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【V】研究成果の刊行物・別刷

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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 (1), 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 erythaema, 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/m² 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/m² day 1, 15	Bet 1.5	Consciousness disorder	Improved consciousness	No follow-up data	18
3	375 mg/m² 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/m² day 1, 8	m-PSL 20	Headache	Resolution of headache	Improved IgG index (1.05 →0.84/4 w)	29
5	375 mg/m² 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/m² 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/m² 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.

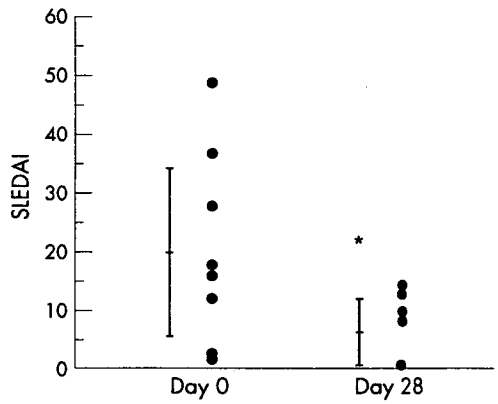

Figure 1 Systemic lupus erythaematosus disease activity index (SLEDAI) score before and 28 days after rituximab treatment. A decrease in SLEDAI score was detected in 9 of the 10 patients. Data are mean (SD). *$p<0.05$.

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

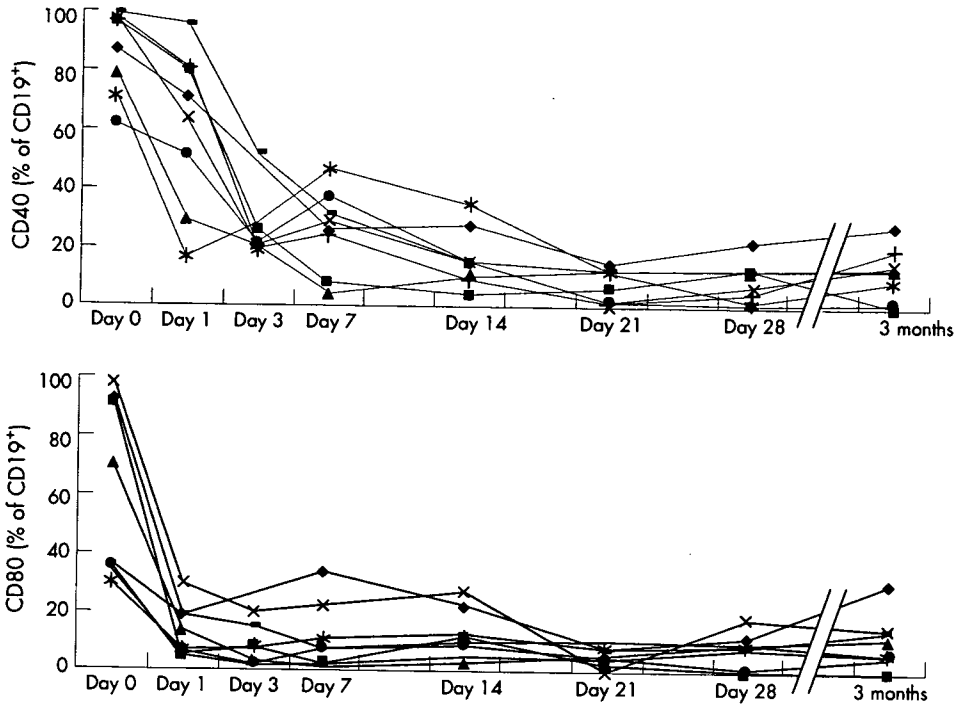

Figure 2 Serial changes in CD40 and CD80 expression on CD19-positive cells after rituximab treatment in eight patients with systemic lupus erythaematosus. CD40 and CD80 expression was measured before and 28 days after rituximab treatment.

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*[33] 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.[34] These results suggest that the target of rituximab treatment is activated B cells. Anolik *et al*[35] 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

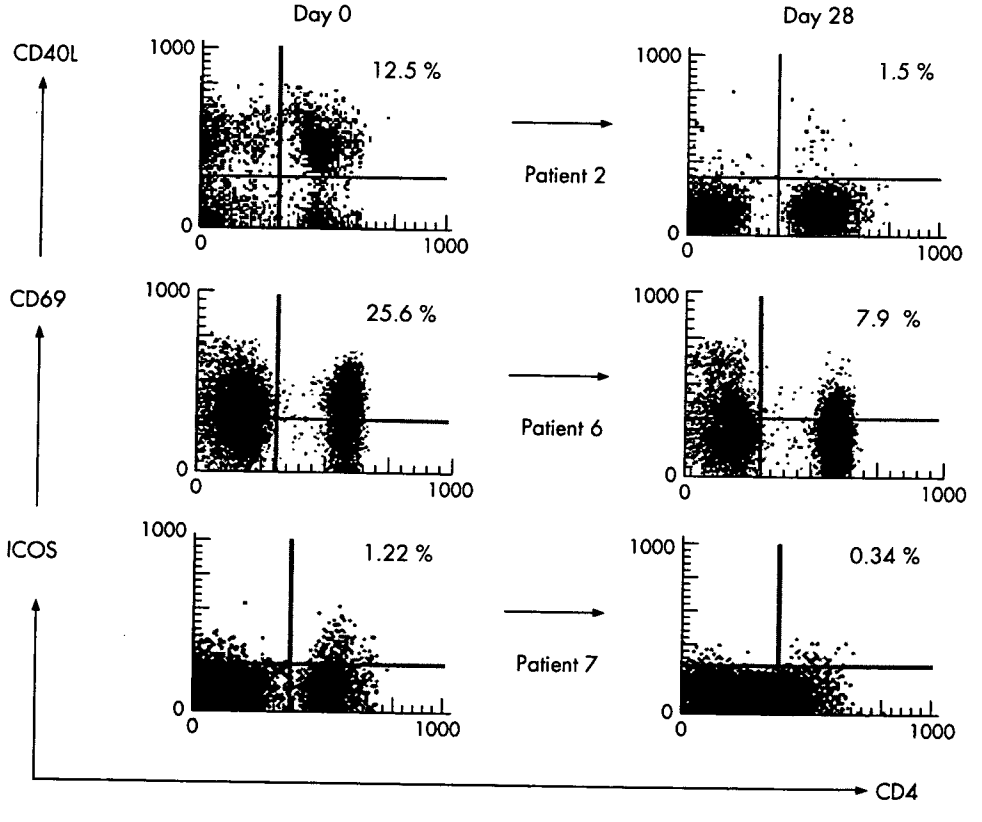

Figure 3 Changes in expression of functional molecules on CD4-positive cells induced by rituximab treatment. The expression of CD40L (patient 2), CD69 (patient 6) and ICOS (patient 7) on CD4-positive cells was measured before (day 0) and 28 days after rituximab treatment. Percentages represent the percentage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

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A polymorphism in human platelet antigen 6b and risk of thrombocytopenia in patients with systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder characterized by circulating autoantibodies directed against nuclear, cytoplasmic, cell membrane, and other autoantigens. Possible manifestations of SLE include the thrombocyte-associated diseases autoimmune thrombocytopenia and thrombosis.

The platelet surface glycoprotein IIb-IIIa (GPIIb-IIIa) complex plays an important role in platelet aggregation and hemostasis, since it is a receptor for fibrinogen, von Willebrand factor, and fibronectin. This complex constitutes a major target for antiplatelet antibodies responsible for immune-related thrombocytopenia (1).

GPIIIa is highly polymorphic, and 5 human platelet antigen (HPA) systems (HPA-1, HPA-4, HPA-6, HPA-7, and HPA-8) are known to be located in this glycoprotein. Polymorphisms in this region have been associated with neonatal alloimmune thrombocytopenia, posttransfusion purpura, and refractoriness to platelet transfusion in European American populations (2). Thus, these polymorphisms may be important as alloantigen determinants. In addition, evidence has been found to support a significant association between HPA-1 polymorphism and thrombosis (3,4), suggesting that HPA is involved in platelet aggregation.

In the Japanese population, polymorphisms in HPA-1, HPA-7, and HPA-8 are very rare. In a previous study, only 1 of 331 random donors was found to be A/B heterozygous in HPA-1, and no polymorphisms were detected in HPA-7 or HPA-8 in any of the subjects (5). The frequencies of polymorphisms in other HPA systems were 2.2% for A/B in HPA-4 and 5.1% for A/B and 0.3% for B/B in HPA-6 (5).

In the present study, we analyzed HPA-1, HPA-4, and HPA-6 polymorphisms in Japanese patients with SLE to determine the association of these polymorphisms with thrombocytopenia and thrombosis. A total of 134 Japanese patients with SLE (121 women and 13 men) with a mean age of 41.4 years (range 20–72 years), who presented to our Rheumatic and Connective Tissue Disease Unit, were recruited for the study. All of them fulfilled the American College of Rheumatology criteria for SLE (6). Fifteen patients (11.2%) fulfilled the Sapporo criteria for definite antiphospholipid syndrome (7). Sixty-seven healthy Japanese individuals were included as a control group. The study was conducted in accordance with the Declaration of Helsinki and the principles of good clinical practice. Approval was obtained from the local ethics committee, and written informed consent was obtained from each patient before enrollment.

Polymorphism status in HPA-1, HPA-4, and HPA-6 was determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), as previously described (5), with minor modifications. Briefly, 50 ng of genomic DNA was amplified in a total volume of 10 μl containing dNTPs (200 μM each) in a standard buffer with 1.5 mM $MgCl_2$, 0.5 units of DNA *Taq* polymerase, and 10 pmoles of each primer. The gene-specific primer sequences were as follows: for HPA-1, forward 5′-CTTA GCTATTGGGAAGTGGTAGGGCCTGC-3′ and reverse 5′-ACCTCAGATCTTCTGACTCAAGTCCTAACGTC TCTTATT-3′; for HPA-4, forward 5′-CCTGTGG-ACATCTACTACTTGATGGACC-3′ and reverse 5′-GCCAATCCGCAGGTTACTCGTGAGCATT-3′; and for HPA-6, forward 5′-CTGGCTGGCTGGGATCCCAGTG-3′ and reverse 5′-CCCTGCAGTTCTCCTCACCTGAG-3′. PCRs were performed as follows: for HPA-1, 27 cycles of 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 45 seconds; for HPA-4, 31 cycles of 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 45 seconds; and for HPA-6, 27 cycles of 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. After all cycles were completed, a final extension step of 72°C for 7 minutes was performed in all PCRs.

The amplified products (486 bp, 126 bp, and 240 bp for HPA-1, HPA-4, and HPA-6, respectively) were digested by *Msp* I (New England Biolabs, Beverly, MA) at 37°C for 16 hours for HPA-1, *Bsm* I (New England Biolabs) at 65°C for 1 hour for HPA-4, and *Mva* I (Takara Shuzo, Shiga, Japan) at 37°C for 16 hours for HPA-6. RFLP products were resolved in 9% polyacrylamide gels, stained with ethidium bromide, and visualized under ultraviolet light. For HPA-1, two bands of 274 bp and 206 bp were visualized for the A/A homozygous genotype, and 4 bands of 274 bp, 206 bp, 173 bp, and 101 bp were identified for the heterozygous genotype. For HPA-4, a 104-bp band corresponded to the A/A genotype, and a 126-bp band to the B/B genotype. For HPA-6, A/A single band of 240 bp was visualized for the A/A homozygous genotype. Two bands of 165 bp and 75 bp and 3 bands of 240 bp, 165 bp, and 75 bp were identified for the B/B homozygous genotype and the heterozygous genotype, respectively.

All patients were tested for antiphospholipid antibodies (aPL), including anticardiolipin antibodies (aCL) and lupus anticoagulant (LAC). The aCL (IgG, IgM, and IgA) were assayed as previously described (8), and for the detection of LAC the guidelines of the Subcommitte for Standardization of the International Society of Thrombosis and Haemostasis were followed (9).

The records of the patients were reviewed retrospectively, and autoimmune thrombocytopenia, defined as platelet count $<100 \times 10^9$/liter, was detected in 35 patients (26.1%). Transient thrombocytopenia associated with disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, hemophagocytotic syndrome, drug-induced thrombocytopenia, and myelodysplastic syndrome were not considered to be autoimmune thrombocytopenia. Twenty-one patients (15.7%) had a history of thrombotic events. LACs were detected in 43 patients (32.1%) and aCL in 26 (19.4%).

HPA-1 polymorphism was not present in the study population. The A/A genotype was detected in HPA-1 in 100% of SLE patients and healthy individuals, in accordance with the results of a previous study (5).

The HPA-4 A/A genotype was found in 98.3% of SLE patients and in 97% of healthy individuals. The heterozygous

Table 1. Distribution of the HPA-6 genotypes*

Group (n)	A/A genotype	A/B genotype	B/B genotype
Healthy controls (67)	66 (98.5)	1 (1.5)	0 (0)
All SLE patients (134)†	124 (92.5)	10 (7.5)	0 (0)
Patients with thrombocytopenia (35)‡	28 (80)	7 (20)	0 (0)
Patients without thrombocytopenia (99)	96 (97)	3 (3)	0 (0)
Patients with thrombosis (21)	21 (100)	0 (0)	0 (0)
Patients without thrombosis (113)	103 (91.2)	10 (8.8)	0 (0)
Patients with aPL (52)	50 (96.2)	2 (3.8)	0 (0)
Patients without aPL (82)	74 (90.2)	8 (9.8)	0 (0)

* Values are the number (%) of subjects. HPA-6 = human platelet antigen 6; SLE = systemic lupus erythematosus; aPL = antiphospholipid antibody.
† Odds ratio (OR) 5.32 (95% confidence interval [95% CI] 0.66–42.48).
‡ OR 8 (95% CI 1.9–32.9), $P < 0.0028$.

genotype was observed in 2 patients and in 2 controls (1.5% and 3%, respectively), a slightly higher frequency than that previously found by Tanaka et al in 331 random donors (0.3%) (5).

The HPA-6 A/B genotype was found in 7.5% of patients with SLE, compared with 1.5% of healthy controls. There was no significant deviation from Hardy-Weinberg equilibrium in either group. The HPA-6 A/B genotype was more frequently observed in patients with thrombocytopenia than in those without (odds ratio 8, [95% confidence interval 1.94–32.98], $P = 0.0028$). There was no difference in genotype distribution based on the presence or absence of thrombotic events or aPL (Table 1).

The first recognized platelet alloantigen was seen in 1959 in a patient with clinical features of posttransfusion purpura (10). Since then, >20 alloantigens have been described and attributed to different antigen systems. The HPA-6 polymorphism is due to an Arg–Gln variation at position 489. We found the HPA-6 A/B genotype in 5.5% of subjects, a frequency similar to that previously found in Japanese donors (4.8%) (11), and significantly higher than that observed in a Finnish population (0.7%) (12).

The initial aim of our study was to identify a risk factor for thrombosis, apart from the presence of aPL, in patients with SLE. The strong correlation between HPA-1 polymorphism and coronary events (4) drove us to investigate a link between HPA polymorphisms and thrombosis in SLE. In the present study there was no statistically significant difference between the frequency of the HPA-6 A/B genotype in patients and that in healthy controls. None of the HPA polymorphisms investigated in this study were identified as a risk factor for thrombosis in the study population. Interestingly, the HPA-6 A/B genotype occurred significantly more frequently in SLE patients with autoimmune thrombocytopenia than in those without.

Autoimmune thrombocytopenia, one of the most common features of SLE, occurs in 7–30% of patients during the course of the disease, but its pathogenesis is still controversial. One postulated mechanism of platelet destruction in SLE involves the presence of antiplatelet antibodies, mainly directed against the GPIIb-IIIa complex, which bind to circulating platelets and facilitate the clearance of platelets by the reticuloendothelial system (1,13). Alternatively, aPL may interact with platelet phospholipids, leading to platelet destruction by the reticuloendothelial system (14,15)

The significance of antigen polymorphisms in the development of autoimmune thrombocytopenia is far from clear. Polymorphisms in GPIIb-IIIa may potentially promote the production of autoantibodies. Platelet GPIIb-IIIa is the main antigenic target recognized by antiplatelet antibodies in autoimmune thrombocytopenia, and anti–GPIIb-IIIa antibodies have been measured in SLE-associated thrombocytopenia using an indirect monoclonal-specific immobilization of platelet antigens assay (1) or an enzyme-linked immunosorbent assay, which detects peripheral blood B cells secreting IgG anti–GPIIb-IIIa (16). It would be of interest to analyze the association between anti–GPIIb-IIIa and HPA-6 polymorphism. Unfortunately, our study design did not include the detection of antiplatelet antibodies, and neither of these detection methods was performed.

In our thrombocytopenia patients with the HPA-6 A/B genotype, platelet count ranged from 3.2×10^9 to 9.8×10^9/liter, implying that the association between HPA-6 A/B genotype and thrombocytopenia has an immunogenetic impact rather than clinical significance as a predictor of bleeding tendency.

In conclusion, our preliminary findings show that the HPA-6 A/B genotype is a risk factor for autoimmune thrombocytopenia in SLE. Further investigations are needed to clarify the relationship between this polymorphism and thrombocytopenia in SLE.

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AUTHOR CONTRIBUTIONS

Dr. Atsumi 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. Amengual, Atsumi, Komano, Kataoka, Horita, Yasuda, Koike.

Acquisition of data. Amengual, Atsumi, Komano, Kataoka, Horita, Yasuda, Koike.

Analysis and interpretation of data. Amengual, Atsumi, Komano, Kataoka, Horita, Yasuda, Koike.