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## Novel adenosine triphosphate (ATP)-binding cassette, subfamily A, member 12 (*ABCA12*) mutations associated with congenital ichthyosiform erythroderma

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MADAM, Autosomal recessive congenital ichthyosis (ARCI) is a keratinization disorder, characterized by general desquamation. ARCI is a heterogeneous entity, including harlequin ichthyosis (HI, MIM 242500), lamellar ichthyosis type 2 (LI2, MIM 601277) and congenital ichthyosiform erythroderma (CIE, MIM 242100). The reported mutations in CIE include adenosine triphosphate (ATP)-binding cassette, subfamily A, member 12 (*ABCA12*),[1] transglutaminase 1 (*TGM1*),[2] lipoxygenase-3, 12(R)-lipoxygenase,[3] *NIPAL4*[4] and *CYP4F22*.[5] Mutations in *ABCA12* also result in LI2 and HI.[6,7] We report *ABCA12* mutations in four unrelated Japanese patients with CIE and identified five unreported and two recurrent mutations.

Patient 1 is a 3-year-old girl with generalized scales on erythroderma, ectropion, eclabium, severely deformed ears and alopecia (Fig. 1a–c). Her elder sister displayed similar symptoms and died after dehydration and infection. Patient 2 is a 9-year-old girl with generalized scales on an erythrodermic skin, mild ectropion, alopecia of the forehead and mild auricular malformation. Her younger sister died after severe skin symptoms and subsequent complications. Patient 3 is a 4-month-old boy, born as a collodion baby, with systemic whitish scales and generalized erythrodermic skin. There is no family history. Patient 4 is a 3-month-old boy, born as a collodion baby, with generalized whitish scales on a mild erythrodermic skin (Fig. 1d,e). Ectropion, eclabium and auricular malformation were not seen. There is no family history. Pathological findings of all patients revealed hyperkeratosis, mild acanthosis and perivascular lymphocytic infiltration.

We initially examined for *ABCA12* mutation, because *ABCA12* mutations have been found frequently in Japanese patients with CIE. For analysis of the *ABCA12* gene, polymerase chain reaction (PCR) fragments were amplified with 53 primer pairs, as previously reported.[6] We identified five unreported and two recurrent mutations (Table 1). Patient 1 had compound heterozygosity of missense/small deletion mutations [(p.Thr1575Pro)+(c.6031delG)]. Patients 2 and 3 had compound heterozygosity of missense/splice-site mutations [(p.Arg986Trp)+(c.5940–1G>C), (p.Asn1380Ser)+(c.5128+3A>G), respectively]. Patient 4 had compound heterozygosity

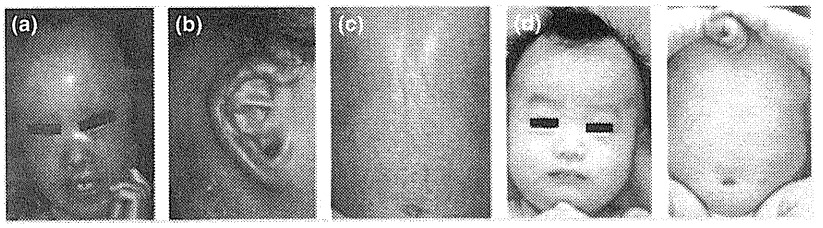


Fig 1. (a–c) Clinical features of patient 1. The whole body was covered with whitish scales on the erythrodermic skin. Ectropion, eclabium and alopecia of the forehead were seen. (d,e) Clinical features of patient 4. Whitish scales and generalized erythrodermic skin were seen.

Table 1 Summary of mutation analysis of *ABCA12* in the present study

| Patient | Age, sex | Mutation | Maternal | Paternal |
|---|---|---|---|---|
| 1 | 3 years, girl | Compound heterozygous | p.Thr1575Pro (c.4723A>C) | c.6031delG |
| 2 | 9 years, girl | Compound heterozygous | p.Arg986Trp (c.2956C>T) | c.5940–1G>C |
| 3 | 4 months, boy | Compound heterozygous | p.Asn1380Ser (c.4139A>G) | c.5128+3A>G |
| 4 | 3 months, boy | Compound heterozygous | p.Thr1575Pro (c.4723A>C) | p.Gly1651Ser (c.4951G>A) |

of missense mutations [(p.Thr1575Pro)+(p.Gly1651Ser)]. Each of the parents was a heterozygous carrier. Five mutations (p.Thr1575Pro, c.6031delG, p.Arg986Trp, c.5940−1G>C and c.5128+3A>G) have not been reported previously. Two recurrent mutations (p.Asn1380Ser and p.Gly1651Ser) have been reported previously in LI2.[6] These mutations were not found in 200 normal, unrelated Japanese alleles.

In cDNA from the skin of patient 2, reverse transcriptase-PCR (RT-PCR) across the c.5940−1G>C mutation site showed a single band of 526 bp. Subcloning and direct sequencing

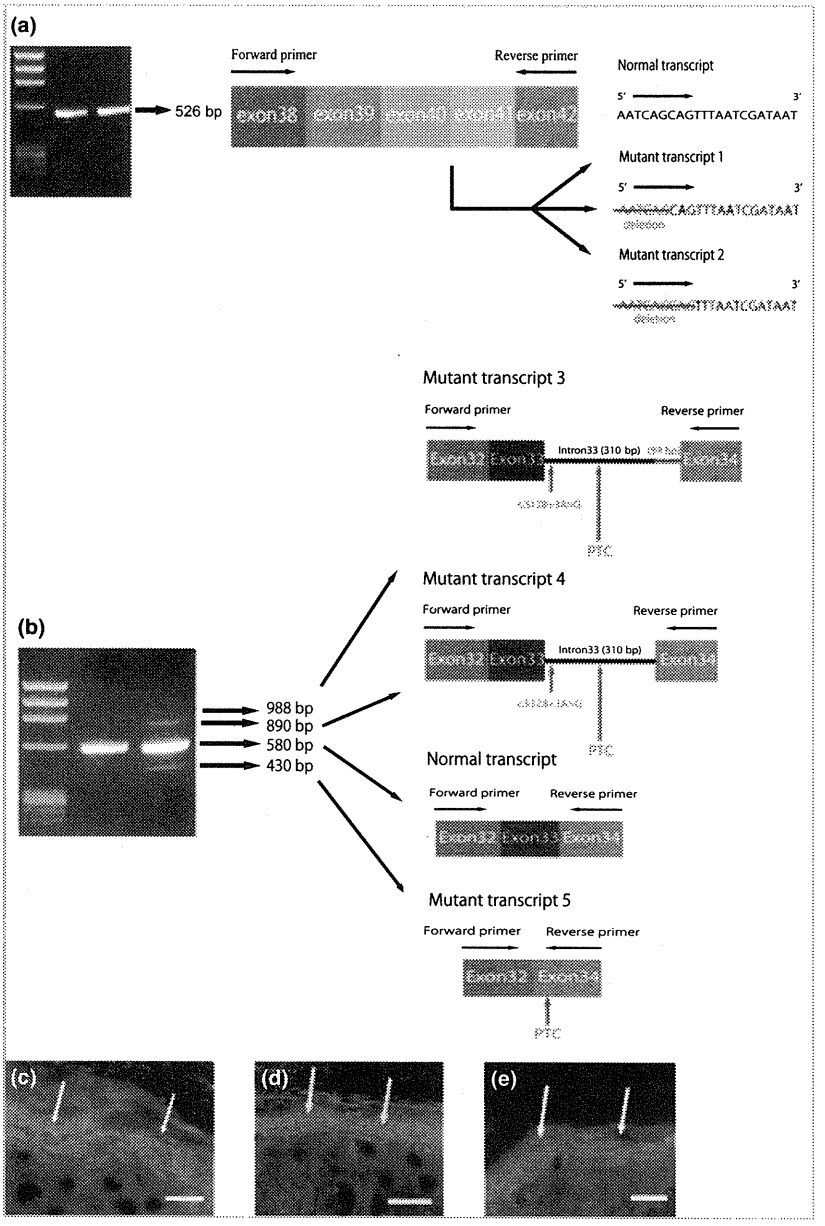


Fig 2. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mRNA fragments around the splice-site mutations and immunofluorescent analysis. (a) In patient 2, RT-PCR, subcloning and direct sequencing through the exon 40–41 boundary revealed two mutant transcripts as well as a normal transcript. Mutant transcript 1 had lost 6-bp nucleotides from exon 41, which resulted in a 2-amino acid deletion (Ile1981_Ser1982del). Mutant transcript 2 had lost 9-bp nucleotides from exon 41, which resulted in a 3-amino acid deletion (Ile1981_Ser1983del). Both mutant transcripts were within-frame deletions. (b) In patient 3, three aberrant mutant transcripts, all of which led to a premature termination codon, were identified by RT-PCR, subcloning and direct sequencing through the exon 33–34 boundary. Mutant transcript 3 was 988 bp in length with the inclusion of 310 bp and another 99 bp of intron 33. Mutant transcript 4 was 890 bp in length with the inclusion of 310 bp of intron 33. Mutant transcript 5 had exon 33 skipping. (c–e) Immunofluorescent labelling of ABCA12 in the skin. (c,d) A dot-like pattern of ABCA12 staining was seen in the cytoplasm of keratinocytes in the upper epidermis in patient 1 (c) and patient 2 (d). (e) In the normal control epidermis, ABCA12 staining was relatively strong in the granular layers and seemed to be dominant at the cell periphery. Bar = 5 μm.

revealed two mutant transcripts with in-frame deletions (Fig. 2a). In cDNA from the skin of patient 3, RT-PCR across the c.5128+3A>G mutation site identified four bands of 988, 890, 580 and 430 bp, with a single 580-bp band in the control sample (Fig. 2b). Subcloning and direct sequencing revealed three aberrant mutant transcripts, all of which led to premature termination codons. Immunofluorescence using anti-ABCA12 antibody revealed a diffuse staining of ABCA12 in the granular layers of control skin (Fig. 2e) and of the non-ABCA12 form (TGM1) from patient CIE (data not shown), while a dot-like staining in the cytoplasm was observed in patients 1 and 2 (Fig. 2c,d).

ABCA12 is a membrane lipid transporter that functions in the lipid transport from the *trans*-Golgi network to lamellar granules.[8] *ABCA12* mutations result in heterogeneity, including LI2, HI and CIE.[1,6,7] LI2 is characterized by generalized scales without serious erythroderma, and caused by either homozygote or compound heterozygote for missense mutations within the first nucleotide-binding folds of *ABCA12*.[6] HI is the severest form of ARCI, characterized by generalized large, plate-like scales with ectropion, eclabium and flattened ears.[7] HI is usually caused by homozygous or compound heterozygous truncation mutations in *ABCA12*.[7] In contrast, CIE with *ABCA12* mutation clinically shows milder manifestations.[1] Thus far, 17 different mutations in *ABCA12* have been reported in 12 cases of CIE. Eleven of 12 cases have at least one missense mutation. Only three of 17 mutations (p.Asn1380Ser, p.Ile1494Thr and p.Arg1514His) were located in the first nucleotide-binding folds. Other mutations were located outside ABCA12 active transporter sites: two nucleotide-binding folds and 12 transmembrane domains. The mutation p.Thr1575Pro was identified in two unrelated patients with different clinical severity. Patient 1 with severer features had a heterozygous truncation mutation (c.6031delG) on another allele, while patient 4, with a milder phenotype, had another heterozygous missense mutation (p.Gly1651Ser). We suggest that the phenotypic variability in these two patients was caused by different mutations.

We identified two *ABCA12* splice-site mutations, which were not reported in CIE: c.5128+3A>G and c.5940−1G>C. RT-PCR analysis across the site of the c.5940−1G>C mutation in patient 2 revealed two mutant transcripts. These findings demonstrate expression of the in-frame shorter transcript lacking two or three amino acids due to this splice-site mutation, which may account for the mild phenotype. In contrast, RT-PCR analysis across the site of the c.5128+3A>G mutation in patient 3 revealed three aberrant mutant transcripts, all of which led to premature termination codons. Therefore, patient 3 had a compound heterozygosity for missense/truncated combinations of mutations.

Using high-throughput sequencing analyses, screening of all ARCI-related genes is currently possible, but the cost is still expensive.[9] Once this is overcome, the elucidation of the pathogenesis of ARCI will greatly progress in the near future.

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Morikazu Medical Science Promotion Foundation, the Japan Lydia O'Leary Memorial Foundation, the Cosmetology Research Foundation, the Japanese Dermatological Association (Shiseido Award), the Fukuoka Foundation for Sound Health, and Galderma K.K. (Galderma Award).

Conflicts of interest: None declared.

## Iatrogenic androgenetic alopecia in a male phenotype 46XX true hermaphrodite

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MADAM, Androgenetic alopecia (AGA) is a term that describes the androgen-dependent and genetically determined nature of the disease.[1] However, although it is known that androgen replacement therapy can induce AGA, no report has previously been issued regarding the development of iatrogenic AGA in a hermaphrodite undergoing androgen therapy. Herein, we describe a unique case of a castrated male phenotype 46XX true hermaphrodite receiving exogenous androgen supplementation who developed male-type hair loss.

A 21-year-old male phenotype 46XX true hermaphrodite presented with a 3-year history of progressive hair loss. At the age of 16 years he was diagnosed as a 46XX true hermaphrodite with bilateral ovotestis, and subsequently underwent bilateral orchiectomy and testis prosthesis insertion. In addition, he was then given testosterone replacement therapy (testosterone enanthate, Jenasteron®; Jenapharm, Jena, Germany) for surgically induced andropausal status, which halted the development of secondary sexual characteristics. After 3 years of androgen therapy, progressive hair thinning developed on the scalp. Hair examination revealed nonscarring Norwood–Hamilton type III vertex alopecia with frontotemporal recession or BASP classification M1V2 alopecia (Fig. 1a, b).[2] Digital microscopy (Folliscope®; LeadM Corporation, Seoul, Korea) showed miniaturized hair shafts, and hair shaft size variation over the vertex scalp (Fig. 2). Serum testosterone, at the time, was 4·1 ng $mL^{-1}$ (normal 2·7–10·7) and serum dehydroepiandrosterone sulphate was 1845 ng $mL^{-1}$ (normal 800–5600). Under a diagnosis of iatrogenic androgen-induced alopecia, finasteride (1 mg daily) therapy was started. After 4 months of treatment, the hair loss stabilized and scalp hair regrowth was observed, despite the continuance of testosterone replacement therapy (Fig. 1c, d).

True hermaphroditism is an extremely rare disorder, which is defined as the coexistence of testicular and ovarian tissue in the same subject. The most frequent karyotype of true hermaphrodites is 46XX.[3] Gender assignments for hermaphrodites are made according to genetic, gonadal, social and psychologically determined sex, and the requests of patients and their relatives.[4] To be reared as male or female, surgical correction of ambiguous external genitalia, surgical removal of dysgenetic gonads, and sex hormone replacement for the surgically induced andropausal or menopausal state are required. The unwanted dermatological side-effects of testosterone replacement therapy include acne, excessive hair growth and male pattern baldness. As in our case, to be reared

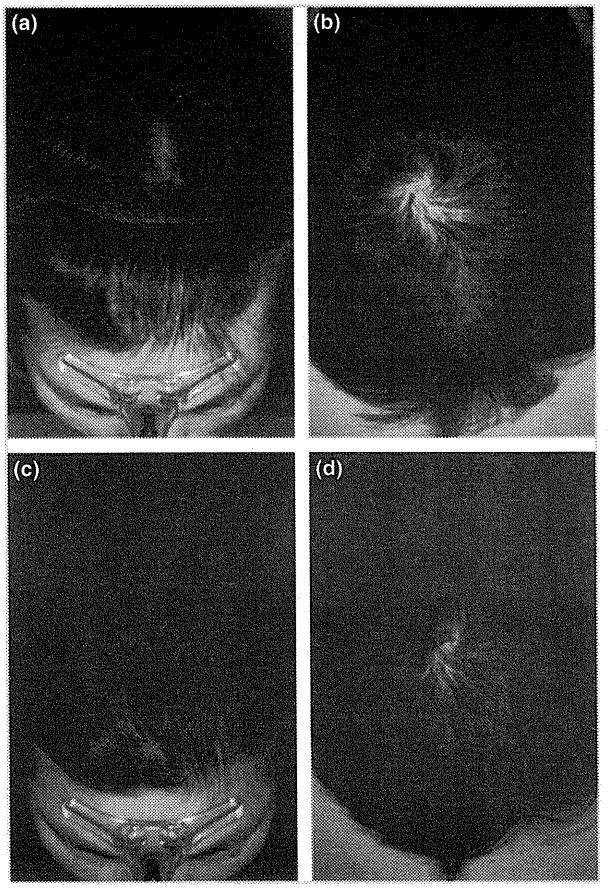


Fig 1. Iatrogenic androgenetic alopecia in a male phenotype 46XX true hermaphrodite showed a great improvement compared with baseline (a, b) after 4 months of finasteride treatment (c, d).

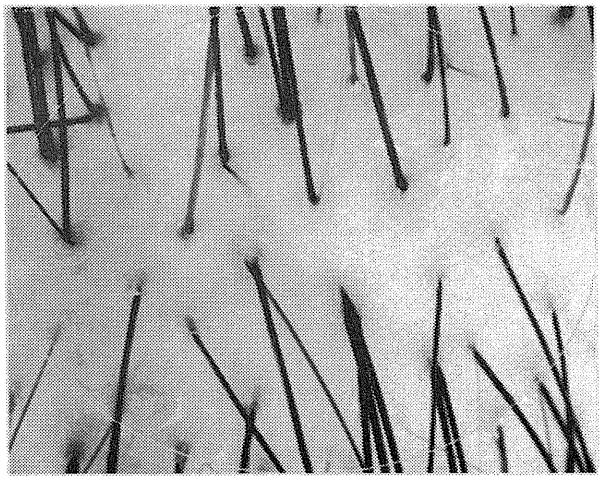

Fig 2. Photomicrograph showing miniaturized hair shafts, and variations in hair shaft size over the vertex scalp (original magnification ×50).

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# Five Japanese cases of antidesmoglein 1 antibody-positive and antidesmoglein 3 antibody-negative pemphigus with oral lesions

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## Summary

Background Oral mucosal lesions develop in pemphigus vulgaris, but not in pemphigus foliaceus. This clinical phenomenon is explained by the 'desmoglein (Dsg) compensation theory'. Dsg3 and Dsg1 are major autoantigens for pemphigus vulgaris and pemphigus foliaceus, respectively. Dsg3 is overexpressed and Dsg1 is weakly expressed on the oral mucosa. Thus, on the oral mucosa, suppression of Dsg3 function by anti-Dsg3 autoantibodies is not compensated by weakly expressed Dsg1 in pemphigus vulgaris, while suppression of Dsg1 function by anti-Dsg1 autoantibodies is perfectly compensated by richly expressed Dsg3 in pemphigus foliaceus.

Objectives We present five Japanese patients with pemphigus who deviate from this theory, i.e. all patients showed oral lesions (three also had cutaneous lesions) and reacted only with Dsg1, but not with Dsg3, by enzyme-linked immunosorbent assay.

Methods To confirm whether the unique clinical phenotypes in our patients were due to a different immunological profile from that in classical pemphigus, we examined the reactivity of the patient sera by immunoprecipitation-immunoblotting analysis using five Dsg1/Dsg2 domain-swapped molecules.

Results The sera of two patients who had only oral lesions tended to react with the extracellular (EC) 5 domain of Dsg1, the domain that is considered nonpathogenic in classical pemphigus foliaceus. Sera of three patients with mucocutaneous lesions reacted with EC1 domain or with both EC1 and EC2 domains of Dsg1, like classical pemphigus foliaceus.

Conclusions These results indicate that antigenic diversity of anti-Dsg1 antibodies in these patients may cause the unique oral mucosal and cutaneous lesions, although further studies are required to elucidate the pathomechanisms.

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### Conflicts of interest
None declared.

H.K and B.O. are joint first authors.

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Pemphigus foliaceus (PF) is an autoimmune bullous disease, which clinically shows superficial blisters on the skin, but no oral mucosal lesions. PF is characterized immunologically by the presence of IgG autoantibodies reacting with desmoglein (Dsg) 1, a cell adhesion molecule of keratinocytes. In contrast, oral lesions occur frequently in patients with pemphigus vulgaris (PV) who have anti-Dsg3 antibodies. The difference in clinical phenotype between PF and PV is often explained using the 'Dsg compensation theory'.[1–3] According to this theory, anti-Dsg3 antibodies, but not anti-Dsg1 antibodies, cause oral mucosal lesions, as Dsg1 is only weakly expressed and Dsg3 is overexpressed on the oral mucosa.

In this report, we describe five Japanese patients with pemphigus who deviate from this theory. Three patients had both oral mucosal and cutaneous lesions, and two patients had only oral lesions. Enzyme-linked immunosorbent assay (ELISA) showed only anti-Dsg1 antibodies. Novel immunoprecipitation-immunoblotting (IP-IB) methods using Dsg1/Dsg2 domain-swapped molecules demonstrated diverse antigenic sites in our cases.

## Materials and methods

### Patient background

The patient backgrounds are summarized in Table 1. Of the five patients, two had only oral lesions and three had both oral and cutaneous lesions. Biopsy specimens were taken from oral mucosa in patient 1 and from skin in patients 3 and 4. We examined ELISA indices of Dsg1 and Dsg3 in all patients at regular intervals and at exacerbation of their symptoms using commercially available Mesacup DSG-1/DSG-3 Test (MBL Co. Ltd, Nagoya, Japan) according to the protocol recommended by the supplier. ELISA detected only anti-Dsg1 antibodies, but not anti-Dsg3 antibodies, in all patients throughout our observation period.

Patient 1 was a 70-year-old Japanese woman who had shown erosive lesions on the tongue and buccal mucosa without any cutaneous lesions for 8 years (Fig. 1a). Histopathological examination of a buccal mucosal lesion revealed acantholytic suprabasal clefting (Fig. 1c). Direct immunofluorescence for the biopsied buccal mucosal lesion demonstrated IgG deposits on the cell surfaces of the epithelium (Fig. 1d), whereas no IgA and IgM deposits were seen. Combination therapy of tetracycline and nicotinamide was effective, but some extent of oral lesions continued. Three years after her first visit, she was diagnosed as having breast cancer, for which she underwent surgery. Her oral mucosal symptoms did not improve even after resection of cancer.

Patient 2 was a 54-year-old Japanese man with a 1-year history of erosions in the oral cavity, particularly on the tongue and pharynx, without any skin lesions (Fig. 1b). Oral prednisolone 20 mg daily successfully improved the lesions, and was thereafter tapered off with no relapse. We followed him for 18 months but no cutaneous lesions appeared.

Patient 3 was a 57-year-old Japanese man who had had oral lesions for 5 months and developed cutaneous lesions 10 days before his first visit. Histopathological examinination of a chest lesion showed acantholysis. Direct immunofluorescence demonstrated deposits of IgG and IgA on the cell surfaces of the epidermis. Dsg1 ELISAs for both IgG and IgA antibodies were positive. Dapsone 50 mg daily partly improved his symptoms.

Patient 4 was a 62-year-old Japanese woman with oral and cutaneous lesions. Histopathological examination of a trunk lesion showed a suprabasilar acantholytic blistering. Oral prednisolone controlled skin lesions, while oral mucosal lesions continued. Five years after her first visit, she complained of

**Table 1** Summary of the present cases

| Patient | Age (years)/ sex | Observation period (months) | Lesions: oral/skin | ELISA indices: Dsg1 (cut off < 14) | ELISA indices: Dsg3 (cut off < 7) | Epitope on Dsg1 | Histopathology (location of biopsy) | DIF and IIF | Complications | Others |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 70/F | 60 | +/− | 46 | <5 | EC3<br>EC5<br>(EC4) | Suprabasal acantholytic cleft (oral mucosa) | DIF: IgG CS+<br>IIF: − | Breast cancer | |
| 2 | 54/M | 18 | +/− | 21 | <5 | EC5 | Not performed | DIF: not performed<br>IIF: − | | |
| 3 | 57/M | 9 | +/+ | 146 | <5 | EC1<br>EC2 | Acantholysis on stratum spinosum (skin) | DIF: IgG CS+, IgA CS+<br>IIF: IgG CS x 160 | | IgA ELISA: OD for Dsg1 0·693 (cut off < 0·15) |
| 4 | 62/F | 144 | +/+ | 157 | <5 | EC1<br>EC2 | Acantholysis on stratum spinosum (skin) | DIF: IgG CS+<br>IIF: IgG CS × 160 | Thymoma (AchR Ab+), dermatomyositis | IgG Dsc3 Ab (+) |
| 5 | 58/F | 2 | +/+ | 81 | <5 | EC1 | Not performed | DIF: not performed<br>IIF: IgG CS × 40 | | |

Ab, antibody; AchR, acetylcholine receptor; CS, cell surface; DIF, direct immunofluorescence; Dsg, desmoglein; EC, extracellular; ELISA, enzyme-linked immunosorbent assay; IIF, indirect immunofluorescence; OD, optical density.

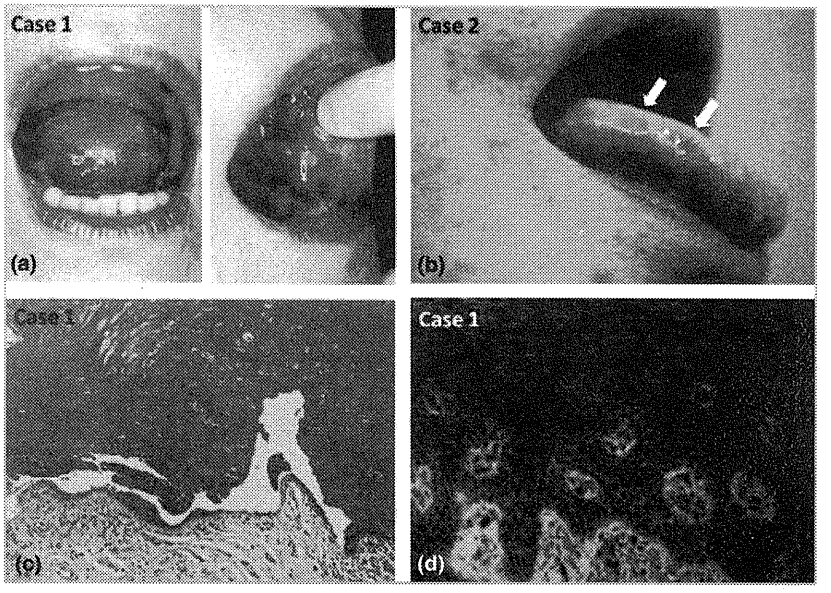


Fig 1. (a, b) Clinical appearance of patient 1 and patient 2. Erosions on the tongue and the buccal mucosa in patient 1 (a) and erosions on the tongue in patient 2 (b, arrows). (c) Histopathological findings for buccal mucosal lesion in patient 1, showing acantholytic suprabasal cleft (haematoxylin and eosin; original magnification × 100). (d) Direct immunofluorescence in patient 1, demonstrating IgG deposits in the cell surfaces of the epithelium.

myalgia with serological elevation of creatinine kinase and aldolase, and was diagnosed as having dermatomyositis. Antinuclear antibodies were positive with no evidence of Jo-1 antibodies. One and a half years later, thymoma was detected and resected. However, the oral mucosal lesions still continued.

Patient 5 was a 58-year-old Japanese woman with erosions in the oral cavity and on the back, suggesting mucocutaneous type PV. However, ELISA detected only anti-Dsg1 antibodies, but not anti-Dsg3 antibodies. She was treated with oral prednisolone 20 mg daily, but her symptoms did not improve.

### Production of recombinant proteins

We have previously constructed recombinant swapped molecules containing one each of extracellular (EC) 1–5 domains of Dsg1 on the backbone of Dsg2.[4] Recombinant proteins were produced in baculovirus-infected insect cells.[4–6] The reactivities of patients with nonepidemic PF were 88%, 50%, 13%, 22% and 0% with EC1–5, respectively.[4]

### Immunoprecipitation-immunoblotting analysis

IP was carried out by mixing a 20 μL bed volume of Protein G Sepharose 4 Fast Flow (GE Healthcare, Uppsala, Sweden), 500 μL of culture medium containing baculoproteins and 2 μL anti-E tag monoclonal antibody (mAb) (Amersham Bioscience, Piscataway, NJ, U.S.A.) or 15 μL of patient sera. The mixture was incubated at 4°C overnight with rotation, and then washed three times by Tris-buffered saline with 0·5 mmol $L^{-1}$ $CaCl_2$. The immunoprecipitated proteins were then resuspended in sodium dodecyl sulphate (SDS) sample buffer with 5% 2-mercaptoethanol and incubated for 3 min at 96°C. They were fractionated by 10% SDS–polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA, U.S.A.). To visualize the protein, anti-E tag mAb at 1 : 5000 dilution was used as primary antibody, and then alkaline phosphatase-conjugated antimouse IgG antibody (Zymed, San Francisco, CA, U.S.A.) at 1 : 4000 dilution was used as secondary antibody. Precipitated proteins were visualized by using 1-Step NBT/BCIP (Thermo Fisher Scientific, Rockford, IL, U.S.A.). The concentrations of baculoproteins were adjusted to show similar density by a preliminary IP-IB experiment using anti-E tag mAb for immunoprecipitation (Fig. 2, inset).

### Desmocollin cDNA transfection study

A cDNA transfection method to detect IgA or IgG antidesmocollin (Dsc) antibodies was performed using eukaryotic expression cDNA clones of human Dsc1–3 and cultured COS-7 cells, as described previously.[7]

## Results

We analysed sera from five patients having oral lesions with anti-Dsg1 antibodies and without anti-Dsg3 antibodies. For initial studies of IP-IB and cDNA transfection, we used the sera from the patients on their first visit, except for patient 1, whose serum was taken at the exacerbation of her symptoms, 3 years after her first visit. In IP-IB studies, the sera from three patients (patients 3–5 with both oral mucosal and cutaneous lesions) reacted with EC1 domain or with both EC1 and EC2 domains of Dsg1 (Fig. 2). In contrast, the serum from patient 1 reacted with EC3 and EC5 domains, and slightly with EC4 domain of Dsg1 (Fig. 2). The serum from patient 2 reacted exclusively with EC5 domain of Dsg1 (Fig. 2). We also examined sera at different time points in all five patients throughout our observation period of 2 months to 12 years, when clinical symptoms were milder. However, Dsg1 ELISA indices were still high, and domain profiles of Dsg1 were unchanged in all cases (data not shown).

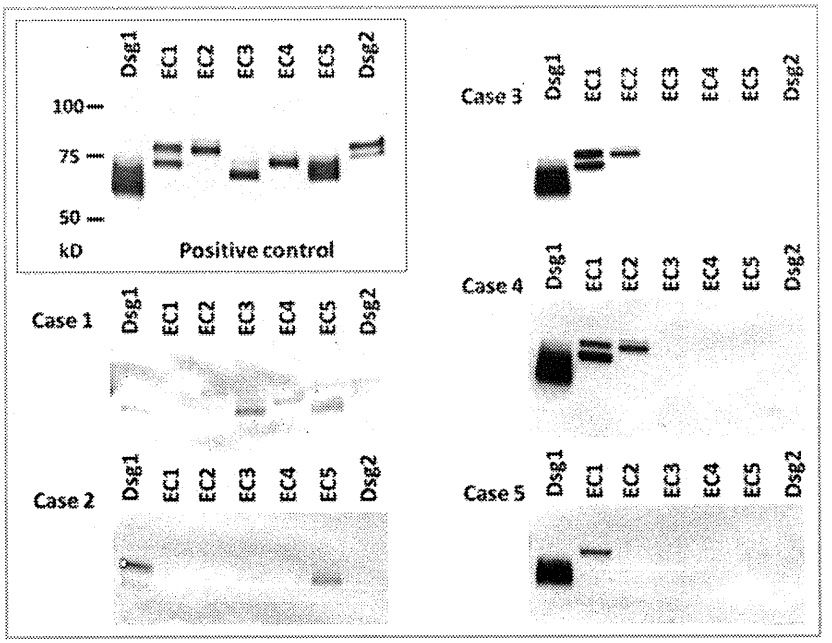


Fig 2. Immunoprecipitation-immunoblotting analysis. Patient 1 sera reacted with the extracellular (EC) 3 and EC5 domains of desmoglein (Dsg) 1 (weakly with EC4 domain), and patient 2 sera reacted exclusively with EC5 domain. Sera from patients 3 and 4 reacted with EC1 and EC2 domains, and patient sera 5 reacted only with EC1 domain. Inset, upper left: preliminary control immunoprecipitation-immunoblotting study using anti-E tag monoclonal antibody for the first immunoprecipitation step, instead of patient sera.

All patients underwent detection of both IgG and IgA antibodies using COS-7 cells transfected with cDNAs of human Dsc1, Dsc2 and Dsc3. The results revealed that IgG antibodies, but not IgA antibodies, in patient 4 reacted only with Dsc3. Other patients showed no anti-Dsc reactivity for either IgG or IgA antibodies. In patient 3 the optical density value of IgA anti-Dsg1 antibodies was elevated in IgA ELISA performed as described previously.[8]

## Discussion

In this study, we examined the precise epitope profile of anti-Dsg1 antibodies in five Japanese patients who showed oral lesions, but not anti-Dsg3 antibodies. Patients 1 and 2 showed only oral mucosal lesions, while patients 3–5 had both oral mucosal and cutaneous lesions. Patients 3–5 reacted with EC1 domain and/or EC2 domain of Dsg1, major target domains in classical PF.[4] Strikingly, patients 1 and 2 showed distinct patterns: patient 1 reacted with EC3 and EC5 domains, and patient 2 reacted only with EC5 domain.

Regarding the immune mechanisms for oral lesions in our patients, we suggest the following possibilities: (i) anti-Dsg1 antibodies caused oral mucosal lesions, (ii) patients had circulating anti-Dsg3 antibodies at an undetectable level, (iii) patients had paraneoplastic pemphigus, as patients 1 and 4 had associated internal tumours,[9,10] and (iv) antibodies to non-Dsg molecules targeted the oral mucosal epithelium.

The first possibility may be supported by the fact that detection of anti-Dsg1 antibodies in normal sera is quite rare and cannot be accidental.[11,12] In this context, it is intriguing that patients 1 and 2 showing only oral lesions reacted with EC3–5 domains, that are considered nonpathogenic in patients with ordinary PF. Such unique anti-Dsg1 antibodies may overcome the 'Dsg compensation theory' and produce oral lesions in our patients. The second possibility is unlikely because the sensitivity of Dsg3 ELISA is quite high and false-negatives are rare.[11] In addition, we performed Dsg3 ELISA at several points throughout our observation period of 2 months to 12 years, and the results were always negative.

The third possibility is also unlikely, because the patient sera did not react with plakins in our IB study using normal human epidermal extracts, and indirect immunofluorescence using rat bladder cryosections showed negative results. Moreover, oral lesions in our patients were much milder than those in patients with typical paraneoplastic pemphigus. However, paraneoplastic pemphigus may be a T cell-mediated disease, in which autoantibodies are not detected, and cytotoxic T cells mediate oral lesions.[13] The fourth speculation is possible. Previous studies reported the existence of autoantibodies in pemphigus sera against acetylcholine receptor,[14] mitochondria,[15] plakoglobin,[16] envoplakin, periplakin[17] and E-cadherin.[18] Moreover, anti-Dsc antibody is a possible causative antibody for oral mucosal lesions, because anti-Dsc3 antibodies were reported to cause loss of cell adhesion by *in vivo* and *in vitro* studies,[19,20] and because conditional Dsc3 knockout mice developed blisters on the oral mucosa and on the skin.[21] From these results, oral lesions in patient 4 could be produced by anti-Dsc3 antibodies. In patient 3, IgA anti-Dsg1 antibodies may play some pathogenic role. IgA autoantibodies in linear IgA bullous dermatosis and dermatitis herpetiformis induce inflammatory reaction by activating complement and granulocytes.[22] Histopathological examination in patient 3 actually showed neutrophilic infiltration. Therefore, the first and fourth possibilities might explain the oral lesions in our patients.

Patients 3–5, but not patients 1 and 2, showed skin lesions. The autoantibodies against EC1 and EC2 domains of Dsg1 are known to be pathogenic for skin blister formation in several

experiments.[23,24] On the other hand, no blister formation was induced in mice passively transferred with anti-EC5 domain antibodies, which were prepared from endemic PF sera. In addition, anti-EC5 domain antibodies show no reactivity by indirect immunofluorescence,[23] which is in agreement with negative indirect immunofluorescence results in our patients 1 and 2. These results may confirm the findings in our study that anti-Dsg1 EC1 and EC2 domain antibodies in patients 3–5, but not anti-Dsg1 EC3 and EC5 domain antibodies in patients 1 and 2, were pathogenic in blister formation in the skin.

In conclusion, the results in this study provide us with several interesting questions, which will be answered by further studies in accumulated similar cases.

**What's already known about this topic?**

- In autoimmune bullous diseases, autoantigens known to play a causative role in oral lesions are laminin 332, type VII collagen, integrin α6, desmoglein (Dsg) 3 and desmocollin (Dsc) 3, but not Dsg1 and Dsc1.

**What does this study add?**

- We report five atypical pemphigus cases that cannot be explained by the 'Dsg compensation theory'.

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ORIGINAL ARTICLE

# Epitope Spreading Is Rarely Found in Pemphigus Vulgaris by Large-Scale Longitudinal Study Using Desmoglein 2–Based Swapped Molecules

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Epitope spreading is involved in inducing and maintaining self-reactivity. Epitope spreading in pemphigus vulgaris (PV), caused by IgG autoantibodies to desmoglein 3 (Dsg3) and Dsg1, was previously analyzed using Dsg3/Dsg1 extracellular domain–swapped molecules. However, precise identification of the responsible epitopes in each molecule by using only this method was problematic. In this study, we studied epitope spreading in PV by a novel immunoprecipitation–immunoblot method using Dsg3 (or Dsg1)/Dsg2 domain–swapped molecules, which overcomes the problems associated with the previous approaches. We analyzed the antigenic epitopes recognized by 212 sera collected from 53 PV patients at multiple disease stages. The major epitopes were present at the N-terminal region of Dsgs and were unchanged over the course of the disease in both anti-Dsg3 mucosal dominant-type PV and anti-Dsg3/Dsg1 mucocutaneous-type PV. These N-terminal epitopes were calcium dependent. Circulating antibodies in paraneoplastic pemphigus and pemphigus herpetiformis had unique epitope distributions, although the Dsg N-termini still contained the major epitopes. These results suggest that, after onset, intramolecular and intermolecular epitope spreading among extracellular domains on Dsg3 and Dsg1 is rare in PV and has no correlation with disease course.

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## INTRODUCTION

Epitope spreading is a phenomenon in which immune responses can spread over the disease course to recognize epitopes that are different from the original target. If it occurs in the same molecule, this is termed "intramolecular epitope spreading" (Lehmann *et al.*, 1992). When responses begin to target epitopes on other proteins, this is termed "intermolecular epitope spreading" (Steinman and Conlon, 1997). Accumulating evidence supports the epitope-spreading hypothesis (McRae *et al.*, 1995; Robinson *et al.*, 2003; McMahon *et al.*, 2005; Chen *et al.*, 2006). However, a pathological role for epitope spreading was not demonstrated in human diseases. One reason may be that it is difficult to evaluate the responsible epitopes (Vanderlugt and Miller, 2002). In addition, published studies were small in scale (Tuohy *et al.*, 1997, 1999; Goebels *et al.*, 2000; Jones *et al.*, 2003; O'Connor *et al.*, 2005).

Pemphigus is caused by IgG autoantibodies against desmogleins (Dsgs) (Amagai, 2003). Four isoforms of Dsgs were described. Dsg1 is the autoantigen in pemphigus foliaceus (PF) and mucocutaneous-type pemphigus vulgaris (PV; Amagai *et al.*, 1995; Stanley and Amagai, 2006). Dsg3 is the autoantigen in mucocutaneous-type PV and mucosal dominant-type PV (Amagai *et al.*, 1991, 1994; Koch *et al.*, 1997; Mahoney *et al.*, 1999). Pemphigus sera occasionally contain Dsg4/Dsg1 cross-reacting IgG autoantibodies (Kljuic *et al.*, 2003; Whittock and Bower, 2003; Nagasaka *et al.*, 2004). No reactivity against Dsg2 was found in PF or PV sera (Ota *et al.*, 2003).

There were no definitive investigations elucidating intramolecular epitope spreading in tissue-specific autoimmune diseases, although some attempts were made for epitopes within each domain of Dsg3 and Dsg1 in PV. These relied on molecules constructed by combining the extracellular (EC) domains of Dsg1 and Dsg3 (Futei *et al.*, 2000; Sekiguchi *et al.*, 2001; Li *et al.*, 2003; Salato *et al.*, 2005). However, these analyses had a limitation. Although most of

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*Abbreviations: Dsg, desmoglein; EC, extracellular; HP, herpetiform pemphigus; IP-IB, immunoprecipitation–immunoblotting; PF, pemphigus foliaceus; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris*

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the anti-Dsg3 IgG autoantibodies in PV did not cross-react with Dsg1, one could not analyze anti-Dsg3 and anti-Dsg1 antibodies simultaneously in mucocutaneous-type PV by using this method. We therefore generated domain-swapped molecules comprising Dsg3 (or Dsg1) with Dsg2 as the backbone, taking advantage of the fact that PV sera show no reactivity with Dsg2. We defined epitope distribution and intramolecular epitope spreading by immunoprecipitation–immunoblotting (IP–IB) using 212 sera collected from 53 PV patients, and showed that epitope spreading is rare in PV.

## RESULTS

### Generation of new domain-swapped molecules

We first generated Dsg1, Dsg2, and Dsg3 molecules with full-length EC domains (Figure 1a). By replacing the five EC domains of Dsg2 with the corresponding Dsg3, we obtained a set of five new Dsg3/Dsg2 domain-swapped molecules as secreted proteins, using the previously reported technique (Chan *et al.*, 2010; Figure 1a). The primers used in this study are shown in Supplementary Table S1 online. Characterization of Dsg1/Dsg2 domain-swapped molecules has been detailed previously (Chan *et al.*, 2010).

### Immunoblotting of culture supernatants by anti-E tag mAb

Culture supernatants containing full-length EC domains of Dsg1, Dsg2, and Dsg3, as well as the five Dsg3/Dsg2 and the five Dsg1/Dsg2 domain-swapped molecules, were fractionated by SDS-PAGE and immunoblotted with anti-E tag mAb. Protein bands of the expected sizes were observed for the unmodified Dsg1–3 and all 10 Dsg3/Dsg2 and Dsg1/Dsg2 domain-swapped molecules (Figure 1b and c).

### Domain-swapped molecules of the N-terminal (EC1, EC2, and EC3) but not C-terminal regions (EC4 and EC5) of Dsg1 and Dsg3 are calcium dependent

We investigated whether domain-swapped molecules were recognized by anti-Dsg1 or anti-Dsg3 IgG autoantibodies in a calcium-dependent manner using EDTA treatment. Domain-swapped molecules were left untreated or were treated with EDTA, and then immunoprecipitated with anti-Dsg1 or anti-Dsg3 IgG antibodies recognizing each of the five EC domains of Dsg1 or Dsg3. EDTA treatment abolished the reactivity of both anti-Dsg1 and anti-Dsg3 IgG to the respective EC1–3, but not to the EC4–5 domains of Dsg1 and Dsg3. (Figure 1d. Note the diminishment of the EC1-swapped molecule without prosequence). Thus, anti-Dsg1 and anti-Dsg3 IgG antibodies recognize calcium-dependent epitopes on the EC1–3 domains of Dsg1 and Dsg3, whereas recognition of epitopes on the EC4–5 domains is calcium independent.

### Most anti-Dsg3 IgG autoantibodies recognize N-terminal regions on the Dsg3 EC domains

We analyzed the Dsg3 epitopes recognized by 212 serum samples from 53 cases of mucosal dominant-type PV and mucocutaneous-type PV over the disease course. All 212 sera reacted with the full-length EC domain of Dsg3, but none with that of Dsg2 (Figure 2a). Of these 212 PV sera, 193 (91.0%) reacted with EC1, 151 (71.2%) with EC2, 107 (50.5%) with EC3, 40 (18.9%) with EC4, and 26 (12.3%) with the EC5 domain of Dsg3. The reactivity of PV sera to the EC1 domain of Dsg3 was significantly higher than that to the EC2–EC5 domains ($P<0.0001$). The reactivity of PV sera to the EC2 domain of Dsg3 was also significantly higher than that to the EC3–5 domains ($P<0.0001$).

Further, we determined the epitope profiles recognized by these 212 PV sera obtained at different disease stages: active, moderate, and remission (Table 1a). The results revealed that the major epitope remained on the N-terminal domain of Dsg3 at every clinical stage. A total of 164 sera (77.4%) recognized multiple EC domains of Dsg3 (Table 1b). Of these, 63 (29.7%) reacted with two, 64 (30.2%) with three, 34 (16.0%) with four, and only 3 (1.4%) with all five EC domains. In contrast, the remaining 48 sera (22.6%) reacted with only one of the EC domains of Dsg3; specifically, 40 (18.9%) reacted with the EC1 domain, 5 (2.4%) only with EC2, and 3 (1.4%) only with EC3 (Table 1c). No sera reacted only with the EC4 or EC5 domain of Dsg3. The number of epitopes recognized by each anti-Dsg3 IgG autoantibody correlated with an increasing ELISA index score ($P=0.0011$), but not with clinical disease activity ($P=0.2730$; Supplementary Tables S2–S4 online).

### Most anti-Dsg1 IgG autoantibodies in mucocutaneous-type PV sera recognize the N-terminal region of Dsg1 EC domains

Next, we analyzed the recognition of Dsg1 epitopes by autoantibodies in 46 serum samples from 15 cases of mucocutaneous-type PV with different clinical disease activity levels (Supplementary Table S3 online). All 46 serum samples reacted with the full-length EC domain of Dsg1, but not with Dsg2 (Figure 2b). Of these PV sera, 45 (97.8%) reacted with the EC1 domain of Dsg1, 12 (26.1%) with EC2, 4 (8.7%) with EC3, 2 (4.3%) with EC4, and 3 (6.5%) with EC5. The reactivity of mucocutaneous-type PV sera to the EC1 domain of Dsg1 was significantly higher than that to the EC2–5 domains ($P<0.0001$), whereas the reactivity to the EC2 domain of Dsg1 was not. Thus, the EC1 domain of Dsg1 was the major epitope recognized by anti-Dsg1 IgG autoantibodies. We also established the Dsg1 epitope profiles for these 46 PV sera obtained at different stages of clinical disease activity (Table 1d). Again, the major epitopes always resided on the N-terminal domain (EC1 domain) of Dsg1 at every stage of clinical disease activity, and their distribution was almost the same no matter which clinical disease activity level was tested. We found that 14 sera (30.4%) recognized epitopes on the multiple EC domains of Dsg1, of which 10 (21.7%) reacted with two, 2 (4.3%) with three, and 2 (4.3%) with four EC domains (Table 1e). No sera were found to react with all five EC domains. A total of 32 sera (69.6%) reacted with only one of the EC domains of Dsg1, 31 (67.4%) reacted only with the EC1 domain, and 1 (2.2%) only with the EC2 domain. No sera reacted only with the Dsg1 EC3, EC4, or EC5 domain (Table 1f).

### Epitope profiles of mucocutaneous-type PV

We compared the epitope profiles of anti-Dsg1 and anti-Dsg3 IgG autoantibodies in 15 cases (46 sera) of mucocutaneous-

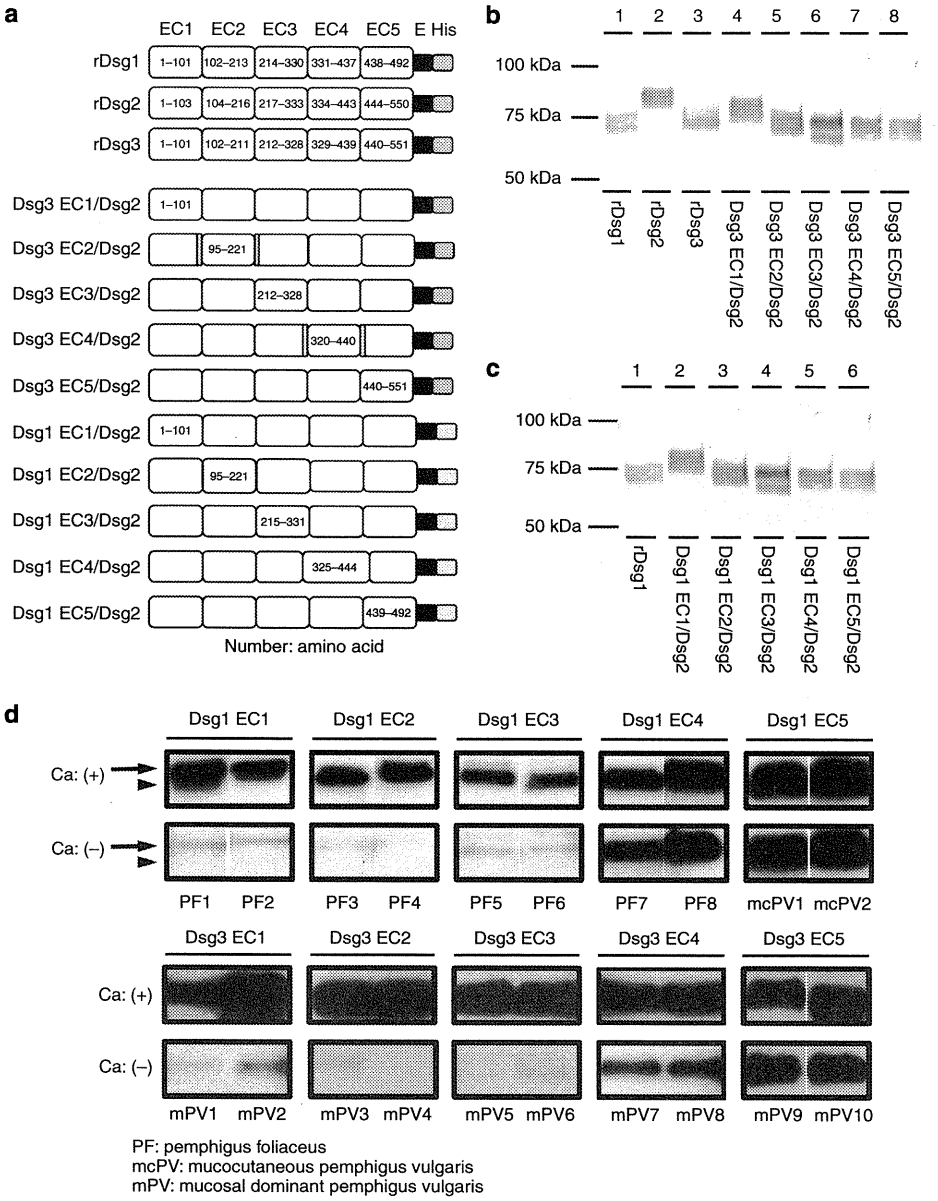


**Figure 1. Calcium-dependent pemphigus foliaceus (PF) and pemphigus vulgaris (PV) epitopes analyzed by new domain-swapped molecules.** (**a**) Full-length recombinant proteins for the extracellular (EC) domains of human desmoglein 1 (Dsg1) (rDsg1), Dsg2 (rDsg2), Dsg3 (rDsg3), and 10 domain-swapped molecules (Dsg3 EC1/Dsg2–Dsg3 EC5/Dsg2 and Dsg1 EC1/Dsg2–Dsg1 EC5/Dsg2). (**b**) Culture supernatants stained with anti-E tag mAb: rDsg1 (lane 1), rDsg2 (lane 2), rDsg3 (lane 3), Dsg3 EC1/Dsg2 (lane 4), Dsg3 EC2/Dsg2 (lane 5), Dsg3 EC3/Dsg2 (lane 6), Dsg3 EC4/Dsg2 (lane 7), and Dsg3 EC5/Dsg2 (lane 8). (**c**) Culture supernatants stained with anti-E tag mAb: rDsg1 (lane 1), Dsg1 EC1/Dsg2 (lane 2), Dsg1 EC2/Dsg2 (lane 3), Dsg1 EC3/Dsg2 (lane 4), Dsg1 EC4/Dsg2 (lane 5), and Dsg1 EC5/Dsg2 (lane 6). (**d**) Dsg1/Dsg2 and Dsg3/Dsg2 domain-swapped molecules pretreated with calcium (Ca: (+)) or EDTA (Ca: (–)) detected by immunoprecipitation–immunoblotting. EC1-swapped molecules with (arrows) and without (arrowheads) prosequence are marked.

type PV (Supplementary Table S3 online). Both anti-Dsg1 and anti-Dsg3 IgG autoantibodies reacted predominantly with the EC1 domain, whereas epitope profiles for the other domains did not always show the same pattern for Dsg1 or Dsg3. Of the 46 sera, 38 reacted with the EC1 domain of both Dsg1 and Dsg3. Only five sera showed the same epitope pattern with Dsg1 and Dsg3.

**Epitope spreading is rare in PV**

We next analyzed whether epitope spreading occurred at any time over the disease course. At every clinical disease activity level, most anti-Dsg3 IgG autoantibodies predominantly recognized the EC1 domain, and no intramolecular epitope shift was apparent in 51 (96.2%) of 53 PV cases (Figure 3a and Supplementary Table S2 online). We found only two

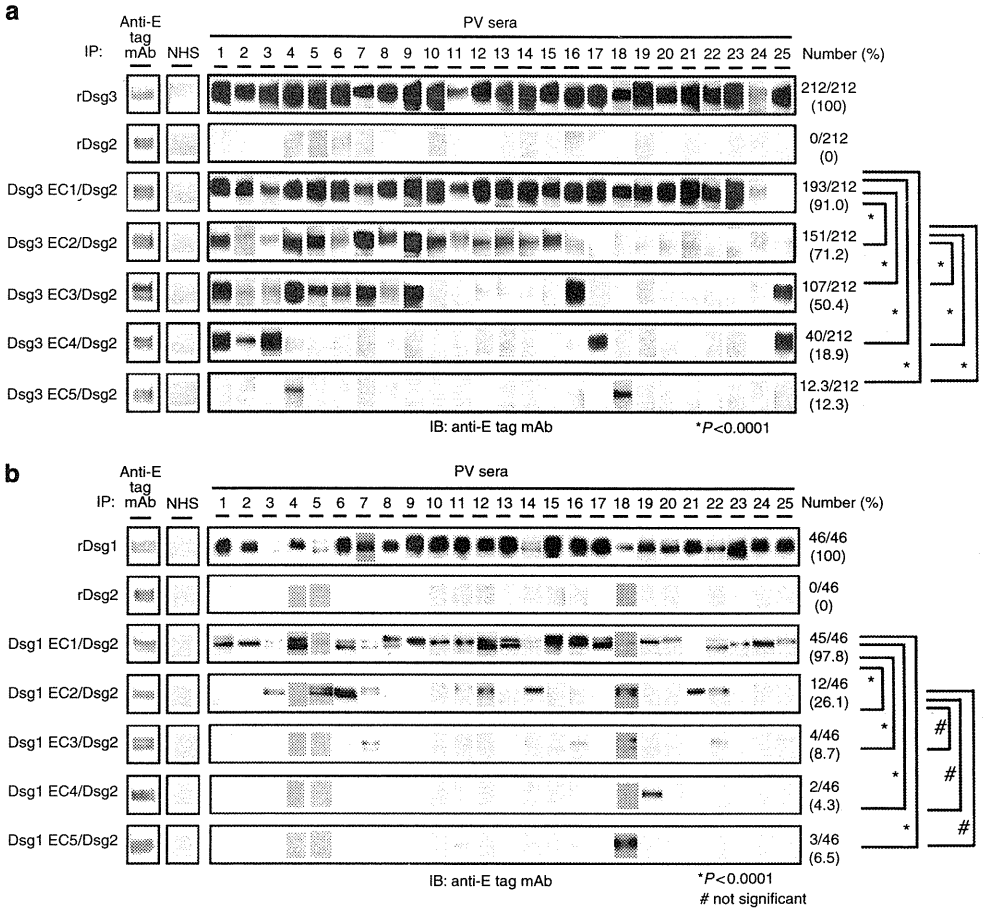


**Figure 2. Epitope profiles of pemphigus vulgaris (PV).** (**a**) Anti-desmoglein 3 (Dsg3) IgG antibodies preferentially bind to the N-terminal extracellular (EC) domains of Dsg3. All 212 pemphigus vulgaris (PV) sera reacted with the full-length EC domain of Dsg3, but none with the full-length Dsg2 EC domain. Of the 212 PV sera, 193 reacted with the Dsg3 EC1 domain, 151 with the EC2 domain, 107 with the EC3 domain, 40 with the EC4 domain, and 26 with the EC5 domain. (**b**) Anti-Dsg1 IgG antibodies preferentially bind to the N-terminal EC domains of Dsg1. Of the 46 PV sera, 45 reacted with the EC1 domain of Dsg1, 12 with the EC2 domain, 4 with the EC3 domain, 2 with the EC4 domain, and 3 with the EC5 domain. *$P<0.0001$, #not significant. IB, immunoblotting; IP, immunoprecipitation; NHS; normal human serum.

patients (3.8%) manifesting apparent Dsg3 epitope spreading during the disease course (Figure 3b), and none for Dsg1 in any of the 15 PV cases (Supplementary Tables S2 and S3 online). In 13 mucocutaneous-type PV cases (44 sera), in which both anti-Dsg3 and anti-Dsg1 IgG autoantibodies were present, no intermolecular epitope spreading between Dsg1 and Dsg3 was apparent (Supplementary Table S3 online). In addition, we found that, in some PV patients, Dsg3 ELISA reactivity and clinical activity did not correlate well (Supplementary Tables S2–S4 online). Therefore, we have analyzed the possible epitope spreading from pathogenic (i.e., EC1–3) to nonpathogenic epitopes (EC4–5) for cases in which Dsg ELISA index score did not decrease even after clinical improvement was obtained. However, statistically, it was not the case ($P=1.000$ for both anti-Dsg3 and anti-Dsg1 antibodies).

### Epitope profiles of paraneoplastic pemphigus

Paraneoplastic pemphigus (PNP) is a rare autoimmune blistering disease associated with neoplasia (Amagai *et al.*, 1998; Ohyama *et al.*, 2001). All 14 PNP sera tested reacted with the EC1 domain of Dsg3. Intriguingly, 12 (85.7%) PNP sera reacted with EC4 (Figure 4a and Table 1g). The reactivity to the EC4 domain of Dsg3 in PNP was comparable to that to the EC1 domain ($P=1.00$), but was significantly higher than that to the EC5 domain ($P=0.0092$).

### Epitope profiles of pemphigus herpetiformis

Herpetiform pemphigus (HP) is a pemphigus variant with clinical features of dermatitis herpetiformis and immunological features of pemphigus (Kubo *et al.*, 1997; Ishii *et al.*, 1999; Seitz *et al.*, 1999). Of the 19 HP cases, 15 possessed only anti-Dsg1 IgG autoantibodies, 4 had only anti-Dsg3 IgG autoantibodies, and 2 had both; although one serum (#15) did not react to the full-length Dsg3, it reacted to its EC1–3 domains (Figure 4b). The major epitopes in 15 Dsg1-reactive HP sera tested were present on the EC1 domain of Dsg1, whereas EC2, EC3, and EC4 domains were also recognized by one-third of these sera (Figure 4b and Table 1h). All six sera from Dsg3-reactive HP cases reacted with both EC1 and

## Table 1. Epitope profiles of Dsg3 and Dsg1 in PV, PNP, and HP

**(a) Epitope profile of Dsg3 at all clinical stages**

| | Recombinant proteins | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | EC domains of Dsg3 | | | | |
| Clinical activity | Dsg3 | Dsg2 | EC1 | EC2 | EC3 | EC4 | EC5 |
| Active (%) (*n*=85) | 85 (100) | 0 (0) | 78 (91.8) | 61 (71.8) | 38 (44.7) | 14 (16.5) | 12 (14.1) |
| Moderate (%) (*n*=60) | 60 (100) | 0 (0) | 52 (86.7) | 42 (70.0) | 29 (48.3) | 11 (18.3) | 6 (10.0) |
| Remission (%) (*n*=67) | 67 (100) | 0 (0) | 63 (94.0) | 48 (71.6) | 40 (59.7) | 15 (22.4) | 8 (11.9) |
| Total (%) (*n*=212) | 212 (100) | 0 (0) | 193 (91.0) | 151 (71.2) | 107 (50.5) | 40 (18.9) | 26 (12.3) |

**(b) PV sera recognizing on multiple EC domain of Dsg3**

| | Number of domains recognized epitopes | | | |
|---|---|---|---|---|
| Clinical activity | Two | Three | Four | Five |
| Active (%) (*n*=64) | 25 | 25 | 13 | 1 |
| Moderate (%) (*n*=46) | 22 | 15 | 8 | 1 |
| Remission (%) (*n*=54) | 16 | 24 | 13 | 1 |
| Total (%) (*n*=164) | 63 | 64 | 34 | 3 |

**(c) PV sera recognizing on single EC domain of Dsg3**

| | Number of domains recognized epitopes | | | | |
|---|---|---|---|---|---|
| Clinical activity | EC1 | EC2 | EC3 | EC4 | EC5 |
| Active (%) (*n*=21) | 18 | 2 | 1 | 0 | 0 |
| Moderate (%) (*n*=14) | 11 | 2 | 1 | 0 | 0 |
| Remission (%) (*n*=13) | 11 | 1 | 1 | 0 | 0 |
| Total (%) (*n*=48) | 40 | 5 | 3 | 0 | 0 |

**(d) Epitope profile of Dsg1 at all clinical stages**

| | Recombinant proteins | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | EC domains of Dsg1 | | | | |
| Clinical activity | Dsg1 | Dsg2 | EC1 | EC2 | EC3 | EC4 | EC5 |
| Active (%) (*n*=20) | 20 (100) | 0 (0) | 19 (95.0) | 4 (20.0) | 1 (5.0) | 0 (0) | 2 (1.0) |
| Moderate (%) (*n*=18) | 18 (100) | 0 (0) | 18 (100) | 6 (33.0) | 0 (0) | 0 (0) | 0 (0) |
| Remission (%) (*n*=8) | 8 (100) | 0 (0) | 8 (100) | 2 (25.0) | 3 (37.5) | 2 (25.0) | 1 (12.5) |
| Total (%) (*n*=46) | 46 (100) | 0 (0) | 45 (97.8) | 12 (26.1) | 4 (8.7) | 2 (4.3) | 3 (6.5) |

**(e) PV sera recognizing on multiple EC domain of Dsg1**

| | Number of domains recognized epitopes | | | |
|---|---|---|---|---|
| Clinical activity | Two | Three | Four | Five |
| Active (%) (*n*=4) | 3 | 0 | 1 | 0 |
| Moderate (%) (*n*=6) | 6 | 0 | 0 | 0 |
| Remission (%) (*n*=4) | 1 | 2 | 1 | 0 |
| Total (%) (*n*=14) | 10 | 2 | 2 | 0 |

Table 1 continued on following page

**Table 1. Continued**

**(f) PV sera recognizing on single EC domain of Dsg1**

| | Number of domains recognized epitopes | | | | |
|---|---|---|---|---|---|
| **Clinical activity** | **EC1** | **EC2** | **EC3** | **EC4** | **EC5** |
| Active (%) (*n*=16) | 15 | 1 | 0 | 0 | 0 |
| Moderate (%) (*n*=12) | 12 | 0 | 0 | 0 | 0 |
| Remission (%) (*n*=4) | 4 | 0 | 0 | 0 | 0 |
| Total (%) (*n*=32) | 31 | 1 | 0 | 0 | 0 |

**(g) Epitope distributions on Dsg3 in 14 PNP cases**

| | Recombinant proteins | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | EC domains of Dsg3 | | | | |
| | **Dsg3** | **Dsg2** | **EC1** | **EC2** | **EC3** | **EC4** | **EC5** |
| PNP (%) (*n*=14) | 14 (100) | 0 (0) | 14 (100) | 8 (57.1) | 10 (71.4) | 12 (85.7) | 3 (21.4) |

**(h) Epitope distributions on Dsg1 for 15 sera and Dsg3 for 6 sera in HP cases**

| | Recombinant proteins | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | EC domains of Dsg1 | | | | |
| | **Dsg1** | **Dsg2** | **EC1** | **EC2** | **EC3** | **EC4** | **EC5** |
| HP (%) (*n*=15) | 15 (100) | 0 (0) | 12 (80.0) | 5 (33.3) | 6 (40.0) | 5 (33.3) | 1 (6.7) |

| | | | Extracellular domains of Dsg3 | | | | |
|---|---|---|---|---|---|---|---|
| | **Dsg2** | **Dsg3** | **EC1** | **EC2** | **EC3** | **EC4** | **EC5** |
| HP (%) (*n*=6) | 0 (0) | 5 (83.3) | 6 (100) | 6 (100) | 5 (83.3) | 1 (16.7) | 0 (0) |

Abbreviations: Dsg, desmoglein; EC, extracellular; HP, herpetiform pemphigus; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris.

EC2 domains, and five reacted with the EC3 domain (Figure 4b). One serum reacted with the EC1, EC2, EC3, and EC4 domains of Dsg3. The reactivity to the EC1 domain of Dsg1 (or Dsg3) in HP was comparable to that to the EC2–4 domains of Dsg1 (or Dsg3) (Dsg1 EC1 vs. EC2: $P=0.1013$, EC1 vs. EC3: $P=0.2417$, EC1 vs. EC4: $P=0.1013$; Dsg3 EC1 vs. EC2: $P=1.0000$, EC1 vs. EC3: $P=1.0000$, EC1 vs. EC4: $P=0.0606$), but was significantly higher than that to the EC5 domain of Dsg1 (or Dsg3; Dsg1 EC1 vs. EC5: $P=0.0005$, Dsg3 EC1 vs. EC5: $P=0.0087$) (Figure 4). Thus, HP sera recognized a broader range of epitopes than PV.

## DISCUSSION

This study documented the rarity of epitope spreading after the onset of disease in PV. At every clinical activity level, the major Dsg3 epitopes remained in the EC1–2 domains, and the epitope profile was essentially identical in most cases. Furthermore, in mucocutaneous-type PV, dominant epitopes of Dsg1 were present in EC1 but not in the EC2–5 domains. We concluded that there was no significant alteration in epitope profiles over the disease course. In addition, we showed that PNP and HP showed broader epitope distribution compared with classical pemphigus. Moreover, we showed for the first time that calcium-dependent conformational epitopes on human Dsg1 and Dsg3 recognized by pemphigus IgG autoantibodies are located within the EC1–3 domains, whereas EC4 and EC5 are calcium stable, as had been suggested in animal models (Tsunoda *et al.*, 2003).

The new domain-swapped molecules described here are useful for epitope mapping and for analyzing epitope spreading. Our new strategy has several advantages. First, the swapped domains have similar structures but distinct epitopes. Hence, they allow precise mapping of the conformational epitopes embedded in the three-dimensional molecular structure (Müller *et al.*, 2006, 2008). Second, by using a disease-irrelevant Dsg2 as the backbone of the domain-swapped molecules, one can analyze the epitopes recognized by anti-Dsg3 IgG autoantibodies (or anti-Dsg1 IgG autoantibodies) without the influence of the presence of anti-Dsg1 IgG autoantibodies (or anti-Dsg3 IgG autoantibodies, respectively). Previously, Dsg/Dsg1-swapped molecules were used to analyze intra- and intermolecular epitope spreading (Futei *et al.*, 2000; Sekiguchi *et al.*, 2001; Li *et al.*, 2003; Salato *et al.*, 2005). However, although Dsg3 and Dsg1 do not have common structure, the Dsg3/Dsg1-swapped molecule system did not allow us to analyze intra- and

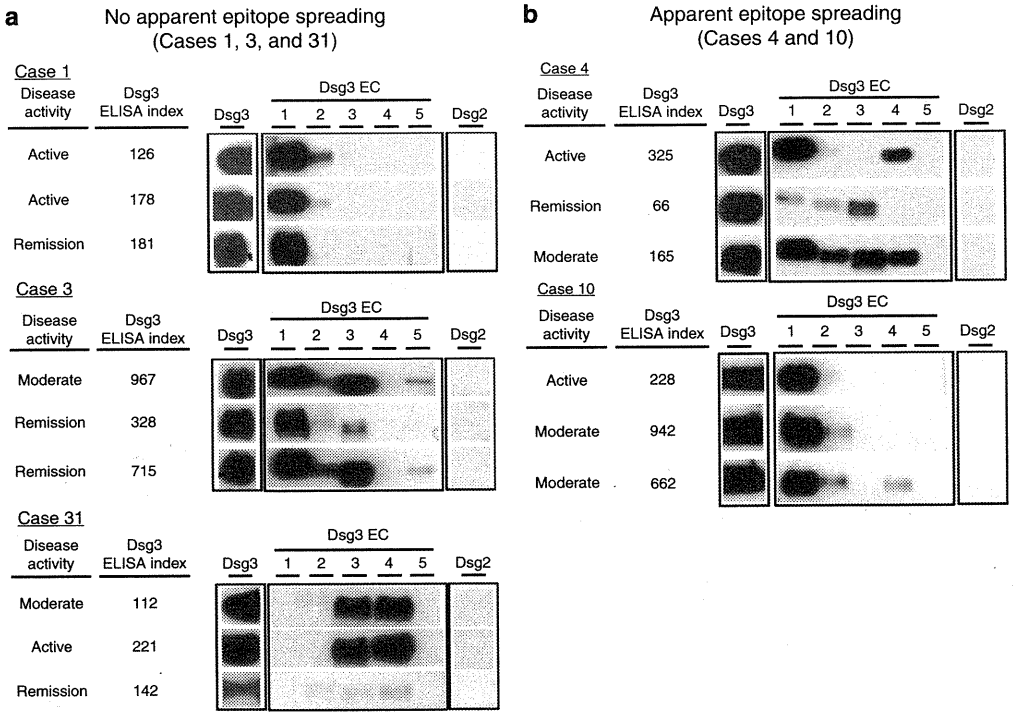


**Figure 3. Epitope shift in the target antigens for anti-desmoglein 3 (Dsg3) IgG antibodies occurs rarely over the disease course.** (**a**) Most cases ($n=51$) had no epitope shift. (**b**) Epitope profiles for anti-Dsg3 IgG antibodies from pemphigus vulgaris (PV) patients 4 and 10. Only these two PV sera showed apparent epitope shift for anti-Dsg3 IgG target antigens. Disease activity and anti-Dsg3 ELISA index for each serum are also shown at the left of each figure. EC, extracellular.

intermolecular epitope spreading precisely, namely in mucocutaneous-type PV, in which anti-Dsg1 and anti-Dsg3 antibodies are present simultaneously in the blood. The advantage of the method reported here is depicted in Figure 5.

Our results suggest that human autoimmunity conforms to the concept of "original antigenic sin", i.e., domination of the immune response by the initial epitope, which does not change over time. We recently also demonstrated that epitope spreading is rare in PF (Chan *et al.*, 2010). Our results are in agreement with a previous study in which epitope shift rarely occurred in PV (Futei *et al.*, 2000). Our results are not in agreement with previous studies that support epitope spreading in pemphigus (Li *et al.*, 2003; Salato *et al.*, 2005). Li *et al.* (2003) showed the possible intramolecular epitope spreading from EC5 at onset to EC1 and EC2 in active stage, and then toward EC5 in remission in endemic PF. As Chan *et al.* (2010) suggested that epitope shift did not occur in nonendemic PF, the discrepancy may be due to the difference between endemic and nonendemic PF. Salato *et al.* (2005) reported that intramolecular epitope spreading from EC2–5 to the EC1 domain of Dsg3 occurred and was a critical step for intermolecular epitope shift from Dsg3 to Dsg1. However, it is a questionable idea, because their claim was based on the result of only one representative case out of three mucosal dominant-type PV patients who transitioned to mucocutaneous-type PV, and they used the Dsg3/Dsg1-swapped system. The present study does not exclude the possibility of autoimmunity initiated by bystander activation, molecular mimicry, or release of cryptic epitopes before disease onset (Miller *et al.*, 1997; Horwitz *et al.*, 1998; Olson *et al.*, 2001).

Our present results revealed a unique Dsg3 epitope distribution in PNP and HP. The EC4 domain in PNP and EC1–3 domains in HP were frequently recognized by autoantibodies, although major Dsg3 epitopes in both PNP and HP were still found in N-terminal EC domains. The result for PNP is consistent with a previous study using competition ELISA with domain-swapped Dsg3/Dsg1 molecules (Futei *et al.*, 2003). The different autoantibody profiles between these diseases and PV may contribute to their unique clinicohistopathological characteristics.

There are limitations to our study. First, it is impossible to evaluate epitope spreading before the onset of disease. Spreading from viral to self-epitopes has been shown in a virus-induced autoimmune disease model (Miller *et al.*, 1997). However, there is no consensus that tissue-specific autoimmune diseases develop during persistent viral or other infections (Horwitz *et al.*, 1998; Zhao *et al.*, 1998; Olson *et al.*, 2001). Second, we cannot exclude the fact that treatment influenced epitope spreading, when using immunosuppressives, apheresis, or intravenous immunoglobulin, all of which could impair epitope spreading. Third, there are technical issues in quantitative evaluation of autoantibodies when using IP-IB on sera with low Dsg titers. Fourth, it is

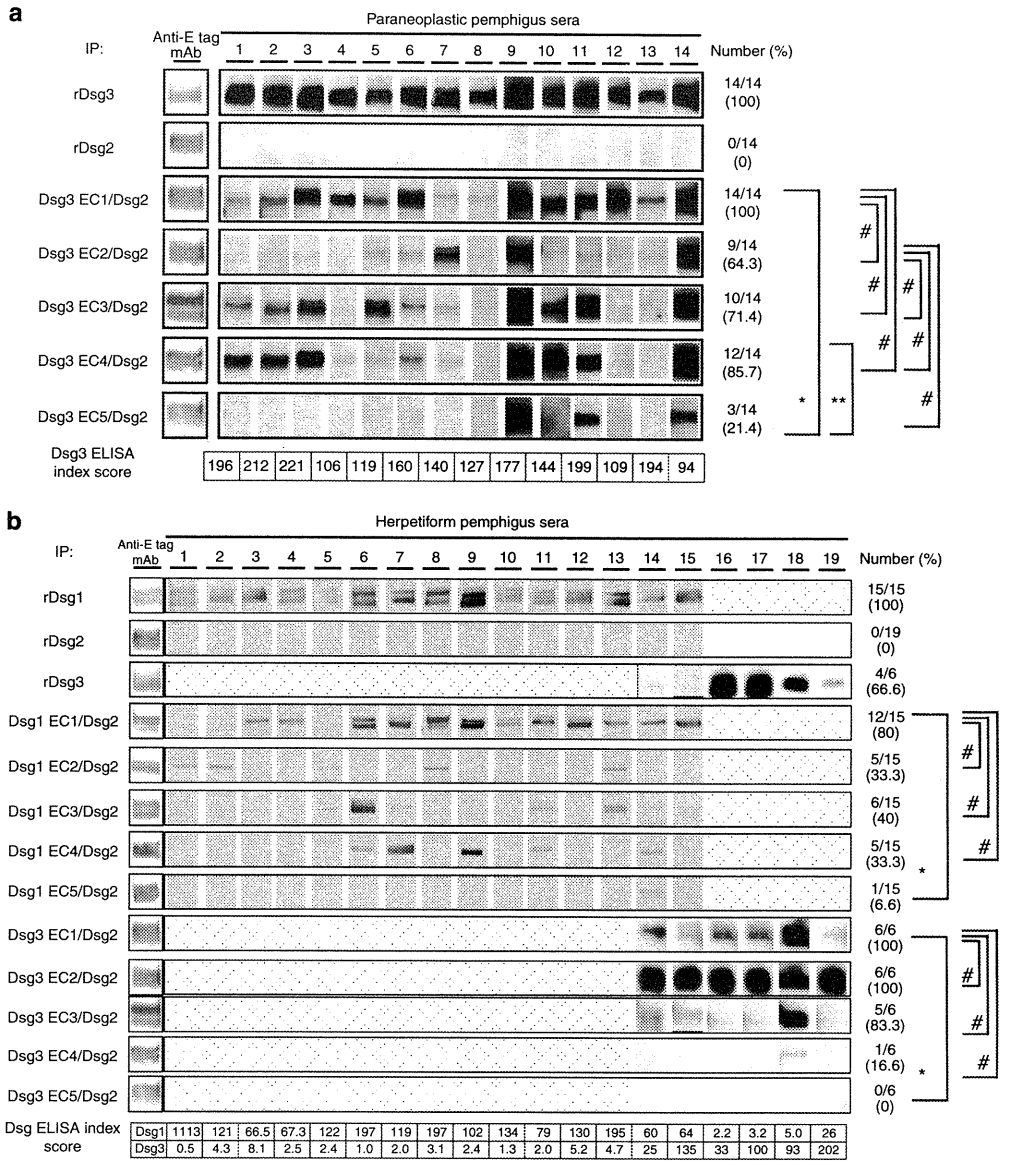


**Figure 4. Epitope profiles of atypical pemphigus.** (**a**) All 14 PNP sera reacted with the full-length desmoglein 3 (Dsg3) extracellular (EC) domain, but none with the full-length EC domain of Dsg2. $^{*}P=0.0001$, $^{**}P<0.001$, and $^{\#}$not significant. All 14 PNP sera reacted with the EC1 domain of Dsg3, 9 with EC2, 10 with EC3, 12 with EC4, and 3 with EC5 (**b**). In the 15 sera from herpetiform pemphigus (HP) cases, the major epitopes were present on the Dsg1 EC1 domain. However, six HP patients had anti-Dsg3 IgG autoantibodies, and all six sera reacted with EC1 and EC2 domains; five reacted with the EC3 domain. All six sera reacted most strongly with the EC2 domain of Dsg3. $^{*}P<0.001$, $^{\#}$not significant. IP, immunoprecipitation.

impossible to analyze the intra-domain epitope spreading within each EC domain by this study.

In conclusion, we have documented the rarity of epitope spreading in PV. Therefore, targeting treatments to the N-terminal domains of Dsg3/Dsg1 should be promising to control PV.

## MATERIALS AND METHODS

### Human sera

All studies followed the guidelines of the Medical Ethics Committees of both Kurume University School of Medicine and Keio University School of Medicine, conducted according to the Declaration of Helsinki Principles. All participants provided informed consent. A total of 212 sera were obtained from 31 patients with mucocutaneous-type PV and 22 with mucosal dominant-type PV confirmed by clinical, histological, and immunological findings. Clinical disease activity was defined according to an arbitrary score as follows: active, >30% of the skin affected by lesions, and functional impairment; moderate, 10–30% skin involvement but no functional disability; remission, no active clinical skin lesions for at least 1 month under treatment with <5 mg per day prednisolone or without any treatment. The interval between measurements was 376 days on average (SD 526).

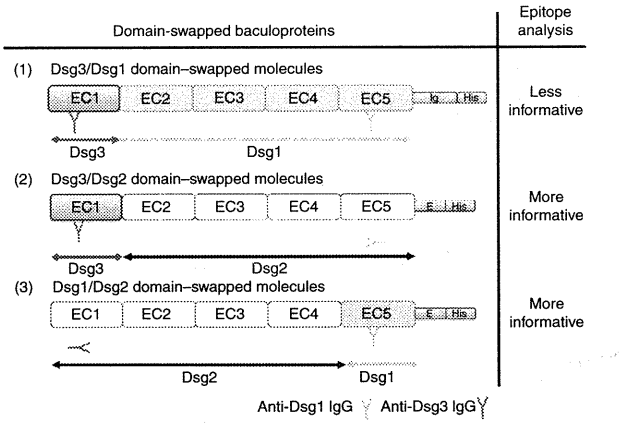


**Figure 5. Schematic diagram of the advantages of new desmoglein 1 (Dsg1)/Dsg2 and Dsg3/Dsg2 domain-swapped molecules.** In mucocutaneous-type pemphigus vulgaris, it is difficult to analyze more informative epitopes using Dsg3/Dsg1 domain-swapped molecules, because both anti-Dsg1 autoantibodies and anti-Dsg3 autoantibodies bind to the same swapped molecules (1). Dsg2 backbone new domain-swapped molecules are able to analyze the epitopes without the influence of other antibodies (2) and (3).

In all, 19 HP and 14 PNP cases were diagnosed by clinical, histological, and immunological findings. All 14 PNP cases possessed IgG autoantibodies against envoplakin and periplakin as assessed by immunoblotting using human epidermal extracts (Hashimoto *et al.*, 1995). All sera were assayed by ELISA using recombinant Dsg1 and Dsg3 baculoprotein as substrates (Ishii *et al.*, 1997; Amagai *et al.*, 1999) and 1,600 dilutions of patients' sera (Cheng *et al.*, 2002).

### Preparation of the plasmid constructs

Production of recombinant Dsg1 (rDsg1), Dsg2 (rDsg2), and Dsg3 (rDsg3) has been described elsewhere (Amagai *et al.*, 1994; Ishii *et al.*, 1997; Ota *et al.*, 2003). To produce the plasmid constructs, we used an overlap-extension PCR technique. For example, to prepare the Dsg3 plasmid construct with the EC3 domain swapped, first we amplified the EC1–2 domains of Dsg2, the EC3 domain of Dsg3, and the EC4–5 domains of Dsg2. The cDNAs for the different domains of Dsg2 and Dsg3 were amplified with appropriate primers (Supplementary Table S1 online) using pQE-hDsg2 and pQE-hDsg3 (Qiagen, Hilden, Germany) as templates. Next, we annealed the PCR products of EC1–2 of Dsg2 and EC3 of Dsg3 several (5 or 7) times, and amplified them with appropriate primers. We then annealed the annealed/amplified products of EC1–2 of Dsg2 and EC3 of Dsg3 and the PCR product of EC4–5 of Dsg2 several times, and amplified them with appropriate primers. Plasmid constructs of the EC1 and EC5 domains were produced by the two-step method of annealing and extension, whereas the constructs of EC2, EC3, and EC4 domains were produced by a three-step method of annealing and extension. The PCR products of Dsg3 domain-swapped molecules were digested with *Ncol*/*Xhol* and ligated to *Ncol*/*Xhol*-cut pQE-Tri expression vector (pQE-hDsg2). These constructs contained an E-tag and a His-tag at their C-terminal region. Sequences were confirmed using an ABI310 genetic analyzer (Applied Biosystems, Carlsbad, CA). Plasmid constructs were designated pQE-Dsg3 EC1/Dsg2, pQE-Dsg3 EC2/Dsg2, pQE-Dsg3 EC3/Dsg2, pQE-Dsg3 EC4/Dsg2, and pQE-Dsg3 EC5/Dsg2.

Domain-specific swapped Dsg1 constructs were prepared according to the previous report (Chan *et al.*, 2010), and designated pQE-Dsg1 EC1/Dsg2, pQE-Dsg1 EC2/Dsg2, pQE-Dsg1 EC3/Dsg2, pQE-Dsg1 EC4/Dsg2, and pQE-Dsg1 EC5/Dsg2.

### Protein production by baculovirus expression

Plasmids were co-transfected with Sapphire baculovirus DNA (Orbigen, San Diego, CA) and Cellfectin reagent (Invitrogen, San Diego, CA) into cultured insect Sf9 cells. A high titer of recombinant baculovirus stock was obtained after several rounds of re-amplification. High Five cells (Invitrogen) cultured in serum-free EX Cell 405 medium (JRH Biosciences, Lenexa, KS) were infected with the recombinant viruses and incubated at 27 °C for 3 days; domain-swapped molecules were secreted into the culture supernatant and stored at −80 °C after cell debris removal by centrifugation. The Dsg3 domain-swapped baculoproteins were designated Dsg3 EC1/Dsg2, Dsg3 EC2/Dsg2, Dsg3 EC3/Dsg2, Dsg3 EC4/Dsg2, and Dsg3 EC5/Dsg2. The Dsg1 domain-swapped baculoproteins were designated Dsg1 EC1/Dsg2, Dsg1 EC2/Dsg2, Dsg1 EC3/Dsg2, Dsg1 EC4/Dsg2, and Dsg1 EC5/Dsg2.

### Immunoblotting

Culture supernatants containing recombinant baculoproteins were fractionated by SDS-PAGE. Mouse anti-E tag mAb (Pharmacia Biotech, Uppsala, Sweden) was used as a primary antibody and alkaline phosphatase–conjugated goat anti-mouse IgG antibody (Zymed Laboratories, San Francisco, CA) as the secondary antibody.

### IP–IB

Culture supernatants containing recombinant baculoproteins were incubated with PV sera and then precipitated with protein G-Sepharose (Amersham Biosciences, Uppsala, Sweden) overnight. After centrifugation, the precipitates were resuspended in SDS sample buffer and boiled for 2 minutes. After centrifugation, the supernatants were applied to SDS-PAGE, and proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The precipitated recombinant baculoproteins were reacted with mouse horseradish peroxidase–conjugated anti-E tag mAb (Amersham Biosciences), and then with the chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA). Finally, the film (Kodak Biomax, Rochester, NY) was exposed to the polyvinylidene difluoride membrane.

### EDTA treatment

A volume of 50 μl of 0.5 M EDTA was added to 400 μl culture supernatants containing the five domain-swapped molecules and incubated at 4 °C for 1 h. The culture supernatants with EDTA were dialyzed against TBS (−). For positive controls with calcium, 400 μl of culture supernatants was added to 50 μl of TBS (−) supplemented with 0.5 mM $CaCl_2$, and dialyzed in TBS (−) with 0.5 mM $CaCl_2$. Subsequently, culture supernatants with or without calcium treatment were analyzed by IP–IB.

### Statistical analysis

The associations among distributions of reactivity of ECs were assessed by the $\chi^2$ test with Bonferroni correction. The associations

between clinical improvement with high ELISA index score and shift from EC1–3 to EC4–5 were assessed by the Fisher exact test. Relationships of reactivity of ECs with clinical disease activity and ELISA index score were assessed by the generalized estimation equation method with AR1 working correlation matrix. Disease activity was treated as an ordinal variable. A $P$-value $<0.01$ was considered statistically significant.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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