasted for >1 year in five patients. Flow cytometric analysis showed that rituximab down regulated CD40 and CD80 on B cells and CD40L, CD69 and inducible costimulator on CD4+ T cells.

Conclusions: Rituximab rapidly improved refractory NPSLE, as evident by resolution of various clinical signs and symptoms and improvement of radiographic findings. The down regulation of functional molecules on B and T cells suggests that rituximab modulates the interaction of activated B and T cells through costimulatory molecules. These results warrant further analysis of rituximab as treatment for NPSLE.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterised by multiple lesions induced by activation of autoreactive T cells and overproduction of autoantibodies by B cells. The involvement of the central nervous system (CNS) in SLE is often intractable, complicating the course of the disease in about 12–75% of patients with SLE. The involvement of the CNS has a negative clinical impact with a 5-year survival of 55–85% and is associated with poor prognosis.[1 2] Neuropsychiatric systemic lupus erythaematosus (NPSLE) exhibits a wide range of symptoms unrelated to SLE activation, which include organic and mental disorders, often associated with impairment of consciousness and/or convulsions. These organic disorders may become permanent, eventually leading to long-term or irreversible decline in higher mental functions.

CNS immune abnormalities have an important role in such disease states. Therefore, a trial of intensive treatment, including the combination of potent immunosuppressive treatment and plasma exchange (PE), depending on the disease type and its severity, may be advisable in an effort to control autoreactive lymphocytes.[3–10] Although the severity of NPSLE correlates with prognosis, there is no established treatment protocol and many cases are resistant to treatment making this condition difficult to control.

This study describes the results of treatment of patients with NPSLE who had previously failed to respond to various immunosuppressants. Our approach was based mainly on the use of anti-CD20 antibody (rituximab), a chimeric antibody that directly targets B cells.[11 12] Rituximab is a biological preparation that eliminates B cells through a variety of mechanisms such as antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity and apoptosis. Rituximab has recently been used for the treatment of a variety of SLE disease conditions and good therapeutic response has been reported.[13–16] We investigated the short-term and long-term responses to rituximab treatment in 10 patients with NPSLE, and report that some showed marked improvement following rituximab treatment. Moreover, the results showed that rituximab modulated the functional molecules of activated lymphocytes, implying the efficacy of anti-CD20 antibody treatment for CNS lesions in patients with SLE, otherwise resistant to other treatments.

MATERIALS AND METHODS

Patients

The study subjects were 10 patients who had been previously diagnosed with SLE based on the American College of Rheumatology criteria.[17] The inclusion criteria were (1) the presence of a highly active disease and (2) CNS lesions resistant to conventional treatment. None of the patients showed improvement in CNS-related symptoms in response to conventional immunosuppressive treatment such as intravenous cyclophosphamide pulse treatment (IV-CY), cyclosporine A (CsA), PE and immunoadsorption therapy. All patients completed the course of anti-CD20 antibody treatment described in this study. Patients 1–8, and patients 9 and 10 were treated at the University of Occupational and Environmental Health Hospital and Kyoto University Hospital, respectively, from 2000 to 2005. Informed consent was obtained from all patients in accordance with the

Abbreviations: CNS, central nervous system; FACS, fluorescence-activated cell sorter; NPSLE, neuropsychiatric systemic lupus erythematosus; PBS, phosphate-buffered saline; PE, plasma exchange; SLE, systemic lupus erythematosus; SLEDAI, SLE Disease Activity Index; SPECT, single-photon-emission computed tomography

regulations of the aforementioned two hospitals, and rituximab was administered in accordance with the study protocol approved by the ethics committee of each hospital.

Treatment protocol

Patients 1–5 and 10 were treated with 375 mg/m^2 rituximab once a week for 2 weeks, and patient 9 received a single administration of the same dose. Patients 6 and 7 received 500 mg rituximab once a week for 4 weeks, while patient 8 was treated with 1000 mg once biweekly for 4 weeks. Blood pressure and ECG were monitored within the first 3.5 h of the administration to check for any reaction to the drug infusion.

Assessment

Clinical symptoms and treatment-induced adverse reactions were assessed before treatment, every week during treatment, every week within 1 month after treatment and once monthly thereafter. Laboratory tests included blood count, erythrocyte sedimentation rate, liver and renal function tests, urinary protein, serum complement titre and autoantibody level (such as anti-ds-DNA antibody). To evaluate the impact of rituximab on CNS lesions, we measured the immunoglobulin (Ig)G index and interleukin (IL)6 level in the cerebrospinal fluid, MRI, cerebral flood flow scintillator (single-photon-emission computed tomography (SPECT), and ^{18}FTG-positron emission tomography. To assess SLE activity, the SLE Disease Activity Index (SLEDAI) was determined before and after treatment. The level of expression of functional molecules on the lymphocyte cell surface was assessed by flow cytometry.

Flow cytometry

Mononuclear cells were isolated from peripheral blood using lymphocyte separation medium (ICN/Cappel Pharmaceuticals, Aurora, Ohio, USA). After washing twice with phosphate-buffered saline (PBS), the cells were incubated in blocking buffer (0.25% human globulin, 0.5% human albumin (Yoshitomi, Osaka, Japan), and 0.1% NaN_3 (Sigma Aldrich, St Louis, Missouri, USA) in PBS) and left to stand in a 96-well plate at 4℃ for 15 min. In the next step, the cells were incubated in 100 μl of fluorescence-activated cell sorter (FACS) solution (0.5% human albumin and 0.1% NaN_3 in PBS) and then treated with fluorescein isothiocyanate-labelled mouse IgG_1 and antihuman CD40, CD69, inducible costimulator (ICOS), CD19, CD4 (Pharmingen, San Diego, California, USA), CD80 (Chemicon Europe, Chandlers Ford, UK), or CD40L (Ancell, Bayport, USA) antibody, and left to react for 30 min at 4℃. The cells were washed three times with FACS solution and analysed using FACScalibar (Becton–Dickinson, San Jose, California, USA).

Statistical analysis

All data were expressed as mean (SD). Differences between data collected before and after treatment were examined for statistical significance using the Student's t test. $p<0.05$ denoted the presence of a significant difference.

RESULTS

Characteristics of patients

Table 1 summarises the NPSLE classification and laboratory data of the 10 patients. All patients were females with a mean (range) age of 31 (20–55) years. The mean (range) duration of illness from the onset of SLE to administration of rituximab was 9.6 years (3 months to 25 years). Immunosuppressants used for treatment before enrollment in the rituximab protocol included CsA, cyclophosphamide, mizoribine, and azathioprine. In addition, five patients with intractable disease did not respond to the combination treatment, and thus received PE as well.

With regard to CNS-related symptoms, acute confusional state was noted in 5, psychosis in 4, seizures in 2, mood disorders in 2, and one patient each had headache, demyelinating syndrome, myelopathy, anxiety disorder and cognitive dysfunction, based on the NPSLE classification of the American College of Rheumatology.[18 19] MRI findings included abnormal signals in the cerebral white matter in six patients. SPECT showed reduced cerebral blood flow in eight patients. Although a high IgG index[20] was noted in five patients (>0.66), an increase in IL6 was confirmed in only one patient.

Serious haemolytic anaemia, cardiomyopathy-associated decreased cardiac function, muscle pain, mucocutaneous disorders, peripheral neural deficits such as abnormal sensation and neurogenic bladder were also seen in these patients, in addition to the CNS-related changes (tables 1 and 2). In all participants, conventional immunosuppressive therapy produced either no improvement of symptoms or only a poor response. The SLEDAI values (range, 2–49) reflected the presence or absence of organ system-specific activity, with large scores representing involvement of CNS and low scores reflecting haematological activity. In the present study, involvement of organs was limited to those that could be confirmed objectively, while subjective signs such as fatigue and paresthesia were not recorded. Thus, using this approach, the SLEDAI scores of patients with objective signs reflecting multiple involvement of CNS were high whereas those of patients with subjective symptoms only were low. In our study, patients 1 and 3 had multiple CNS signs, patients 1 (49 points) and 3 (37 points) had seizures, psychosis and organic brain syndrome. On the other hand, patient 2 had MRI abnormality in the medulla oblongata but had only paresthesia as a subjective symptom (2 points), and patient 7 had MRI abnormality in the dorsal medulla spinalis and paralysis of the lower extremities, mood and anxiety disorders. However, the SLEDAI scores of both patients were based on subjective symptoms, and thus the scores were low (2 and 3, respectively).

Clinical outcome

At the start of rituximab treatment, patients were treated with low to moderate doses of corticosteroids (15–40 mg of prednisolone, 1–3 mg betamethasone), and continued to use this treatment during the rituximab arm of the study. However, immunosuppressants were stopped at entry to the study in all patients except for patient 8 who continued her treatment of 50 mg azathioprine. The postrituximab follow-up period was 7–45 months. Table 2 provides details of the clinical symptoms and laboratory tests before and 28 days after rituximab treatment (unless otherwise indicated in the table). Improvement in the skin and mucocutaneous lesions was fast, and the ejection fraction recovered from 44% to 72.1% in patient 4. All patients showed improvement in haematopenia and complement titre and marked falls in PE-resistant autoantibodies after treatment. Analysis of SLE activity before and after the treatment showed a significant decrease in SLEDAI from 19.9 (range, 49–2) before treatment to 6.2 (range, 15–0) after treatment ($p = 0.013$, fig 1). Moreover, SLEDAI decreased to 0 in 9 of the 10 patients at 1–6 months after rituximab treatment.

Rituximab treatment was also effective against CNS lesions in all patients. In particular, the consciousness state of all the five patients who were in acute confusional state before treatment, improved rapidly after the treatment. For example, the GCS score of patient 1 improved from 7–11 to 15 after 5 days of treatment, and that of patient 2 from 3 to 14 after 2 days of treatment. This rapid recovery was clinically significant. In addition, even in three patients who were in a dazed state and needed to be woken up before rituximab

Table 1 Characteristics of 10 female patients with neuropsychiatric systemic lupus erythaematosus at study entry

Patient	Age (years)	Duration of disease	Previous treatment	NP classification	MRI/SPECT	IgG index /IL6 (pg/ml)	Clinical manifestations	SLEDAI
1	35	19 years	CS (40 mg, pulse 14), IV-CY (22), VCR (10 mg), CsA (300 mg, 3 years), AZ (100 mg, 2 months), MTX (8 mg/w, 4 months), PE (11), IA (15)	Acute confusional state, seizure, psychosis	Normal/abnormal	Not done/not done	Fever, fatigue, nephritic syndrome, leukopenia, low Hb, high ESR, CH50, anti-ds DNA ↑	49
2	55	25 years	CS (40 mg, pulse 3), IV-CY (7), PE (2)	Acute confusional state	II, III/abnormal	0.73 ↑ / 1.8	Paresthesia of fingers, severe AIHA, anti-ds DNA ↑	2
3	46	3 months	CS (50 mg), IV-CY (11, PE (2), IA (3)	Acute confusional state, seizure	II, III/abnormal	0.46/33.8 ↑	Leukopenia, low Hb, thrombocytopenia, proteinuria, AIH, anti-ds DNA ↑	37
4	20	1 year	CS (50 mg), CsA (175 mg, 1 m)	Headache	Normal/not done	1.05 ↑ /3.1	Fever, fatigue, skin rash, alopecia, cardiomyopathy, polyneuropathy, leukopenia, C4 ↓, anti-ds DNA ↑	16
5	34	3 years	CS (60 mg), IV-CY (8), MZ (150 mg, 25 months)	Demyelinating syndrome	II, III/normal	0.85 ↑ /0.9	Sensory deficit, photosensitivity, mouth ulcer, lymphocytopenia, C4 ↓	16
6	30	22 years	CS (40 mg), MZ (150 mg, 22 years)	Mood disorder	Normal/abnormal	0.54/1.5	Polyneuropathy, muscular pain, skin rash, leukopenia, anti-ds DNA ↑	17
7	21	7 years	CS (60 mg, pulse 3), IV-CY (14), MTX (intrathecal 30 mg), MZ (300 mg, 2 years)	Myelopathy, mood disorder, anxiety disorder	II, III/abnormal	0.80 ↑ /4.7	Periungual erythema, leukopenia	3
8	20	9 months	CS (45 mg), IV-CY (6), AZ (50 mg, 1 month)	Psychosis, cognitive dysfunction	III/abnormal	0.56/1.0	Lymphadenopathy, alopecia, malar rash, lymphocytopenia	18
9	20	8 months	CS (60 mg, pulse 3), IV-CY, DFPP (4)	Acute confusional state, psychosis	III/abnormal	0.98 ↑ /4.2	Fever, lymphadenopathy, low Hb, lymphocytopenia, high ESR, anti-Sm ↑	28
10	29	17 years	CS (40 mg, pulse 2), AZ (100 mg, ly), CsA (300 mg, 1 month), IV-CY (2), PE (4)	Acute confusional state, psychosis	Normal/abnormal	0.60/2.4	Severe AIHA, CH50 ↓	18

The disease activity was high in all patients and none had responded to conventional immunosuppressants.

AIHA, autoimmune haemolytic anaemia; AZ, azathioprine; CS, corticosteroid; CsA, cyclosporine; CY, cyclophosphamide; DFPP, double filtration plasmapheresis; ESR, erythrocyte sedimentation rate; Hb, haemoglobin; IA, immunoadsorption, MTX, methotrexate, MZ, mizoribine; PE, plasma exchange; SLE-DAI, Systemic Lupus Erythaematosus Disease Activity Index; VCR, vincristine.

For IV-CY, PE and IA, numbers in parentheses represent the number of treatments. For CS, CsA, AZ and MZ, the doses in parentheses represent maximum dosage. For VCR in patient 1 and MTX in patient 7, the dose in parentheses expresses total dosage. MRI finding: II, small areas of increased signal intensity secondary to microinfarctions; III, focal areas of signal intensity in grey matter (*Am J Roentgenol* 1985;144:1027–31).

Table 2 Clinical outcomes of neuropsychiatric systemic lupus erythaematosus after anti-CD20 antibody treatment

Patient	Dose of rituximab	Other treatments at study entry (mg)	CNS manifestations before	CNS manifestations after	Objective NPSLE findings after treatment	Duration of remission (m)
1	375 mg/m^2 day 1, 8	Bet 1.0	Consciousness disorder, seizure, psychosis	Complete recovery (GCS 7–11→15/5 days)	Improvement of SPECT	22
2	375 mg/m^2 day 1, 15	Bet 1.5	Consciousness disorder	Improved consciousness	No follow-up data	18
3	375 mg/m^2 day 1, 8	Bet 1.0	Consciousness disorder, seizure	Complete recovery (GCS 3→14/2 days)	No improvement in MRI and SPECT	23
4	375 mg/m^2 day 1, 8	m-PSL 20	Headache	Resolution of headache	Improved IgG index (1.05 →0.84/4 w)	29
5	375 mg/m^2 day 1, 8	Bet 1.25	Paresthesia of fingers, toes and left precordial-back	Resolution of paresthesia	Improvement of neck MRI	7
6	500 mg day 1, 8, 15, 22	Bet 2.5	Depressive state, insomnia	Improvement of depressive state	Improvement of SPECT	7
7	500 mg day 1, 8, 15, 22	Bet 1.25	Paresis of both lower limbs, muscle weakness, depressive state	Reduction of paresis, improvement of depressive state (SDS 58→50/2 w)	Improvement of SPECT, improvement of IgG index (0.80 →0.72/3 m)	14
8	1000 mg day 1, 15	Bet 1.25, AZ 50	Psychosis, cognitive dysfunction	Improvement of psychosis (BPRS 26→7/8 w)	Improvement of SPECT	11
9	375 mg/m^2 day 1	PSL 45	Consciousness disorder, psychosis, paresis of both lower limbs, neurological bladder	Complete recovery	Improvement of PET and MRI, improved IgG index (0.98 →0.61/2 w)	10
10	375 mg/m^2 day 1, 8	Bet 3	Consciousness disorder, hallucination, cataplexy	Complete recovery	No significant improvement in objective findings	4

Bet, betamethasone; BPRS, brief psychiatric rating scale; CNS, central nervous system; GCS, Glasgow Coma Scale; m-PSL, methylprednisolone; MRI, magnetic resonance imaging; NPSLE, neuropsychiatric systemic lupus erythaematosus; PET, 18FTG-positron emission tomography; PSL, prednisolone; SDS, self-rating depression scale; SPECT, single photon emission computed tomography. For other abbreviations, see table 1.

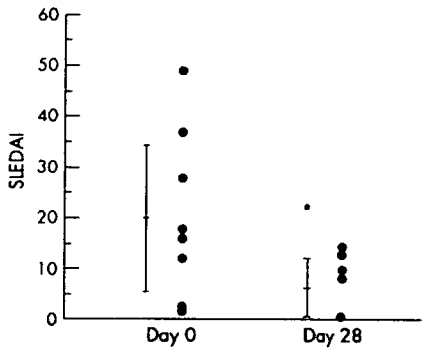

Figure 1 Systemic lupus erythaematosus disease activity index (SLEDAI) score before and 28 days after rituximab treatment. A decrease in SLEDAI score was detected in 9 of the 10 patients. Data are mean (SD). *$p<0.05$.

treatment, became alert the next day (patient 2) or after a few days of treatment (patients 9 and 10). Furthermore, rituximab also improved neuropsychiatric symptoms such as psychosis and mood disorder within a few weeks to a few months after treatment. For example, the Brief Psychiatric Rating Scale, which is used for the assessment of schizophrenia, markedly decreased in patient 8 from 26 to 7 points within 2 months, together with recovery of communication skills. In addition, patients 1 and 9 showed rehabilitation into society after rituximab treatment although they had serious neuropsychiatric symptoms before treatment. In addition to the improvement in SLE activity and clinical symptoms, rituximab also improved the quality of life of the patients.

We also assessed the effects of rituximab treatment by comparing the findings of MRI and SPECT before and after treatment. In four patients (patients 1, 6, 7 and 8), rituximab treatment improved cerebral blood flow as determined by SPECT; in patient 1, such improvement was noted at the early stage of treatment and paralleled the improvement in clinical symptoms. For patient 5, rituximab treatment resulted in improvement in the abnormal findings in T2-weighted images of the cervical cord on MRI, along with the improvement in sensory deficits due to inflammation at the same site. For patient 9, rituximab treatment resulted in reduction of the high-intensity lesion in the head MRI T2-weighted image.

Four of our patients had peripheral neuropathies in addition to CNS lesions. Treatment with rituximab resulted in remission or marked improvement of paresthesia in patient 2, radiculopathy in patient 4, ulnar neuropathy in patient 6, and neurological bladder in patient 9. Rituximab also improved quality of life based on improvement of peripheral neuropathy-related symptoms although such symptoms tended to persist after treatment.

While the overall therapeutic effect of rituximab was excellent, some patients developed relapse after long-term remission. Six of the 10 patients showed reactivation of SLE including reappearance of CNS-related symptoms. For patient 1, remission was maintained with low-dose steroid for 22 months after rituximab treatment. However, the patient showed recurrence associated with an increase in autoantibodies and proteinuria. Recurrence was also noted 18 months after treatment in patient 2, associated with haemolysis. Both patients 1 and 2 required retreatment with rituximab. At 23 months after completion of rituximab treatment, patient 3 showed worsening of the head MRI findings and cerebrospinal fluid abnormalities and developed witnessed seizure attacks. In patient 5, a reduction in the steroid dose was followed by recurrence of CNS-related symptoms after 7 months. Generalised skin rashes appeared in patient 9 after 10 months and patient 10 reported worsening of lupus headache after 4 months. Patients 3 and 5 received IV-CY treatment, and patient 9 and 10 required an increase in the steroid dose. However, four patients (patients 4, 6, 7 and 8) maintain a remission state at the time of writing this report (at 35 months in patient 4, at 7 months in patient 6, at 19 months in patient 7 and 16 months in patient 8) after the completion of rituximab treatment.

Adverse effects

Of the 10 patients, two developed pneumonia, one had herpes zoster, one developed chickenpox and one had intractable infection of decubitus ulceration. These infections were successfully controlled with antibiotics.

Phenotypic analysis of SLE lymphocytes

T cells and B cells are activated by antigen stimulation via T cell receptors and signals from costimulatory molecules. The responsible costimulatory molecules, such as CD40/40L, CD80, CD86/CD28 and ICOS/B7h, are known to be expressed in patients with active SLE.[21-26]

We performed serial analysis of the expression of functional molecules in eight patients with SLE before and after rituximab treatment by flow cytometry. Rituximab treatment resulted in rapid disappearance of CD20, a specific antigen to B cells, marked decrease in CD19-positive cells, within several days to 2 weeks after treatment. Rituximab also resulted in rapid falls in the percentages of CD40-expressing and CD80-expressing CD19 cells within 1 day and both were hardly detected after the second day (fig 2). The expression levels of these molecules were still low at 3 months after completion of rituximab treatment.

We also assessed the effects of treatment on the expression levels of CD40L (a costimulatory molecule on CD4-positive cells), ICOS and CD69 (an early activation antigen). While only three patients showed high expression of these molecules before treatment, rituximab treatment reduced the expression levels of these molecules in all three patients (fig 3), suggesting that rituximab does not only affect B cells but also T cells in patients with SLE.

DISCUSSION

To date, reports on rituximab treatment for autoimmune diseases have covered various conditions, including RA, SLE, dermatomyositis, Sjögren's syndrome and vasculitis.[27-30] Rituximab treatment resulted in improvement, manifested by a decrease in the British Disease Activity score and SLE DAI score, of arthropathy, nephropathy, thrombocytopenia and haemolytic anaemia.[11-16]

Although few reports described the efficacy of rituximab treatment in patients with SLE with CNS lesions,[11 14 31] to our knowledge, there are no published reports that provide detailed analysis of the effects of such treatment in a large group of patients. Rituximab has a large molecular weight of 146 kDa, and hence cannot readily cross the blood–brain barrier; therefore, it is unlikely to reach the cerebrospinal fluid following systemic administration. We measured rituximab concentration in the cerebrospinal fluid of patient 8 at 24 h after treatment. The value (0.3 μg/ml) was slightly higher than the lower detection limit of the assay, whereas the serum concentration was 279 μg/ml. Based on this finding, we assume that the central effects of rituximab are mediated through another mechanism, not through antibody-dependent cellular cytotoxicity and/or complement-dependent cytotoxicity.[32]

To assess autoreactive lymphocyte activity, we determined the expression of various functional molecules on the surface of peripheral blood lymphocytes before and after rituximab treatment by using flow cytometry. We previously proposed that

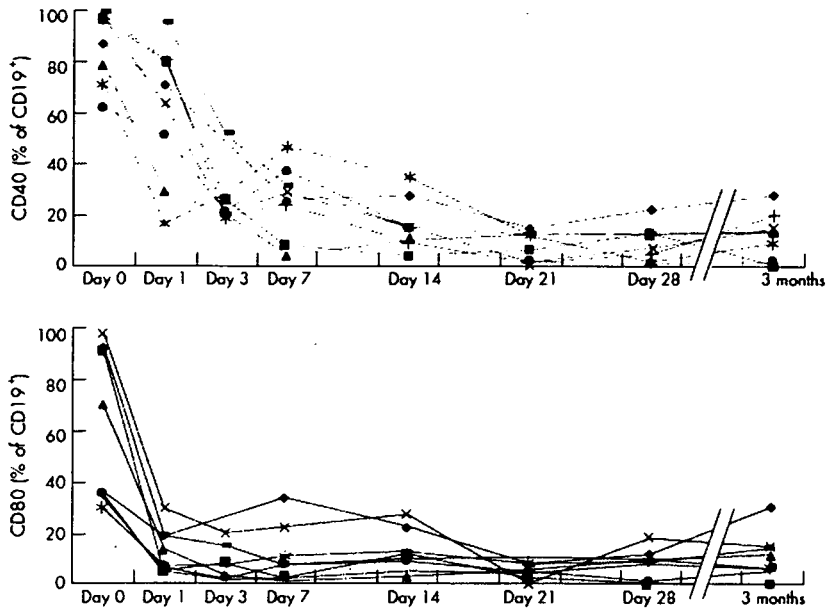

Figure 2 Serial changes in CD40 and CD80 expression on CD19-positive cells after rituximab treatment in eight patients with systemic lupus erythaematosus. CD40 and CD80 expression was measured before and 28 days after rituximab treatment.

rituximab could regulate SLE disease activity and correct autoimmune abnormalities.[12] The present results showed a rapid decrease in the expression of functional surface molecules and maintenance of long-term control following rituximab treatment (fig 2). Specifically, a marked decrease in the proportion of CD40-expressing and CD80-expressing cells was detected on the day after initiation of rituximab treatment. In this regard, Leng *et al*[13] found CD40 overexpression in CD19 cells in patients with rheumatoid arthritis compared with healthy controls. Others also reported that the percentage of CD80-positive cells among activated B cell subset was higher in SLE than the controls.[14] These results suggest that the target of rituximab treatment is activated B cells. Anolik *et al*[15] examined B cell phenotypes after rituximab treatment and reported that the proportion of autoreactive memory B cells was decreased after rituximab treatment. Considered together, the above results and those of the present study suggest that T cell activation is negatively influenced by a rapid decrease in B cell to T cell stimulation in

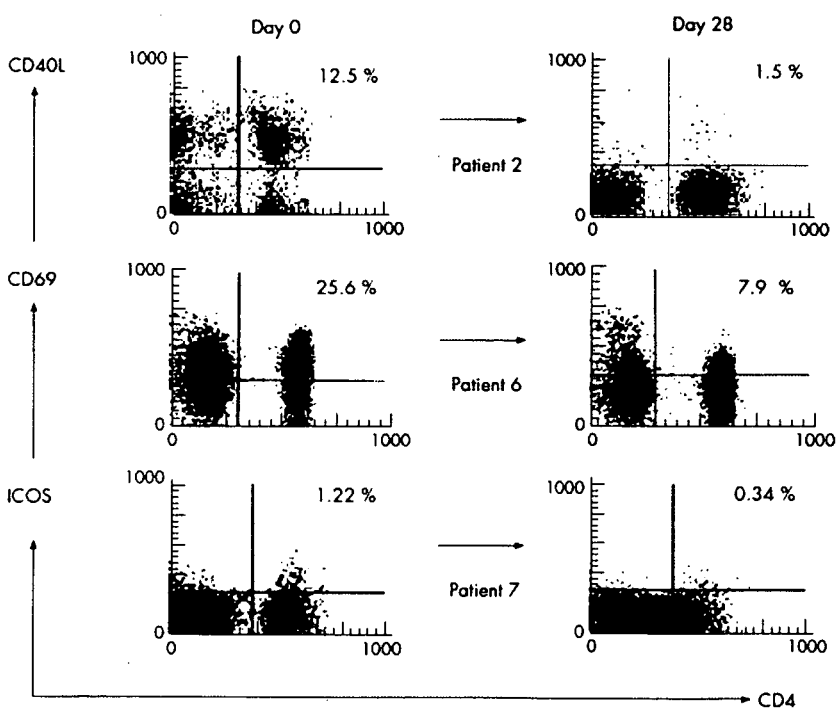

Figure 3 Changes in expression of functional molecules on CD4-positive cells induced by rituximab treatment. The expression of CD40L (patient 2), CD69 (patient 6) and ICOS (patient 7) on CD4-positive cells was measured before (day 0) and 28 days after rituximab treatment. Percentages represent the percentage of CD4-positive cells expressing the functional molecules.

parallel with the loss of B cells. Our results also showed that rituximab down regulated CD40L, ICOS and CD69 on CD4-positive cells in patients with active SLE (fig 3). Sfikakis *et al*[36] also reported that rituximab treatment decreased CD40L and CD69 expression in patients with SLE. These results imply that rituximab could eliminate B cells bearing functional molecules and inhibit the interaction between these B cells and activated T cells by down regulating costimulatory molecules, and also possibly by reducing the production of certain cytokines and complement activation, which could lead to rapid improvement of CNS manifestations of the disease.

At present, there is no treatment strategy for patients with NPSLE who fail to respond to conventional therapies. In such patients, large doses of steroids are provided on long-term basis, and IV-CY is administered continuously. Our study showed that rituximab is useful as a new treatment for such cases. However, recurrence after rituximab treatment was noted in our patients, as has been reported previously in patients with rheumatoid diseases.[28] Two of our patients who experienced recurrence received rituximab re-treatment. However, these patients experienced recurrence at 18 and 22 months after rituximab treatment, suggesting that remission could be maintained for a comparatively long period of time with rituximab treatment. Further studies are needed to develop strategies for the prevention of recurrence and counter measures for inhibiting the production of antichimeric antibodies.[37 38] There is also a need to investigate the long-term effects of rituximab treatment and its organ specificity.

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

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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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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

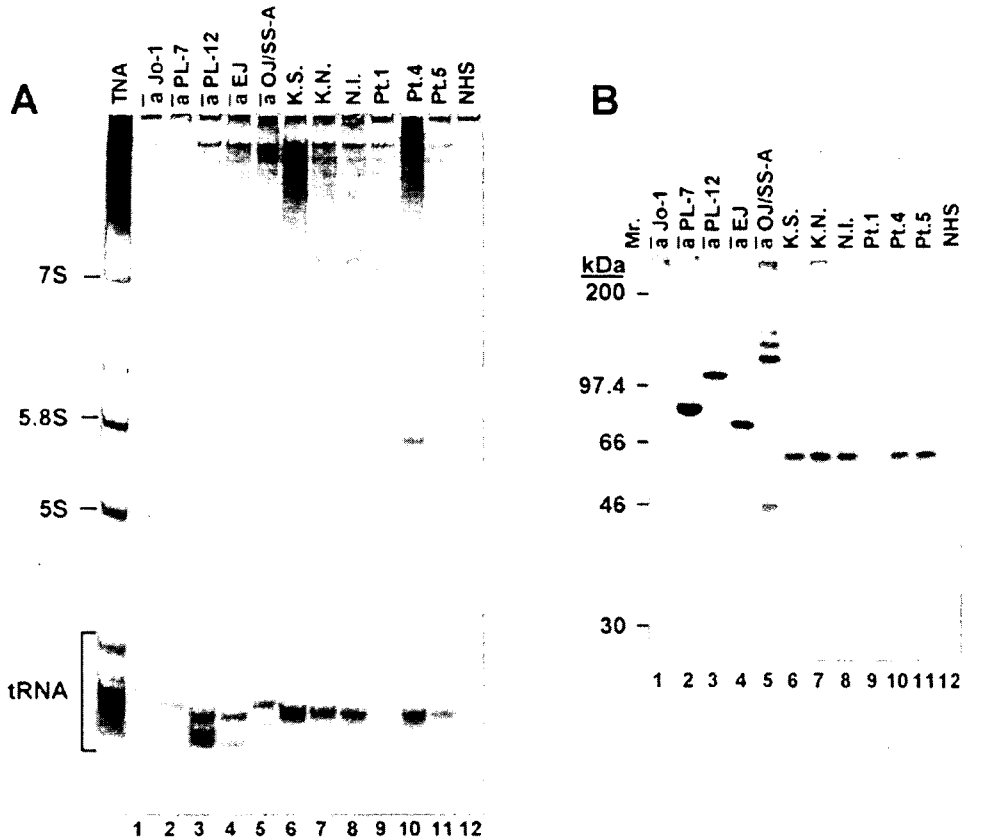

Figure 1. A, Immunoprecipitation (IP) for nucleic acids with anti-KS and control sera. Shown are patterns of transfer RNA (tRNA) resulting from 7*M* urea–10% polyacrylamide gel electrophoresis (PAGE) of phenol-extracted immunoprecipitates from HeLa cell extract, developed with silver stain. TNA = total 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	I
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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