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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-

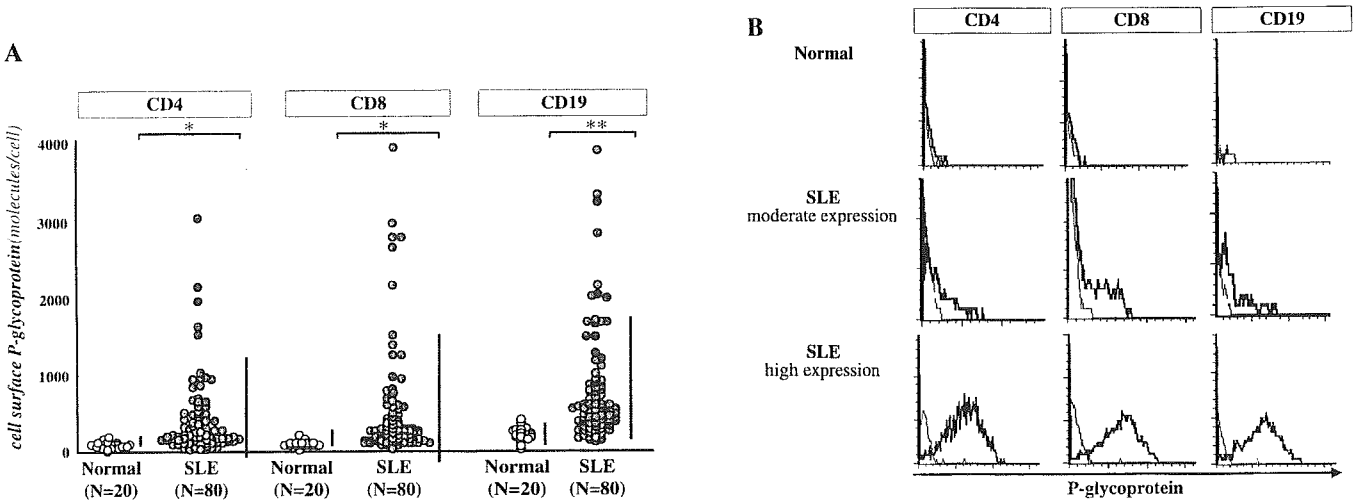

Figure 1. Expression of P-glycoprotein (P-gp) on lymphocytes from patients with systemic lupus erythematosus (SLE), as determined by flow cytometry. **A,** P-gp expression on CD4+, CD8+, and CD19+ peripheral blood lymphocytes from 20 normal volunteers and 80 SLE patients. Results were calculated with 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

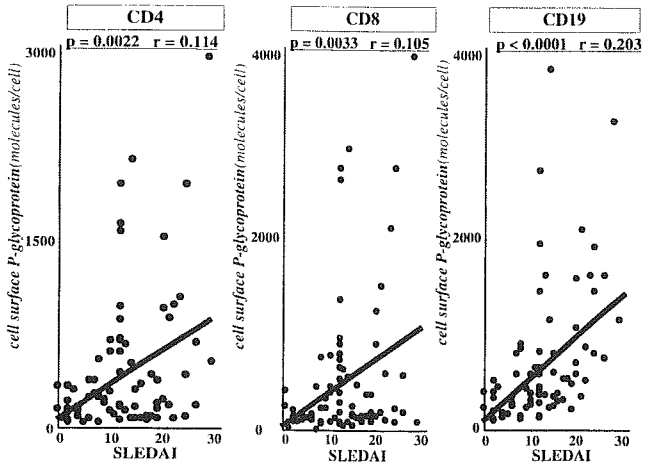

Figure 2. Correlation of the expression of P-glycoprotein (P-gp) on lymphocytes and scores on the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) in 80 patients with systemic lupus erythematosus (SLE). Levels of P-glycoprotein expression on SLE lymphocytes correlated closely with disease 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.

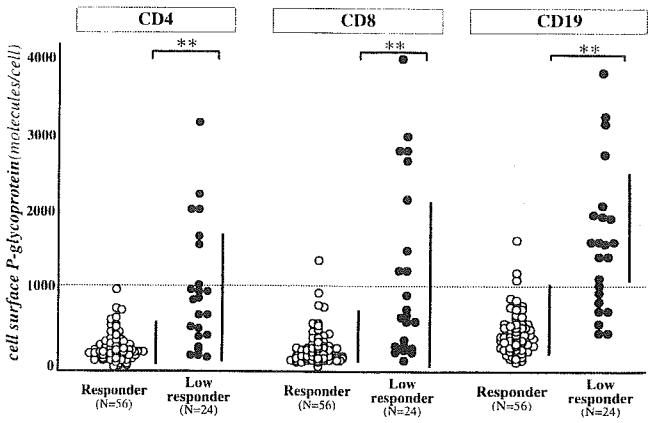

Figure 3. Relationship between responsiveness to corticosteroids and expression of P-glycoprotein (P-gp). Flow cytometric analysis was used to determine P-gp expression on the CD4+, CD8+, and CD19+ peripheral blood lymphocytes from systemic lupus erythematosus patients who were responders (n = 56) or 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.

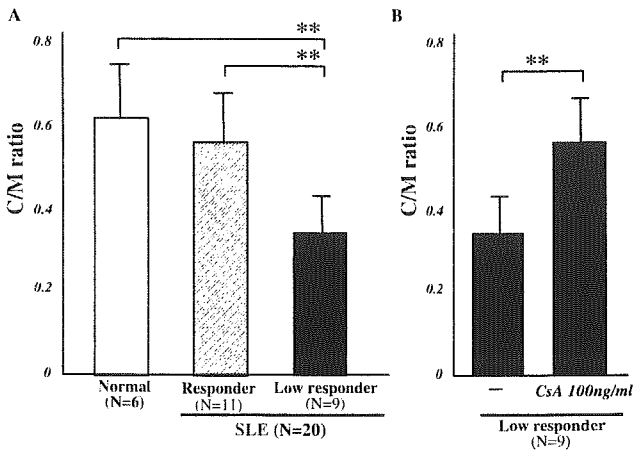

Figure 4. Decrease in intracellular dexamethasone levels in peripheral blood mononuclear cells (PBMCs) from systemic lupus erythematosus (SLE) patients who were low responders to corticosteroid therapy and inhibition of dexamethasone excretion by competitive binding of cyclosporin A to P-glycoprotein. **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.

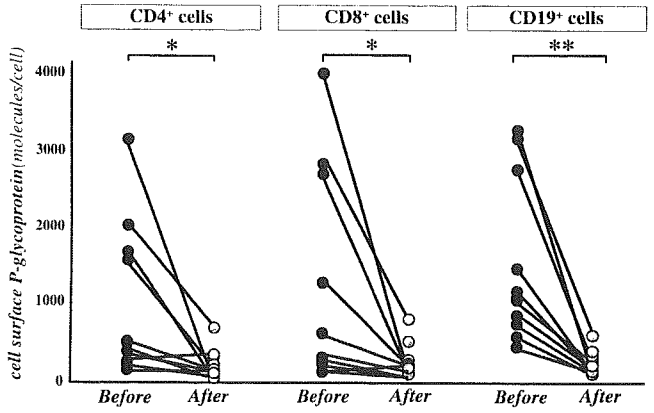

Figure 5. Effects of intensive immunosuppressive therapy on P-glycoprotein (P-gp) expression. Flow cytometric analysis was used to determine P-gp expression on peripheral blood lymphocytes from 10 low responders before and after intensive therapy with immunosuppressive agents (see Table 2). Results were 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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Significance of Valine/Leucine^{247} Polymorphism of β_2-Glycoprotein I in Antiphospholipid Syndrome

Increased Reactivity of Anti–β_2-Glycoprotein I Autoantibodies to the Valine^{247} β_2-Glycoprotein I Variant

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Objective. **To clarify the consequences of the valine/leucine polymorphism at position 247 of the β_2-glycoprotein I (β_2GPI) gene in patients with antiphospholipid syndrome (APS), by investigating the correlation between genotypes and the presence of anti-β_2GPI antibody. The reactivity of anti-β_2GPI antibodies was characterized using recombinant Val^{247} and Leu^{247} β_2GPI.**

Methods. **Sixty-five Japanese patients with APS and/or systemic lupus erythematosus who were positive for antiphospholipid antibodies and 61 controls were analyzed for the presence of the Val/Leu^{247} polymorphism of β_2GPI. Polymorphism assignment was determined by polymerase chain reaction followed by restriction enzyme digestion. Recombinant Val^{247} and Leu^{247} β_2GPI were established to compare the reactivity of anti-β_2GPI antibodies to β_2GPI between these variants. The variants were prepared on polyoxygenated plates or cardiolipin-coated plates, and the reactivity of a series of anti-β_2GPI antibodies (immunized anti-human β_2GPI monoclonal antibodies [Cof-19–21] and autoimmune anti-β_2GPI monoclonal antibodies [EY1C8, EY2C9, and TM1G2]) and IgGs purified from patient sera was investigated.**

Results. **A positive correlation between the Val^{247} allele and the presence of anti-β_2GPI antibodies was observed in the patient group. Human monoclonal/polyclonal anti-β_2GPI autoantibodies showed higher binding to recombinant Val^{247} β_2GPI than to Leu^{247} β_2GPI, although no difference in the reactivity of the immunized anti-β_2GPI between these variants was observed. Conformational optimization showed that the replacement of Leu^{247} by Val^{247} led to a significant alteration in the tertiary structure of domain V and/or the domain IV–V interaction.**

Conclusion. **The Val^{247} β_2GPI allele was associated with both a high frequency of anti-β_2GPI antibodies and stronger reactivity with anti-β_2GPI antibodies compared with the Leu^{247} β_2GPI allele, suggesting that the Val^{247} β_2GPI allele may be one of the genetic risk factors for development of APS.**

The antiphospholipid syndrome (APS) is characterized by arterial/venous thrombosis and pregnancy morbidity in the presence of antiphospholipid antibodies (aPL) (1–3). Among the targets of aPL, β_2-glycoprotein I (β_2GPI), which bears epitopes for anticardiolipin antibodies (aCL), has been extensively studied (4–6). APS-related aCL do not recognize free β_2GPI, but do recognize β_2GPI when it is complexed with phospholipids or negatively charged surfaces, by exposure of cryptic epitopes (7) or increment of antigen density (8).

The significance of antigen polymorphism in the production of autoantibodies or the development of autoimmune diseases is now being widely discussed. It is speculated that amino acid substitution in antigens can lead to differences in antigenic epitopes of a given protein. In particular, β_2GPI undergoes conformational

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alteration upon interaction with phospholipids (9). β_2GPI polymorphism on or near the phospholipid binding site can affect the binding or production of aCL (anti-β_2GPI autoantibodies), the result being altered development of APS. Polymorphism near the antigenic site, or which leads to alteration of the tertiary structure of the whole molecule, may affect the binding of autoantibodies. Five different gene polymorphisms of β_2GPI attributable to a single-nucleotide mutation have been described: 4 are a single amino acid substitution at positions 88, 247, 306, and 316 (10), and the other is a frameshift mutation associated with β_2GPI deficiency found in the Japanese population (11). In particular, the Val/Leu^{247} polymorphism locates in domain V of β_2GPI, between the phospholipid binding site in domain V and the potential epitopes of anti-β_2GPI antibodies in domain IV, as we reported previously (12). Although anti-β_2GPI antibodies are reported to direct to domain I (13) or domain V (14) as well, it should be considered that a certain polymorphism alters the conformation of the molecule, affecting function or antibody binding at a distant site.

We previously reported that, in a group of British Caucasian subjects, the Val^{247} allele was significantly more frequent in primary APS patients with anti-β_2GPI antibodies than in controls or in primary APS patients without anti-β_2GPI antibodies (15), but the importance of the Val^{247} allele in patients with APS is still controversial. In this study, we analyzed the correlation between the β_2GPI Val^{247} allele and anti-β_2GPI antibodies in the Japanese population. We also investigated the reactivity of anti-β_2GPI antibodies to recombinant Val^{247} β_2GPI and Leu^{247} β_2GPI, using a series of monoclonal anti-β_2GPI antibodies and IgGs purified from sera of patients with APS. Finally, to investigate the difference in anti-β_2GPI binding to those variants, we conformationally optimized to domain V and the domain IV–V complex of β_2GPI variants at position 247, referring the crystal structure of β_2GPI.

PATIENTS AND METHODS

Patients and controls. The study group comprised 65 patients (median age 38 years [range 18–74 years]; 57 women and 8 men) who attended the Hokkaido University Hospital, all of whom were positive for aPL (IgG, IgA, or IgM class aCL, and/or lupus anticoagulant). Thirty-four patients had APS (16 had primary APS, and 18 had secondary APS), and 31 patients did not have APS (24 had systemic lupus erythematosus [SLE], and 7 had other rheumatic diseases). Among all subjects, 19 had a history of arterial thrombosis, and 6 had venous thrombosis. Of the 31 patients with a history of pregnancy, 8 experienced pregnancy complications (some patients had more than 1 manifestation of pregnancy morbidity). Anti-β_2GPI antibodies were detected by enzyme-linked immunosorbent assay (ELISA) as β_2GPI-dependent aCL (16). IgG, IgA, or IgM class β_2GPI-dependent aCL were found in 30, 14, and 21 patients, respectively (some patients had >1 isotype), and 34 patients had at least 1 of those isotypes. Lupus anticoagulant, detected by 3 standard methods described previously (17), was found in 51 patients. The diagnoses of APS and SLE, respectively, were based on the preliminary classification criteria for definite APS (18) and the American College of Rheumatology criteria for the classification of SLE (19). Informed consent was obtained from each patient or control subject. The control group comprised 61 healthy individuals with no history of autoimmune, thrombotic, or notable infectious disease.

Determination of β_2GPI gene polymorphism. Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using a standard phenol–chloroform extraction procedure or the DnaQuick kit (Dainippon, Osaka, Japan). Polymorphism assignment was determined by polymerase chain reaction (PCR) followed by allele-specific restriction enzyme digestion (PCR–restriction fragment length polymorphism) using *Rsa* I (Promega, Southampton, UK) as described previously (15).

Purification of patient IgG. Sera from 11 patients positive for IgG class β_2GPI-dependent aCL were collected. The mean (±SD) titer of aCL IgG from these patients was 29.0 ± 21.5 IgG phospholipid (GPL) units (range 12.4 to >98 GPL units). IgG was purified from these sera using a protein G column and the MAbTrap GII IgG purification kit (Pharmacia Biotech, Freiburg, Germany), as recommended by the manufacturer.

Monoclonal anti-β_2GPI antibodies. Two types of anti-β_2GPI monoclonal antibodies were used. Cof-19, Cof-20, and Cof-21 are mouse monoclonal anti-human β_2GPI antibodies obtained from immunized BALB/c mice, directed to domains V, III, and IV of β_2GPI, respectively. These monoclonal antibodies recognize the native structure of human β_2GPI (12).

EY1C8, EY2C9, and TM1G2 are IgM class autoimmune monoclonal antibodies established from patients with APS (20). These antibodies bind to domain IV of β_2GPI, but only after interaction with solid-phase phospholipids or with a polyoxygenated polystyrene surface. EY1C8 and EY2C9 were established from a patient whose genotype of β_2GPI was heterozygous for Val/Leu^{247}. The genotype of the patient with TM1G2 was not determined.

Preparation of recombinant β_2GPI. As previously reported, genes were expressed in *Spodoptera frugiperda* Sf9 insect cells infected with recombinant baculoviruses (12). A full-length complementary DNA of human β_2GPI coding Val^{247} was originally obtained from Hep-G2 cells (21), and the valine residue was replaced by leucine, using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega, Madison, WI). The sequence of the primers for a mutant Val^{247}→Leu (GTA→TTA) is as follows: 5′-GCATCTTGTAAATTACCTGTGAAAAAAG-3′. A DNA sequence of the mutant was verified by analysis using ABI Prism model 310 (PE Applied Biosystems, Foster City, CA).

Binding assays of monoclonal anti-β_2GPI antibodies and purified IgGs to the recombinant β_2GPI (cardiolipin-coated plate). The reactivity of a series of monoclonal anti-β_2GPI antibodies and IgG fractions (purified from the sera of APS patients positive for IgG class anti-β_2GPI) against 2 β_2GPI variants was investigated using an ELISA. ELISAs were performed using a cardiolipin-coated plate as previously reported (16) but with a slight modification. Briefly, the wells of Sumilon Type S microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were filled with 30 μl of 50 μg/ml cardiolipin (Sigma, St. Louis, MO) and dried overnight at 4°C. After blocking with 2% gelatin in phosphate buffered saline (PBS) for 2 hours and washing 3 times with 0.05% PBS–Tween, 50 μl of 10 μg/ml recombinant β_2GPI and controls were distributed and incubated for 30 minutes at room temperature. Wells were filled with 50 μl of serial dilutions of monoclonal antibodies (Cof-19–21, EY1C8 and EY2C9, and TM1G2) or purified patient IgG (100 μg/ml), followed by incubation for 30 minutes at room temperature. After washing 3 times, 50 μl of alkaline phosphatase–conjugated anti-mouse IgG (1:3,000), anti-human IgM (1:1,000), or anti-human IgG (1:6,000) was distributed and incubated for 1 hour at room temperature. The plates were washed 4 times, and 100 μl of 1 mg/ml *p*-nitrophenyl phosphate disodium (Sigma) in 1*M* diethanolamine buffer (pH 9.8) was distributed. Optical density (OD) was read at 405 nm, with reference at 620 nm. One percent fatty acid–free bovine serum albumin (BSA) (A-6003; Sigma)–PBS was used as sample diluent and control.

Binding assays of monoclonal anti-β_2GPI antibodies to recombinant β_2GPI (polyoxygenated plate). Anti-β_2GPI antibody detection assay using polyoxygenated plates was performed as previously reported (22), with minor modifications. Briefly, the wells of polyoxygenated MaxiSorp microtiter plates (Nalge Nunc International, Roskilde, Denmark) were coated with 50 μl of 1 μg/ml recombinant β_2GPI in PBS and incubated overnight at 4°C. After blocking with 3% gelatin–PBS at 37°C for 1 hour and washing 3 times with PBS–Tween, 50 μl of monoclonal antibodies, diluted with 1% BSA–PBS, were distributed and incubated for 1 hour at room temperature. The following steps were taken, in a similar manner.

Conformational optimization of domain V and the domain IV–V complex in human β_2GPI variants at position 247. A conformation of domain V in the valine variant at position 247 was first constructed from the crystal structure of the leucine variant (implemented in Protein Data Bank: 1C1Z) (23). Replacement of leucine by valine at position 247 was performed using the Quanta system (Molecular Simulations, San Diego, CA), and the model was optimized by 500 cycles of energy minimization by the CHARMm program (24), with hydrophilic hydrogen atoms and TIP3 water molecules (25). Molecular dynamics simulation (5 psec) of the model was then performed with 0.002 psec time steps. The cutoff distance for nonbonded interactions was set to 15Å, and the dielectric constant was 1.0. A nonbonded pair list was updated every 10 steps. The most stable structure of each domain in the dynamics iterations was then optimized by 500 cycles of energy minimization. The final structures of domain V consisted of 2,616 atoms, including 603 TIP3 water molecules, and had a total energy of -1.63×10^4 kcal/mole with a root-mean-square force of 0.869 kcal/mole.

Molecular models of a domain IV–V complex (leucine and valine variants at position 247) were further constructed by considering the location of the oligosaccharide attachment site in domain IV, the location of epitopic regions of the Cof-8 and Cof-20 monoclonal antibodies, the junction between domains IV and V, and molecular surface charges of both domains. These models were again optimized by molecular dynamics simulation and by energy minimization as described above. The final structures of the complex in the leucine and valine variants consisted of 3,773 and 3,778 atoms, respectively, including hydrophilic hydrogen atoms and 806 and 808 TIP3 water molecules, respectively, and had total energy of -2.07×104 and -2.03×104 kcal/mole with a root-mean-square force of 0.985 and 0.979 kcal/mole, respectively.

Statistical analysis. Correlations between the allele frequencies and clinical features such as the positiveness of β_2GPI-dependent aCL were expressed as odds ratios (ORs) and 95% confidence intervals (95% CIs). *P* values were determined by chi-square test with Yates' correction. *P* values less than or equal to 0.05 were considered significant.

RESULTS

Val/Leu^{247} polymorphism of β_2GPI and the presence of β_2GPI-dependent aCL. As shown in Table 1, the Leu^{247} allele was dominant in the population of healthy Japanese individuals, compared with Caucasians, which is consistent with a previous report (26). Japanese patients with anti-β_2GPI had a significantly increased frequency of the Val^{247} allele, compared with Japanese patients without anti-β_2GPI ($P = 0.0107$) or Japanese controls ($P = 0.0209$).

The binding of autoimmune anti-β_2GPI to recombinant Val^{247} and Leu^{247} β_2GPI. Representative binding curves using cardiolipin-coated plates and polyoxygenated plates are shown in Figure 1. Regardless of the type of plates, Cof-20 bound equally to valine and leucine variants of β_2GPI (Figures 1a and c), in any concentration of Cof-20. The binding curves of Cof-19 and Cof-21 were similar to that of Cof-20 (results not

Table 1. Frequency of the Val^{247} allele of β_2GPI in patients with APS*

Group	Japanese	British Caucasians
Patients with anti-β_2GPI	23/68 (33.8)†	48/56 (85.7)‡
Patients without anti-β_2GPI	9/62 (14.5)	39/58 (67.2)
Controls	23/122 (18.9)	55/78 (70.5)

* Values are the number (%). β_2GPI = β_2-glycoprotein I; APS = antiphospholipid syndrome.
† $P = 0.0107$ versus patients without anti-β_2GPI (odds ratio [OR] 3.01, 95% confidence interval [95% CI] 1.26–7.16), and $P = 0.0209$ versus controls, by chi-square test (OR 2.15, 95% CI 1.09–4.23).
‡ $P = 0.204$ versus patients without anti-β_2GPI (OR 2.92, 95% CI 1.16–7.39), and $P = 0.0396$ versus controls, by chi-square test (OR 2.51, 95% CI 1.03–6.13).

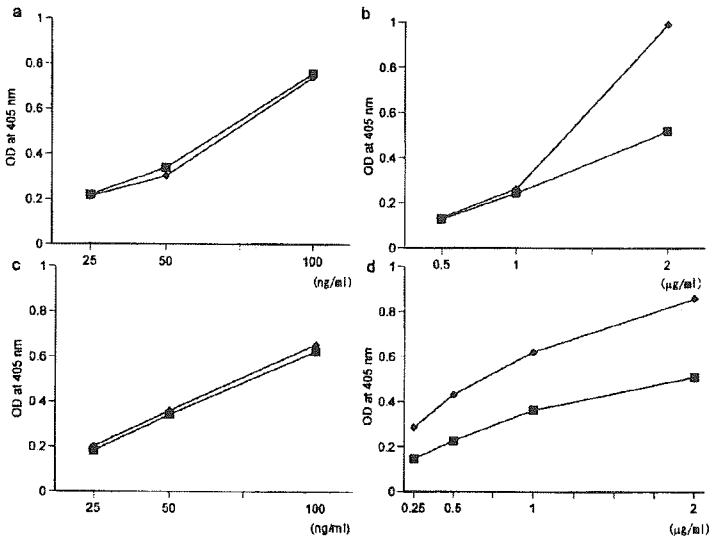

Figure 1. Representative binding curves of monoclonal anti-β_2-glycoprotein I (anti-β_2GPI) antibodies to recombinant valine/leucine^{247} β_2GPI. **a,** Binding curve of Cof-20 using cardiolipin-coated plate. **b,** Binding curve of EY2C9 using cardiolipin-coated plate. **c,** Binding curve of Cof-20 using polyoxygenated plate. **d,** Binding curve of EY2C9 using polyoxygenated plate. Binding to Val^{247} β_2GPI and Leu^{247} β_2GPI are indicated with diamonds and squares, respectively. OD = optical density.

shown). In contrast, EY2C9 showed stronger binding to Val^{247} β_2GPI than to Leu^{247} β_2GPI (Figures 1b and d). EY1C8 and TM1G2 also showed stronger binding to Val^{247} β_2GPI. Figure 2a shows the binding of the monoclonal antibodies, on cardiolipin-coated plates, in the following concentrations: for Cof-19–21, 100 ng/ml;

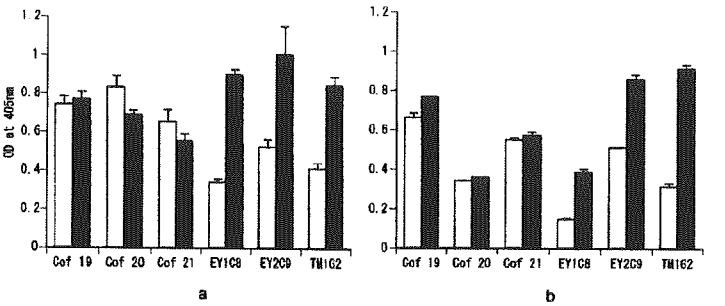

Figure 2. Reactivity of anti-β_2-glycoprotein I (anti-β_2GPI) antibodies to β_2GPI variants. **a,** The binding of monoclonal anti-β_2GPI antibodies to the recombinant valine/leucine^{247} β_2GPI was investigated using enzyme-linked immunosorbent assay (ELISA) on cardiolipin-coated plates. Concentrations of antigens and antibodies were as follows: for recombinant β_2GPI, 10 μg/ml; for Cof-19–21, 100 ng/ml; for EY1C8 and EY2C9, 2 μg/ml; for TM1G2, 5 μg/ml. **b,** The binding of monoclonal anti-β_2GPI antibodies to the recombinant Val/Leu^{247} β_2GPI was investigated using ELISA on polyoxygenated plates. Concentrations of antigens and antibodies were as follows: for recombinant β_2GPI, 1 μg/ml; for Cof-19–21, 50 ng/ml; for EY1C8 and EY2C9, 2 μg/ml; for TM1G2, 5 μg/ml. Results were presented as the optical density (OD) at 405 nm. Open columns indicate binding activity to Leu^{247} β_2GPI, and solid columns indicate binding activity to Val^{247} β_2GPI. Bars show the mean and SD.

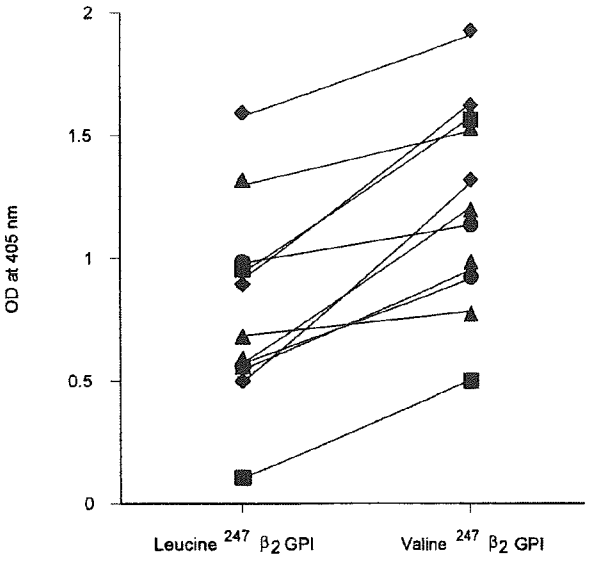

Figure 3. Reactivity of purified IgG from patients (100 μg/ml) to recombinant Val/Leu^{247} β_2-glycoprotein I (β_2GPI) (10 μg/ml), presented as the optical density (OD) at 405 nm. Squares, circles, and triangles indicate patients homozygous for the Leu^{247} allele, homozygous for the Val^{247} allele, and heterozygous for the Val/Leu^{247} allele, respectively. Diamonds indicate patients whose genotypes were not available.

for EY1C8 and EY2C9, 1 μg/ml; and for TM1G2, 2.5μg/ml. In contrast with the close reactivity of Cof-19, Cof-20, and Cof-21 between Val^{247} β_2GPI and Leu^{247} β_2GPI, autoimmune monoclonal antibodies (EY1C8, EY2C9, and TM1G2) showed higher binding to Val^{247} β_2GPI than to Leu^{247} β_2GPI. The autoimmune monoclonal antibodies also showed a higher binding to Val^{247} β_2GPI directly coated on polyoxygenated plates (Figure 2b). IgG in sera collected from 11 patients (100 μg/ml) also showed higher binding to Val^{247} β_2GPI than to Leu^{247} β_2GPI on cardiolipin-coated plates, regardless of the patients' genotypes (Figure 3).

Conformational alteration by leucine replacement by valine at position 247. Each domain V conformation in 2 variants at position 247 is shown in Figure 4a. The root-mean-square deviations for matching backbone atoms and equivalent atoms in the leucine and valine variants were 0.76 and 1.11 Å, respectively. The largest shift was observed at Val^{303}, one of the residues located on the backbone neighboring position 247. The shift seemed to be caused by weak flexibility of side chains consisting of Val^{247}, Pro^{248}, and Val^{249} and the electrostatic interactions between Lys^{250}, Lys^{251}, Glu^{307}, and Lys^{308}.

The molecular models of the IV–V complex in leucine and valine variants are shown in Figure 4b. The root-mean-square deviations for matching these backbone atoms and equivalent atoms were 1.72 and 2.03 Å, respectively. Electrostatic interactions and hydrogen bonds between Asp^{193} and Lys^{246}/Lys^{250}, Asp^{222} and Lys^{305}, and Glu^{228} and Lys^{308} appeared in the IV–V complex, but the interaction between Glu^{228} and Lys^{308} was disrupted by the leucine replacement by valine, because direction of the Lys^{308} side chain was significantly changed in the complex. As a result, Trp^{235} of domain IV, located on the contact surface with domain V, was slightly shifted.

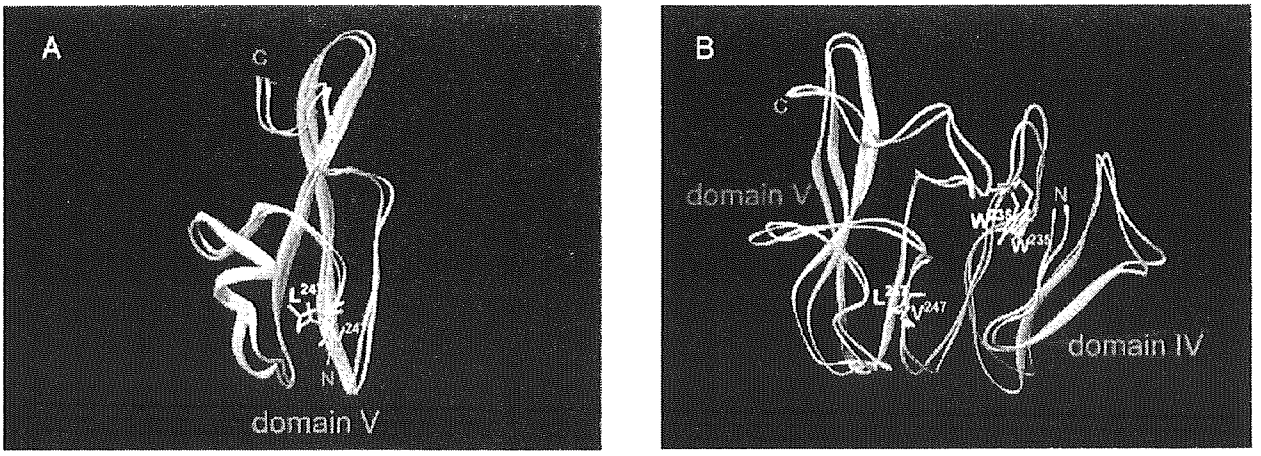

Figure 4. Conformational alterations in domain V (A) and in the domain IV–V complex (B), replacing leucine by valine at position 247. Structure of the valine (light blue) and leucine (white) variants was shown by a ribbon representation with the secondary structure.

DISCUSSION

This study shows the positive correlation between the Val^{247} β_2GPI allele and anti-β_2GPI antibody production in a Japanese population, confirming the correlation observed in a British Caucasian population in our previous report (15). A positive correlation between the Val^{247} allele and the presence of anti-β_2GPI antibodies was also reported in Asian American (26) and Mexican patients (27). However, this correlation was not observed in other American populations (26) or in patients with thrombosis or pregnancy complications in the UK (28). This discrepancy may be the result of the difference in the frequency of the Val^{247} allele among races, or the difference in the background of investigated patients. Another possibility is that the relationship between the Val^{247} allele and thrombosis in Caucasians may be controversial due to underpowered studies or to differences in the procedure used to detect anti-β_2GPI antibodies. Methods for the detection of anti-β_2GPI antibodies differ among laboratories. For example, cardiolipin-coated plates or oxygenated plates are used in some methods, whereas unoxygenated plates are used in others. In addition, bovine β_2GPI is used instead of human β_2GPI in some assays. The antibodies used for standardization also differ, although monoclonal antibodies such as EY2C9 and HCAL (29) have been proposed as international standards of calibration materials.

β_2GPI is a major target antigen for aCL, and, according to our previous investigation, B cell epitopes reside in domain IV and are considered to be cryptic and to appear only when β_2GPI interacts with negatively charged surfaces such as cardiolipin, phosphatidylserine, or polyoxygenated polystyrene surface (7), although other studies indicate that the B cell epitopes are located on domain I (13) or domain V (14). According to another interpretation for the specificity of aCL, increment of the local antigen density on the negatively charged surface also contributes to anti-β_2GPI detection in ELISA (8,30). Studies on the crystal structure of human β_2GPI revealed that the lysine-rich site and an extended C-terminal loop region on domain V are crucial for phospholipid binding. Position 247 is located at the N-terminal side of domain V, and, around this position, Lys^{242}, Ala^{243}, and Ser^{244} were suggested to play a role in the interaction between domains IV and V (9,23,31).

Although the Val/Leu^{247} polymorphism may not be very critical for the autoantibody binding, the amino acid substitution at this point was revealed to affect the affinity of monoclonal aCL established from patients with APS and that of purified IgG from patients positive for β_2GPI-dependent aCL. We conformationally optimized to domain V and the domain IV–V complex of β_2GPI variants at position 247, referring the crystal structure of β_2GPI. IgG aCL was screened using the standardized aCL ELISA, in which both the Leu^{247} and the Val^{247} allele of β_2GPI are contained as antigen. Although biochemical characteristics and structure are similar between valine and leucine, the replacement of Leu^{247} by Val^{247} leads to a significant alteration in the tertiary structure of domain V and/or the domain IV–V interaction (Figure 4). It is likely that the structural alteration affects the affinity between anti-β_2GPI autoantibodies and the epitope(s) present on its molecule. One explanation for this phenomenon is that this β_2GPI polymorphism affects the electrostatic interaction between domain IV and domain V or the protein–protein interaction, resulting in differences in the accessibility of the recognition site by the autoantibodies, or the local density of β_2GPI.

Another possible explanation of the correlation between the Val/Leu^{247} polymorphism of β_2GPI and anti-β_2GPI antibodies is T cell reactivity. Ito et al (32) investigated T cell epitopes of patients with anti-β_2GPI autoantibodies by stimulating patients' PBMCs with a peptide library that covers the β_2GPI sequence. Four of 7 established CD4+ T cell clones reacted to peptide fragments that include amino acid position 244–264, then position 247 is included among the candidate epitopes. Arai et al (33) found preferred recognition of peptide position 276–290 by T cell clones from patients with APS. They also found high reactivity to peptide 247–261 in one patient. We speculate that a small alteration in the conformation arising from the valine/leucine substitution at position 247 may affect the susceptibility to generate autoreactive T cell clones in patients with APS.

Our results in this study indicate that the Val/Leu^{247} polymorphism affects the antigenicity of β_2GPI for anti-β_2GPI autoantibodies, and that the Val^{247} allele can be a risk factor for having autoantibodies against this molecule. Therefore, the Val/Leu^{247} variation of β_2GPI may be crucial for autoimmune reactivity against β_2GPI. We further show the significance of the Val/Leu^{247} polymorphism of β_2GPI in the strength of the binding between β_2GPI and anti-β_2GPI autoantibodies. The significance of antigen polymorphisms in the production of autoantibodies or in the development of autoimmune diseases is not well understood. To our knowledge, this report is the first to present a genetic polymorphism of

autoantigen directly affecting its interaction with autoantibodies.

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ORIGINAL ARTICLE

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Clinical characteristics of *Pneumocystis carinii* pneumonia in patients with connective tissue diseases

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Abstract The characteristics of *Pneumocystis carinii* pneumonia (PCP) in patients with connective tissue diseases (CTDs) were examined retrospectively. Nine patients were enrolled in this study. Their mean age was 57.1 years. All the patients received a high-dose steroid or immunosuppressant. The onset (mean 6.6 days) of fever, cough, breathlessness, and geographical ground-glass opacities revealed by chest computed tomography was acute. The serum β-D-glucan level increased with a simultaneous increase in the Krebs von den Lungen (KL)-6 or surfactant protein D level. The serum immunoglobulin G (IgG) and albumin levels and the peripheral blood lymphocyte count at the onset of PCP were low, but only the serum IgG level decreased significantly. The patients were treated with trimethoprim-sulfamethoxazole or pentamidine isetionate. Six patients died eventually: two patients of progressive respiratory failure, two probably due to a recurrence of the PCP, and two with microbial respiratory infections other than PCP. Five of the six patients required mechanical ventilation. Three patients received secondary prophylaxis and survived. In conclusion, the acute onset was characteristic of PCP in patients with CTDs. High-dose steroids, immunosuppressants, and hypogammaglobulinemia are risk factors; and respiratory failure requiring mechanical ventilation, severe secondary infections, and a lack of secondary prophylaxis are poor prognostic factors. Secondary prophylaxis is recommended for all of these patients.

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Key words Connective tissue diseases (CTDs) · Hypogammaglobulinemia · *Pneumocystis carinii* pneumonia (PCP) · Prognostic factors · Risk factors

Introduction

Pneumocystis carinii pneumonia (PCP) is one of the most prevalent opportunistic infections in immunocompromised hosts.[1] Genetic analyses established that *Pneumocystis carinii* (PC) is not a protozoan but a fungus.[2] Host-specific *Pneumocystis* organisms are identified in every mammalian species, and a new name, *Pneumocystis jiveroci*, is suggested for a human-specific organism.[3] The risk factor for PCP in patients infected with human immunodeficiency virus (HIV) is a CD4 lymphocyte count lower than 200 μl.[4] Poor prognostic factors include mechanical ventilation, increased lactate dehydrogenase and C-reactive protein (CRP) levels, hypoalbuminemia, hypoxemia, and a low CD4 lymphocyte count.[5–7] Patients with connective tissue diseases (CTDs) are also at high risk for PCP.[8] PCP has been documented in patients receiving immunosuppressive therapy for systemic lupus erythematosus (SLE), dermatomyositis,[9,10] Wegener's granulomatosis,[11,12] and rheumatoid arthritis (RA).[13] PCP accounted for 4% of all microbial pneumonitis in patients with CTDs, and its mortality rate was 45.7%.[14] The risk factors in patients with CTDs included steroid or cytotoxic therapy, interstitial pneumonitis, or lymphocytopenia.[9–12,15,16] Primary prophylaxis for PCP was effective in patients with CTDs receiving steroid therapy.[17]

In this study, we retrospectively analyzed the clinical characteristics, laboratory data, radiological findings, treatment courses, and outcomes of PCP in patients with CTDs to determine whether these patients have any characteristics different from those of HIV patients with PCP.