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【V】研究成果の刊行物・別刷

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Clinical Relevance of the Expression of P-Glycoprotein on Peripheral Blood Lymphocytes to Steroid Resistance in Patients With Systemic Lupus Erythematosus

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Objective. **P-glycoprotein (P-gp) of membrane transporters leads to drug resistance by the exclusion of intracellular drugs, including corticosteroids. Some patients with highly active systemic lupus erythematosus (SLE) show poor response to corticosteroids; however, the mechanisms of steroid resistance remain unclear. The aim of this study was to elucidate the clinical relevance of P-gp expression on lymphocytes to steroid resistance in patients with active SLE.**

Methods. **Flow cytometric analyses of the expression of P-gp on peripheral blood lymphocytes from 20 normal volunteers and 80 SLE patients were performed. Steroid-exclusion analysis of peripheral blood mononuclear cells (PBMCs) was conducted by using radioisotope-labeled dexamethasone.**

Results. **P-gp was expressed at significantly high levels on most of the peripheral blood lymphocytes from SLE patients, whereas normal lymphocytes had only marginal expression. The quantity of P-gp on SLE lymphocytes correlated with the disease activity in each patient, as estimated by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI). Furthermore, in SLE patients whose SLEDAI scores were >12 despite taking >0.5 mg/kg/day of prednisolone, P-gp expression on lymphocytes was markedly increased, and intracellular dexamethasone in their PBMCs was significantly decreased. However, intensive immunosuppressive treatment in these SLE patients resulted in successful control of disease activity, which occurred in parallel with a marked reduction of P-gp on lymphocytes.**

Conclusion. **The overexpression of P-gp on lymphocytes might lead to exclusion of corticosteroids from lymphocytes, resulting in steroid resistance in patients with highly active SLE. Reduction of P-gp expression achieved by intensive immunosuppressive treatment overcame the steroid resistance. We therefore propose that measurement of P-gp expression on lymphocytes is useful in the assessment of steroid resistance and is a good marker for indicating the need for intensive immunosuppressive treatment in patients with highly active SLE.**

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibody production by activated B cells and autoreactive T cells. The main treatment strategy is to control the autoreactive lymphocytes with corticosteroids or other immunosuppressive agents. However, we often encounter patients with highly active SLE who do not respond to corticosteroid treatment, and this lack of response to corticosteroids is an important obstacle to overcome in the treatment of SLE.

Among the multiple mechanisms of resistance to multiple drugs, overexpression of P-glycoprotein (P-gp), a 170-kd product of the multidrug resistance 1 (MDR-1) gene, has emerged as the major molecule involved in multidrug resistance during chemotherapy for various malignancies (1–4). P-gp is a member of the ATP binding cassette transporter superfamily of genes, and it

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Table 1. Characteristics of the study subjects*

	Normal volunteers (n = 20)	SLE patients (n = 80)
Age, mean (range) years	35 (26–42)	36 (10–67)
Sex, no. of females/no. of males	17/3	75/5
Disease duration, mean (range) years	–	4.5 (0.1–27)
SLEDAI score, mean (range)	–	12 (0–29)
SLE involvement, no. of patients		
Lupus nephritis	–	53
CNS lupus	–	23
Pulmonary hypertension	–	5
Serositis	–	14
Prednisolone (or equivalent) treatment		
No. taking	–	63
Dosage, median (range) mg/day	–	7.5 (0–60)
No. of patients taking combination therapy at study enrollment		
Cyclophosphamide	–	24
Cyclosporin A	–	26
Mizoribine	–	20

* SLE = systemic lupus erythematosus; SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; CNS = central nervous system.

functions as an energy-dependent transmembrane efflux pump. Overexpression of P-gp results in reduction of intracellular concentrations of xenobiotics, drugs, and poisons, such as vinca alkaloids, anthracyclines, verapamil, colchicines, antimalarials, cyclosporine, and corticosteroids (5–8). Thus, P-gp appears to be a double-edged sword, being involved both in protecting cells from these drugs and in developing resistance to them.

Previous studies have shown that resistance to chemotherapy induced by P-gp is closely associated with the prognosis of human malignancies (1–4). In this regard, P-gp is expressed on various types of cells, including leukemic cells and CD34+ hematopoietic stem cells as well as epithelial cells in the liver, kidney, pancreas, gut, and adrenal glands (9–13). Treatment resistance is common not only in patients with hematopoietic malignancies, but also in those with systemic autoimmune diseases, including SLE. In this context, the expression of P-gp on immune cells such as T cells and B cells, the functional relevance of P-gp to lymphocytes, and the regulatory mechanisms of the induction of P-gp on these cells are not clear in SLE.

We previously found that the transcription of MDR-1 and the expression of P-gp are mediated through the human Y-box binding protein 1 (YB-1), an MDR-1 transcription factor, following lymphocyte activation by typical immune stimuli such as interleukin-2 (IL-2) (14). Furthermore, studies both from our laboratories and others have demonstrated increases in the number of cytokine-producing lymphocytes as well as increases in serum levels of these cytokines in patients with active SLE (15–17).

The present study was designed to elucidate the relationship between P-gp expression on lymphocytes from SLE patients with high levels of disease activity and clinical response to corticosteroids. We also determined the significance of evaluating the expression of P-gp on peripheral blood lymphocytes in clinical decision-making in relation to treatment strategies.

MATERIALS AND METHODS

Isolation of peripheral blood mononuclear cells (PBMCs). PBMCs from 20 normal volunteers and from 80 SLE patients who fulfilled the American College of Rheumatology revised criteria for SLE (18) were isolated by density-gradient centrifugation using Lymphocyte Separation Medium 50494 (Pharmacia Biotech, Uppsala, Sweden) as described previously (19,20). The clinical activity of SLE was assessed by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (21). Table 1 summarizes the demographic characteristics and clinical features of the SLE patients and normal volunteers.

This study was approved by the human subjects research committee of our university. Informed consent was obtained from all subjects who were enrolled in the study.

Flow cytometry. Staining and flow cytometric analysis of PBMCs were conducted according to standard procedures, using a FACScan (Becton Dickinson, Mountain View, CA), as described previously (19,20). Briefly, PBMCs were initially plated in a 96-well culture dish (2×10^5 cells/well) and incubated with polyclonal gamma globulin (10 μg/ml; Mitsubishi Welpharma, Osaka, Japan) to block Fc receptors. These cells were then incubated with MRK-16 (Kyowa Medex, Tokyo, Japan), a specific monoclonal antibody (mAb) against P-gp (22), followed by the addition of fluorescein isothiocyanate–conjugated anti-mouse IgG antibody (Fujisawa, Osaka, Japan) in fluorescence-activated cell sorter me-

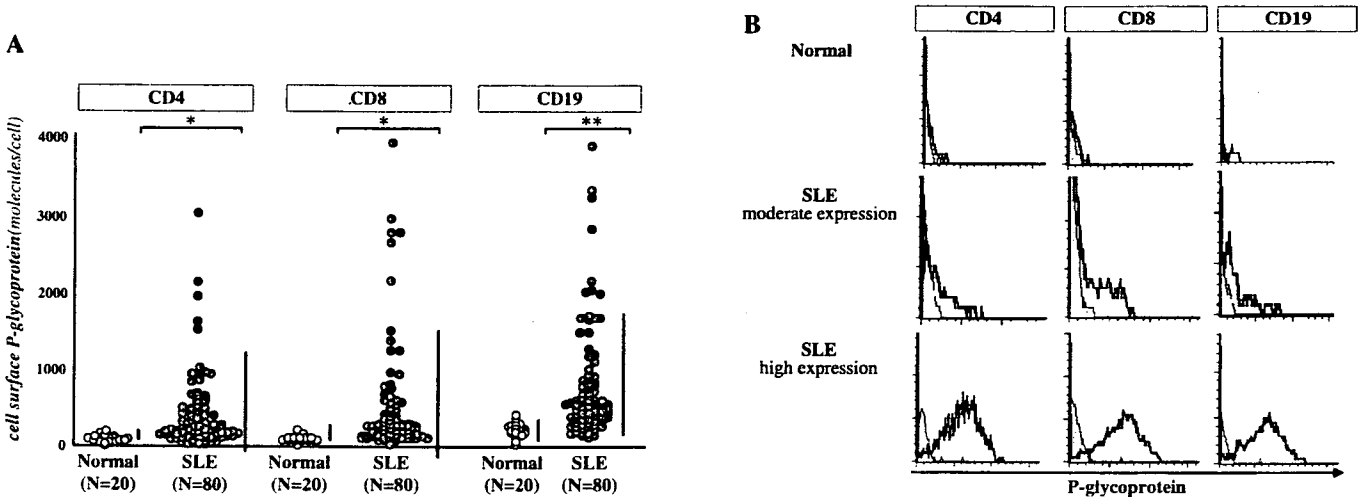

Figure 1. Expression of P-glycoprotein (P-gp) on lymphocytes from patients with systemic lupus erythematosus (SLE), as determined by flow cytometry. **A**, P-gp expression on CD4+, CD8+, and CD19+ peripheral blood lymphocytes from 20 normal volunteers and 80 SLE patients. Results were calculated with the use of standard QIFIKIT beads. Values are the mean and SD of independent experiments. * = $P < 0.05$; ** = $P < 0.01$ by unpaired t-test. **B**, Typical P-gp expression on CD4+, CD8+, and CD19+ peripheral blood lymphocytes from a normal volunteer, an SLE patient with moderate P-gp expression, and an SLE patient with high P-gp expression. Open histograms represent cells stained with MRK-16 and fluorescein isothiocyanate (FITC)–conjugated anti-mouse IgG antibody in each linear scale on a fluorescence amplifier. Shaded histograms represent profiles of FITC-conjugated anti-mouse IgG antibody used as a negative control.

dium, which consisted of phosphate buffered saline (PBS), 0.5% human serum albumin (Mitsubishi Welpharma), and 0.2% NaN_3 (Sigma-Aldrich Japan, Tokyo, Japan), for 30 minutes at 4°C.

For the 2-color analysis, we incubated PBMCs with phycoerythrin-conjugated CD4 mAb, CD8 mAb, or CD19 mAb (Fujisawa) after blocking free anti-mouse IgG binding sites with irrelevant antibodies. The 2-color–stained cells were detected by electronic gating based on their CD4, CD8, or CD19 expression using a FACScan. Quantification of the cell surface antigens on a single cell was performed using QIFIKIT beads (Dako, Kyoto, Japan), as previously described (23).

Dexamethasone accumulation. ^{14}C-labeled *n*-butanol (1.61 mCi/mmole; Toho Biochemical, Tokyo, Japan) was diluted with unlabeled butanol (Sigma-Aldrich Japan) at a concentration of 0.5 MBq/ml. ^{3}H-labeled dexamethasone (40.0 Ci/mmole; Perkin Elmer, Boston, MA) was dissolved in DMSO (Nacalai Tesque, Tokyo, Japan) and then diluted with PBS (final concentration of DMSO 0.1%). PBMCs were suspended in PBS with 7 m*M* dextrose for the ATP supply, which is dispensable in this assay (24), at a density of 5×10^6 cells/ml. The PBMCs were then incubated with 5.0×10^{-5}*M* ^{14}C-labeled *n*-butanol and 3.0×10^{-8}*M* ^{3}H-labeled dexamethasone for 20 minutes at 37°C.

For competitive studies with cyclosporin A, PBMCs were incubated with 100 ng/ml of cyclosporin A (Novartis, Tokyo, Japan) for 15 minutes and then incubated with ^{14}C-*n*-butanol and ^{3}H-dexamethasone. Cyclosporin A was dissolved in DMSO before diluting with PBS (final DMSO concentration 0.03%). After incubation, 100-μl aliquots were layered on 80 μl of a mixture of lauryl bromide and silicone oil (2:1 ratio; Nacalai Tesque) in an Eppendorf tube (Assist, Tokyo, Japan). After centrifugation at 10,000 revolutions per minute for 2 minutes, the aliquots were rapidly frozen in liquid nitrogen, and the frozen tube was cut at the medium–mixture boundaries, thereby obtaining the upper layer as the medium fraction and the lower layer as the cell fraction.

The cell fractions were melted with Soluene-350, and 10 ml of Hionic-Fluor (Packard, Meriden, CT) was added. The medium fractions were mixed with 10 ml of a mixture of toluene (Wako, Osaka, Japan), methanol (Wako), ethylene glycol monoethyl ether (Nacalai Tesque), and PermaFluor (200:50:50:12 ratio; Packard). The radioactivity of each fraction was counted with a scintillation counter. The cell to medium ratio, which is an index of the intracellular and extracellular dexamethasone concentration ratio, was computed using the following formula: cell:medium ratio = [(^{3}H in the cell fraction/^{14}C in the cell fraction)/(^{3}H in the medium fraction/^{14}C in the medium fraction)].

Statistical analysis. Results are expressed as the mean ± SD. Student's t-test was used to compare data between 2 groups. One-way analysis of variance and Bonferroni correction were used to compare data among 3 or more groups. Correlations between 2 variables were examined using Pearson's correlation analysis. In the figures, a linear regression line is shown together with Pearson's correlation coefficient (r) and the respective correlation P value. P values less than 0.05 were considered statistically significant.

RESULTS

Expression of cell surface P-gp on peripheral blood lymphocytes from SLE patients. We examined the expression of P-gp using mAb against the MRK-16

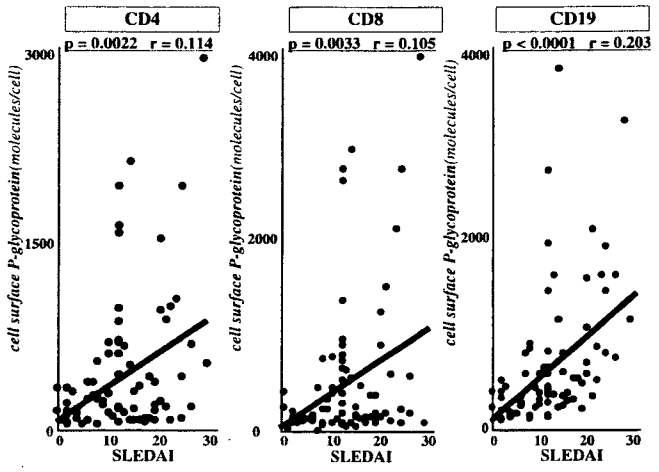

Figure 2. Correlation of the expression of P-glycoprotein (P-gp) on lymphocytes and scores on the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) in 80 patients with systemic lupus erythematosus (SLE). Levels of P-glycoprotein expression on SLE lymphocytes correlated closely with disease activity in each patient, as estimated by the SLEDAI score. Numbers of P-gp molecules per cell were calculated with the use of standard QIFIKIT beads. Pearson's correlation analysis was used to determine statistical significance.

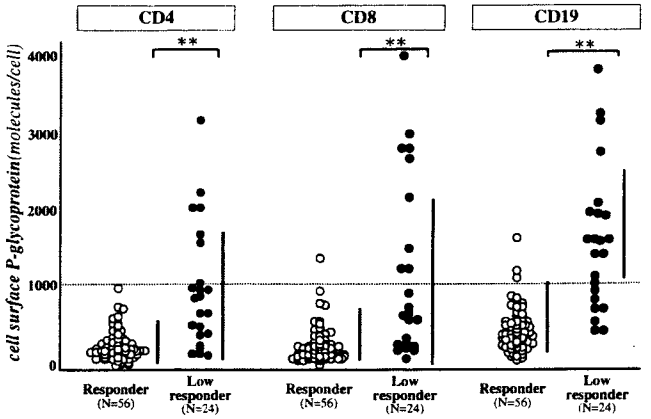

Figure 3. Relationship between responsiveness to corticosteroids and expression of P-glycoprotein (P-gp). Flow cytometric analysis was used to determine P-gp expression on the CD4+, CD8+, and CD19+ peripheral blood lymphocytes from systemic lupus erythematosus patients who were responders (n = 56) or low responders (n = 24) to corticosteroid therapy. Results were calculated with the use of standard QIFIKIT beads. Horizontal line indicates 1,000 molecules/cell. Values are the mean and SD of independent experiments. ** = $P <$ 0.01 by unpaired t-test.

epitope of P-gp on peripheral blood lymphocytes from 80 SLE patients and 20 normal volunteers. P-glycoprotein was highly expressed on most of the peripheral CD4+, CD8+, and CD19+ lymphocytes from the SLE patients. Levels of P-gp expression on lymphocytes from SLE patients ranged from marginal to extremely high, with most expressing moderate levels (Figure 1). Expression on normal lymphocytes was only marginal.

Relationship between disease activity and expression of P-gp. SLE patients with high levels of disease activity who do not respond to initial treatment with high-dose oral corticosteroids have been encountered in our clinical practices, but the mechanisms of this steroid resistance are not clear. We postulated that both SLE disease activity and P-gp expression correlate with corticosteroid resistance. We therefore investigated the relationship between SLEDAI scores and P-gp expression on peripheral blood lymphocytes from patients with SLE.

The level of expression of P-gp on SLE lymphocytes correlated closely with the disease activity in each patient, as estimated by the SLEDAI score (Figure 2).

Table 2. P-glycoprotein expression and involvement in SLE patients*

Involvement	SLEDAI score	P-glycoprotein, molecules/cell		
		CD4	CD8	CD19
Lupus nephritis				
Patients with (n = 53)	14.4 ± 7.3	427.5 ± 590.0	508.7 ± 808.7	849.3 ± 827.0
Patients without (n = 27)	13.0 ± 6.8	452.7 ± 445.7	515.2 ± 645.7	670.6 ± 582.6
P	NS	NS	NS	NS
CNS lupus				
Patients with (n = 23)	14.3 ± 7.1	402.1 ± 439.5	429.6 ± 619.8	932.8 ± 796.4
Patients without (n = 57)	13.8 ± 7.2	449.7 ± 582.3	543.7 ± 803.8	731.0 ± 736.1
P	NS	NS	NS	NS
Serositis				
Patients with (n = 14)	15.8 ± 7.9	673.6 ± 865.3	757.4 ± 1139.1	924.2 ± 964.8
Patients without (n = 66)	13.5 ± 6.9	385.6 ± 440.0	458.6 ± 643.9	760.3 ± 707.7
P	NS	NS	NS	NS

* *P* values are for comparisons between systemic lupus erythematosus (SLE) patients with versus those without specific involvement. Values are the mean ± SD of independent experiments. SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; NS = not significant; CNS = central nervous system.

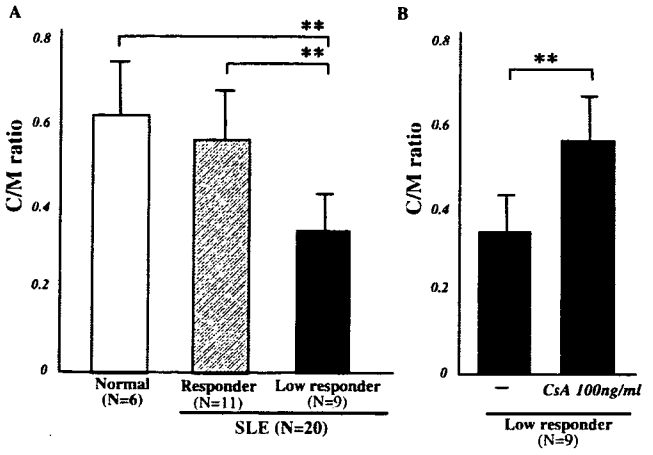

Figure 4. Decrease in intracellular dexamethasone levels in peripheral blood mononuclear cells (PBMCs) from systemic lupus erythematosus (SLE) patients who were low responders to corticosteroid therapy and inhibition of dexamethasone excretion by competitive binding of cyclosporin A to P-glycoprotein. **A,** Intracellular dexamethasone levels were evaluated by determining the cell to medium (C/M) ratio in PBMCs from 6 normal volunteers, 11 responders, and 9 low responders. **B,** Intracellular dexamethasone levels were evaluated by determining the C/M ratio in PBMCs from 9 low responders in the absence and presence of 100 ng/ml of cyclosporin A. Values are the mean and SD. ** = $P < 0.01$ by paired t-test.

Among the 3 subsets of lymphocytes, P-gp was most strongly expressed on CD19+ cells and showed the best correlation with disease activity ($P < 0.0001$). In addition, P-gp expression on SLE lymphocytes correlated significantly with general disease activity, but not with specific organ involvement (Table 2).

Relationship between responsiveness to corticosteroids and expression of P-gp. The SLE patients were divided into 2 groups according to their responses to corticosteroids. The low responders were patients whose SLEDAI scores were >12 despite taking >0.5 mg/kg/day of prednisolone (or equivalent). The responders were patients who responded well when taking <1.0 mg/kg/day of prednisolone (or equivalent).

We then analyzed the relationship between clinical responsiveness to corticosteroid therapy and the level of expression of P-gp on lymphocytes. The levels of P-gp expression on CD4+, CD8+, and CD19+ lymphocytes were markedly increased in the low responders (Figure 3). In almost all lymphocytes from the responders, cell surface P-gp expression was <1,000 molecules per cell, whereas in the low responders, cell surface P-gp expression was >1,000 molecules per cell for more than 1 of the 3 lymphocyte subsets. Furthermore, we demonstrated that intracellular dexamethasone levels in PBMCs from the low responders were significantly decreased compared with the levels in responders and in normal volunteers (Figure 4A).

To confirm the functional involvement of P-gp in the decreased levels of intracellular dexamethasone, we added cyclosporin A, a competitive inhibitor of P-gp, to PBMCs from low responders and evaluated dexamethasone excretion. The excretion of dexamethasone from PBMCs obtained from the low responders was inhibited by cyclosporin A (Figure 4B).

Effects of intensive immunosuppressive therapy on P-gp expression. Intensive therapy with immunosuppressive agents was initiated in 10 of the low responders, all of whom had high levels of disease activity, as demonstrated by SLEDAI scores >12 points, despite taking >0.5 mg/kg/day of prednisolone. In addition to the prednisolone treatment, the patients received either intravenous pulse cyclophosphamide, plasmapheresis,

Table 3. Intensive immunosuppressive therapy in 10 low responders who had highly active systemic lupus erythematosus*

Patient	Age, years	Treatment received prior to intensive therapy	Intensive immunosuppressive therapy (no. of courses)	SLEDAI score		
				Before	After	Time required
1	28	Pred. 1 mg/kg/day	Pulse MP (3) + plas. (2)	28	2	5 weeks
2	19	Pred. 1 mg/kg/day	Cyclosporin A	20	16	2 weeks
3	67	Pred. 1 mg/kg/day	Pulse MP (3)	20	8	4 weeks
4	24	Pred. 0.5 mg/kg/day	IV pulse CYC (1) + vincristine (1)	24	12	2 weeks
5	20	Pred. 1 mg/kg/day	IV pulse CYC (1) + plas. (6) + pulse MP (2)	25	19	4 weeks
6	33	Pred. 0.8 mg/kg/day	Pred. 1 mg/kg/day + plas. (3)	21	12	6 weeks
7	23	Pred. 1 mg/kg/day	IV pulse CYC (2)	29	12	5 weeks
8	50	Pred. 1 mg/kg/day	IV pulse CYC (1) + plas. (4)	26	13	5 weeks
9	68	Pred. 0.8 mg/kg/day	IV pulse CYC (4)	12	2	6 months
10	39	Pred. 1 mg/kg/day	IV pulse CYC (1) + plas. (1)	20	16	2 weeks

* Low responders were patients with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scores >12 despite taking >0.5 mg/kg/day of prednisolone. Pred. = prednisolone (or equivalent); MP = methylprednisolone; plas. = plasmapheresis; IV = intravenous; CYC = cyclophosphamide.

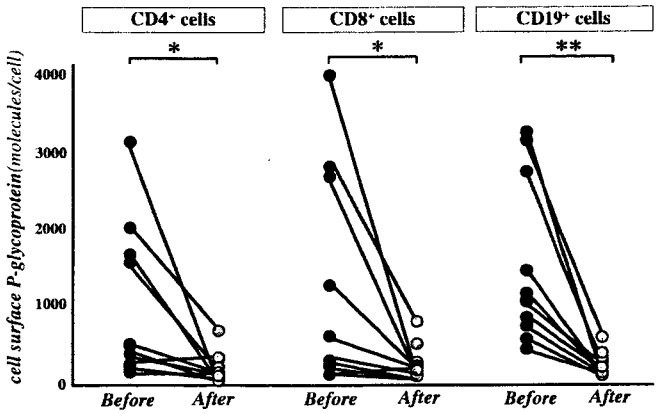

Figure 5. Effects of intensive immunosuppressive therapy on P-glycoprotein (P-gp) expression. Flow cytometric analysis was used to determine P-gp expression on peripheral blood lymphocytes from 10 low responders before and after intensive therapy with immunosuppressive agents (see Table 2). Results were calculated with the use of standard QIFIKIT beads. * = $P < 0.05$; ** = $P < 0.01$ by paired t-test.

oral cyclosporin A, or a 1-gm pulse of methylprednisolone. All 10 patients responded to the intensive therapy, showing clinical improvement as demonstrated by a decrease in the SLEDAI score (Table 3).

We also evaluated the expression of P-gp on lymphocytes from these 10 patients before and after institution of intensive immunosuppressive therapy. Prior to administration of the intensive immunosuppressive agents, there was overexpression of P-gp on lymphocytes from almost all of these low responders. Clinical improvement induced by the intensive therapy was associated with a marked reduction in P-gp expression on lymphocytes from these patients (Figure 5). After withdrawal of the intensive immunosuppressive therapy, these 10 patients were successfully treated with oral prednisolone alone. These results indicate that the resolution of disease activity in patients with highly active SLE induced by intensive treatment with immunosuppressive agents was associated with recovery of responsiveness to corticosteroids and was mediated by a down-regulation of P-gp expression on their lymphocytes.

DISCUSSION

Corticosteroid treatment in patients with active SLE, an autoimmune disease characterized by autoantibody production by activated B cells and autoreactive T cells, is used to suppress the highly activated lymphocytes (25). Although most patients with SLE respond to high-dose corticosteroids, some show poor response. Therefore, it is important to elucidate the mechanisms of steroid-unresponsiveness in order to overcome the refractory status. Several mechanisms for the lack of response to corticosteroids have been considered. Patients with SLE often develop peritonitis or hypoalbuminemia, which could cause malabsorption of corticosteroid from the intestine (26,27). Another possible reason for a poor response is a rapid degradation of corticosteroids (28,29). However, both malabsorption and degradation of corticosteroids can be rapidly corrected by intravenous infusion of high doses of the drugs.

The results of our studies showed high levels of expression of P-glycoprotein on lymphocytes from patients with SLE. We also demonstrated high levels of P-gp expression in patients with highly active disease, and we found that P-gp overexpression on lymphocytes correlated with a lack of response to corticosteroids. Intracellular levels of dexamethasone were found to decrease significantly in PBMCs obtained from 9 patients with highly active SLE who did not respond to high-dose corticosteroid therapy. Other investigators have reported that decreased cytoplasmic glucocorticoid concentrations are the result of increased P-glycoprotein–mediated efflux of glucocorticoid from lymphocytes and is one of the mechanisms of glucocorticoid resistance in inflammatory bowel disease and asthma (30,31). We therefore propose that P-gp acts as a "hydrophobic vacuum cleaner"; that is, P-gp captures drugs like a vacuum cleaner when they pass through the cell membrane and then releases them outside the cell. Thus, when the number of P-gp molecules expressed on the lymphocyte cell surface increases, corticosteroids (a P-gp substrate) cannot reach the cytoplasm, and this results in unresponsiveness to corticosteroid therapy. Our results imply that high levels of P-gp expression on lymphocytes might lead to active efflux of corticosteroids from the cytoplasm to the cell exterior, resulting in the development of steroid unresponsiveness and failure to control disease activity in SLE patients with highly active disease.

In recent studies, we found that IL-2, a potent lymphocyte stimulus (32,33), up-regulated the expression of P-gp on lymphocytes via activation of the transcription factor YB-1 and that this up-regulation markedly reduced the intracellular corticosteroid concentration in vitro (14). The increased IL-2 levels in SLE patients usually fall below a threshold level following corticosteroid therapy, but remain at high levels in patients who respond poorly to treatment and who continue to have highly active disease (15,16,34,35). Therefore, lymphocytes activated by IL-2 and other cytokines in SLE patients with highly active disease

apparently acquire MDR-1–mediated multidrug resistance, including poor response to corticosteroids and probably other drugs as well, such as disease-modifying antirheumatic drugs.

It is noteworthy that intensive immunosuppressive therapy that included intravenous pulse cyclophosphamide and pulse methylprednisolone successfully controlled disease activity at the same time as a marked reduction of P-gp expression on CD4+, CD8+, CD19+ cells was noted in 10 of the SLE patients with highly active disease that was resistant to therapy with corticosteroids alone. We therefore propose that down-regulation of P-gp by intensive therapy with immunosuppressive agents might be important in overcoming corticosteroid resistance.

The sequence of changes in the clinical course of SLE in these 10 patients and the changes in levels of P-gp expression on lymphocytes occurred as follows: 1) the patients had high levels of P-gp expression on lymphocytes and high levels of disease activity that was resistant to treatment with oral prednisolone (low responder); 2) intensive therapy with immunosuppressive agents, including pulse methylprednisolone, intravenous pulse cyclophosphamide, cyclosporin A, and repeated plasmapheresis, was initiated; 3) marked reduction in P-gp expression on lymphocytes occurred; and 4) there was recovery of responsiveness to oral prednisolone (responder) and marked improvement in disease activity (Table 3 and ref. 36, where patient 1 is described in detail). Therefore, we also propose that the disappearance of P-gp expression causes a recovery of steroid responsiveness and leads to successful subsequent treatment with oral prednisolone.

These results suggest that intensive therapy with immunosuppressive agents should be initiated in SLE patients with highly active disease in order to overcome steroid-unresponsiveness due to overexpression of P-gp. Our results also suggest that the threshold number of P-gp molecules is ~1,000 per cell. Monotherapy with conventional oral corticosteroids, even at high dosages (1 mg/kg/day), predictably fails to control disease activity if the P-gp expression on lymphocytes is >1,000 molecules per cell at initiation of treatment. In such patients, we propose that combination therapy with prednisolone and another immunosuppressive agent, such as cyclophosphamide, be given in order to avoid possible steroid-unresponsiveness and delays in controlling disease activity.

In the present study, we also identified 1 patient who failed to respond to high-dose corticosteroid treatment and whose disease activity decreased within 2 weeks after the addition of cyclosporin A. Accumulation of more patients similar to this one should allow the proper evaluation of the use of cyclosporin A in patients with resistance to corticosteroids (37,38). Cyclosporin A is a P-gp substrate and is also a competitive inhibitor of P-gp (3,14). We demonstrated that levels of intracellular dexamethasone in the PBMCs from low responders were increased to levels as high as those in the responders by cyclosporin A treatment. We therefore suggest that cyclosporin A could be used not only to inhibit nuclear factor of activated T cell–dependent IL-2 transcription in lymphocytes, but also as a competitive inhibitor of P-gp. In a previous study, we documented that cyclosporine and its derivatives caused a recovery of intracellular corticosteroids in cultured lymphocytes by competitively binding to P-gp (14). In fact, in chemotherapy of malignancies, several clinical trials of competitive P-gp antagonists, such as cyclosporine and its derivatives, examined their effect on overcoming the multidrug resistance induced by P-gp overexpression (2,3). Therefore, we propose that cyclosporine, as a competitor of P-gp, is a useful treatment for highly active SLE in patients who do not respond to corticosteroids.

In conclusion, we demonstrated in the present study that reduction of P-gp expression achieved by intensive therapy with immunosuppressive agents resulted in an overcoming of steroid resistance. P-gp appears to be involved in the lack of response to corticosteroids in patients with highly active SLE. Accordingly, we propose that measurement of levels of P-gp expression on lymphocytes is useful for the assessment of steroid resistance and is a good marker for indicating the need for intensive immunosuppressive therapies in patients with highly active SLE.

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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.

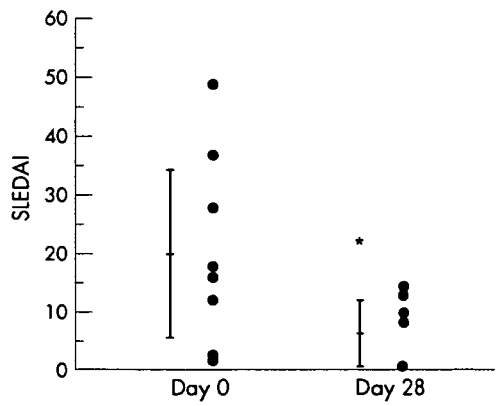

Figure 1 Systemic lupus erythaematosus disease activity index (SLEDAI) score before and 28 days after rituximab treatment. A decrease in SLEDAI score was detected in 9 of the 10 patients. Data are mean (SD). *p<0.05.

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

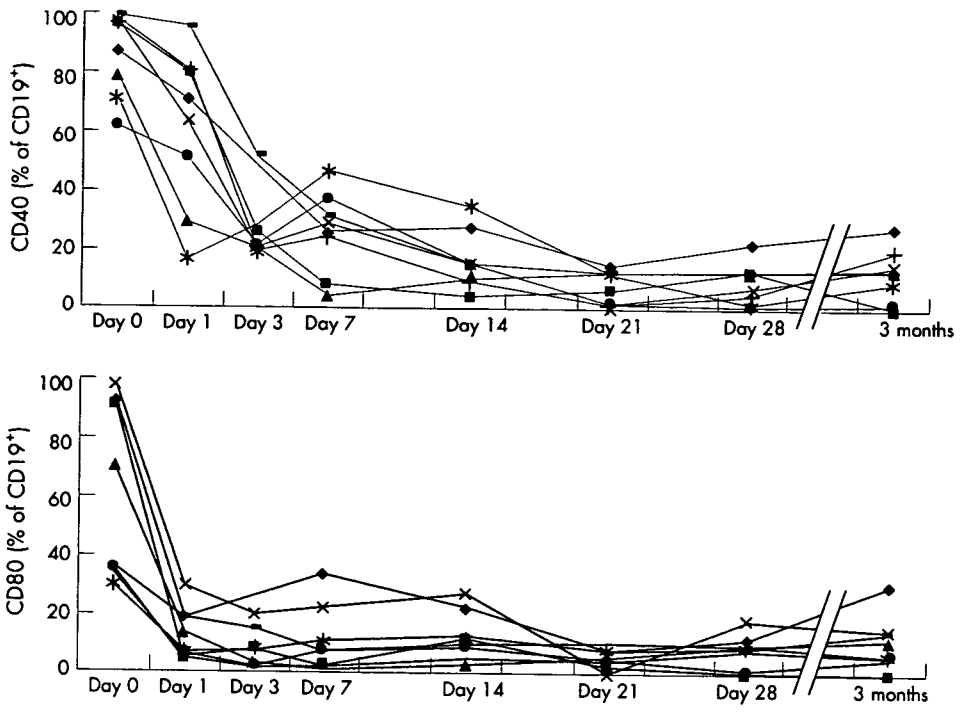

Figure 2 Serial changes in CD40 and CD80 expression on CD19-positive cells after rituximab treatment in eight patients with systemic lupus erythaematosus. CD40 and CD80 expression was measured before and 28 days after rituximab treatment.

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

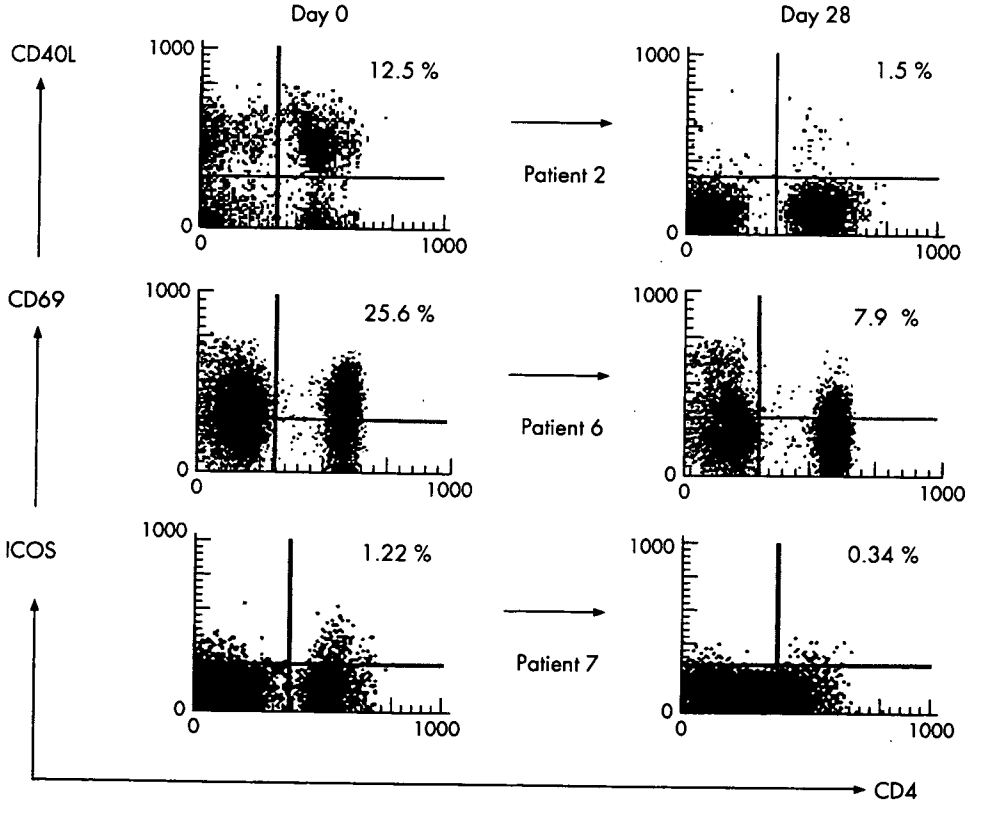

Figure 3 Changes in expression of functional molecules on CD4-positive cells induced by rituximab treatment. The expression of CD40L (patient 2), CD69 (patient 6) and ICOS (patient 7) on CD4-positive cells was measured before (day 0) and 28 days after rituximab treatment. Percentages represent the percentage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