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SHORT COMMUNICATION

Characterization of Two Cases of Bullous Pemphigoid Reactive Only with BP230 on Japanese Enzyme-linked Immunosorbent Assays

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Bullous pemphigoid (BP) is characterized clinically by oedematous erythemas and tense, histopathologically subepidermal blisters with eosinophilic infiltration, and immunologically by immunoglobulin G (IgG) autoantibodies to BP230 and BP180 (1, 2). BP230 mediates the interaction between intermediate filaments and BP180 (3). Since BP230 has a cytoplasmic localization, and IgG antibodies cannot access this in intact cells, the production of IgG autoantibodies to BP230 is thought to be a secondary event. In contrast, as BP180 is a transmembrane protein, IgG autoantibodies to BP180 are considered pathogenic (1).

The aim of this study was to characterize 2 BP patients, whose sera originally reacted only with BP230.

CASE REPORTS

Patient 1. An 82-year-old Japanese woman developed diffuse erythemas and multiple tense blisters over her whole body (Fig. 1a). Indirect immunofluorescence (IF) showed circulating IgG autoantibodies reactive with the epidermal side of 1M NaCl-split skin (Fig. 1b). Skin biopsy was not performed. A clinical diagnosis of generalized BP was made. A combination of topical corticosteroid and oral doxycycline and epinastine hydrochloride resulted in complete remission.

Patient 2. An 84-year-old Japanese woman presented with oedematous erythemas and tense blisters exclusively on the dorsal side of her feet, which had been present for 1 month (Fig. S1c[1]). IgG antibodies reacted with the epidermal side of 1M NaCl-split skin (Fig. S1d[1]). A clinical diagnosis of localized BP was made. However, patient 2 was lost to follow-up. Skin biopsy was not performed.

Both patients were negative for BP180, but positive for BP230 on IgG ELISA kits for BP230 and BP180 produced in Japan (Mesacup, Medical & Biological Laboratories (MBL), Nagoya, Japan) (4, 5), (Table SI[1]). To verify this result, the sera was also examined with different IgG ELISA kits produced in Germany (Euroimmun Co. Ltd, Luebeck, Germany) (6, 7). Using these ELISA kits, patient 1 was positive for both BP180 and BP230, whereas patient 2 was negative for both antigens (Table SI[1]).

The BP230 ELISA kit produced by MBL uses full-length bacterial recombinant proteins (RPs) of both N- and C-terminal domains of BP230, while the ELISA kit produced by Euroimmun uses bacterial RP of partial C-terminal domain of BP230. Therefore, to study the reactivity with BP230 in more detail, we performed novel BP230-domain-specific ELISAs (Hayakawa et al., manuscript in preparation) (Table SI[1]). Bacterial RPs of N- and C-terminal domains of BP230 were prepared, as described previously (8). In these ELISAs, patient 1 was negative for the N-terminal domain, but strongly positive for the C-terminal domain, of BP230 (Table SI[1]). Patient 2 was strongly positive for the N-terminal domain and intermediately positive for the C-terminal domain.

The presence of IgE antibodies was further examined by novel techniques (Ohzono et al., manuscript in preparation) (Table SI[1]), utilizing commercially available BP180 and BP230 ELISA kits for IgG antibodies (MBL). Two additional BP patients (patients 3 and 4) and 2 healthy individuals were used as controls. This study showed that, while IgE anti-BP180 antibodies were negative in both patients 1 and 2, IgE anti-BP230 antibodies were slightly positive in patient 1 and strongly positive in patient 2.

Immunoblotting of normal human epidermal extract showed positive IgG reactivity with the 230 kDa BP230 in patient 2, but not in patient 1 (Fig. S1e[1]). In immunoblotting analyses of RPs of monomeric NC16a domain and C-terminal domain of BP180, both patients did not react with either RP (Fig. S1f and g[1]). IgG antibodies in both patients did not show any positive results in immunoblotting analyses using normal human dermal extract, or concentrated HaCaT cell culture supernatant and purified human laminin-332.

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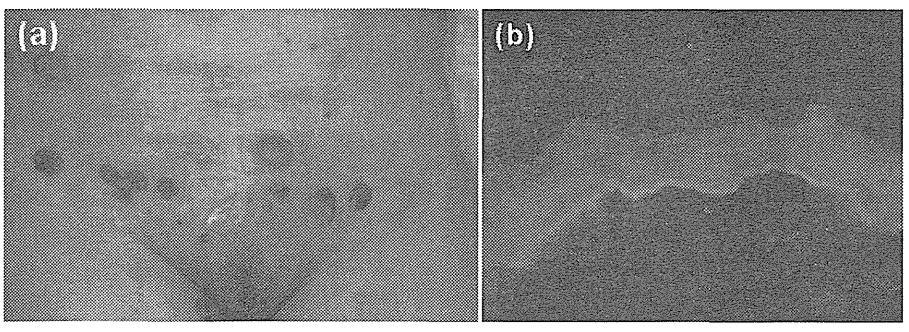

Fig. 1. (a) Clinical features on the abdomen of patient 1. (b) Indirect immunofluorescence (IF) using 1M NaCl-split skin in patient 1 (×100) (For complete figure see Fig. S1[1]).

DISCUSSION

The 2 patients with BP showed distinct results between 2 different commercially available ELISA kits for both BP230 and BP180.

Taking into account the difference in RPs used in the N- and C-terminal BP230 ELISA and C-terminal BP230 ELISA, one can explain the discrepant results that patient 2 was negative in the C-terminal BP230 ELISA, but positive in the N- and C-terminal BP230 ELISA, while patient 1 was positive on both ELISAs. Namely, patient 1 may have high antibodies to the partial C-terminal domain RP of BP230 used in the C-terminal BP230 ELISA, resulting in high relative units in the C-terminal BP230 ELISA. In contrast, patient 2 may have high antibodies to the N-terminal domain of BP230, but low antibodies to the C-terminal domain RP used in the C-terminal BP230 ELISA, resulting in a high index in the N- and C-terminal BP230 ELISA and negative relative units in the C-terminal BP230 ELISA.

Concerning BP180 ELISA kits, MBL ELISA uses monomeric NC16a RP of only one NC16a domain, while Euroimmun ELISA uses tetrameric NC16a RP of 4 tandem repeats of NC16a domain, indicating higher sensitivity in tetrameric NC16a ELISA. This explains well the discrepant result that patient 1 was negative in monomeric NC16a ELISA, but weakly positive in tetrameric NC16a ELISA.

As a mechanism of subepidermal blister formation in BP, binding of anti-BP180 antibodies to BP180 is considered to cause inflammation, internalization of BP180 and signalling events, resulting in dermo-epidermal separation (1, 9, 10). In contrast, circulating IgG anti-BP230 antibodies are thought to be secondary antibodies.

In the present study, patient 2 showed autoantibodies to BP230, but not to BP180, in 2 different ELISAs. Negative reactivity with BP180 was also supported by the results in immunoblotting using epidermal extract, concentrated culture supernatant of HaCaT cells and 2 BP180 RPs. These results tempted us to speculate that anti-BP230 antibodies, at least in patient 2, were pathogenic. Thus far, 3 BP cases with generalized skin lesions, similar to case 1 in this study, were reported to show only anti-BP230 antibodies (11). In addition, several cases of localized BP or pemphigoid nodularis, similar to case 2 in this study, were reported to react only with BP230 (12, 13).

A pathogenic role of anti-BP230 autoantibodies was also suspected in patient 1 when reactivity was first found only with BP230 in the study using the C-terminal BP230 ELISA and monomeric NC16a ELISA. However, the tetrameric NC16a ELISA, with higher sensitivity to BP180, detected relatively low reactivity with BP180 in patient 1. Thus, IgG anti-BP180 autoantibodies may cause the extensive skin lesions in patient 1.

The results of this study indicate that simultaneous use of 2 different ELISA kits may be helpful in making diagnoses and understanding the pathophysiology in individual patients with BP. Further extensive comparative studies between the 2 commercially available ELISA kits for both BP180 and BP230 are in progress.

Pathogenic IgE antibodies to BP antigens were reported to be present in BP sera (14, 15). Therefore, we also performed a preliminary study for IgE antibodies to BP180 and BP230 using a novel technique that detected weak and strong IgE reactivity with BP230 in patients 1 and 2, respectively, while both patients showed negative IgE reactivity with BP180. Interestingly, this reactivity profile for IgE antibodies was similar to that for IgG antibodies. The high IgE antibodies to BP230 may play a role in blister formation in patient 2. However, while patient 1 showed extensive skin lesions, patient 2 showed minimum skin lesions restricted to the dorsal side of the feet, indicating a relatively low pathogenic activity of IgE anti-BP230 autoantibodies. A more extensive study of IgE antibodies to BP180 and BP230 in patients with BP, which may elucidate the pathogenic role of IgE antibodies in BP, is in progress in our institute.

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Homozygous deletion of six genes including corneodesmosin on chromosome 6p21.3 is associated with generalized peeling skin disease

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ABSTRACT

Background: Peeling skin syndrome (PSS) is a rare autosomal recessive form of ichthyosis showing skin exfoliation. PSS is divided into acral and generalized PSS, and the latter is further classified into non-inflammatory type (PSS type A) and inflammatory type (PSS type B). PSS type B is now called peeling skin disease (PSD). Different loss-of-function mutations in the corneodesmosin (*CDSN*) gene have been reported to cause PSD.

Objective: The aim of this study was to determine genetic basis of disease in a 14-year-old Japanese patient with PSD.

Methods and results: Immunohistochemical study showed lack of corneodesmosin (CDSN) in the skin, and standard PCR for genomic DNA failed to amplify *CDSN* product, suggesting CDSN defect. Multiplex ligation-dependent probe amplification and genomic quantitative real-time PCR analyses detected large homozygous deletion of 59,184 bp extending from 40.6 kb upstream to 13.2 kb downstream of *CDSN*, which included 6 genes (*TCF19*, *CCHCR1*, *PSORS1C2*, *PSORS1C1*, *CDSN* and *C6orf15*). The continuous gene lost did not result in additional clinical features. Inverted repeats with 85% similarity flanking the deletion breakpoint were considered to mediate the deletion by non-homologous end joining or fork stalling and template switching/microhomology-mediated break-induced replication. Parents were clinically unaffected and were heterozygote carriers of the same deletion, which was absent in 284 ethnically matched control alleles. We also developed simple PCR method, which is useful for detection of this deletion.

Conclusion: Although 5 other genes were also deleted, homozygous deletion of *CDSN* was considered to be responsible for this PSD.

1. Introduction

Peeling skin syndrome (PSS) is a rare autosomal recessive type of ichthyosis characterized by lifelong peeling of the skin [1,2]. PSS is classified into two major forms; i.e. acral PSS and generalized PSS, and the latter is further divided into non-inflammatory type (PSS type A) and inflammatory type (PSS type B, OMIM #270300) [3,4]. PSS type B nomenclature has recently been modified to peeling skin disease (PSD) [5]. Histopathological examination of skin biopsy of PSS patients often reveal hyperkeratosis and an exfoliation between stratum corneum and stratum granulosum of skin [6]. PSD patients often show raised IgE levels, pruritus, growth failure and food allergies [7]. PSD shows several clinical, histopathological and ultrastructural similarities to Netherton syndrome (NS) [8,9]. However, unlike NS, PSD is not caused by *SPINK5* mutations, does not show gross hair anomalies, and has different immunochemical features [9].

PSD has recently been reported to be caused by mutations in the corneodesmosin (*CDSN*) gene on chromosome 6p21.3, which encodes CDSN [7]. CDSN is a specialized component of corneodesmosomes that play an important role in maintaining structural integrity of stratum corneum [10]. *Cdsn* ablation was found to induce lethal skin-barrier disruption related to desmosome dysfunction in

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mice [11,12]. Using 3-dimensional skin models, Oji et al. demonstrated that lack of CDSN in human causes an epidermal barrier defect [7]. Thus, CDSN is vastly important for the epidermal barrier integrity, and its absence may result in strong predisposition to atopic manifestations [7]. The *CDSN* gene is located approximately 160 kb telomeric to HLA-C on chromosome 6p21.3, spans 5.4 kb and contains 2 exons, both of which are located within *PSORS1C1*, also known as *SEEK1* [13,14]. To date, more than 5 different loss-of-function *CDSN* mutations have been reported in PSD [7,15–18].

In this study, we performed a comprehensive genetic analysis of the *CDSN* region in a Japanese boy with PSD and his clinically unaffected parents to determine the genetic basis of the disease. We here report the identification and characterization of a homozygous large genomic deletion of the entire *CDSN* gene in PSD.

2. Patient and methods

2.1. Patient

The proband was a 14-year-old Japanese male, who referred to ur hospital with peeling skin condition resembling NS. He presented with generalized peeling of skin (Fig. 1A and B), high IgE levels, pruritus, short stature and easily removed hair, consistent with PSD. He was the only child of healthy parents who showed no skin abnormalities. According to the information from the parents, the marriage was not consanguineous. All described studies were performed following guidelines of ethical committee of Kurume University School of Medicine. Written informed consent was obtained from each individual and the study was conducted in accordance with Declaration of Helsinki Principles. Skin biopsy was taken from the patient and blood samples were also collected from the patient and parents.

2.2. Immunohistochemical studies

Deparaffinized and rehydrated sections of formalin-fixed, paraffin-embedded skin tissues mounted on glass slides were autoclaved for 20 min in 10 mM of sodium citrate buffer, pH 6.0, for antigen retrieval. After pretreatment with 3% H_2O_2 in PBS and then with 1% skim milk PBS, the sections were incubated overnight with the primary antibody against CDSN (R&D Systems, Minneapolis, MN, USA) at 4 °C in moist chamber following manufacturer's

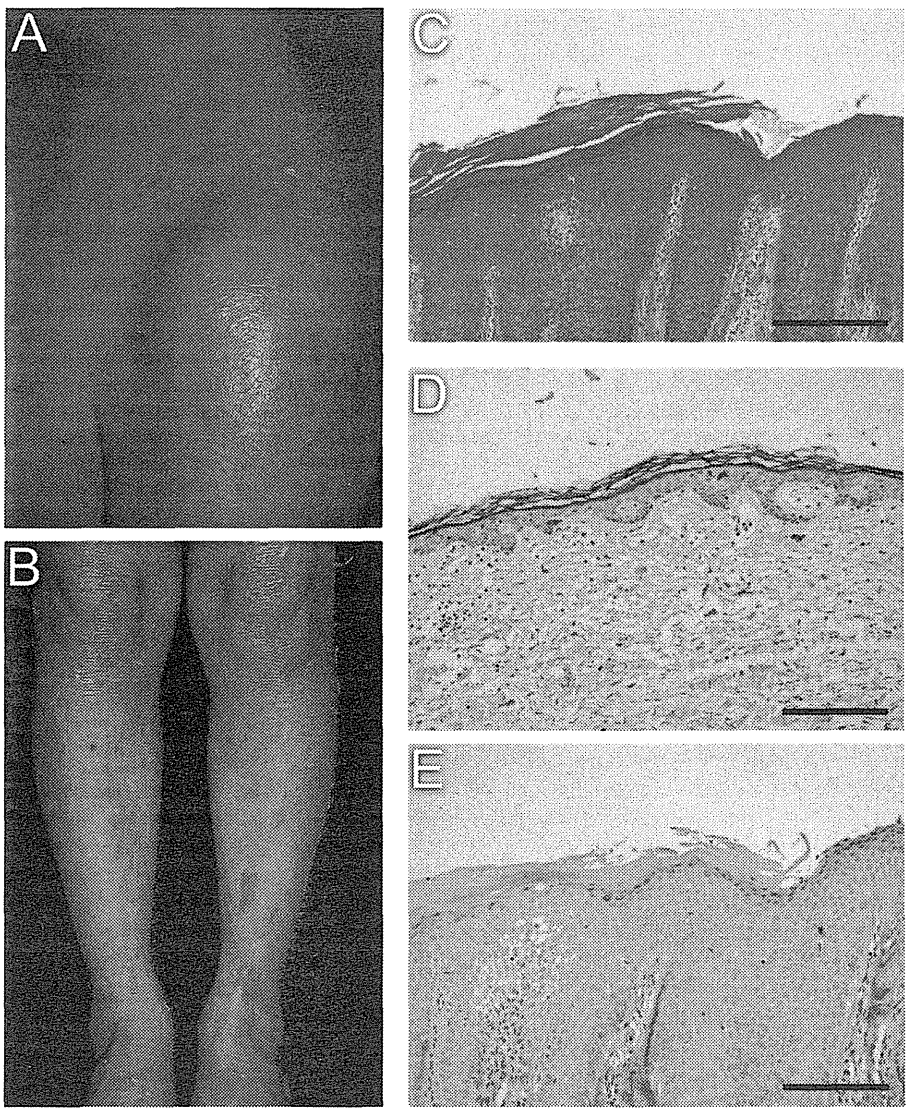

Fig. 1. Clinical presentation and histopathological and immunohistochemical analysis of the patient with PSD. Clinical features on the shoulder (A) and on the legs (B). (C) Histopathology (H&E staining) of skin biopsy showing extensive acanthosis and exfoliation between the stratum corneum and the stratum granulosum. Immunohistochemical studies showing positive staining of CDSN in epidermis of normal human control (D) but lack of CDSN in epidermis of the patient (E). Scale bars; C–E, 200 μm.

recommendation. After three washes with PBS, sections were incubated with 1:100 diluted anti-sheep IgG-HRP (DAKO, Glostrup, Denmark) at room temperature for 30 min and color was developed with 3,3-diaminobenzidine. Slides were counterstained with hematoxylin, dehydrated, cover-slipped, and observed.

2.3. Genomic DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes using QIAamp DNA Blood Kit (Qiagen, Hilden, Germany), according to manufacturer's protocol. For quantitative real-time PCR (qRT-PCR) and multiplex ligation-dependent probe amplification (MLPA) analyses, genomic DNA was cleaned-up with Genomic DNA Clean-up Kit (Macherey-Nagel, Diiren, Germany). Concentration and purity of genomic DNA was analyzed by Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA).

2.4. MLPA analysis

MLPA probes within 100-kb genomic DNA segment on chromosome 6p21.3 (Fig. 3A), which includes *CDSN*, were designed using online MAPD tool [19], with several modifications to default settings, including minimum free energy change (ΔG), GC content of hybridization sequence and maximum perfect matches allowed in genome. Sequences and characteristics of probes are shown in Supplementary Table S1. MLPA analysis was performed with SALSA MLPA P200 Reference Kit (MRC-Holland, Amsterdam, Netherlands) following manufacturer's instructions. Amplified products were separated using ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed by the use of Gene Mapper Software (Applied Biosystems).

2.5. Gene dosage analysis by qRT-PCR

Primers within 100-kb genomic DNA segment on chromosome 6p21.3 (Fig. 3A) for qRT-PCR analysis were designed using online Primer3plus tool [20]. Sequences and characteristics of primers are shown in Supplementary Table S2. qRT-PCR was performed with 4 ng of genomic DNA per reaction in total volume of 20 μl of qRT-PCR mixture containing 0.3 mM of each primer using Thunderbird SYBR Green qRT-PCR Mix (Toyobo, Osaka, Japan) and Light Cycler 480 II Real-Time PCR System (Roche Diagnostics, Mannheim, Germany). Experiments were performed in duplicates and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as internal control for normalization. Gene copy number of normal control was arbitrarily set to 2.

2.6. PCR and DNA sequencing for deletion breakpoint and size

Following qRT-PCR analysis, PCR to analyze gene deletion was performed using forward primer (P6k) upstream and reverse primer (P67k) downstream of *CDSN* (Supplementary Table S2) and ExTaq DNA polymerase (Takara, Shiga, Japan). PCR products were cloned into pGEM-T vector (Promega, Madison, WI, USA) and several clones from the patient and parents were sequenced. Resultant sequence was submitted to BLAST search against human genome. To analyze gene deletion, forward primer, which was common to both normal and deleted alleles (P6k) and reverse primers within (Del-allele-R) and outside (WT-allele-R) deleted region were designed (Table 1). Three primers were used in single tube to perform PCR to differentiate normal, heterozygote and homozygote alleles.

Table 1
Characteristics of primers used for deletion analysis in PSD.

Primer	Sequence	Product size (bp)	
		Wild-type allele	Deletion allele
Forward	CCCTAGACCGTGGGATGGTA[a]		
WT-allele-R	GCTGCCAGACTTATTGACGC[b]	60,679	1495
Del-allele-R	TGCCCAGGGTCACATAACTG[c]	847	0

[a] Same as primer P6k, located in *TCF19*.
[b] Located outside deleted region.
[c] Located in *TCF19* within deleted region.

3. Results

3.1. Histopathological and immunohistochemical findings

Histopathological examination of the patient skin biopsy revealed extensive acanthosis and exfoliation between stratum corneum and stratum granulosum (Fig. 1C). Because clinical and histopathological findings were consistent with PSD, we first performed immunohistochemical study to examine CDSN expression. As expected, CDSN was detected in cornified layers of epidermis in normal human skin (Fig. 1D). In contrast, skin biopsy taken from the patient showed no positive staining (Fig. 1E). These results suggested defect of CDSN in the patient.

3.2. Standard PCR for genomic DNA

To screen for *CDSN* mutations, standard genomic DNA PCR for both exons of *CDSN* was performed using primers shown in Supplementary Table S2. *CDSN* fragments were successfully amplified in both 2 normal controls and the parents (Fig. 2A and B). In contrast, no *CDSN* fragment for both exons was obtained in the patient (Fig. 2A and B). To exclude possibility of genomic DNA degradation, we performed PCR for filaggrin (*FLG*). *FLG* was amplified successfully in all subjects, suggesting that there was no gross degradation genomic DNA (Fig. 2C). Taking immunohistochemical staining and genomic PCR results together, the entire *CDSN* gene was considered to be deleted in the patient.

3.3. MLPA analysis

Since MLPA has been proved to be useful tool for wide range genomic rearrangements and deletion analysis [21,22], we performed MLPA analysis in the patient, parents and 4 normal

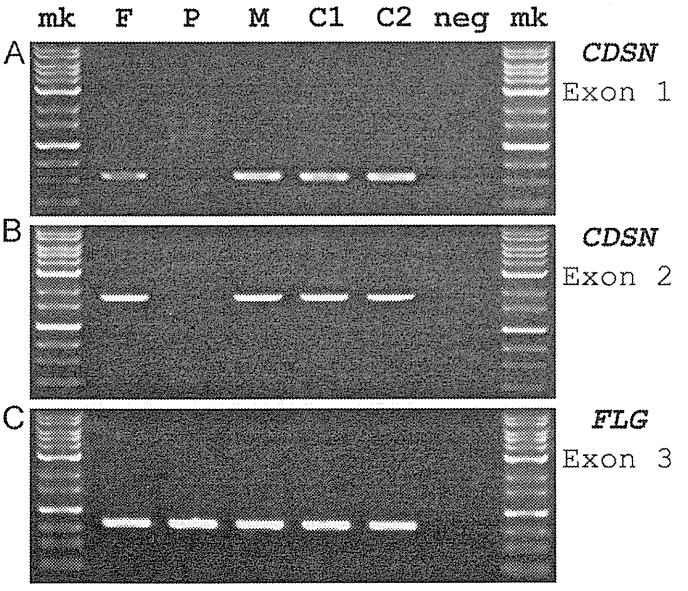

Fig. 2. PCR analyses. Standard genomic DNA PCR analyses for *CDSN* exon 1 (A), *CDSN* exon 2 (B) and *FLG* exon 3 (C). F, father; P, patient; M, mother; C1, control 1; C2, control 2; neg, no DNA; mk, 1 kb DNA size marker.

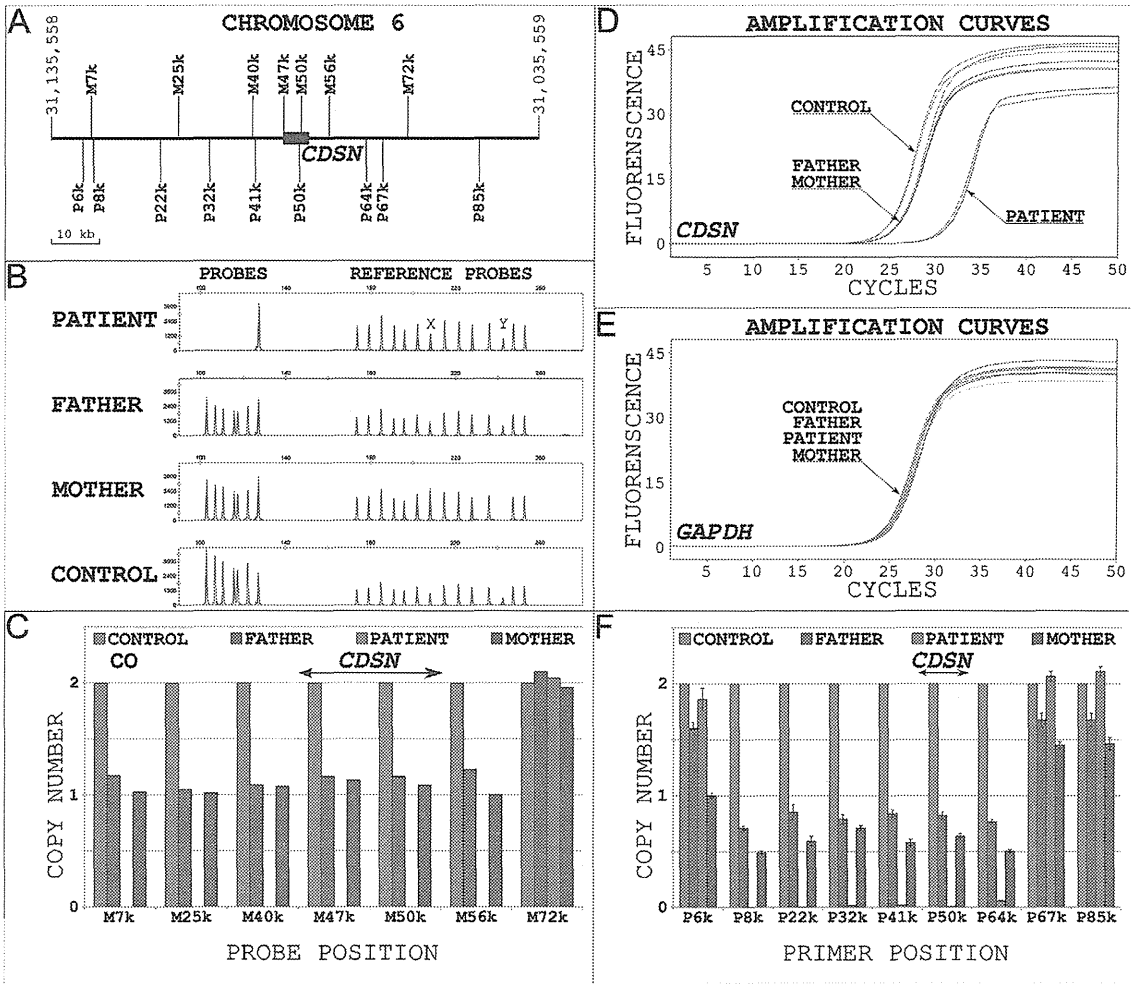

Fig. 3. MLPA analysis and gene dosage analysis by qRT-PCR. (A) Schematic drawing of 100-kb genomic DNA segment on chromosome 6p21.3 and positions of probes and primers used for MPLA and qRT-PCR analyses. *CDSN* is located on opposite strand. The co-ordinates in the genome (Chr6:31,135,558-31,035,559) are indicated. Approximate positions in kb of probes for MLPA (M) and primers for qRT-PCR (P) are indicated. (B) Raw MLPA data within 100-kb genomic DNA segment on chromosome 6p21.3. (C) Histogram of MLPA data showing absence of *CDSN* in patient DNA. Representative qRT-PCR amplification curves for wild-type (control) and heterozygote (parents) and homozygote (patient) for *CDSN* (D) and *GAPDH* (E). (F) Histogram of gene dosage analyses.

controls. Approximate positions of probes for MLPA analysis are depicted in Fig. 3A. Raw MLPA data showed absence of signal for *CDSN* and nearby genomic regions in the patient (Fig. 3B). In contrast, raw signals for MLPA reference probes, including those against X and Y chromosomes were normal in all subjects tested (Fig. 3A). When data were analyzed and normalized according to manufacturer's instructions, probes against both *CDSN* exons and nearby regions produced null gene copy number in the patient (Fig. 3C). The results also showed 1 copy number in parents in contrast to 2 copy number in normal control (Fig. 3C). Three other normal controls showed similar results as those shown in both Fig. 3B and C (data not shown). These results confirmed that the entire *CDSN* gene was homozygously deleted in the patient, and parents were in heterozygote states.

3.4. Mapping of genomic deletion around CDSN

The results of MLPA analysis indicated that large genomic deletion was present, because probes at M7k and M56k did not produce any signals in the patient (Fig. 3B and C). To precisely map the deletion and to determine the size of deletion, we developed and performed comprehensive gene dosage analysis by means of qRT-PCR for 100-kb genomic DNA segment on chromosome 6p21.3 around *CDSN*. Approximate locations of primers are depicted in Fig. 3A.

We found that 4 ng of genomic DNA was sufficient to perform genomic DNA-based qRT-PCR utilizing SYBR green I DNA-binding dye. Theoretically, with equal genomic DNA input, normal individual with 2 alleles should produce gene copy number (normalized ratio multiplied by 2) of 2, whereas heterozygote and homozygote should produce 1 and 0, respectively.qRT-PCR analysis with *CDSN* primers showed the differences in amplification curves; i.e. 1 allele in parents and both alleles in patient were deleted, when compared to normal control (Fig. 3D). In these experiments, amplification curves for *GAPDH* did not differ significantly (Fig. 3E), confirming that similar amounts of genomic DNA were used in each reaction. Amplification curves and Cp values (Fig. 3D and E) yielded calculated gene copy number of 2, 0.82 and 0.01 for control, parents (father) and patient, respectively. Results of our comprehensive qRT-PCR analysis indicated that patient's genomic DNA sample contained neither *CDSN* nor nearby regions (Fig. 3F). Our analysis also revealed that the patient could produce only 2 copy number at primer positions P6k and P67k, suggesting deletion breakpoint was between these two positions and before P8k and after P64k (Fig. 3F).

3.5. Identification of gene deletion breakpoint and size

Using primers upstream and downstream of *CDSN* that produced 2 gene copy number in the patient (P6k forward and

P67k reverse, Supplementary Table S2, Fig. 3A and F), we performed conventional PCR to determine the deletion breakpoint and size. PCR amplification product size was estimated to be between 250 and 5500 bp, if deletion was present. Approximately 2500 bp PCR product was obtained in both the patient and parents, whereas no specific product was obtained in controls (Fig. 4A). Although primers used in this study (Fig. 3A and Supplementary Table S2) would produce over 60 kb product in samples without deletion, the amplification was not feasible under the PCR conditions employed.

By cloning into T-vector and sequencing, we found size of amplified product to be 2465 bp, suggesting that 59 kb sequence was missing. BLAST search analysis revealed deletion breakpoint (Fig. 4B). The same analysis calculated the deletion size as 59,184 bp, extending from 40.6 kb upstream to 13.2 kb downstream of *CDSN* (Fig. 4C). The deletion was larger than that present in an SSTO haplotype (Fig. 4C). Thus, 6 genes (*TCF19* [partially], *CCHCR1*, *PSORS1C2*, *PSORS1C1*, *CDSN* and *C6orf15*) were considered to be homozygously and heterozygously deleted in the patient and parents, respectively (Fig. 4C). To examine the frequency of this deletion in general population, we performed qRT-PCR using primers P50k (deleted) and P6k (not deleted) for 52 ethnically matched normal individuals. Our analysis revealed that the deletion was absent from 104 control chromosomes (data not shown).

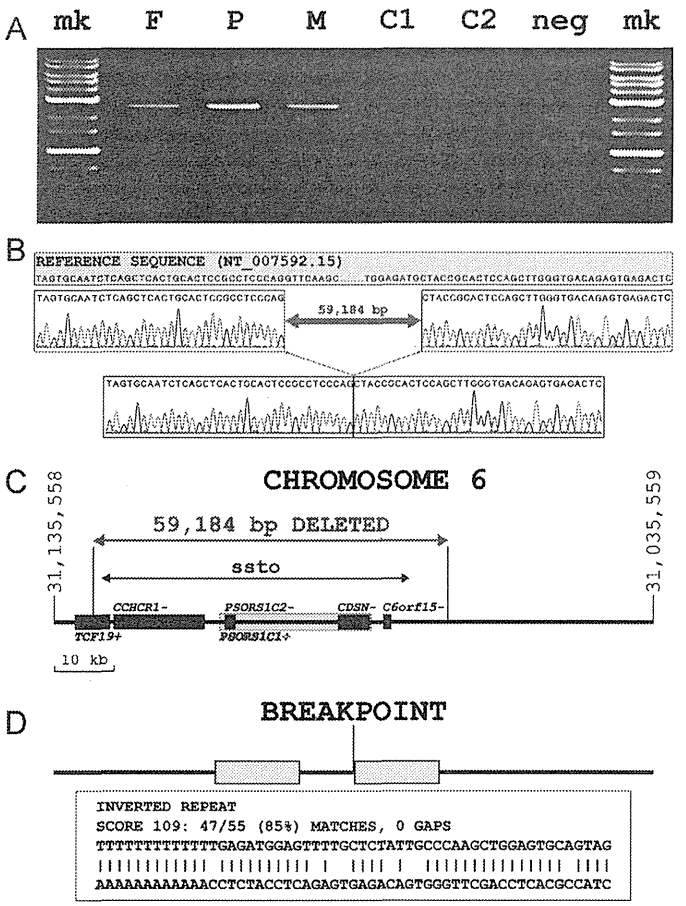

Fig. 4. Characterization of gene deletion, breakpoint and size. (A) PCR for deletion breakpoint. PCR was performed with P6k forward and P67k reverse primers shown in Fig. 3A. F, father; P, patient; M, mother; C1, control 1; C2, control 2; neg, no DNA; mk, 1 kb DNA size marker. (B) BLAST search analysis of patient DNA sequence of the product shown in (A) against human genome. (C) Map of 100 kb genomic DNA segment on chromosome 6p21.3 showing genes deleted in the family. Breakpoints in the family and an SSTO haplotype breakpoints are indicated. (D) Mechanism of gene deletion. Breakpoint flanking sequences were analyzed with EMBOSS software and inverted repeats on both sides of breakpoint are aligned.

3.6. Mechanism of gene deletion

EMBOSS analysis [23] for sequence near deletion breakpoint revealed the presence of large inverted repeat of 55 bp (47/55 matches, equivalent to 85% identity) on both sides of deletion breakpoint (Fig. 4D). In addition, 3 palindrome sequences ranging from 10 to 15 bp were also found on both sides of deletion breakpoint (data not shown). These repeats and palindrome sequences might mediate this deletion in this family.

3.7. Simple PCR to screen for deletion

To analyze this deletion, we developed simple and reliable PCR method. We designed 3 PCR primers: one common forward primer (P6k) and 2 allele specific reverse primers (Table 1 and Fig. 5A). One of the reverse primers (Del-allele-R) is located within deleted region, whereas the other (WT-allele-R) is located outside deleted region (Fig. 5A). Using these three primers in a single tube, subjects with normal alleles produce 847 bp fragment (Table 1). On the other hand, 1495 bp product is produced, when this deletion is present (Table 1). This analysis using the 3 primers showed only 1495 bp band in the patient (Fig. 5B). The parents produced both bands, whereas the normal controls produced only 847 bp band (Fig. 5B). These results confirmed our findings that the patient and parents were homozygote and heterozygotes, respectively, for this deletion. Further analysis using this newly developed PCR method showed that the deletion was absent from 180 additional control alleles (Supplementary Fig. S1).

4. Discussion

In this study, using immunohistochemical, MLPA, qRT-PCR, PCR and DNA sequencing analyses, we identified and characterized a large genomic deletion on chromosome 6p21.3 in a 14-year-old Japanese male with PSD. Size of deletion was calculated to be 59.184 kb and included *TCF19*, *CCHCR1*, *PSORS1C2*, *PSORS1C1*, *CDSN* and *C6orf15*. We also developed simple and reliable conventional PCR method to assess this deletion. By carefully designing 3 primers around breakpoint, 2 products with different sizes were amplified depending on whether subjects were normal, heterozygotes or homozygotes. This diagnostic tool should be useful in

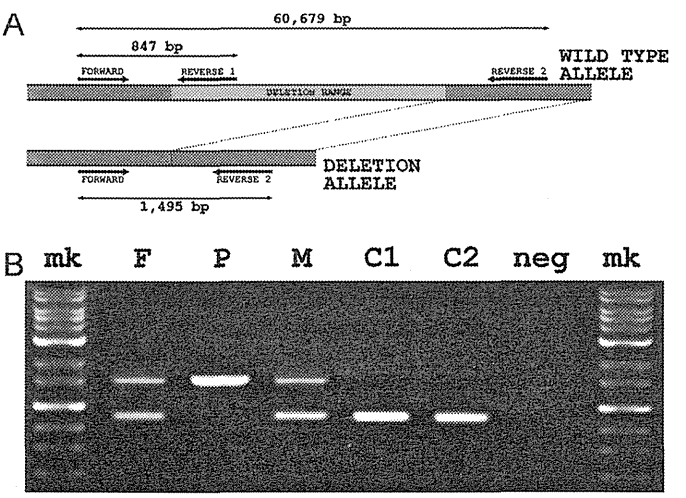

Fig. 5. Development of simple and reliable PCR method to assess the deletion. (A) Strategy used to develop simple PCR method to screen for deletion. PCR was performed with a common forward primer (P6k) and 2 allele specific reverse primers. (B) Three primers were used in single PCR tube to amplify both wild-type and deleted alleles. F, father; P, patient; M, mother; C1, control 1; C2, control 2; mk, 1 kb DNA size marker.

screening for this deletion in PSD patients and may be incorporated into genetic counseling schemes.

Of the deleted genes, *CDSN* was most likely candidate gene for PSD. Recent studies for patients from various geographic regions have identified nonsense or frame-shift mutations at the beginning of *CDSN* coding region, which resulted in complete absence of CDSN and caused PSD [7,15–17]. More recently, expression of non-functional truncated CDSN has also been reported in PSD [18].

It should be noted that individuals carrying just one functional allele usually showed no skin anomalies. In contrast, nonsense heterozygous *CDSN* mutations have been reported in several cases with hypotrichosis simplex of the scalp, in which toxic truncated CDSN aggregates in the dermis caused the abnormality through dominant negative mechanism [24–26]. Furthermore, *CDSN* SNPs have been reported to play a role in the pathogenesis of psoriasis [27,28]. These findings indicate that CDSN is involved in maintaining structural integrity of hair and skin and that different types of mutation in *CDSN* show distinct phenotypes.

Genomic deletion of entire *CDSN* gene in human with PSD has only recently been described [29,30]. There are three major proposed models in mechanisms for genomic rearrangements in human genome; i.e. non-allelic homologous recombination (NAHR), non-homologous end joining (NHEJ) and fork stalling and template switching/microhomology-mediated break-induced replication (FoSTeS/MMBIR) [31]. NAHR is mediated by low copy repeats with recombination hotspots and accounts for most recurrent rearrangements [31,32]. NHEJ breakpoints contain peculiar repetitive genomic architectural elements, including palindromic DNA, stem-loop structures and repeats [31,32]. In contrast to NAHR and NHEJ, FoSTeS/MMBIR events occur only during DNA replication, when active replication fork stalls and newly formed DNA strand switches template [31,32]. When microhomology is present, newly formed DNA anneals to different DNA template, which can be part of completely different open fork [31,32].

In this study, in addition to 3 possible palindrome sequences, we found that inverted sequences with 85% similarity were present on both sides of deletion breakpoint. Such repetitive sequences have been reported in many conditions, including families with incontinentia pigmenti with *NEMO* deletion [33]. The deletion in our case appears to be produced by NHEJ or FoSTeS/MMBIR event at inverted repeats or palindrome sequences. Wada et al. proposed that the deletion in their case may have been caused by Alu-mediated recombination event [30].

We do not yet know how widespread the deletion is. However, the deletion was not found in 284 Japanese normal control chromosomes, suggesting that it segregates with PSD. Screening larger cohorts of control samples from various populations by our simple PCR method would shed light on frequency and mechanism of the deletion. At final stages of preparation of this manuscript, we came across two new reports on Japanese PSD patients apparently with the same or similar deletion as that in our patient [29,30]. This suggests that the deletion is relatively frequent in Japanese PSD patients. In our study, both maternal and paternal DNAs showed the same type of deletion. The deletion may have originated from a common ancestor and may result from a founder effect. On the other hand, it may be a recurrent mutation.

We also noticed that *CDSN* is located in chromosomal region where at least 7 alternative haplotypes are documented in human genome database [34]. They give rise to a variety of sequences with different gaps similar to HLA alleles. An SSTO haplotype did not include *CDSN* and at first we thought this is the case in our family. However, our comprehensive analyses using qRT-PCR and conventional PCR in combination with DNA sequencing showed that the deletion in this family is 9.2 kb larger than that present in the SSTO haplotype. Of note, one of the deleted genes, *TCF19*, is fully present in the SSTO haplotype but it is partially deleted in the studied family. Therefore, this sequence may represent a novel haplotype or an unassigned variant of the SSTO haplotype in the HLA region.

Our study raises an important insight into genes or diseases. Currently, our patient does not show additional clinical features associated with the contiguous gene loss. Some genetic and non-genetic diseases appear later in life. Our patient is only 14-year old and may develop other late onset conditions associated with absence of one or more deleted genes. On the other hand, absence of certain genes may be beneficial. Other genes deleted in this case are not well characterized, except for association of *PSORS1* genes with psoriasis [27,28]. *TCF19*, also known as *SC1*, encodes transcription factor that was postulated to play an important role in later stages of cell cycle progression [35]. There is no known human disease associated with TCF19. Defect of *CCHCR1* upregulated cytokeratins 6, 16 and 17 and changed expression of other genes related to terminal differentiation and cornified cell envelope formation [36,37]. This may cause abnormal keratinocyte proliferation as seen in psoriasis [36,37]. Our patient showed short stature, allergy to foods and easily removed hair, which are all phenotypes of PSD. However, histological finding of thickened epidermis may indicate relationship to hyper-proliferation of keratinocytes and acanthosis, which may predispose to psoriasis [38]. Our study raises the possibility that the patients should be followed-up and may benefit from personalized and systematic therapy to prevent late onset diseases, which may occur due to the absence of one or more of these deleted genes. This also calls for thorough investigation of functions of these other genes and their products.

In this study, we demonstrated usefulness of gene dosage analysis by qRT-PCR, because conventional PCR cannot differentiate between normal and heterozygote states. Although there are limitations, including reduced ratios for heterozygotes in some cases in our analysis, SYBR green I dye-based qRT-PCR proved useful for gene copy analysis and for deletion mapping. Optimizations in stringent primer design, annealing temperature, DNA concentration or primer concentration should improve the qRT-PCR as quicker and more useful tool for gene deletion analysis.

In conclusion, we determined that, genetic basis of PSD in a Japanese patient was due to homozygous deletion of entire *CDSN* gene. Although 5 other genes were absent in this patient, *CDSN* deletion was considered to be responsible for the skin condition by the results of previous reports of PSD [7,15–18]. Our study expanded mutation database of *CDSN* and confirmed important role of CDSN in maintaining stratum corneum structural integrity.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2014.04.003.

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Clinical and immunological profiles in 17 Japanese patients with drug-induced pemphigus studied at Kurume University

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Summary

Background Drug-induced pemphigus (DIP) shows clinical, histopathological and immunological features of pemphigus. However, little is known about immunological profiles in DIP.
Objectives To characterize clinical and immunological profiles in patients with DIP.
Methods We studied 17 Japanese patients with DIP who were treated at Kurume University Hospital or who consulted from other hospitals between 1997 and 2012. Complicated diseases, clinical and histopathological manifestations, responsible drugs and findings in immunofluorescence, enzyme-linked immunosorbent assays (ELISAs), immunoblotting (IB) and prognosis were analysed.
Results Eight of the 17 patients with DIP showed pemphigus foliaceus-like appearance, three showed pemphigus herpetiformis-like appearance, and six showed atypical bullous lesions. Responsible drugs were thiol-containing drugs in 16 patients (bucillamine in nine cases, D-penicillamine in four cases, and cetapril, thiopronine and captopril in one patient each), and a nonthiol drug, sulfasalazine, in one patient. By ELISAs and/or IB analyses, nine patients reacted only with desmoglein 1 (Dsg1), four reacted with Dsg1 and Dsg3, and four showed no specific reactivity. By IB of normal human epidermal extracts, in addition to positive reactivity with Dsg1, four patients with no detectable malignancy showed paraneoplastic pemphigus-like reactivity with the 210-kDa envoplakin and the 190-kDa periplakin. Four cases showed anti-Dsg3 antibodies without mucosal lesions. While 11 cases recovered after discontinuation of the causative drugs, six patients had a very protracted or intractable disease course, and might develop true pemphigus.
Conclusions The present study indicated that the majority of the patients with DIP studied showed a pemphigus foliaceus-type phenotype with anti-Dsg1 autoantibodies, caused by thiol-containing drugs.

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Conflicts of interest
None declared.

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What's already known about this topic?

- Drug-induced pemphigus (DIP) is a rare type of drug eruption. Most patients with DIP show pemphigus foliaceus-like clinical and histopathological features.
- To date, approximately 200 cases of DIP have been described.
- It has been reported that thiol-containing drugs cause pemphigus foliaceus, while nonthiol drugs cause pemphigus vulgaris.

What does this study add?

- We report characteristics of 17 patients with DIP for their background, clinical and histopathological manifestations, causative drugs and the results of immunofluorescence, enzyme-linked immunosorbent assays and immunoblotting.
- The present study indicated that the majority of the patients with DIP studied showed a pemphigus foliaceus-type phenotype with antidesmoglein 1 autoantibodies, caused by thiol-containing drugs.

Drug-induced pemphigus (DIP) is a rare type of drug eruption, which clinically, histopathologically and immunologically mimics pemphigus.[1] Drugs that induce DIP are categorized into thiol-containing drugs and nonthiol drugs.[2] Thiol-containing drugs include D-penicillamine, captopril and thiopronine, while nonthiol drugs include enalapril, penicillin and cephalosporins.[2,3] Some nonthiol drugs contain sulfur, which may undergo metabolic processing to thiols.[4] Active amide and phenol groups also induce DIP.[3–5] It was reported that thiol-containing drugs cause pemphigus foliaceus (PF), while nonthiol drugs cause pemphigus vulgaris (PV).[4]

To date, although about 200 cases of DIP have been described, no systematic studies of large numbers of patients with DIP have been reported, probably because of the rarity of the disease. Moreover, only a few studies characterized autoantigen profiles by immunoblotting (IB) or immunoprecipitation.[4] In this study, we report the characteristics of 17 patients with DIP for their background, clinical and histopathological manifestations, causative drugs and the results of immunofluorescence (IF), enzyme-linked immunosorbent assays (ELISAs) and IB.

Material and methods

This study was approved by the Ethical Committee of Kurume University. Seventeen Japanese patients, who were treated in our hospital or consulted from other hospitals between 1997 and 2012, were enrolled in this study. Patient information included sex, age, time of onset, clinical manifestations, causative drugs and prognosis. Diagnosis of pemphigus was confirmed by clinical and histopathological findings and IF studies.

Type of pemphigus was classified mainly by clinical appearance. Although PV is clinically characterized by flaccid blisters, deep erosions and oral lesions, this study had no cases with PV-like clinical features. The cases with clinical characteristics of superficial blisters, shallow erosions and scaly crusted erythematous patches were categorized as PF-type DIP. Pemphigus herpetiformis (PH) clinically shows pruritic annular erythemas with vesicles in the periphery, and the cases with these features were categorized as PH-type DIP. Atypical bullae and erosions were observed in some cases, which were categorized as unclassified-type DIP.

Direct IF of biopsy skin was performed using the standard method. Indirect IF of normal human skin was performed by the standard method using sera diluted 10–640×. Indirect IF of monkey oesophagus was also performed at serum dilution of 10× only. ELISAs for human desmoglein 1 (Dsg1) and Dsg3 were performed as described previously.[6] In addition, recently developed ELISAs using eukaryotic recombinant proteins of human desmocollin 1 (Dsc1), Dsc2 and Dsc3 were also performed (N. Ishii *et al.*, submitted for publication). Finally, we performed IB of normal human epidermal extracts as described previously.[7]

That all 17 cases were really induced by drugs was evidenced by a combination of drug history and clinical, histopathological and immunological findings. Thiol drugs that are already known to induce pemphigus were administered before the development of skin disease in all cases, except for patient 8. All patients, except for patients 8, 10–12, 14 and 16, recovered after the drugs were stopped. The skin disease was reproduced by readministration of the drug in patients 4 and 8.

We present details for seven cases of DIP, which have not been reported in the English literature and were examined extensively.

Patient 1

A woman in her 60s with a 10-year-history of schizophrenia, who had taken captopril, diltiazem hydrochloride, spironolactone and furosemide for hypertension for 8 years, developed skin lesions 6 months ago. Physical examination revealed vesicles within erythemas on the face, trunk and extremities (Fig. 1a). Histopathology showed subcorneal acantholytic blister (Fig. 1b).

Direct IF showed IgG deposition to the entire epidermis, being stronger in the upper epidermis (Fig. 1c). Indirect IF of both normal human skin and monkey oesophagus detected circulating IgG anticell-surface antibodies. IB of normal human epidermal extracts detected the 160-kDa Dsg1 and weakly the 190-kDa periplakin. At this time, ELISAs were not available. Drug-lymphocyte stimulation test (DLST) for capto-pril showed a negative result.

The diagnosis of captopril-induced PF-type DIP was made. Two months after captopril was discontinued, the skin lesions disappeared.

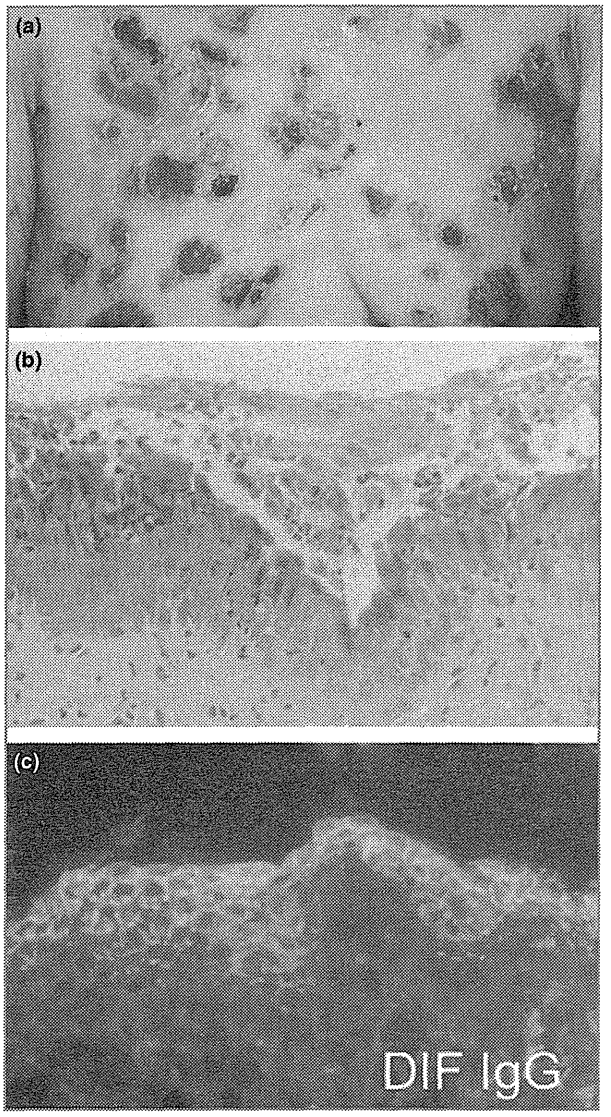

Fig 1. Pemphigus foliaceus-type case. Clinical (a), histopathological (b) and direct immunofluorescence results for IgG (c) of patient 1.

Patient 7

A woman in her 60s taking prednisolone 3 mg daily and bucillamine 100 mg daily for 1 year for rheumatoid arthritis (RA) developed oedematous erythemas 2 months ago. Physical examination revealed multiple exudative erythemas with superficial flaccid bullae and erosions on the lower back and waist (Fig. 2a). Laboratory tests including rheumatoid factor showed normal results, except for positive antinuclear antibodies (80×). Histopathology showed typical PF-like subcorneal acantholytic bullae (Fig. 2b). Direct IF showed deposition of IgG (Fig. 2c) and C3 (Fig. 2d) to the entire epidermis.

Indirect IF of normal human skin and monkey oesophagus detected no circulating IgG antibodies. IB showed relatively weak reactivity only with periplakin. ELISAs detected anti-Dsg1 antibodies (index 26·0, cut-off 14) but not anti-Dsg3 antibodies (index < 5, cut-off 7). DLST was not performed.

The diagnosis of bucillamine-induced PF-type DIP was made. Two months after bucillamine was discontinued, her skin lesions cured leaving pigmentation, and Dsg1 ELISA index became negative. Salazosulfapyridine for RA treatment did not induce any skin lesions.

Patient 10

A woman in her 70s taking D-penicillamine for the last 5 years for RA of 15 years' duration developed pruritic skin lesions 2 months ago. Physical examination revealed vesicles within annular exudative erythemas sized 1–20 cm on the trunk and extremities (Fig. 3a). Laboratory tests showed serum eosinophilia (828 cells mm^{-3}), positive antinuclear antibodies (320×) and slightly elevated rheumatoid factor (37, normal < 30), but were otherwise normal. Histopathology showed typical eosinophilic spongiosis with bullae in the entire epidermis (Fig. 3b). Direct IF showed cell surface deposition to the entire epidermis for IgG and to the lower epidermis for C3.

Indirect IF of normal human skin, but not monkey oesophagus, showed anticell-surface antibodies at a titre of 160×. IB showed no positive reaction. ELISAs showed negative results for both Dsg1 and Dsg3 for initial serum. However, only anti-Dsg1 antibodies later became positive (index 37·0). DLST for D-penicillamine showed negative results.

The diagnosis of D-penicillamine-induced PH-type DIP was made. Three weeks after D-penicillamine was discontinued and oral prednisolone 30 mg daily was started, skin lesions reduced significantly. Then prednisolone was reduced to 20 mg daily and diaphenylsulfone 50 mg daily was added. However, annular erythemas recurred, which were not suppressed by the addition of salazosulfapyridine 1500 mg daily or a combination of minocycline 200 mg daily and niacinamide 900 mg daily. Therefore, prednisolone was increased to 30 mg daily and the other drugs discontinued, leading to remission. The patient still had a few skin lesions 2 years later when taking prednisolone 5 mg daily.

Patient 11

A woman in her 80s taking bucillamine 300 mg daily for RA for the last 8 months developed cutaneous lesions 1 month ago. Physical examination revealed vesicles within the annular exudative erythemas on the trunk and extremities (Fig. 4a). Histopathology showed typical eosinophilic spongiosis with intraepidermal blisters (Fig. 4b). Direct IF showed deposition of IgG (Fig. 4c) and C3 (Fig. 4d) both to the entire epidermis and to the epidermal basal membrane zone (BMZ).

Indirect IF of human skin showed antibodies to cell surfaces but not to the BMZ. Indirect IF of monkey oesophagus showed weak cell-surface reactivity. IB showed relatively weak reactivity only with periplakin. The results of IB using normal human dermal extracts and recombinant proteins of BP180 NC16a and C-terminal domains were all negative. Thus, we considered that the deposition of IgG and C3 to the BMZ in direct IF was lupus bands. ELISAs detected anti-Dsg1

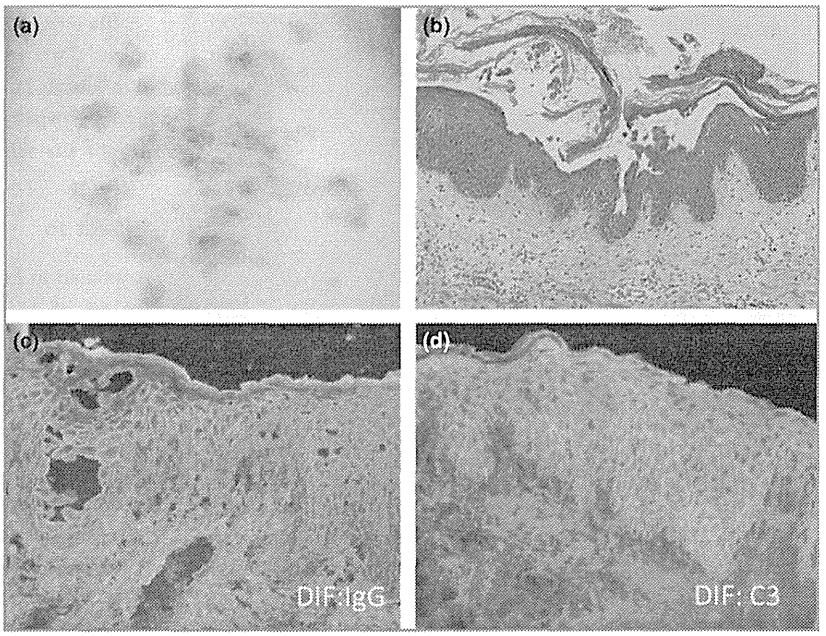

Fig 2. Pemphigus foliaceus-type case. Clinical (a) and histopathological (b) features and direct immunofluorescence results for IgG (c) and C3 (d) of patient 7.

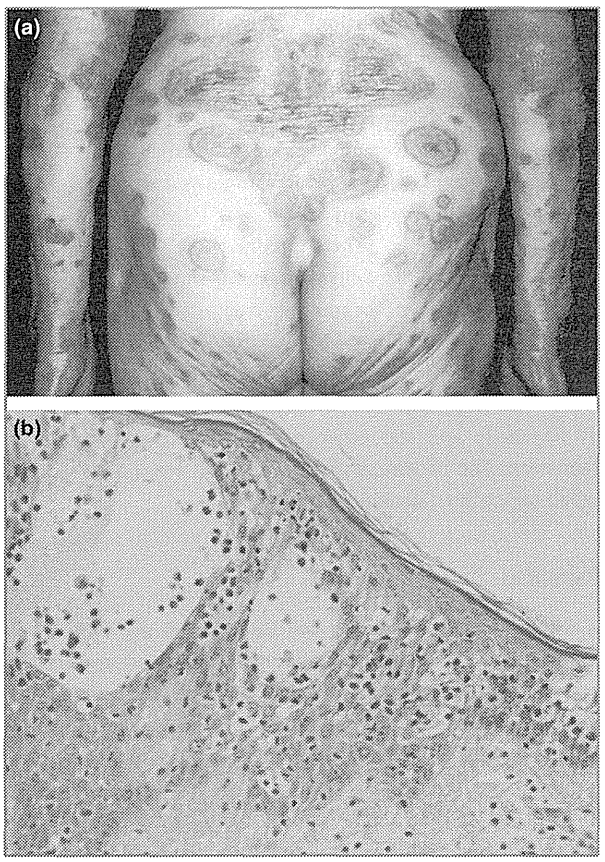

Fig 3. Pemphigus herpetiformis-type case. Clinical (a) and histopathological (b) features of patient 10.

antibodies (index 22·0), but antibodies to Dsg3, BP180 and BP230 were negative. DLST for bucillamine showed a negative result.

The diagnosis of bucillamine-induced PH-type DIP was made. As skin lesions remained 2 weeks after discontinuation of bucillamine, prednisolone 30 mg daily was started, resulting in gradual disappearance of the skin lesions. ELISA indices of Dsg1 decreased to normal levels.

Patient 12

A man in his 50s taking prednisolone 20 mg daily and D-penicillamine 100 mg daily for the last year for RA developed skin lesions 2 weeks ago. Physical examination revealed multiple exudative erythemas with vesicles, erosions and pustules on the scalp, trunk and extremities (Fig. 5a). Histopathology showed suprabasal acantholysis (Fig. 5b). Direct IF showed IgG deposition to the entire epidermis (Fig. 5c). Indirect IF of normal human skin showed anticell-surface antibodies at a titre of 320× and antinuclear antibodies at a titre of 80×. Indirect IF of monkey oesophagus showed only antinuclear antibodies. IB showed relatively weak reactivity only with periplakin. ELISAs detected antibodies to both Dsg1 (index 142·23) and Dsg3 (index 35·84). The stimulation index of DLST for D-penicillamine was 185% (positive > 180%).

The diagnosis of D-penicillamine-induced unclassified-type DIP was made. As skin lesions remained 2 weeks after discontinuation of D-penicillamine, prednisolone 40 mg daily and diaphenylsulfone 75 mg daily were started, resulting in the disappearance of skin lesions. ELISA indices for both Dsg1 and Dsg3 became negative.

Patient 15

A man in his 70s with RA of 10 years' duration was treated with prednisolone 5 mg daily and salazosulfapyridine 1000 mg daily for 4 years. Six months after the initiation of thiopronine 300 mg daily, pruritic skin lesions developed and

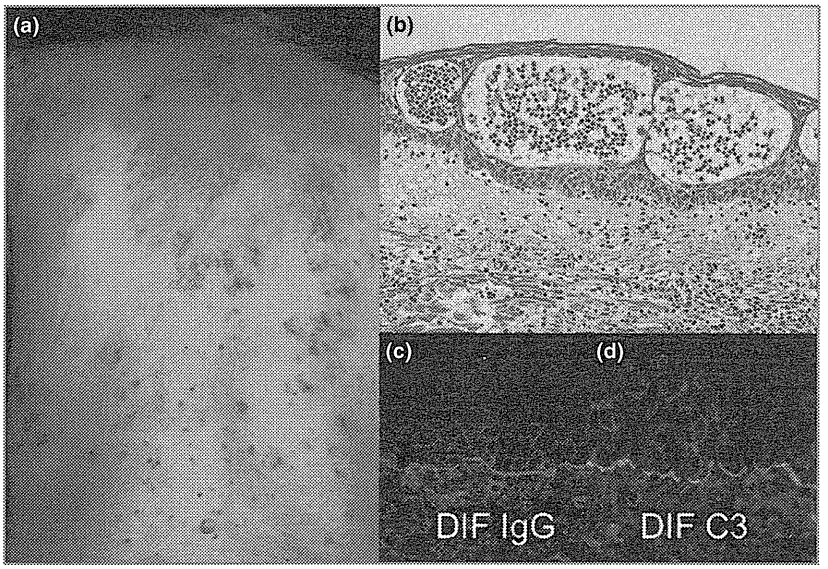

Fig 4. Pemphigus herpetiformis-type case. Clinical (a) and histopathological (b) features and direct immunofluorescence results for IgG (c) and C3 (d) of patient 11.

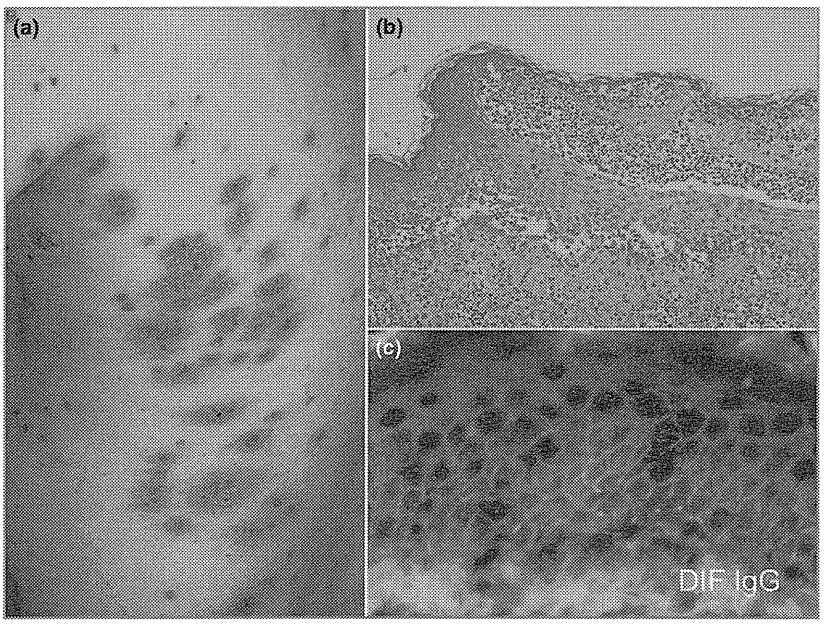

Fig 5. Unclassified-type case. Clinical (a) and histopathological (b) features and direct immunofluorescence results for IgG (c) of patient 12.

gradually worsened. Physical examination revealed erythemas with erosions and scales on the trunk and extremities (Fig. 6a). Clinical features suggested PF or bullous pemphigoid (BP). Histopathology showed eosinophilic spongiosis (Fig. 6b). Direct IF revealed cell-surface deposition to the entire epidermis for IgG (Fig. 6c) and to the lower epidermis for C3 (Fig. 6d).

Indirect IF of both human skin and monkey oesophagus detected no IgG reactivity. IB showed relatively weak reactivity with both envoplakin and periplakin. ELISA indices for Dsg1, Dsg3 and BP180 were all negative. Stimulation indices of DLST were 173% (high but not positive) for thiopronine and 26% (negative) for salazosulfapyridine.

This patient was diagnosed with thiopronine-induced unclassified-type DIP. Discontinuation of thiopronine and salazosulfapyridine with continued prednisolone 5 mg daily led to complete disappearance of his skin lesions within 1 month. Taking prednisolone 5 mg daily and meloxicam 10 mg daily, there was no recurrence.

Patient 17

A man in his 70s taking prednisolone 2·5 mg daily and bucillamine 100 mg daily for 2 years for her RA developed pruritic skin lesions 1 year later, which gradually worsened. Physical examination revealed vesicles, erosions and crusts with erythemas on the trunk and extremities (Fig. 7a). Mucous membranes were not involved. Clinical features suggested PV or BP. Histopathology showed suprabasal acantholysis (Fig. 7b). Direct IF showed cell-surface deposition to the entire epidermis for IgG (Fig. 7c) and to the lower epidermis for C3 (Fig. 7d).

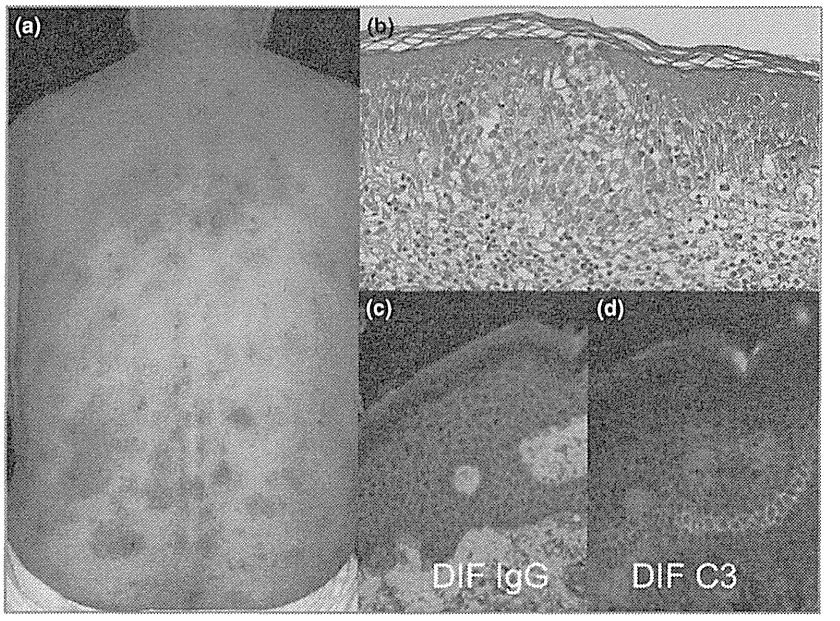

Fig 6. Unclassified-type case. Clinical (a) and histopathological (b) features and direct immunofluorescence results for IgG (c) and C3 (d) of patient 15.

Indirect IF of monkey oesophagus, but not human skin, showed weak cell-surface reactivity. IB showed no specific reactivity. By ELISAs, anti-Dsg1 antibodies were negative, but the anti-Dsg3 antibody index was 11 (intermittent range 7–14). The stimulation index of DLST for bucillamine was 101%.

The diagnosis of bucillamine-induced unclassified-type DIP was made. One month after bucillamine was discontinued and prednisolone 60 mg daily and minocycline 100 mg daily were started, the patient's skin lesions disappeared and his Dsg3 ELISA index became negative. With prednisolone 10 mg daily, there was no recurrence.

Results

All clinical, histopathological and immunological results are summarized in Table 1.

Patient background

This study included eight male and nine female Japanese patients. The average age was 65·9 years (range 26–89). Disease complications are as follows: 12 patients had RA, and one each had systemic lupus erythematosus, systemic sclerosis and adult Still disease.

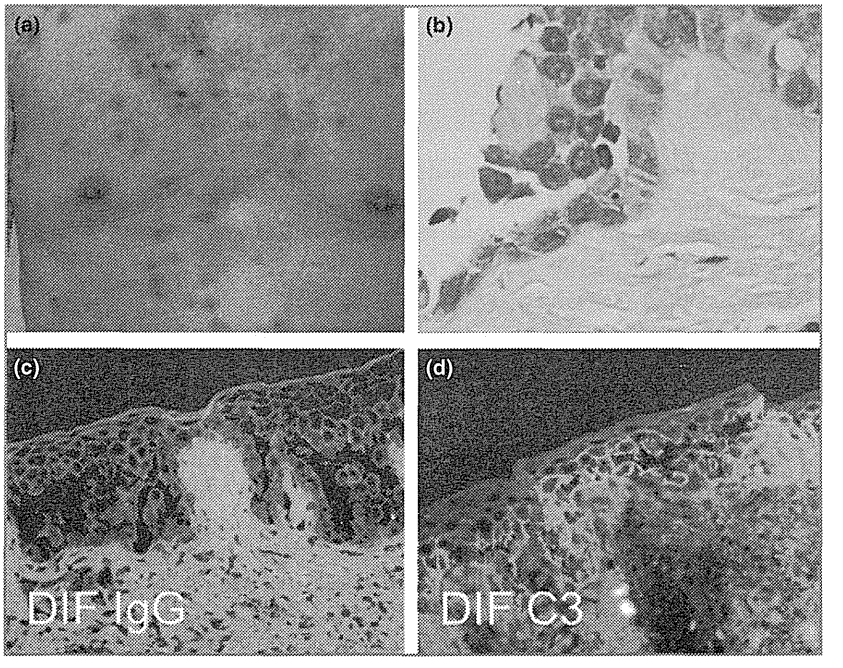

Fig 7. Unclassified-type case. Clinical (a) and histopathological (b) features and direct immunofluorescence results for IgG (c) and C3 (d) of patient 17.

Table 1 Clinical, histopathological and immunological features for 17 patients with DIP

Patient no.	Age (years) /sex	Associated disease	Body sites	Skin lesions	Type	Causative drugs	Duration	Histopathology	DIF	IIF		ELISA indices		ELISA			IB	DLST (SI%)	Prognosis
										HS	ME	Dsg1	Dsg3	Dsc1	Dsc2	Dsc3			
1	65/F	Hypertension, schizophrenia	Face, trunk, limbs	E, V	PF	Captopril	8 years	Subcorneal acantholysis	IgG (e), C3 (ND)	640×	(+)	187	(−)	(−)	(−)	(−)	Dsg1, PPL	(−)	Recover
2	64/M	Hypertension, coniosis, alcoholism	Face, trunk	E, V	PF	Cetapril	Unknown	Liquefaction degeneration	IgG (e), C3 (l)	10×	(+)	18	25·2	0·359	0·163	0·256	Dsg1	ND	Recover
3	26/F	Still disease	Face, trunk, limbs	E, V	PF	Bucillamine	4 months	Subcorneal acantholysis	IgG (e), C3 (l)	80×	ND	ND	ND	ND	ND	ND	Dsg1	ND	Recover
4	64/F	RA	Unknown	E, V	PF	Bucillamine	20 days	Subcorneal acantholysis	IgG (ND), C3 (l)	10×	(+)	29·75	(−)	(−)	(−)	(−)	PPL, EPL	ND	Recover
5	74/M	RA	Trunk	E, V	PF	Bucillamine	9 months	Subcorneal acantholysis	IgG (e), C3 (ND)	160×	(+)	70·68	(−)	(−)	(−)	(−)	PPL, EPL	ND	Recover
6	60/M	RA	Trunk, limbs	E, V	PF	D-penicillamine	10 months	Subcorneal acantholysis	ND	10×	(−)	32·68	(−)	(−)	(−)	(−)	PPL	ND	Recover
7	62/F	RA	Trunk	E, V	PF	Bucillamine	11 months	Subcorneal acantholysis	IgG (e), C3 (e)	(−)	(−)	26	(−)	(−)	(−)	(−)	PPL	ND	Recover
8	68/F	RA	Trunk, limbs	E, V	PF	Azalfidine	5 years	Subcorneal acantholysis	IgG (e), C3 (ND)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	ND	Intractable
9	65/F	Systemic lupus erythematosus	Trunk, limbs	E, V, H	PH	Bucillamine	18 months	Eosinophilic spongiosis	IgG (e), C3 (l)	(−)	(−)	42·07	(−)	(−)	(−)	(−)	(−)	ND	Recover
10	74/F	RA	Trunk, limbs	E, V, H	PH	D-penicillamine	4 years and 8 months	Eosinophilic spongiosis	IgG (e), C3 (l)	160×	(−)	37	(−)	(−)	(−)	(−)	(−)	(−)	Intractable
11	89/F	RA	Trunk, limbs	E, V, H	PH	Bucillamine	7 months	Eosinophilic spongiosis	IgG (e, b), C3 (e, b)	40×	(+)	22	(−)	(−)	(−)	(−)	PPL	(−)	Protracted
12	58/M	RA	Scalp, trunk, limbs	E, V	U	D-penicillamine	12 months	Suprabasal acantholysis	IgG (e), C3 (ND)	320×	(+) ANA	142·23	35·84	(−)	(−)	0·507	PPL	185%	Protracted
13	62/M	RA, renal failure	Trunk, limbs	E	U	Bucillamine	8 months	Subcorneal acantholysis, suprabasal acantholysis	IgG (e), C3 (e)	(−)	(+)	55·2	15·4	(−)	(−)	(−)	PPL, EPL	ND	Recover
14	53/F	Systemic sclerosis	Trunk, limbs	E, V	U	D-penicillamine	16 months	Suprabasal acantholysis	IgG (e), C3 (l)	160×	(−)	75·4	46	(−)	(−)	(−)	Dsg1	ND	Protracted
15	75/M	RA, respiratory failure	Trunk, limbs	E	U	Thiopronine	6 months	Eosinophilic spongiosis	IgG (e), C3 (l)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	PPL, EPL	173%	Recover
16	83/M	RA	Scalp, trunk, limbs	E	U	Bucillamine	17 months	Suprabasal acantholysis	IgG (e), C3 (l)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	Protracted
17	79/M	RA	Trunk, limbs	E, V	U	Bucillamine	12 months	Suprabasal acantholysis	IgG (e), C3 (l)	(−)	(+)	(−)	11	(−)	(−)	(−)	(−)	(−)	Recover
Average age	65·9 years								IgG (15/15), C3 (12/12)	10/17; 58·8%	7/16, 43·7%	12/16, 75%	4/16, 25%	1/16, 6%	1/16, 6%	2/16, 12%	12/17, 70·6%	1/7, 14·3%	

F, female; M, male; RA, rheumatoid arthritis; E, erythema, V, vesicles; H, herpetiform; PF, pemphigus foliaceus; PH, pemphigus herpetiformis; U, unclassified; DIF, direct immunofluorescence; e, entire cell surface; l, lower cell surface; b, basal membrane zone; IIF, indirect immunofluorescence; HS, human skin; ME, monkey oesophagus; ANA, antinuclear antibodies; Dsc, desmocollin; Dsg, desmoglein; ELISA, enzyme-linked immunosorbent assay (Dsg1: positive index value >14, negative < 14, Dsg3: positive index value > 14, intermittent 7–14, negative < 7), Dsc1 ELISA, IgG optical density (OD) < 0·200; Dsc2 ELISA, IgG OD < 0·070; Dsc3 ELISA, IgG OD < 0·120; IB, immunoblotting; PPL, periplakin; EPL, envoplakin; IB of epidermal extracts (Dsg1, 160 kDa; PPL, 190 kDa; EPL, 210 kDa); DLST, drug-lymphocyte stimulation test; SI, stimulation index; ND, not done.

Causative drugs and the time for skin development

Sixteen (94%) of 17 patients with DIP took thiol-containing drugs: bucillamine in nine cases, D-penicillamine in four cases, and cetapril, thiopronine and captopril in one case each. One patient took a nonthiol drug, azalfidine. Nine patients developed skin lesions 20 days to 18 months (mean 9·6 months) after bucillamine was started. Four cases developed skin lesions 10–56 months (mean 23·5 months) after D-penicillamine was started.

Clinical and histopathological characteristics

Mainly from the clinical characteristics for all 17 patients with DIP, we diagnosed eight (47%) patients as PF type, three (18%) as PH type and six (35%) as unclassified type. No patients showed oral mucosal lesions.

Histopathological findings were obtained in all cases. Apparent subcorneal and suprabasal acantholysis was seen in eight and five cases, respectively. Four cases showed eosinophilic spongiosis and one case showed liquefaction degeneration.

Immunological studies

Direct IF and ELISAs were performed at the first visit for each case, except that ELISAs were performed later for patient 1. All indirect IF studies and IB of epidermal extracts were repeated and showed exactly the same results, except for patient 3, for whom sera were not available. Dsc ELISAs were not performed at the first visit in any case.

Immunofluorescence

Direct IF showed deposition to keratinocyte cell surfaces of IgG in all 16 cases examined, and of C3 in all 12 cases examined. Intriguingly, patients 7, 11 and 13 showed C3 deposits to the entire epidermis, while other C3-positive skin biopsies showed C3 deposits to the lower-most epidermis.

By indirect IF of normal human skin, 10 (59%) of 17 cases, including six PF-type cases, two PH-type cases and two unclassified-type cases, showed antikeratinocyte cell-surface antibodies at titres of 10–640×, showing 59% sensitivity. Indirect IF titres showed no statistically significant relation with either DIP groups or disease severity. Indirect IF of monkey oesophagus detected anticell-surface antibodies in seven (44%) of 16 DIP sera with slightly lower sensitivity than that of normal human skin.

Enzyme-linked immunosorbent assays

By ELISAs, five PF-type patients with DIP (patients 1, 4, 5, 6 and 7) showed only anti-Dsg1 antibodies, which is the diagnostic criterion for PF. In contrast, one PF-type patient (patient 2) showed positive results for both Dsg1 (index 18) and Dsg3 (index 25·2). Three PH-type patients (patients 9, 10 and 11) reacted only with Dsg1. Three unclassified-type patients (patients 12, 13 and 14) were positive for both Dsg1 and Dsg3. However, one PF-type and two unclassified-type patients (patients 8, 15 and 16) showed negative results for both Dsg1 and Dsg3, although direct IF revealed cell-surface deposition of IgG. Patient 17 with the unclassified type showed only anti-Dsg3 antibodies (index 11). However, because the ELISA index was very low and no mucous lesion was observed, this case was categorized into unclassified type.

In addition, novel Dsc1–3 ELISAs for 16 sera showed that patient 2 reacted with all desmocollins (Dsc1–3), and patient 12 reacted only with Dsc3, indicating low prevalence of anti-Dsc antibodies in DIP.

Immunoblotting studies

IB of normal human epidermal extracts detected the 160-kDa Dsg1 protein band only in four patients (patients 1, 2, 3 and 14) (Fig. 8). The intensity was generally weaker than in classical PF. We found no reactivity with Dsg3.

Patients 4, 5, 13 and 15 reacted with both the 210-kDa envoplakin and the 190-kDa periplakin, with strong reactivity in patient 4 and minimum reactivity in patient 15 (Fig. 8). This reactivity indicated the diagnosis of paraneoplastic pemphigus (PNP), although none of these cases showed any malignant tumour or PNP-like clinical manifestations. In addition, patients 6, 7, 11 and 12 reacted relatively weakly with periplakin, but not envoplakin, which we considered nonspecific reactivity.

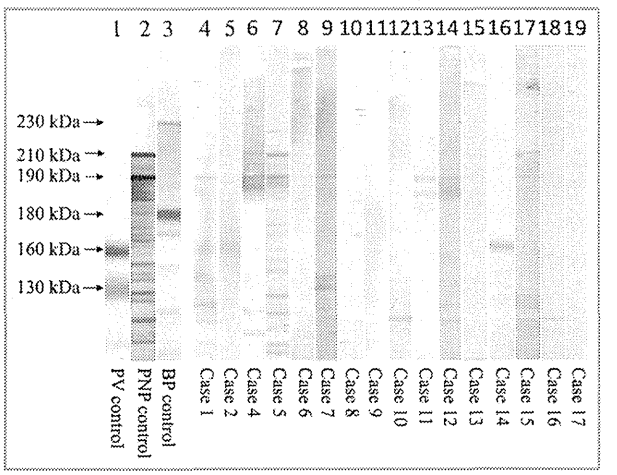

Fig 8. Results of immunoblotting of normal human epidermal extracts. Control pemphigus vulgaris (PV) serum reacted with the 160-kDa desmoglein 1 (Dsg1) and the 130-kDa Dsg3 (lane 1), control paraneoplastic pemphigus (PNP) serum reacted with the 210-kDa envoplakin and the 190-kDa periplakin (lane 2), and control bullous pemphigoid (BP) serum reacted with the 230-kDa BP230 and the 180-kDa BP180 (lane 3). The results of patient 3 with drug-induced pemphigus are not shown, because the sera were not available for this experiment.

Discussion

The results of this study clearly indicate that DIP is basically a PF-like condition. Eight (47%) of 17 patients with DIP presented PF-like clinical manifestations. Seven of the eight cases showed reactivity with Dsg1 by ELISA and/or IB. Although patient 8's serum showed no reactivity with Dsg1, it might have had low anti-Dsg1 antibodies or reacted with epitopes on Dsg1, which were undetectable by ELISA and IB.

Three patients presented with PH-type clinical and histopathological manifestations, and reacted exclusively with Dsg1 by ELISA but not by IB. Our previous study showed that 75% of patients with PH reacted exclusively with Dsg1.[8] Thus, the PH type may represent the second group of Dsg1-related DIP. Therefore, in total, 11 of 17 patients with DIP were considered to have a PF-like condition.

Interestingly, four patients with DIP (patient 2 with PF type and patients 12, 13 and 14 with unclassified type) showed antibodies to both Dsg1 and Dsg3, which is the pattern indicating mucocutaneous-type PV. Although the reason for this discrepant result is unknown, patients 2, 12 and 13 might have nonpathogenic anti-Dsg3 antibodies, and patient 14 might be close to PV. In particular, patient 2 with PF type showed PF-like skin lesions, further indicating that the anti-Dsg3 antibodies were nonpathogenic.

Among six patients with DIP of unclassified-type, ELISA results indicated that patients 12, 13, 14 and 17 are close to PV. In contrast, patients 15 and 16 showed no possible autoantigens.

Direct IF usually shows IgG deposit to the epidermis and C3 deposit to the lower epidermis in common pemphigus cases. Indeed, most patients with DIP showed this pattern. However, patients 7, 11 and 13 showed C3 deposits to the entire epidermis, suggesting that these patients might have a different type of complement activation.

The sensitivity of ELISA and IB for anti-Dsg1 antibodies were 75% and 24%, respectively, consistent with the results in previous studies where about one-third of PF sera reacted with Dsg1 by IB,[9] while ELISA detected anti-Dsg antibodies in almost all PF and PV cases.[6]

IgG anti-Dsc3 autoantibodies induced loss of keratinocyte adhesion,[10] and IgG and IgA anti-Dsc1–3 antibodies were found mainly in atypical pemphigus, PNP or PH.[11,12] Our new IgG ELISA for Dsc1–3 revealed that patient 2 with the PF type reacted with all of Dsc1–3, while patient 12 with the unclassified type reacted only with Dsc3. However, the pathogenic significance of anti-Dsc antibodies in DIP is currently unknown.

Nine cases were given bucillamine. Bucillamine is a disease-modifying drug, which is commonly used to treat RA in Japan. Bucillamine has structural similarities to D-penicillamine but contains one more free sulfhydryl residue, resulting in a therapeutic effect different from D-penicillamine.

Four patients had D-penicillamine, confirming previous studies.[2,3] Three patients took other thiol-containing drugs, cetapril, thiopronine and captopril, which were also known to induce DIP.[2,3] Thus, the responsible drugs for 16 of 17 DIP cases were thiol-containing drugs. Because most of our patients showed PF-related phenotypes, this result may support the previous proposal that thiol-containing drugs preferentially cause PF-type DIP.[4]

By IB, four patients reacted with envoplakin and periplakin, which are detected in PNP.[13] However, these cases showed no clinical features of PNP. Although the pathological significance is unknown, these plakin family proteins may be possible autoantigens in DIP.[14]

DLST was performed in seven of our patients (patients 1, 10, 11, 12, 15, 16 and 17). Only patient 12 showed a positive DLST result for D-penicillamine (stimulation index 185%). Although a positive DLST result helps to define the causative drug, a negative result cannot exclude a possible allergic role of the drugs. Therefore, diagnosis of DIP should be made based on a combination of drug history and clinical, histopathological and immunological findings.

Information of prognosis was available for 11 of the 17 patients with DIP and showed rapid recovery from pemphigus after the causative drugs were discontinued. In contrast, six patients (patients 8, 10, 11, 12, 14 and 16) showed a protracted or intractable disease course and required prednisolone, long after the discontinuation of causative drugs, suggesting the development of true pemphigus. One patient took azalfidine, a nonthiol drug, and might develop true pemphigus. The other five cases had either D-penicillamine (patients 10, 12 and 14) or bucillamine (patients 11 and 16). Seven of nine bucillamine-induced cases showed quick recovery after drug discontinuation, while three of four D-penicillamine-induced cases showed a protracted disease course, indicating that D-penicillamine tends to induce true pemphigus by an unknown mechanism.

Acknowledgments

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