Race Differences in Immunogenetic Features and Photosensitivity of Cutaneous Lupus Erythematosus from the Aspect of Japanese Studies

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Skin lesions of collagen diseases are influenced by environmental triggers, such as UV light, and are variable in cutaneous lupus erythematosus (LE), such as systemic LE (SLE), chronic discoid LE (CDLE), subacute cutaneous LE (SCLE), and LE tumidus (LET). Although there are a few conflicting reports on photosensitivity in collagen diseases, many Japanese dermatologists feel there are photosensitivity differences in LE between Asians and Caucasians with SCLE and LET. To address this issue, we have carried out genetic studies of Japanese SLE and CDLE patients and reviewed the race differences in photosensitivity of cutaneous LE from Japanese studies. Human leukocyte antigen (HLA) studies in Japanese patients revealed that HLA-DRB1*1501 association was with CDLE and SLE. The association between HLA-Cw6 and CDLE was first reported in a Japanese population, and a HLA-A33-B44-DRB1*1302 haplotype showed a positive association in CDLE. However, these results are not compatible with those from Caucasian subjects. There are no significant associations among HLA studies, photosensitivity, and anti-Ro/SS-A antibodies in Japanese CLE patients. Photosensitivity will be a key factor to dissolve multifactorial complexes of LE etiopathogenesis. An axis of photosensitivity, anti-Ro/SS-A antibodies, and apoptosis via tumor necrosis factor-α is the best marker to verify the contribution of genetics in CLE patients. The incidence and degree of photosensitivity of SCLE and LET are much lower in Japanese than in Caucasians. This discrepancy may lead to investigations of CLE pathogenesis through global collaborations.

Key words: **lupus erythematosus; photosensitivity; genetics; HLA; autoimmune mouse model**

Introduction

The etiopathogenesis of systemic lupus erythematosus (SLE) remains poorly understood. However, we can say that susceptibility to SLE is largely dependent on genetic predisposition and that only by understanding the genetic basis for SLE will it be possible to define the pathogenesis of lupus.[1] For understanding the genetic background, many strains of spontaneous SLE-prone mice have contributed to organ studies, including the skin.[2] Genetic differences induce certain skin manifestations, such as LE-like skin lesions in MRL/Mp-lpr/lpr (MRL/lpr) mice and mixed connective tissue disease (MCTD)-like skin lesions in MRL/Mp-+/+ (MRL/n) mice.[3] In MRL mice, only the lpr (lymphoproliferation) mutation (Fas defect) is responsible for different skin eruptions from MRL/n

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mice. Fibroblasts and keratinocytes cultured from MRL/lpr mice are susceptible to UVB-induced cytotoxicity, which associates with intrinsic SLE phenomena, and might be regulated by an individual's genetic background.[4]

We easily speculate that skin color may reflect the degree, severity, and manifestation pattern of photo-related skin lesions in LE patients. In this review, we report the relationship between HLA and disease susceptibility in chronic discoid LE (CDLE) and SLE Japanese patients and summarize ethnic differences in photosensitivity in cutaneous LE, such as CDLE or subacute cutaneous LE (SCLE).

Genetic Basis of Japanese Patients with Cutaneous LE

In cases of neonatal LE (NLE), it is well known that the human leukocyte antigen (HLA)-DR3 phenotype, which is found in the great majority of Caucasian mothers of NLE infants, is absent in Japanese mothers. Significantly high relative risk of HLA-DRw12 was reported in Japanese infants with NLE,[5] which is different from that of Caucasian patients reported in the literature.[6] Miyagawa studied HLA-DR and -DQ distributions in NLE and demonstrated that maternal HLA-DR5 haplotype bearing DRB1*1101-DQA1*0501-DQB1*0301 was associated with neonatal cutaneous lupus but not congenital heart blocks (CHB), which associated with HLA- DR2 haplotype (including HLA-DQB1*0602).[7]

Table 1 shows the recent results of an HLA study of Japanese CDLE and SLE patients.[8] The frequency of HLA-DRB1*1501(DR2) was significantly increased in both CDLE and SLE, whereas there was a weakly negative association between HLA-DRB1*1502(DR2) and CDLE. Furthermore, HLA-DRB1*1302(DR6) showed a positive association with CDLE. HLA-A33 and -B44 are in linkage disequilibrium with DRB1*1302 among the Japanese population. The increased frequencies of HLA-A33 and -B44 seem to be secondary. Interestingly, HLA-Cw6 and -Cw7 associated with DLE and SLE, respectively.

In this study, HLA-DRB1*1501 associated with both CDLE and SLE. Together with a previous report on a Japanese population,[9] the possibility that common genetic factors linked to HLA-DRB1*1501 might be present in subjects with CDLE and those with SLE deserves further study. CDLE patients tended to negatively associate with HLA-DRB1*1502, a split of HLA-DR2, although the statistical significance was marginal. HLA-Cw7 positively associated with SLE, which was inconsistent with a previous report from Hashimoto *et al.* in which the authors described a negative association between HLA-Cw7 and oral ulcers, pneumonitis, and anti-Sm antibody formation.[10]

In CDLE, an association between HLA-Cw6 and CDLE has not been documented in the Japanese population, and the HLA-A33-B44-DRB1*1302 haplotype showed a positive association. Reports from European countries suggest that combinations were found in 17.2% of HLA-Cw7, DR3, DQw1 and HLA-B7, Cw7 and DR3 were found in 17.2% of CDLE and in 2.3% of the controls,[11] and the association of CCLE with certain HLA class II alleles points to an involvement of HLA-DQ and/or -DR molecules (HLA-DRB1*1601-DQA*0102) in the pathogenesis of CDLE,[12] which is different from the present Japanese result.

Photosensitivity in Cutaneous LE

The most common presentation in SLE cutaneous lesions is the erythema over photolocalizing areas. In CDLE, skin lesions in most cases are limited to the face, where malar areas and the nose are predominantly affected. Our recent survey of Japanese patients revealed that 59% (49/83 cases) of SLE and 39% (12/31 cases) of CDLE patients had photosensitivity, which was determined by standard clinical examinations (including medical histories of patients). These incidences seem to be in the average range based on a review by Walchner

TABLE 1. Association Between Human Leukocyte Antigen and Chronic Discoid Lupus Erythematosus/Systemic Lupus Erythematosus in Japanese Patients

				CDLE vs. Controls			SLE vs. Controls		
HLA	CDLE	SLE	Controls	RR	$\chi 2$	P	RR	$\chi 2$	P
	$n = 45$	$n = 81$	$n = 120$						
HLA-A33	20.0%	9.9%	9.2%	2.5	3.60	NS			
B44	22.2%	11.1%	10.0%	2.6	4.23	<0.05			
Cw6	6.7%	0%	0.8%	8.5	4.70	<0.05			
Cw7	20.0%	37.0%	18.0%				2.6	8.82	<0.005
	$n = 23$	$n = 58$	$n = 120$						
HLA-DRB1* 1501	26.1%	22.4%	8.3%	3.4	6.17	<0.02	3.2	6.89	<0.02
DRB1* 1502	4.3%	20.7%	21.7%	0.2	3.78	NS			
DRB1* 1302	30.4%	12.1%	13.3%	2.8	4.18	<0.05			

CDLE, chronic discoid lupus erythematosus; HLA, human leukocyte antigen; NS, not significant; RR, relative risk; SLE, system lupus erythematosus.[8]

et al.[13] Our preliminary experiments, using the modified method noted in our previous reports,[14] showed that there are no differences in UVB-induced cytotoxicity in cultured keratinocytes from suction blister roofs of normal Caucasian and Japanese subjects.[8]

The positive ratio for Japanese SLE patient phototesting was 33%, but there was no statistically significant association between clinical photosensitivity and phototesting[2] [in which we used a method of determining the minimal erythema dose (MED)]. Even if a photo-provocation method is applied to Caucasian patients with SLE, the positive ratio is under 30%.[15] In this respect there are few differences between Caucasians and Asians. However, in cases of LE subgroups, such as SCLE, lupus erythematosus tumidus (LET), and CDLE, there are marked differences observed between Caucasians and Japanese. The positive ratio from photo-provocation tests in CDLE was less than 20% in Japanese but over 40% in Caucasians.[15]

Among LE subsets, SCLE and LET are characterized by photosensitivity, irrespective of autoantibodies.[16,17] In textbooks and literature, written in English, 9–10% of all LE patients are reported as being SCLE or SCLE-like, but there are six patients with SCLE, which is 2% of all LE patients, who visited Wakayama Medical University from 1984 to 2007. In a report from Kuhn *et al.*, SCLE patients are more than 65% positive in photo-provocation tests,[17] but our experience and Japanese case reports in the literature suggest that the shortening of MED is found in less than 40% of SCLE. LET has a much higher photosensitivity. Lehmann reported almost 80% of LET patients showed photosensitivity by photo-provocation tests.[15] However, there are very few reports of LET in Japan.

This discrepancy of the incidence of photosensitivity may depend on the genetic background, methods, skill of investigators, form of questioning, and the age of subjects.[13,15] Hence, new and more reliable methods should be developed for examining photosensitivity.

Genetics of Photosensitivity in Cutaneous Lupus Erythematosus

Recent surveys for susceptibility to SLE have shown how genetic plasticity affects complex phenotypes, as seen in SLE,[18] because SLE is a multifactorial disease in that both genetics and environmental factors play crucial roles in pathogenesis. In Japanese SLE patients, photosensitivity associates with DRB1*0405 and/or DQB1*0401,[9] which was not confirmed by our present study. Because SLE is heterogeneous, it is difficult to identify strict gene associations

with clinical manifestations. However, a report from Nath *et al.*[19] is very promising from dermatological and ethnic views because these researchers found a candidate region on 11p13 for discoid lesions of SLE in African-American families.

Investigations with SCLE and LET may be better suited for studying ethnic differences than SLE because these diseases are not as immunoserologically complex. Anti-Ro/SS-A antibodies are disease markers, and photosensitivity is a clinical marker in both diseases.[16,17] Apoptosis and cytokines [especially tumor necrosis factor-α (TNF-α)] could be the best clues for linking anti-Ro antibodies and photosensitivity.[14,20] In SCLE, the HLA-A1, B8, DR3 haplotype has subsequently been markedly extended and is referred to as the 8.1 ancestral haplotype [i.e., the common caucasoid haplotype (HLA-A1, Cw7, B8, TNFAB* a2b3, TNFN*S, C2*C, Bf*s, C4A*Q0, C4B*1, DRB1*0301, DRB3*0101, DQA1*0501, DQB1*0201)] carried by most people who type for HLA-B8, DR3. Japanese studies failed to demonstrate such associations among patients who have annular erythema associated with LE/ Sjogren's syndrome (reviewed from Ref. 8).

Interestingly, there is an association of the −308A TNF-α promoter polymorphism with one form of SCLE.[20] This polymorphism is associated with an extended HLA A*01, B*08, DRB1*0301 haplotype. Contrary to this report, several investigations on the Ro60 gene or apoptosis gene have been carried out but there have been no definite results in cutaneous LE. Even if polymorphisms in cutaneous LE are found, the role of the polymorphism may be unclear in terms of etiology or disease association.

The genetic basis of cutaneous LE and photosensitivity is far from being understood. Anti-Ro/SS-A antibody is the best established laboratory marker, and related biological responses such as HLA, TNF-α, and complement have been investigated. Clinical and laboratory phenotypes with ethnic differences remain elusive.

Conflicts of Interest

The authors have no potential conflict of interest with regard to this report.

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A Single Helper T Cell Clone Is Sufficient to Commit Polyclonal Naive B Cells to Produce Pathogenic IgG in Experimental Pemphigus Vulgaris

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A Single Helper T Cell Clone Is Sufficient to Commit Polyclonal Naive B Cells to Produce Pathogenic IgG in Experimental Pemphigus Vulgaris[1]

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The development of naive B cells into IgG-producing memory B cells requires cognate T cell-B cell interaction in Ag-specific immune responses. It is unknown whether a single T cell clone is sufficient or whether multiple clones are necessary to induce polyclonal IgG production in vivo. We addressed this issue using a mouse model of pemphigus vulgaris, a fatal autoimmune blistering skin disease caused by IgG autoantibodies against desmoglein (Dsg) 3. We previously isolated several Dsg3-reactive T cell clones from Dsg3$^{-/-}$ mice. Among these, two pathogenic T cell clones induced anti-Dsg3 IgG production and the development of a pemphigus phenotype when adoptively transferred with unprimed B cells from Dsg3$^{-/-}$ mice. IgG Abs harvested from recipient mice reacted with at least three parts of the extracellular domain of Dsg3, as determined using domain-swapped Dsg3/Dsg1 molecules. The anti-Dsg3 IgGs included at least two subclasses among IgG1, IgG2a, IgG2b, and IgG3 in each mouse. The anti-Dsg3 IgG induced by Dsg3-reactive T cell clones with primed B cells from Dsg3$^{-/-}$ mice also showed reactivity against different parts of the molecule, with a similar epitope distribution. Together, these results indicate that a single potent Dsg3-reactive T cell is sufficient to commit polyclonal naive B cells to produce pathogenic anti-Dsg3 IgG Abs and induce the PV phenotype. These findings provide an important framework for examining immunological mechanisms in Ab-mediated autoimmune diseases. *The Journal of Immunology,* **2009, 182: 1740–1745.**

T cell-B cell interaction is generally important in producing IgG, which is involved in many conditions in patients with infections, organ transplantation, and autoimmune diseases (1). Various key steps in this strictly regulated interaction have been studied. For example, cognate interaction between Ag-specific T and B cells is required to produce Ag-specific IgG (2). Subsequently, CD40-CD154 interaction, which induces the expression of activation-induced cytidine deaminase, is essential for Ig-isotype class switch recombination and somatic hypermutation in IgG production (3). Spatial factors are also required for T-B interaction in vivo such as lymphocyte homing capacity in germinal centers, which is regulated by the appropriate expression of CXCR5 and CXCR4 in lymphocytes (4). Although many such molecules that determine T cell-B cell interaction and the subsequent IgG production have been described, the exact way of T cell-B cell interaction remains to be proven in vivo; i.e., whether a single T cell clone is able to interact with polyclonal B cells or whether several different T cell clones are necessary to commit a single B cell clone in Ag-specific immune responses (5–7). This is largely due to the lack of an appropriate in vivo model to address this fundamental question.

Pemphigus vulgaris (PV)[3] is a life-threatening blistering disease involving IgG autoantibodies directed against desmoglein 3 (Dsg3), a cadherin-type glycoprotein expressed on stratified squamous epithelium, including the skin and oral mucosa, that plays a key role in cell-cell adhesion (8). Anti-Dsg3 autoantibodies bind to keratinocyte cell surfaces and induce cell detachment, resulting in blisters and erosion in the skin and mucous membranes as well as characteristic histological findings such as suprabasilar acantholysis (9, 10). Dsg3-reactive T cells are believed to play an important role in the pathogenesis of PV and have been detected in PBMCs from PV patients and healthy controls (11). However, it has not been demonstrated how Dsg3-reactive T cells interact with B cells and induce anti-Dsg3 Ab production in vivo.

We previously developed a PV mouse model in which lymphocytes from rDsg3-immunized Dsg3$^{-/-}$ mice were adoptively transferred into Rag2$^{-/-}$ mice that express Dsg3 (12). The recipient mice showed stable anti-Dsg3 IgG production and developed a PV phenotype, including mucosal erosions with acantholytic blisters, similar to PV patients. However, there are several differences between human PV and the mouse model. Dsg3 is recognized as a foreign Ag in mouse PV, whereas Dsg3 is a self-Ag in human PV. Anti-Dsg3 IgG Abs produced in the mouse model were predominantly reactive against the C terminus of the Dsg3 extracellular domain (13), whereas human anti-Dsg3 IgG autoantibodies predominantly react with the N-terminal domain (14). Although this mouse model does not mimic the onset of the disease, it does provide an important in vivo tool for dissecting immunological mechanisms for the production of pathogenic anti-Dsg3 IgG Abs that play a key role in the pathogenesis of PV.

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[3] Abbreviations used in this paper: PV, pemphigus vulgaris; Dsg, desmoglein; IB, immunoblotting; IP, immunoprecipitation; m, mouse (prefix).

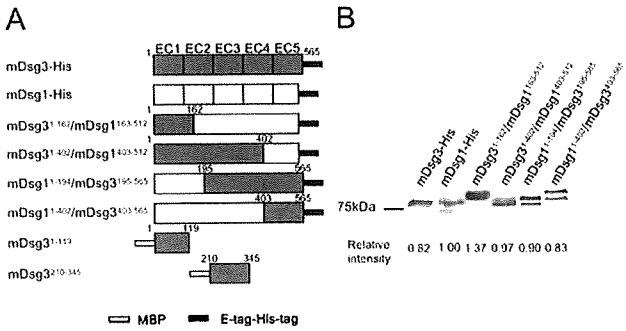

FIGURE 1. Antigenic proteins used. *A*, Schematic illustration of recombinant proteins. The numbers indicate the positions of amino acid residues in Dsg3 or Dsg1. EC, extracellular domain; MBP, maltose-binding protein. *B*, Immunoblot analysis for each baculoprotein. Standardized samples were fractionated by SDS-PAGE and detected using an anti-E-tag Ab and alkaline phosphatase-conjugated anti-mouse IgG Ab. The intensity of each band was measured by ImageJ.

Recently, we established a unique system in which the principle of the PV mouse model was applied, and we used this system to evaluate the in vivo pathogenic capability of Dsg3-reactive T cell clones to induce the PV phenotype via adoptive transfer with primed B cells isolated from rDsg3-immunized Dsg3$^{-/-}$ mice into Rag2$^{-/-}$ mice (15). Some of the T cell clones induced anti-Dsg3 IgG production and the PV phenotype, whereas others did not. These observations indicate that Dsg3-reactive T cells show pathogenic heterogeneity in the production of anti-Dsg3 IgG.

In this study, we examined whether pathogenic T cell clones were able to prime naive B cells and induce anti-Dsg3 IgG production and the PV phenotype. We also characterized the epitopes of the anti-Dsg3 IgG Abs raised in these mice. Through this investigation, we also attempted to determine the numerical requirement of T cell clones for polyclonal Ab production. It remains to be clarified whether several T cells clones specific to a target Ag are necessary for polyclonal Ab production in vivo, or whether a single T cell clone is sufficient to react with several B cell clones with the same Ag specificity, but against different epitopes.

Materials and Methods

Mice

Dsg3$^{-/-}$ mice with a mixed genetic background of 129/SV (H-2^b) and C57BL/6J (H-2^b) were obtained by mating male and female Dsg3$^{-/-}$ mice (Jackson Laboratory) (16). C57BL/6 Rag-2$^{-/-}$ mice were purchased from the Central Institute for Experimental Animals (Tokyo, Japan) The Keio University Ethics Committee for Animal Experiments (Tokyo, Japan) approved all experiments in this study.

Ags

The extracellular domains of Dsg3 and Dsg1 (mouse (m)Dsg3-His and mDsg1-His, respectively) and four domain-swapped molecules (mDsg3$^{1-162}$/mDsg1$^{163-512}$, mDsg3$^{1-402}$/mDsg1$^{403-512}$, mDsg1$^{1-194}$/mDsg3$^{195-565}$, and mDsg1$^{1-402}$/mDsg3$^{403-565}$) were produced as E- and His-tagged fusion proteins using a baculovirus expression system as previously described (13) (Fig. 1*A*). The amount of each baculoprotein in the culture medium was semiquantified by immunoblotting, probed with an anti-E-tag Ab (GE Healthcare) and an alkaline phosphatase-conjugated anti-mouse IgG Ab (Zymed Laboratories) in combination with quantification of band intensity using ImageJ software (rsb.info.nih gov/ij/). The culture medium was diluted to contain equal amounts of each molecule and used for the experiments. mDsg3$^{1-119}$ and mDsg3$^{210-345}$, previously reported as rDsg3-1 and rDsg3-3, respectively (15), were prepared as bacterial fusion proteins with maltose-binding protein and used for T cell stimulation.

Dsg3-reactive T cell clones

T cell clones were established as previously described (15). Briefly, the footpads of Dsg3$^{-/-}$ mice were immunized with mDsg3-His and emulsified with CFA (Sigma-Aldrich). Seven days later, single-cell suspensions were prepared from the popliteal lymph nodes of the mice and cultured with a mixture of rDsg3 fragments. The cells were stimulated twice with rDsg3 fragments in the presence of autologous 40 Gy-irradiated splenocytes and subsequently subjected to limiting dilution to establish T cell clones. Clonality was confirmed by RT-PCR and direct sequencing, using TCRVβ gene-specific primers in combination with Cβ region primers. T cell clones were maintained by repeated antigenic stimulation using rDsg3 fragments once every 10–14 days and supplementation with T-STIM without Con A (BD Biosciences) as a source of growth factors twice weekly.

Adoptive transfer

Unprimed and primed Dsg3$^{-/-}$ B cells were prepared by depleting CD4$^+$ and CD8$^+$ cells from the splenocytes of nonimmunized and mDsg3-His-immunized Dsg3$^{-/-}$ mice, respectively, followed by positive selection of B220$^+$ cells using the MACS cell separation system (Miltenyi Biotech) A T cell clone (0.5–1.0 $\times$ 10^6 cells) was transferred with unprimed or primed B cells (5 $\times$ 10^6) into Rag2$^{-/-}$ mice. Serum samples were collected once weekly after adoptive transfer.

Histopathology and direct immunofluorescent staining

Formalin-fixed tissue was stained with H&E and observed under an inverted microscope (TE2000-U; Nikon). For direct immunofluorescent staining, 10-μm cryosections of the palate were stained directly with Alexa Fluor 488-conjugated anti-mouse IgG Abs (Molecular Probes) and observed using a fluorescence microscope (TE2000-U, Nikon) to detect IgG deposits.

Immunoprecipitation (IP)-immunoblotting (IB)

Serum samples were incubated with each domain-swapped molecule and protein G-Sepharose (GE Healthcare) at 4°C for 18 h Protein G-Sepharose was washed five times with PBS containing 0.1% Nonidet P-40, 500 mM NaCl, and 0.5 mM $CaCl_2$ and subjected to SDS-PAGE. Immunoprecipitated proteins were visualized by chemiluminescence, using Western Lightning Plus (PerkinElmer) after probing with a mouse anti-E-tag Ab and HRP-conjugated anti-mouse IgG Ab (Medical and Biological Laboratories), unless stated otherwise.

ELISA

ELISA was used to determine anti-Dsg3 IgG titer as previously described (12). The amount of anti-Dsg3 IgG Abs absorbed by the domain-swapped molecules was calculated using results from a competition ELISA in which 1 μl of serum was preincubated with 150 μl of culture medium containing a domain-swapped molecule as a competitor at 4°C for 18 h, and then the unabsorbed anti-Dsg3 IgG Abs in serum were quantified by mDsg3 ELISA. A purified monoclonal anti-Dsg3 IgG (AK9) was used as a standard (10). The amount of anti-Dsg3 IgG Abs absorbed by each competitor was calculated using the following formula (amount of unabsorbed anti-Dsg3 IgG Abs in preincubated serum with mDsg1-His) $-$ (amount of unabsorbed anti-Dsg3 IgG Abs in preincubated serum with each competitor) (μg/ml). The competition ratio (percentage of competition) was calculated as the amount of anti-Dsg3 IgG Abs bound to the competitor compared with that bound to Dsg3.

Results

Characterization of Dsg3-reactive T cell clones

Three Dsg3-reactive CD4$^+$ T cell clones were used. All clones were pathogenic in that they induced anti-Dsg3 IgG production and the PV phenotype, including acantholytic blisters and IgG deposition on keratinocyte cell surfaces in vivo, when adoptively transferred with primed B cells isolated from mDsg3-His-immunized Dsg3$^{-/-}$ mice into Rag2$^{-/-}$ mice (15). Other nonpathogenic clones were not suitable for this study, because they did not induce anti-Dsg3 IgG production in vivo. Two T cell clones, 147#48 and 164#2, were reactive to peptides processed by splenic APCs from residues 210–345 of mDsg3, the middle portion of the Dsg3 extracellular domain, whereas another T cell clone, 154#33, was reactive to a peptide processed from the N-terminal residues 1–119 (Fig. 1*A*). T cell epitopes recognized by T cell clones 147#48 and 164#2 were not identified at the peptide level but were clearly different from that of T cell clone 154#33. To genetically examine the difference between T cell clones 147#48 and 164#2,

Table I. *Characteristic features of T cell clones used and results of their adoptive transfer with unprimed/primed B-cells*

T Cell Clone	Antigenic Fragment	Gene Segment of TCRβ-Chain	CDR3 Amino Acid Sequence of TCR β-Chain			Anti-Dsg3 IgG Production	
		Vβ/Jβ	Vβ	N-Dβ-N	Jβ	With unprimed B	With primed B
147#48	$mDsg3^{210-345}$	8.2/1.3	CASGD	WSF	GNTLYFGEG	+	+
154#33	$mDsg3^{1-119}$	8.2/2.4	CASGD	PGQGG	TLYFGAG	NA[a]	+
164#2	$mDsg3^{210-345}$	6/2.3	CASS	MRGG	AETLYFGSG	+	+

[a] Not analyzed.

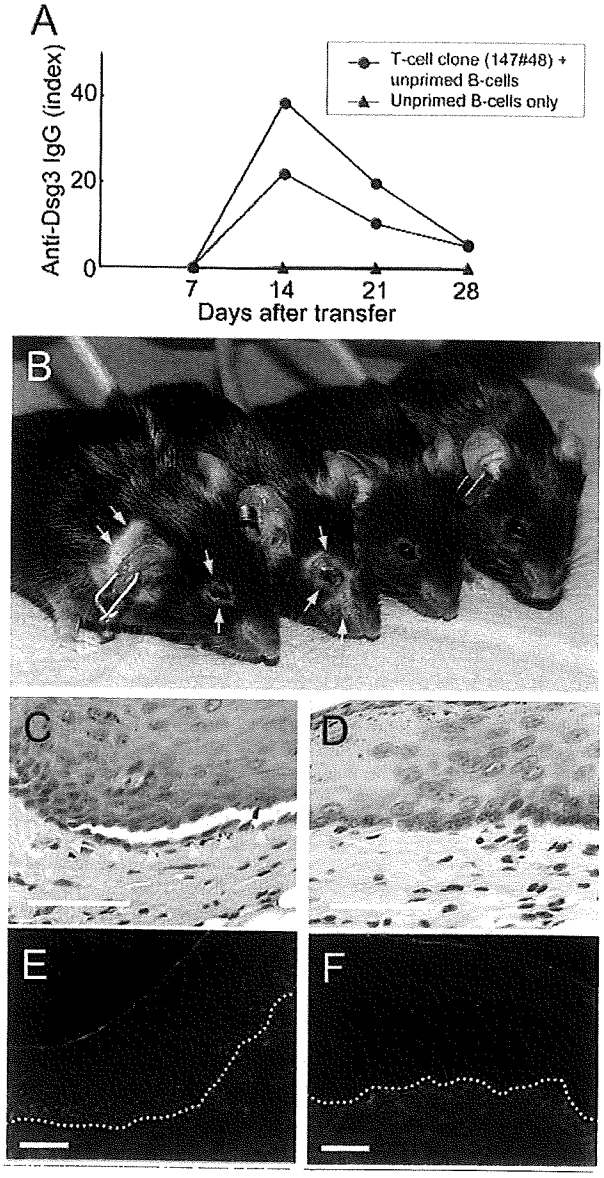

FIGURE 2. PV phenotype induced in $Rag2^{-/-}$ mice that received Dsg3-reactive T cell clones and unprimed $Dsg3^{-/-}$ B cells. *A*, ELISA was used to determine anti-Dsg3 IgG titers in sera from recipient mice that received Dsg3-reactive T cell clone 147#48 with unprimed B cells (closed circles) or B cells alone (closed triangles). *B*, Skin phenotype of the recipient mice. The two mice on the *left* received Dsg3-reactive T cell clone 147#48 and unprimed $Dsg3^{-/-}$ B cells, whereas the two mice on the *right* received only unprimed $Dsg3^{-/-}$ B cells Erosion and hair loss on the periorbital areas and shoulders were noticed in the left two mice (yellow arrows). *C–F*, Histopathological changes (*C* and *D*) and IgG deposition (*E* and *F*) in the palate of $Rag2^{-/-}$ mice that received Dsg3-reactive T cell clone 147#48 and unprimed B cells (*C* and *E*) or B cells alone (*D* and *F*). Yellow triangles indicate acantholytic blisters. White dotted lines indicate the basement membrane zone. Scale bar, 50 μm.

Vβ and Jβ gene usage and the CDR3 regions of TCR β-chains were analyzed, indicating that all three clones possessed different combinations of Vβ and Jβ genes and different CDR3 regions (Table I). Thus, the T cell clones used were distinct from one another.

Development of the PV phenotype with Dsg3-reactive T cell clones and unprimed B cells

The pathogenic capability of Dsg3-reactive T cell clones 147#48 and 164#2 to induce anti-Dsg3 IgG production and the PV phenotype in combination with unprimed B cells was evaluated (Table I). Unprimed B cells were prepared from nonimmunized $Dsg3^{-/-}$ mice in which no anti-Dsg3 IgG was detected by either ELISA or living keratinocyte cell staining (data not shown). The unprimed B cell population should include no Dsg3-specific memory B cells, because B cells derived from $Dsg3^{-/-}$ mice have not had any chance to encounter Dsg3 in vivo. In contrast, the primed B cell population already includes anti-Dsg3 IgG-producing memory B cells. Because cognate T cell help is critical for the development of naive B cells into memory B cells in the Ag-specific immune response in vivo (2), Dsg3-reactive T cell clones ought to interact with Dsg3-specific naive B cells in vivo to help them produce anti-Dsg3 IgG Abs.

After the transfer of 147#48 (n = 3) or 164#2 (n = 1) together with unprimed B cells into $Rag2^{-/-}$ mice, the recipient mice produced anti-Dsg3 IgG with a peak at day 14, whereas no anti-Dsg3 IgG was detected in mice receiving only unprimed B cells (Fig. 2*A*). Even when primed B cells alone isolated from $Dsg3^{-/-}$ mice immunized with mDsg3-His were transferred into $Rag2^{-/-}$ mice,

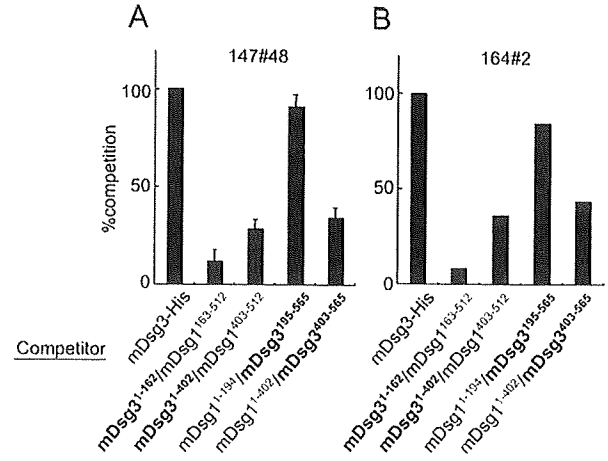

FIGURE 3. Competition ELISA using anti-Dsg3 IgG produced in $Rag2^{-/-}$ mice that received the $Dsg3^{210-345}$-reactive T cell clone 147#48 (n = 3, *A*) or 164#2 (n = 1; *B*) in combination with unprimed $Dsg3^{-/-}$ B cells. Serum samples were preincubated with each domain-swapped molecule, shown on the *bottom*, and then subjected to ELISA for anti-Dsg3 IgG The competition ratios (%) were calculated Data are presented as the mean ± SEM.

Table II. *Result of anti-Dsg3 IgG subclass ELISA (OD_{450})*[a]

Mouse	IgG1	IgG2a	IgG2b	IgG3
T cell clone 147#48[b] (n = 3)	0.328	0.057	0.265	0 175
	0.174	0.019	0.094	0 028
	0.256	0.039	0.119	0.034
T cell clone 164#2[b] (n = 1)	0.247	0.138	0.042	0.045
B6 spleen (n = 4)	0.031	0.030	0.026	0.031
	0.018	0.018	0.018	0.030
	0.015	0.016	0.019	0.031
	0.016	0.015	0.017	0.027

[a] Mean antibody titer + 3SD was 0.042, 0.040, 0.031, and 0.035 for IgG1, IgG2a, IgG2b, and IgG3, respectively. Values > mean + 3SD are underlined
[b] Plus unprimed $Dsg3^{-/-}$ B cells

no anti-Dsg3 IgG was detected in the recipient mice (data not shown). In addition, anti-Dsg1 IgG was not detected in all examined sera from the recipient mice (data not shown), indicating that intermolecular spreading of the B cell epitope from Dsg3 to Dsg1 did not occur. The mice receiving a T cell clone and unprimed B cells developed skin erosions on the neck and periorbital area at day 14 (Fig. 2*B*). The oral palate showed in vivo IgG deposition on keratinocyte cell surfaces and the loss of cell-cell adhesion in keratinocytes just above the basal cell layer, resulting in suprabasilar acantholysis, which is typical of pemphigus vulgaris (Fig. 2, *C* and *E*). In contrast, no such phenotype was observed in the mice receiving only unprimed B cells (Fig. 2, *A*, *B*, *D*, and *F*). These findings indicate that single Dsg3-reactive T cell clones established in vitro were able to induce pathogenic anti-Dsg3 IgG production and the PV phenotype in vivo, even in combination with unprimed $Dsg3^{-/-}$ B cells.

Induction of polyclonal anti-Dsg3 IgG production by single Dsg3-reactive T cell clones

We next characterized the epitopes of anti-Dsg3 IgG Abs by competition ELISA using various parts of the Dsg3 molecule. In these assays, we used four domain-swapped molecules containing portions of the mDsg3 and mDsg1 extracellular domains (Fig. 1*A*). Because none of the serum samples from PV model mice were reactive to mDsg1-His, these domain-swapped molecules were useful in mapping the regions of Dsg3 involved in binding, as previously described (12). When any two molecules consisting of the entire extracellular domain were mixed as competitors ($mDsg3^{1-162}/mDsg1^{163-512}$ and $mDsg1^{1-194}/mDsg3^{195-565}$; $mDsg3^{1-402}/mDsg1^{403-512}$ and $mDsg1^{1-402}/mDsg3^{403-565}$), their competition ratios were almost equal to that of $Dsg3^{1-565}$ (99.7 ± 6.3 and 104.3 ± 24.8%, respectively; n = 6). This finding indicated that these swapped molecules generated most, if not all, major epitopes in Dsg3.

Abs induced by the Dsg3-reactive T cell clone 147#48 reacted to all domain-swapped molecules in varying degrees (Fig. 3*A*; n = 3). They were most strongly reactive to $mDsg1^{1-194}/mDsg3^{195-565}$ and less so to $mDsg3^{1-162}/mDsg1^{163-512}$, $mDsg3^{1-402}/mDsg1^{403-512}$, and $mDsg1^{1-402}/mDsg3^{403-565}$. This finding indicates that the induced Abs were polyclonal and reactive to at least three different regions, namely amino acids 1–162, 195–402, and 403–565 of Dsg3. Another T cell clone, 164#2, induced IgG Abs with a similar epitope distribution, primarily in residues 195–402 but also in 1–162 and 403–565 (Fig. 3*B*; n = 1). These epitope distributions remained the same throughout the experimental time course (data not shown). These results indicated that single Dsg3-reactive T cell clones were able to stimulate unprimed B cells to produce polyclonal anti-Dsg3 IgG in vivo.

Induction of two or more IgG subclasses by Dsg3-reactive T cell clones

The IgG subclasses of anti-Dsg3 Abs induced by Dsg3-reactive T cell clones were examined via ELISA against mDsg3. The IgG subclasses are thought to be determined in vivo based on cytokine production in the transferred T cell clones. In this study, we transferred T cell clone 147#48, which expresses IL-2, IL-4, IL-6, IL-10, IFN-γ, and TGF-β, and clone 164#2, which expresses IL-2, IL-4, IL-10, IFN-γ, and TGF-β (15). All four recipient mice, which received a single Dsg3-reactive T cell clone and unprimed $Dsg3^{-/-}$ B cells, produced IgG1 together with IgG2a, IgG2b, and IgG3 (Table II). The predominance of the IgG1 subclass is consistent with the predominant IgG1 subclass found in PV model mice (17). This finding provides further evidence that single Dsg3-reactive T cell clones interact with polyclonal naive B cells that produce at least two different IgG subclasses.

Similar epitope distribution induced by single Dsg3-reactive T cell clones and primed B cells

We also examined the epitopes of the anti-Dsg3 IgG Abs produced in mice receiving primed B cells in combination with the T cell clones 147#48 and 164#2, which were used with unprimed B cells, as mentioned above (Table I). Primed B cells were obtained from

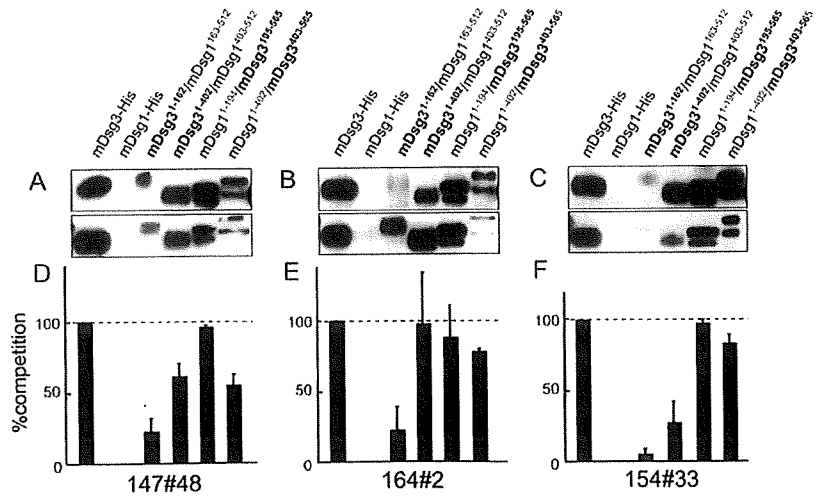

FIGURE 4. Characterization of anti-Dsg3 IgG produced in $Rag2^{-/-}$ mice that received the Dsg3-reactive T cell clones 147#48 (*A* and *D*), 164#2 (*B* and *E*), or 154#33 (*C* and *F*) in combination with primed $Dsg3^{-/-}$ B cells. Serum samples (n = 2 in each clone) were subjected to IP using recombinant proteins, shown on the *top*, in combination with IB using an anti-E-tag Ab and HRP-conjugated anti-mouse IgG Ab (*A–C*). Serum samples were preincubated with each domain-swapped molecule shown on the *top* and subjected to ELISA for anti-Dsg3 IgG (*D–F*). The competition ratios (%) were calculated.

$Dsg3^{-/-}$ mice that were immunized with mDsg3-His. Serum samples from the recipient mice were subjected to both IP-IB and competition ELISA ($n = 4$). IP-IB, in which immunoprecipitated recombinant molecules in serum samples were detected using an anti-E-tag Ab, revealed that the harvested anti-Dsg3 Abs were reactive to all domain-swapped Dsg1/Dsg3 molecules (Fig. 4, *A* and *B*) to varying degrees. The intensities of the bands correlated well with the competition ratios of the corresponding domain-swapped molecules in general (Fig. 4, *D* and *E*).

We also tested another T cell clone 154#33 ($n = 2$) that recognized a clearly different T cell epitope from clones 147#48 and 164#2 (Fig. 1*A* and Table I). The anti-Dsg3 IgG Abs induced by T cell clone 154#33 showed a similar epitope distribution as those induced by clones 147#48 and 164#2 (Fig. 4, *C* and *F*). They all tended to generate Abs to the middle and C-terminal region of Dsg3 extracellular domain, as found in the PV model mice in general, as opposed to the finding in PV patients (13, 14).

Thus, anti-Dsg3 IgG Abs induced from primed B cells by single T cell clones were polyclonal, and their epitope distribution in primed B cells was similar to that in unprimed B cells.

Discussion

We demonstrated that single Dsg3-reactive T cell clones were able to help polyclonal naive B cells produce anti-Dsg3 IgG Abs and to induce the PV phenotype in recipient mice via the adoptive transfer of Dsg3-reactive T cell clones with unprimed B cells isolated from nonimmunized $Dsg3^{-/-}$ mice. Anti-Dsg3 IgG production in this system required T cell help in vivo, because the transfer of unprimed B cells without Dsg3-reactive T cell clones resulted in no production of anti-Dsg3 IgG in vivo. Furthermore, the T cell help was dependent upon Ag specificity based on the results using OVA-reactive T cells in the previous study (15). OVA-reactive T cell lines, which were established by repetitive stimulation of lymphocytes from OT-II-$Rag2^{-/-}$ mice with OVA peptide in vitro, were not able to induce anti-Dsg3 IgG production when transferred with primed B cells from immunized $Dsg3^{-/-}$ mice. Furthermore, a BrdU study demonstrated that OVA-reactive T cell lines could not continue to proliferate in vivo at a late time point (Day 37) after transfer whereas Dsg3-reactive T cell lines could, probably recognizing endogenous Dsg3 (15). At an early time point (day 10) after transfer, OVA-reactive T cells still showed proliferative activity probably due to homeostatic proliferation, and anti-Dsg3 IgG Abs were not detected in those mice whereas anti-Dsg3 IgG Abs were detected on day 14 after transfer with Dsg3-reactive T cell lines and a primed $Dsg3^{-/-}$ B cell. Because the OVA-reactive T cell line failed to exert Ag-specific immune response in vivo, these cells could not induce the PV phenotype even though they were able to express IL-4, which is essential for the PV phenotype induction. Because the frequency of Dsg3-specific B cells should be extremely low in a naive B cell population, Dsg3-specific B cells are thought to receive essential signals from Dsg3-reactive T cells by T cell-B cell interaction, which is selectively achieved via TCR-MHC-peptide complex association. Together with the results from the transfer of OVA-reactive T cells, a Dsg3-specific T cell-B cell interaction is required for anti-Dsg3 IgG production even when using primed B cells for adoptive transfer. However, future experiments with T cell clones reactive to some endogenous Ag are necessary to confirm this Ag-specific stimulation of Ab production by the transferred T cell clones.

To further analyze T cell-B cell interaction, we evaluated the epitopes of the anti-Dsg3 IgG Abs induced by single pathogenic T cell clones and demonstrated that anti-Dsg3 IgG Abs, which were produced in vivo without any additional immunization in the recipient mice, recognized at least three different regions of the Dsg3 extracellular domain, based on the results from IP-IB and competition ELISA using domain-swapped Dsg1/Dsg3 molecules. These results indicate that a single T cell clone, possibly originating from a single T cell, was sufficient to stimulate polyclonal anti-Dsg3 Ab production in naive B cells. This polyclonal IgG production was achieved by at least two sequential biological steps: 1) T cells successfully interacting with polyclonal B cells; and 2) T cells providing essential signals to drive polyclonal Ab production. Several previous experiments demonstrated that B cells play an important role as APCs (18–20) and can control polyclonal T cell activation (21, 22). These reports potentially show that a single B cell can take up an Ag and its complex via membrane-bound Abs and present multiple T cell epitopes in MHC molecules, interact with polyclonal T cells, and provide essential signals for T cell activation. Although a single T cell should theoretically be capable of interacting with polyclonal B cells and exerting helper activity for polyclonal IgG production in reverse, most previous studies have analyzed this in the B cell to T cell direction. Among those, a classical study demonstrated that a single T cell clone was able to promote Ig-isotype class switch recombination and production of Ag-specific IgM, IgG, and IgA in vivo (7). It was demonstrated by only in vitro studies that Ag-specific T cell lines induced polyclonal IgG production with various Ag specificities (23). However, T cells and B cells were obliged to interact with each other in a small well in vitro regardless of their homing capacity, physiological Ag presentation, their interactions with the surrounding environment, and so on. Our study overcame the weaknesses of such in vitro studies and successfully analyzed T-B interaction in the T cell to B cell direction. Based on the unique system described here, this study is the first to demonstrate that a single T cell clone can both interact with polyclonal B cells and drive production of polyclonal IgG recognizing different epitopes in vivo.

What does polyclonal B cell help by a single T cell mean in the immune system? Although T cell activation was achieved by a variety of APCs such as B cells, dendritic cells, and macrophages, B cell help was conducted only by cognate T cells. Thus, this limitation in B cell help by T cells, if any, may be a rate-determining step in Ab production. Whereas T cells and B cells have been demonstrated to show dynamic motility to search for each other in secondary lymphoid organs (24), polyclonal B cell help by a single T cell results in a synergistic effect, together with polyclonal T cell activation by a B cell in Ab production, in which both B cell help and T cell activation are essentially required. These bidirectional results, polyclonal B cell help and T cell activation, demonstrated the rapid diversification of the humoral immune response.

Patients with pemphigus have polyclonal IgG autoantibodies against multiple epitopes on Dsg1 and/or Dsg3 (14). In fogo selvagem, an endemic form of pemphigus foliaceus, intramolecular epitope shifts on Dsg1 were observed depending on the active or remission stage (25). In several cases of pemphigus, it has been described that intermolecular epitope shift between Dsg1 and Dsg3 occurs along with the transition of pemphigus phenotype as rare events (26, 27). Although the exact immunological mechanisms of the epitope spreading in human are largely unknown, our study showed that the specificity of intramolecular B cell epitopes is independent of T cell epitopes on Dsg3. In addition, the mice tested in the short period did not show the intermolecular epitope spreading from Dsg3 to Dsg1, indicating Dsg3-specific T cells are not sufficient to produce Abs against Dsg1 in the present system. It will be intriguing to test whether transfer of Dsg3-specific T cell clones together with B cells from $Dsg1^{-/-}$ mice induces IgG Abs against Dsg1. Although the unavailability of $Dsg1^{-/-}$ mice makes this approach difficult, our approach will provide a valuable tool

for investigating the mechanisms on intramolecular and intermolecular epitope spreading in the future.

How tolerance to Dsg3 is broken in patients with pemphigus still remains unclear. It was reported that Dsg3-reactive T cells could be isolated from healthy individuals with PV-associated HLA alleles (11). In addition, low level of anti-Dsg3 Ab was detected in healthy relatives of PV patients (28, 29). In a BCR transgenic model, a Dsg3-specific B cell escaped from tolerance mechanism (30). Those findings indicate that a central tolerance mechanism does not necessarily eliminate or inactivate self-reactive T cells or B cells and that its break is not sufficient to induce the onset of pemphigus. Further studies with TCR transgenic mice derived from these Dsg3-specific T cell clones and adoptive transfer of the transgenic T cells with B cells from various mice will open a new path to unveil the mysterious issues on tolerance.

Although Dsg3-reactive T cells are thought to be pathogenic in PV, we demonstrated that Dsg3-reactive T cells can be classified into pathogenic and nonpathogenic groups in a previous study (15). Furthermore, the present study demonstrates that single Dsg3-reactive T cell clones can induce polyclonal pathogenic anti-Dsg3 IgG production in vivo, suggesting that pathogenic T cells consist of only a minor and restricted population among general immune cells. The identification and characterization of this pathogenic subpopulation may assist in the development of efficient therapeutic strategies against the key "commander" T cell population that may be the source of the harmful autoimmune response.

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Disclosures

The authors have no financial conflict of interest

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A randomized double-blind trial of intravenous immunoglobulin for pemphigus

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Background: Pemphigus is a rare life-threatening intractable autoimmune blistering disease caused by IgG autoantibodies to desmogleins. It has been difficult to conduct a double-blind clinical study for pemphigus partly because, in a placebo group, appropriate treatment often must be provided when the disease flares.

Objective: A multicenter, randomized, placebo-controlled, double-blind trial was conducted to investigate the therapeutic effect of a single cycle of high-dose intravenous immunoglobulin (400, 200, or 0 mg/kg/d) administered over 5 consecutive days in patients relatively resistant to systemic steroids.

Methods: We evaluated efficacy with time to escape from the protocol as a novel primary end point, and pemphigus activity score, antidesmoglein enzyme-linked immunosorbent assay scores, and safety as secondary end points.

Results: We enrolled 61 patients with pemphigus vulgaris or pemphigus foliaceus who did not respond to prednisolone (≧20 mg/d). Time to escape from the protocol was significantly prolonged in the 400-mg group compared with the placebo group ($P < .001$), and a dose-response relationship among the 3 treatment groups was observed ($P < .001$). Disease activity and enzyme-linked immunosorbent assay scores were significantly lower in the 400-mg group than in the other groups ($P < .05$ on day 43, $P < .01$ on day 85). There was no significant difference in the safety end point among the 3 treatment groups.

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Other investigators in the Pemphigus Study Group are listed in the Appendix.

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Limitation: Prednisolone at 20 mg/d or more may not be high enough to define steroid resistance.

Conclusion: Intravenous immunoglobulin (400 mg/kg/d for 5 d) in a single cycle is an effective and safe treatment for patients with pemphigus who are relatively resistant to systemic steroids. Time to escape from the protocol is a useful indicator for evaluation in randomized, placebo-controlled, double-blind studies of rare and serious diseases. (J Am Acad Dermatol 2009;60:595-603.)

Pemphigus is a life-threatening, rare intractable autoimmune blistering disease caused by IgG autoantibodies to desmoglein (Dsg) (epidermal adhesion factor). It is characterized by the development of blisters and erosions of the skin and mucosa.[1] Currently, oral steroids are the drugs of first choice for pemphigus, and may be used in combination with immunosuppressants or plasma exchange. However, many patients with pemphigus experience cycles of remission and recurrence, and accordingly become unresponsive to conventional therapy. On the other hand, patients with complications such as diabetes mellitus, gastrointestinal disease, osteoporosis, infection, or immunodeficiency are relatively contraindicated for use of high-dose (HD) steroids. For such patients, an alternative effective treatment strategy is required.

Although several reports suggesting the effectiveness of HD intravenous immunoglobulin (IVIG) in the treatment of pemphigus have been published since its introduction as monotherapy in 1989, most are case reports with a low evidence level or involved clinical research with a limited number of patients using multiple treatment cycles.[2] No well-controlled, double-blind clinical study to demonstrate the efficacy of HD-IVIG has been conducted.[3-13] This is because: (1) pemphigus is a rare intractable disease; (2) appropriate treatment must be provided in a timely manner if symptoms are aggravated or unchanged for a certain period of time; (3) inclusion of a placebo group compromises compliance with the study protocol; and (4) it is not ethical to treat patients with pemphigus using placebo because mortality is high.

We developed a novel evaluation end point to solve these problems and verified the usefulness of HD-IVIG in a single treatment cycle for this rare intractable disease.

Abbreviations used:

ADRs:	adverse drug reactions
Dsg:	desmoglein
HD:	high dose
IVIG:	intravenous immunoglobulin
PAS:	pemphigus activity score
PF:	pemphigus foliaceus
PV:	pemphigus vulgaris
TEP:	time to escape from the protocol

METHODS

Patients

This study was conducted in 27 medical institutions in Japan with affiliated dermatologists specialized in autoimmune blistering disease. Patients were given the diagnosis of pemphigus vulgaris (PV) or pemphigus foliaceus (PF) as confirmed based on our national diagnostic criteria as follows: pemphigus was diagnosed when at least one item from every 3 findings, or two items from clinical findings and one item from immunologic findings were satisfied.

1. Clinical findings
   - Multiple, easily rupturing, flaccid blisters of the skin
   - Subsequent progressive, refractory erosions or crust after blisters
   - Noninfectious blisters or erosions of visible mucosa including oral mucosa
   - Nikolsky sign
2. Histologic findings
   - Intraepidermal blisters caused by loss of adhesion between epidermal cells (acantholysis)
3. Immunologic findings
   - IgG (or complement) deposition in the intercellular spaces of the lesional or normal-appearing skin and mucosa as detected by direct fluorescent antibody assay
   - Antiepidermal intercellular IgG autoantibody (anti-Dsg IgG autoantibody) identified by indirect fluorescent antibody assay or enzyme-linked immunosorbent assay

The study patients had to meet all the following inclusion criteria and none of the exclusion criteria.

1. Inclusion criteria: patients aged 20 years or older who provided written informed consent to participate in the study and met all of the following criteria.

Table I. Criteria for pemphigus activity score

Variable score	Skin lesion area*	No. of new blisters/d	Oral mucosal lesions†
3	≥15%	≥5	≥30%
2	≥5% and <15%	1 to 4	≥5% and <30%
1	<5%	Occasionally‡	<5%
0	None	None	None

*Percentage of entire surface area.
†Score is doubled for patients who have only oral mucosal lesions at time of study enrollment.
‡Blisters sometimes newly develop within 1 week but not every day.

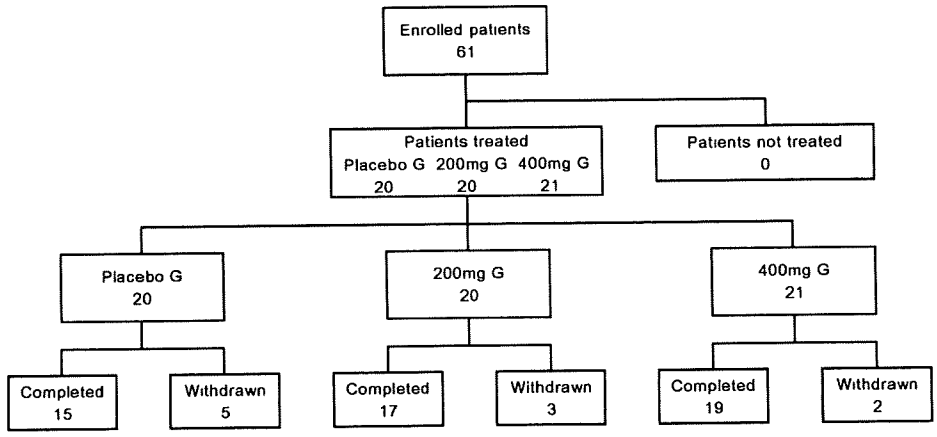

Fig 1. Disposition of patients. *G*, Group.

- Treatment with any steroid at greater than or equal to 20 mg/d (prednisolone equivalent)
- Symptoms (total pemphigus activity score [PAS] [Table I]) did not respond to steroid therapy

2. Exclusion criteria: patients who met any of the following criteria were excluded from the study because efficacy evaluation of the test drug might be affected and to assure the safety of patients.
   - Patients treated with plasma exchange therapy, steroid pulse therapy, or HD-IVIG within 30, 14, or 42 days, respectively, before informed consent and the start of study treatment
   - Patients with a history of shock or hypersensitivity to the test drug
   - Patients with IgA deficiency, hepatic disorder, renal disorder, or hemolytic or blood loss anemia
   - Patients with any previous or existing cerebrovascular or cardiovascular disorder

Study design

This was a multicenter, randomized, placebo-controlled, double-blind, parallel-group study. The study protocol and written informed consent form approved by the institutional review board at each study institution were used in the study. Observation of the first patient was started on November 4, 2004, and that of the last patient was completed on September 25, 2006.

Treatment groups

The IVIG group received IV drip infusion at 200 or 400 mg/kg/d administered in divided dose over 5 consecutive days. The placebo group received IV drip infusion of physiologic saline for 5 consecutive days.

Investigational drugs manufactured by Nihon Pharmaceutical Co Ltd (Higashikanda, Tokyo, Japan) were used in the study.

Methods of allocation

Patients were randomized by a central enrollment system to the treatment groups according to a dynamic allocation scheme to ensure that there were no between-group differences in the dose of prior steroid, total PAS, or disease type.

Blinding

Because the investigational drugs were distinguishable in terms of appearance and viscosity after

Table II. Demographic and other baseline characteristics

Characteristic	Category	Dose: Placebo n = 20	Dose: 200 mg n = 20	Dose: 400 mg n = 21	Between-group comparison
Sex	Male	9	10	8	NS* (P = .766)
	Female	11	10	13	
Age, y	Mean ± SD	53.1 ± 10.9	57.0 ± 14.6	50.1 ± 11.7	NS† (P = .225)
Body weight, kg	Mean ± SD	57.8 ± 11.6	58.0 ± 10.4	57.7 ± 9.1	NS* (P = .686)
Disease type	PV	13	14	13	NS* (P = .942)
	PF	7	6	8	
Disease duration, mo	Mean ± SD	16.1 ± 13.6	28.6 ± 32.3	28.5 ± 46.9	NS† (P = .414)
Baseline PAS	Mean ± SD	3.3 ± 1.4	3.6 ± 1.8	3.7 ± 1.1	NS† (P = .660)
Steroid dose, mg	Mean ± SD	27.6 ± 9.7	23.9 ± 11.1	27.4 ± 11.1	NS† (P = .461)
Immunosuppressants	No. of patients (%)	2 (10.0)	7 (35.0)	5 (23.8)	NS* (P = .179)

NS, Not significant difference; *PAS*, pemphigus activity score; *PF*, pemphigus foliaceus; *PV*, pemphigus vulgaris.
Two-sided test for both analyses.
*Fisher exact test.
†One-way analysis of variance.

reconstitution, independent staff at each study institution separately prepared and administered the dosing solution, and evaluated efficacy and safety in each patient to maintain blinding. The bottles of the investigational drugs were covered with a masking cover and provided to the independent staff member in charge of administration. Each independent staff member involved signed a blinding confirmation form at the end of the study to assure that blinding was maintained.

End points

Time to escape from the protocol (TEP) was used as the primary efficacy end point. TEP was defined as the length of the period until a patient stayed on the protocol without any additional treatment. When symptoms were unchanged for 2 weeks or aggravated, the treatment given was considered to be ineffective and additional treatment was required such as increase in steroid dose, change in steroid type, use of additional immunosuppressive agents, or plasma exchange; these patients were considered escaped from the protocol. This methods allow doctors in charge to have flexibility to rescue patients with other treatment when needed.

The secondary end points used in the study included: (1) PAS over time (scores [0-3 point] for skin lesion area, number of new blisters/d, and oral mucosal lesions, and their total scores [Table I]); and (2) the titers of pemphigus autoantibodies over time (anti-Dsg1 autoantibody titer and anti-Dsg3 autoantibody titer). Titers of pemphigus autoantibodies were determined by enzyme-linked immunosorbent assay.[14,15] As a safety end point, the occurrence of adverse events by 85 days after the start of the study treatment (day 85) was investigated. Adverse events were recorded up to day 43 if patients escaped from the protocol by day 43 or up to TEP if patients escaped from the protocol after day 44.

Statistical analysis

The cumulative rate of TEP, which was estimated by evaluation of the dose-response relationship of TEP and by analysis using the Kaplan-Meier method, was compared among the treatment groups by log rank test. Scores for skin lesion area, number of new blisters/d, and oral mucosal lesions, and total score, the secondary end point, up to day 85 were compared with baseline data by the paired t test for each treatment group. The data after TEP were imputed from the data at the TEP (last observation carried forward). Adverse events occurring up to day 85 for which the causal relationship with HD-IVIG or placebo was judged to be other than "not related" were handled as adverse drug reactions (ADRs). A two-sided significance level of .05 was used for analyses.

RESULTS

Disposition of patients

The disposition of patients enrolled in the study is shown in Fig 1. A total of 61 patients were treated with the investigational drug (placebo, 20; 200 mg, 20; and 400 mg, 21). All the enrolled patients including 10 patients (placebo, 5; 200 mg, 3; and 400 mg, 2) who were withdrawn from the study according to the requirements in the protocol were included in the analyses. The main reasons for study withdrawal were the evaluator's decision to withdraw the patient and the occurrence of adverse events. The demographic and other baseline

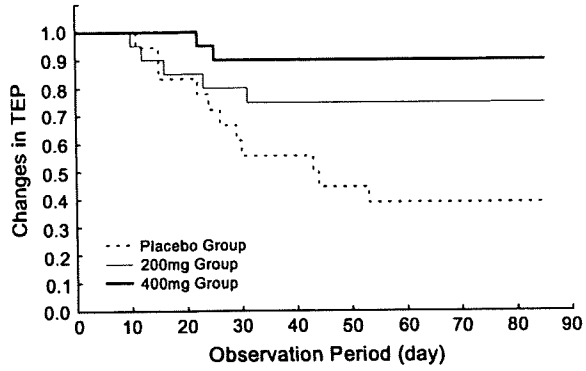

Fig 2. Changes in time to escape from protocol (*TEP*). TEP was significantly prolonged in 400-mg group compared with placebo group with dose-dependent fashion. Cumulative TEP on day 85 was 10.0% in 400-mg group, 25.0% in 200-mg group, and 61.0% in placebo group (log rank test). Between-group comparison demonstrated significant prolongation of TEP in 400-mg group compared with placebo group ($P < .001$, log rank test). In contrast, difference between 200-mg and placebo groups was not significant ($P = .052$). In addition, dose-response relationship was observed in TEP ($P < .001$). Data are stated using TEP ratio.

characteristics are presented in Table II. There were no significant between-group differences in the distribution of baseline characteristics. The average disease durations of 200- and 400-mg groups are longer than in the placebo group, but this is because the former group happened to contain patients with extremely long duration (116 months in 200 mg; 142 and 169 months in 400 mg) and the difference was not statistically significant.

Efficacy (primary end point): TEP

TEP was evaluated as the primary end point (Fig 2). In the 400-mg group, 19 of 21 patients stayed on the protocol during the observation period. Two patients escaped from the protocol with TEPs of 22 and 25 days. In the 200-mg group, 15 of 20 patients stayed on the protocol and the shortest TEP was as early as 10 days among the 5 escaped patients. In the placebo group, only 9 patients stayed on the protocol, and the shortest TEP was as early as 11 days. TEP was within 30 days for 8 patients.

TEP in the active treatment groups was compared with that in the placebo group (log rank test). The TEP in the 400-mg group was significantly longer than that in the placebo group ($P < .001$), whereas the difference between the 200-mg and placebo groups was not significant ($P = .052$). Log rank test of TEP for the 61 patients indicated a dose-response relationship for this parameter ($P < .001$).

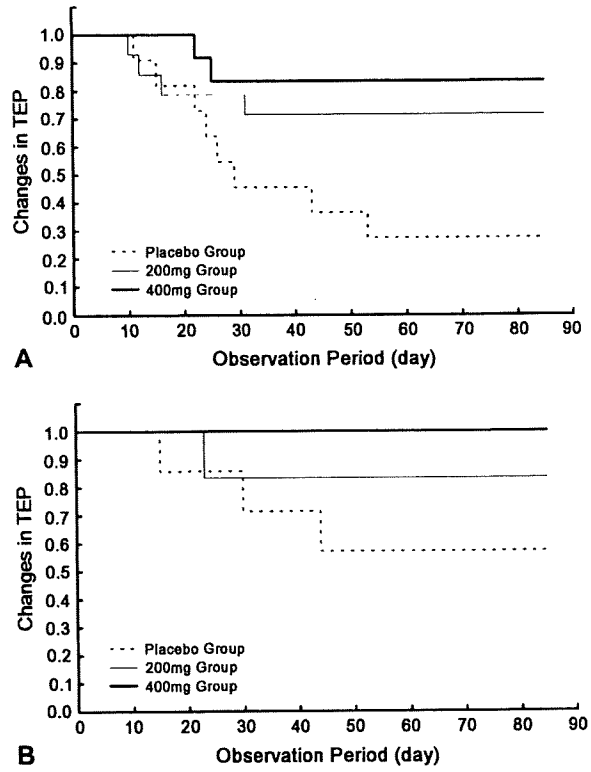

Fig 3. Cumulative time to escape from protocol (*TEP*) shown by pemphigus subtype. Cumulative TEP estimated by Kaplan-Meier method was divided in disease subtype of pemphigus vulgaris (PV) (**A**, n = 13 in 400-mg group, n = 14 in 200-mg group, n = 13 in placebo group) and pemphigus foliaceus (PF) (**B**, n = 8 in 400-mg group, n = 6 in 200-mg group, n = 7 in placebo group). Cumulative TEP in patients with PV on day 85 was 15.0% in 400-mg group, 29.0% in 200-mg group, and 73.0% in placebo group, whereas that of patients with PF was 0.0% in 400-mg group, 17.0% in 200-mg group, and 43.0% in placebo group. Between-group comparison demonstrated significant prolongation of TEP in 400-mg group compared with placebo group (PV, $P = .007$; PF, $P = .044$; log rank test). In contrast, difference between 200-mg and placebo groups was not significant (PV, $P = .055$; PF, $P = .416$). In addition, dose-response relationship was observed in TEP (PV, $P = .007$; PF, $P = .043$).

Analyses stratified by baseline characteristics (disease type and PAS) also demonstrated dose-response relationships and significant differences between the 400-mg and placebo groups, as in the overall analyses (Figs 3 and 4).

Efficacy (secondary end point)

Pemphigus activity score. Efficacy was also evaluated based on the changes in clinical symptoms, ie, changes in PAS determined based on skin lesion area, number of new blisters/d, and oral mucosal lesions. In the 400-mg group, total PAS

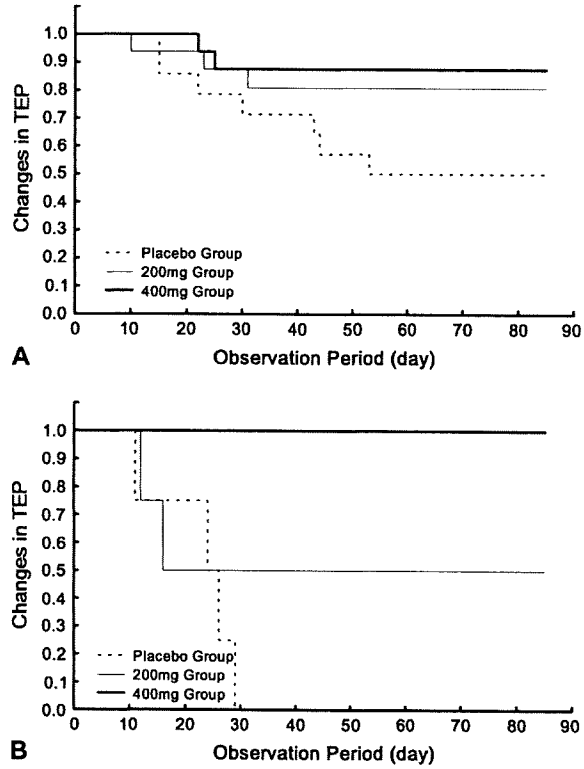

Fig 4. Cumulative time to escape from protocol (*TEP*) shown in different pemphigus activity score (PAS). Data were divided by PAS into two groups: total PAS of 0 to 4 (**A**, n = 17 in 400-mg group, n = 16 in 200-mg group, n = 16 in placebo group) and total PAS of 5 to 9 (**B**, n = 4 in 400-mg group, n = 4 in 200-mg group, n = 4 in placebo group). Cumulative TEP in patients with total PAS of 0 to 4 on day 85 was 12.0% in 400-mg group, 19.0% in 200-mg group, and 50.0% in placebo group, whereas those of patients with total PAS of 5 to 9 was 0.0% in 400-mg group, 50.0% in 200-mg group, and 100.0% in placebo group. Between-group comparison demonstrated significant prolongation of TEP in 400-mg group compared with placebo group (total score 0-4, $P = .028$; total score 5-9, $P = .006$). In contrast, difference between 200-mg and placebo groups was not significant (total score 0-4, $P = .109$; total score 5-9, $P = .345$). In addition, dose-response relationship was observed in TEP (total score 0-4, $P = .024$; total score 5-9, $P = .012$).

was significantly decreased from the baseline score at all points of observation (day 8, $P = .05$; after day 15, $P < .01$). It was decreased from 3.7 on day 1 to 2.0 on day 85 (by 46.8%) (Fig 5). In the 200-mg group, total PAS was significantly decreased from the baseline score at all points of observation after day 15 (day 15-43, $P < .05$; day 57-85, $P < .01$). It was decreased from 3.7 on day 1 to 2.3 on day 85 (by 36.6%). On the other hand, in the placebo group, no significant decrease from baseline score was observed at any of the points of observation. Each PAS

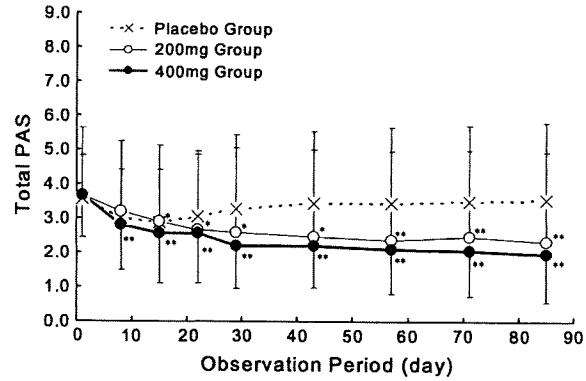

Fig 5. Changes of pemphigus activity score (*PAS*) over time. Total PAS was significantly lower in 400- and 200-mg groups than in placebo group. Significant difference from day 1 at hazard ratio of *0.05 and **0.01.

(skin lesion area, number of new blisters/d, and oral mucosal lesions) also exhibited a significant change from baseline in the 400-mg group ($P < .01$) but not in the placebo group (data not shown).

Titers of anti-Dsg IgG autoantibodies

It has been reported that levels of IgG autoantibodies to Dsg1 and Dsg3 in patients with pemphigus correlate with disease activity.[14,15] Accordingly, efficacy was also evaluated based on the changes in anti-Dsg1 IgG autoantibody titer for patients with PF and PV or in anti-Dsg3 IgG autoantibody titer for patients with PV (Fig 6). In the 400-mg group, anti-Dsg1 and −Dsg3 IgG antibody titers were significantly decreased from baseline on days 43 and 85 (day 43 and 85, $P < .01$). In the 200-mg group, anti-Dsg1 and −Dsg3 IgG antibody titers also exhibited significant decreases on day 85 but not day 43 (day 43, $P < .05$; day 85, $P < .01$). On the other hand, in the placebo group, no significant decrease from baseline was observed in either anti-Dsg1 or −Dsg3 IgG antibody titer.

Safety

The incidence of ADRs was 28.6% (n = 6/21) in the 400-mg group, 35.0% (n = 7/20) in the 200-mg group, and 25.0% (n = 5/20) in the placebo group. No significant difference was observed between the placebo and 200- or 400-mg groups. ADRs reported in the study included: headache in two patients, aggravated chronic hepatitis C, decreased lymphocytes, palpitations, abdominal discomfort, constipation, nausea, pain at the injection site, increased creatinine, increased blood pressure, and decreased platelet count in one patient each in the 400-mg group; and increased alanine aminotransferase in 3 patients; increased γ-glutamyltranspeptitase, hepatic dysfunction, and increased bilirubin in two patients each; and common cold, muscle pain, increased

aspartate aminotransferase, increased blood pressure, decreased lymphocytes, increased neutrophils, decreased white blood cell count, bleeding tendency, anorexia, hypoalbuminemia, hepatic encephalopathy, gastrointestinal bleeding, malaise, fever, increased ammonium, increased C-reactive protein, decreased hematocrit, decreased hemoglobin, decreased platelet count, decreased red blood cell count, and decreased urine volume in one patient each in the 200-mg group. All these ADRs were consistent with the information displayed on the Food and Drug Administration Web site (http://www.fda.gov/cber/gdlns/igivimmuno.htm).

One patient in the 200-mg group died of hepatic failure as a result of aggravation of hepatitis C, which was an underlying complication reported before the start of the study.

This event was judged as probably related to the investigational drug in the evaluator's opinion.

DISCUSSION

Most clinical research involving a rare disease is based on case reports or data from limited samples obtained in open-label studies. In particular, in life-threatening, serious, and intractable diseases, such as pemphigus, appropriate treatment must be provided in a timely fashion if symptoms are aggravated or unchanged for days. This makes performance of a placebo-controlled, double-blind comparison study infeasible. On the other hand, the efficacy of new drugs for malignant tumors or for patients requiring pain relief is evaluated based on the time to recurrence of tumor or the number of patients requiring rescue analgesia.[16-21] Based on these considerations, we developed a novel efficacy indicator (ie, TEP) with reference to the end points used for efficacy evaluation of drugs for malignant tumors or for patients requiring pain relief, to conduct a placebo-controlled, double-blind comparison study in patients with pemphigus who were relatively resistant to systemic steroids. This new efficacy end point provides flexibility for physicians to rescue patients when required and proved to be useful to evaluate the efficacy of a single cycle of HD-IVIG in a double-blind comparison design. However, some concerns remain regarding the rigidity: a period of 3 to 7 days before the start of study treatment was required to confirm the unresponsiveness of patients to steroids, and switching to other treatments was prohibited during the first 5-day treatment period.

The mode of action of HD-IVIG is complex. It is found to exert its effect through modulation of expression and function of Fc receptors, interference with complement activation and the cytokine network, provision of anti-idiotypic antibodies, modulation of dendritic cell, T- and B-cell activation, differentiation, and their effector functions.[22,23] Thus, HD-IVIG has multiple modes of action and is thought to act synergistically. HD-IVIG exerts immunomodulatory effects in autoimmune and inflammatory disorders without suppressing the immune system, which provides a distinctive advantage over conventional treatment.

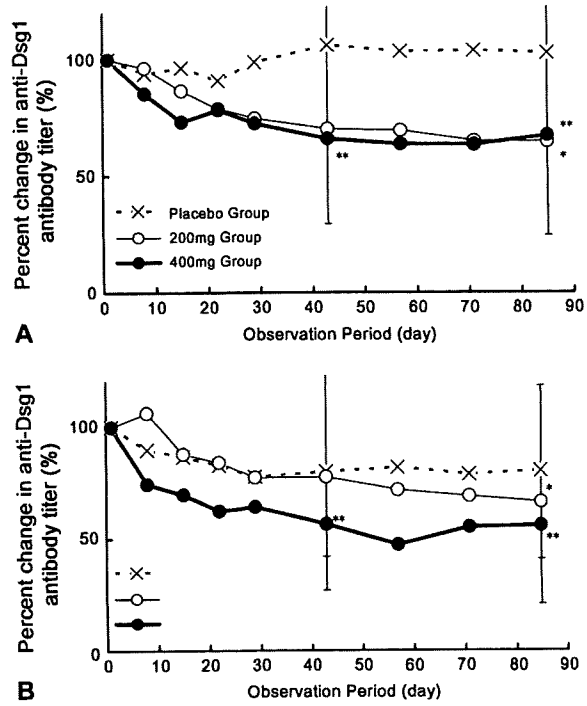

Fig 6. Changes of anti-desmoglein (*Dsg*) IgG titers. Anti-Dsg IgG titers were significantly lower in 400-mg intravenous immunoglobulin group than in placebo group over time. Changes of titers in anti-Dsg1 IgG autoantibodies (**A**) in patients with pemphigus vulgaris (PV) and pemphigus foliaceus and in anti-Dsg3 IgG autoantibodies (**B**) in patients with PV were shown (mean ± SD). Significant difference from day 1 at hazard ratio of *0.05 and **0.01.

Most of the previous studies suggesting efficacy of HD-IVIG for treatment of pemphigus involved multiple treatment cycles. However, our study demonstrated that a single cycle with HD-IVIG for 5 days has a therapeutic benefit to suppress the disease activity of pemphigus. Like rituximab, for which efficacy was recently reported in a single cycle,[24] IVIG is expensive and should be considered for patients who show difficulty with or resistance to conventional treatments.

In conclusion, our study suggests that TEP is a useful indicator for evaluation for rare intractable diseases such as pemphigus, and that a single cycle of HD-IVIG appears to be an effective treatment for

patients with pemphigus who are relatively resistant to systemic steroids.

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APPENDIX

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