antibodies in the patient sera. Sera from patient 5, but not from patients 1, 2, and 8, reacted with COL7 by immunoblotting of the normal human dermal extract.

We next performed immunofluorescence using RDEB skin sections, similar to a previous study for epidermolysis bullosa acquisita (Liu *et al.*, 2003). The results of representative sera are depicted in Figure 1c, and the results of all sera tested are shown in Supplementary Figure S1 online. Anti-COL7 mAb showed no positive reactivity in RDEB skin, confirming complete lack of COL7 expression (Figure 1c). Anti-laminin γ1 pemphigoid patient sera, but not epidermolysis bullosa acquisita sera, reacted with BMZ in RDEB skin (data not shown). Eight of nine sublamina densa–type LABD sera positive in normal skin did not react with BMZ in RDEB skin, whereas a lamina lucida–type LABD serum reacted with BMZ. One sublamina densa–type LABD patient serum (patient 10) reacted with BMZ on RDEB skin (Figure 1c). None of the sublamina densa–type LABD sera negative in normal skin reacted with BMZ in RDEB skin either.

Although the results of immune-electron microscopy and immunofluorescence of RDEB skin suggested that most sublamina densa–type LABD patient sera reacted with COL7, only a few patient sera reacted with COL7 in immunoblotting of normal human dermal extract. We considered that this discrepancy was due to the loss of conformation of COL7 during the immunoblotting procedure.

We then performed IgA ELISA using the commercially available ELISA kit, which used mammalian recombinant protein with combined NC1 and NC2 domains of COL7 (Medical and Biological Laboratories, Nagoya, Japan). However, in this ELISA, only one patient serum showed positive reactivity (data not shown).

Therefore, we developed IgA ELISA. Trimer form recombinant protein of full-length mammalian COL7 (Siprashvili *et al.*, 2010) and Can Get Signal Immunoreaction Enhancer Solution (Toyobo, Tokyo, Japan) were used for detecting autoantibodies to COL7 in patient sera. The bound autoantibodies were detected by the HRP detection system using anti-human IgA-HRP (Medical and Biological Laboratories, Nagoya, Japan) and TMB (Moss, Pasadena, MD). Technical details are described in Supplementary Materials online.

In this ELISA, eight sublamina densa–type LABD patient sera showed positive results, whereas all 16 normal control sera were negative (Table 1). Lamina lucida type–LABD, bullous pemphigoid, pemphigus vulgaris, and pemphigus foliaceus sera did not show positive reaction in the IgA ELISA analyses (Supplementary Figure S2 online). The results of this ELISA for three representative sera at different dilutions indicated that the results were in a linear range (Supplementary Figure S3 online). The different results between commercial ELISA and developed ELISA in this study may indicate that IgA autoantibodies in patient sera reacted with conformational epitopes in the collagenous domain of COL7 but not NC1 and NC2 domains.

The results of all studies in this study are summarized in Table 1. As previous immunoblotting studies, only 3 of 12 sublamina densa–type LABD patient sera reacted with COL7 by conventional immunoblotting of normal human dermal extract. In contrast, most patient sera were confirmed to react with COL7 by the results obtained in indirect immunofluorescence of RDEB skin and ELISA of COL7 trimer recombinant protein. The results between immunofluorescence and ELISA studies were almost consistent.

In this study, in addition to three patients positive with COL7 in conventional immunoblotting, two additional analyses confirmed the reactivity with COL7 in five patients, although autoantigens in four patients were still unknown. The results in this study indicated that COL7 is the major autoantigen in sublamina densa–type LABD.

All studies were conducted under the approval of the Ethics Committee of Kurume University School of Medicine and according to the Declaration of Helsinki Principles. Written informed consent was obtained from all patients and normal control individuals.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We gratefully acknowledge Ms Michiru Kubo and Ms Kyoko Hiromatsu for the technical assistance, and Ms Mihoko Ikeda, Ms Tomoko Tashima, and Ms Mami Nishida for the secretarial work. We thank the patients for their participation. This study was supported by Grants-in-Aid for Scientific Research (nos. 20390308, 20591331, 21659271, 23591634, 23791298, 23791299, 23791300, 23791301, 24659534, 24591672, 24591640, 24791185), by the Supported Program for the Strategic Research Foundation at Private Universities from the Ministry of Education, Culture, Sports, Science and Technology, and by the "Research on Measures for Intractable Diseases" Project: matching fund subsidy (H23-028 to K. Iwatsuki, and H24-038 to T. Hashimoto) from the Ministry of Health, Labour, and Welfare. The study was also supported by grants from the Kaibara Morikazu Medical Science Promotion Foundation, Ishibashi Foundation, Kanae Foundation for the Promotion of Medical Science, Takeda Science Foundation, Chuo Mitsui Trust and Banking Company, Limited, and Nakatomi Foundation.

Atsunari Tsuchisaka[1], Koji Ohara[1], Norito Ishii[1], Ngon T. Nguyen[2], M. Peter Marinkovich[2,3] and Takashi Hashimoto[1]

[1]Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, Fukuoka, Japan; [2]Department of Dermatology, Stanford University School of Medicine, Center for Clinical Sciences Research, Stanford, California, USA and [3]Division of Dermatology, Palo Alto Veterans Affairs Medical Center, Palo Alto, California, USA.
E-mail: hashimot@med.kurume-u.ac.jp

SUPPLEMENTARY MATERIAL

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

REFERENCES

Guide SV, Marinkovich MP (2001) Liner IgA bullous dermatosis. *Clin Dermatol* 19:719–27

Hashimoto T, Ishiko A, Shimizu H *et al.* (1996) A case of linear IgA bullous dermatosis with IgA anti-type VII collagen autoantibodies. *Br J Dermatol* 134:336–9

Ishiko A, Shimizu H, Masunaga T *et al.* (1996) 97-kDa linear IgA bullous dermatosis (LAD) antigen lozalizes to the lamina lucida of the epidermal basement membrane. *J Invest Dermatol* 106:739–43

Ishii N, Yoshida M, Hisamatsu Y *et al.* (2004) Epidermolysis bullosa acquisita sera react with distinct epitopes on the NC1 and NC2 domains of type VII collagen: study using immunoblotting of domain-specific recombinant proteins and postembedding immunoelectron microscopy. *Br J Dermatol* 150:843–51

Ishii N, Ohyama B, Yamaguchi Z *et al.* (2008) IgA autoantibodies against the NC16a domain of

BP180 but not 120-kDa LAD-1 detected in a patient with linear IgA disease. *Br J Dermatol* 158:1151–3

Liu Y, Shimizu H, Hashimoto T (2003) Immunofluorescence studies using skin sections of recessive dystrophic epidermolysis bullosa patients indicates that the antigen of anti-p200 pemphigoid is not a fragment of type VII collagen. *J Dermatol Sci* 32:125–9

Rusenko KW, Gammon WR, Briggaman RA (1989) Type VII collagen is the antigen recognized by IgA anti-sub lamina densa autoantibodies. *J Invest Dermatol* 92:510, (Abstr.)

Siprashvili Z, Nguyen NT, Bezchinsky MY *et al.* (2010) Long-term type VII collagen restriction to human epidermolysis bullosa skin tissue. *Hum Gene Ther* 21:1299–310

Willsteed E, Bhogal BS, Black MM *et al.* (1990) Use of 1M NaCl split skin in the direct immunofluorescence of the liner IgA bullous dermatoses. *J Cutan Pathol* 17:144–8

Zambruno G, Manca V, Kanitakis J *et al.* (1994) Linear IgA bullous dermatosis with autoantibodies to a 290 kd antigen of anchoring fibrils. *J Am Acad Dermatol* 31: 884–8

Zone JJ, Taylor TB, Kadunce DP *et al.* (1990) Identification of the cutaneous basement membrane zone antigen and isolation of antibody in linear immunoglobulin - A bullous dermatosis. *J Clin Invest* 85: 812–20

RNA-seq Studies Reveal New Insights into p63 and the Transcriptomic Landscape of the Mouse Skin

Journal of Investigative Dermatology (2015) **135,** 629–632; doi:10.1038/jid.2014.384; published online 23 October 2014

TO THE EDITOR

For many years now, researchers have utilized mouse genetic models to study various facets of skin biology—from normal developmental programs to diseased states. Often, these studies have included a global examination of changes in skin gene expression. Although microarrays have been the go-to technology in the past, it has been supplanted by genomic methods driven by next-generation sequencing (NGS) such as RNA-sequencing (RNA-seq; Ozsolak and Milos, 2011). RNA-seq experiments yield higher throughput and more precise measurements of mRNA transcript levels. Hence, a growing number of studies are now utilizing RNA-seq to generate transcriptomic maps of cells, tissues, and organs. Although such studies, particularly under the auspices of the ENCODE project, have covered a large number of biological specimens, to our knowledge, data from mouse skin have been lacking (Stamatoyannopoulos *et al.*, 2012).

To better appreciate the wide spectrum of gene expression levels in mouse skin, we isolated total RNA from dorsal skin dissected from 18.5-day-old embryo (E18.5), post-natal day 3 (P3), and 10-week-old animals. The RNA samples, in duplicate, were then subjected to cDNA library preparation using the TruSeq RNA Sample Preparation Kit (Illumina) and sequenced in parallel using an Illumina HiSeq. Reads were mapped to the reference genome sequence of *Mus musculus* (mm9 build), and transcript read counts were calculated as fragments per kilobase of transcript per million (FPKM) mapped reads.

We next examined the mouse skin RNA-seq data in detail to probe the transcriptional regulatory mechanisms in skin keratinocytes and focused specifically on the transcription factor (TF) p63. p63 is a member of the p53/p63/p73 family and a lineage-specific master regulatory factor highly expressed in stratified epithelia including the skin (Crum and McKeon, 2010; Koster, 2010). Mouse knockouts for p63 have revealed a significant epidermal phenotype during embryogenesis (Mills *et al.*, 1999; Yang *et al.*, 1999; Romano *et al.*, 2012). However, understanding the biological role of p63 has often been hampered by the complexity of the multiple p63 isoforms generated because of alternate promoter usage and alternative splicing (Yang *et al.*, 1998). These p63 isoforms include those possessing a transactivation domain in the N terminus (referred to as the TA isoforms) and those lacking this domain (referred to as the ΔN isoforms), as well as three major splice variants (α, β, and γ) that differ in the C-terminal region (Figure 1). The complexity of the p63 gene products has been a source of confusion and debate over the expression and function of these isoforms during skin development. Our mouse skin RNA-seq data have offered several key insights into this topic.

First, consistent with previous studies (Laurikkala *et al.*, 2006; Romano *et al.*, 2009), our RNA-seq analysis confirmed that ΔNp63 is the primary isoform that is expressed in mouse skin keratinocytes, whereas TAp63 isoforms are barely detectable (Figure 1b and c). Second, the most abundant transcript identified in mouse skin is the longer ΔNp63α isoform, which encodes for a protein that is endowed with the sterile alpha motif (SAM) and transactivation-inhibitory domain (TID) domains. Third, there exists an isoform that has not received much attention, which we have referred to as ΔNp63Δ4. This isoform is derived from an alternate splice donor site within exon 8 of the *Trp63* gene and encodes for a slightly smaller protein product, which lacks 4 amino acids in a segment located between the DNA-binding and the olimerization domains of p63 (Figure 1a). Whether the absence of this 4 amino acids stretch distinguishes the activity of ΔNp63αΔ4 from ΔNp63α or ΔNp63γΔ4 from ΔNp63γ remains to be seen. Finally,

Abbreviations: FPKM, fragments per kilobase of transcript per million; NGS, next-generation sequencing; RNA-seq, RNA-sequencing; NGS, next-generation sequencing; TF, transcription factor

Accepted article preview online 9 September 2014; published online 23 October 2014

BJD
British Journal of Dermatology

Clinical and immunological findings in 104 cases of paraneoplastic pemphigus

A. Ohzono,[1] R. Sogame,[1] X. Li,[1] K. Teye,[1] A. Tsuchisaka,[1] S. Numata,[1] H. Koga,[1] T. Kawakami,[2] D. Tsuruta,[1] N. Ishii[1] and T. Hashimoto[1]

[1]Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan

[2]Department of Dermatology, St Marianna University School of Medicine, Kanagawa, Japan

Linked Comment: Sprecher, *Br J Dermatol* 2015; **173**: 1363–64.

Correspondence
Takashi Hashimoto.
E-mail: hashimot@med.kurume-u.ac.jp

The present affiliation for D.T. is the Department of Dermatology, Osaka City University Graduate School of Medicine, Osaka, Japan

Accepted for publication
1 September 2015

Funding sources
This study was supported in part by Grants-in-Aid for Scientific Research (nos 20390308, 20591331, 21659271, 23591634, 23791298, 23791299, 23791300, 23791301, 24659534, 24591672, 24591640 and 24791185) and the Supported Program for the Strategic Research Foundation at Private Universities from the Ministry of Education, Culture, Sports, Science and Technology; and by the 'Research on Measures for Intractable Diseases' Project matching fund subsidy (H23-028 to K. Iwatsuki and H24-038 to T.H.) from the Ministry of Health, Labour and Welfare. The study was also supported by grants from the Kaibara Morikazu Medical Science Promotion Foundation, Ishibashi Foundation, Kanae Foundation for the Promotion of Medical Science, Takeda Science Foundation, Chuo Mitsui Trust and Banking Company, Limited, and Nakatomi Foundation.

Conflicts of interest
None declared.

DOI 10.1111/bjd.14162

Summary

Background Although there are many reports of sporadic patients with paraneoplastic pemphigus (PNP), only a few systematic studies on large cohorts of patients with PNP have been reported.
Objectives To analyse the clinical and immunological findings in a large cohort of patients with PNP.
Methods This retrospective study consisted of 104 patients with PNP. Clinical and histopathological manifestations, associated neoplasms, complicating diseases, prognosis and results of immunofluorescence, immunoblotting and enzyme-linked immunosorbent assays (ELISAs) were analysed.
Results The clinical and histopathological findings in this study were generally similar to those in previous reports. The most common associated neoplasms included malignant lymphomas, malignant solid tumours and Castleman disease, in that order, while 12 patients had no detectable tumours. Novel ELISAs for desmocollins (Dscs) showed that 19 (18·6%), 42 (41·2%) and 62 (60·8%) of 102 patients with PNP showed antibodies to Dsc1, Dsc2 and Dsc3, respectively. Thirty-two (60%) of 53 patients had antibodies to alpha-2-macroglobulin-like protein 1 (A2ML1). We found statistically significant correlations between positive desmoglein 3 reactivity and genital lesions, and between positive desmoglein 3 reactivity and bronchiolitis obliterans.
Conclusions We consider that antibodies to Dscs and A2ML1 are useful for the diagnosis of PNP.

What's already known about this topic?

- Paraneoplastic pemphigus (PNP) is an autoimmune bullous disease with polymorphous mucocutaneous lesions associated with either benign or malignant neoplasms.
- Bronchiolitis obliterans with progressive respiratory failure is a cause of death in PNP.

What does this study add?

- Several patients with PNP with typical clinical and immunological features did not show detectable neoplasms.
- Antidesmocollin autoantibodies and antialpha-2-macroglobulin-like protein 1 antibodies are useful for the diagnosis of PNP.

Paraneoplastic pemphigus (PNP) is an autoimmune bullous disease presenting polymorphous mucocutaneous lesions associated mainly with lymphoproliferative neoplasms.[1] PNP shows clinically severe mucocutaneous lesions that resemble pemphigus vulgaris, erythema multiforme and lichen planus. Associated neoplasms include non-Hodgkin lymphoma, chronic lymphocytic leukaemia, Castleman disease and thymoma.[2] Histopathological findings are variable and include acantholytic blisters and dyskeratosis in the epidermis and subepidermal bullae. Indirect immunofluorescence (IIF) detects IgG antibodies reactive with the cell surfaces of normal human skin and with transitional epithelium of rat bladder.

The main autoantigens in PNP are the plakin family proteins [plectin, desmoplakins I and II, bullous pemphigoid antigen (BP)230, envoplakin and periplakin] and desmogleins (Dsgs) 1 and 3.[3–5] Recently, alpha-2-macroglobulin-like protein 1 (A2ML1) has been identified as the 170-kDa PNP antigen.[6] Progressive respiratory failure caused by bronchiolitis obliterans (BO) frequently leads to a fatal outcome in PNP.

In 1990, Anhalt *et al.*[1] suggested five criteria to define PNP: (i) painful stomatitis and polymorphous cutaneous eruption with lesions that may be blistering or lichenoid or may resemble erythema multiforme or drug eruption; (ii) histopathological findings that reflect the variability of the cutaneous lesions, showing acantholysis and keratinocyte necrosis, and vacuolar interface dermatitis, (iii) direct immunofluorescence (DIF) demonstrating a deposition of IgG and complement in epidermal cell surfaces, and often granular/linear complement deposition along basement membrane zone (BMZ); (iv) serum autoantibodies that bind the cell surfaces of skin and mucosae in a pattern typical for pemphigus, but additionally bind to simple, columnar and transitional epithelia; and (v) serum autoantibodies reacting with Dsg1 and Dsg3, as well as the plakin family proteins, including desmoplakins I and II, envoplakin, periplakin, BP1 and plectin. Furthermore, Anhalt suggested revised criteria for the diagnosis of PNP.[2] However, many reported patients with PNP did not satisfy these criteria. Thus, although several case studies have been reported, only a few systematic studies have been performed with reference to PNP. In this study we collected and retrospectively studied data from 104 patients with PNP for clinical and histopathological findings and the results of various immunological tests, and performed extensive statistical analyses.

Material and methods

This retrospective study of patients with PNP over a period of 16 years was approved by the ethical committee of Kurume University. We examined 104 patients with PNP who visited Kurume University Hospital or were referred for immunoserological tests from other hospitals in Japan, Korea, the U.S.A. and European countries between January 1997 and April 2013. Final diagnoses of PNP were made at either Kurume University Hospital or other hospitals.

Recently, Poot *et al.* proposed that the detection of autoantibodies against envoplakin, periplakin and A2ML1 by immunoprecipitation is sensitive for PNP.[7] We have reported that envoplakin and periplakin were the major PNP autoantigens with high diagnostic significance.[8] We suggested that the detection of envoplakin and periplakin is an important tool to diagnose PNP in immunoblotting (IB). Moreover, we have experienced cases in which an associated neoplasm was not found, but PNP was suspected both clinically and immunologically. Therefore, in this study, we selected patients with PNP who satisfied two inclusion criteria: (i) envoplakin and periplakin were positive by IB of normal human epidermal extract and (ii) severe lesions were present on at least one mucous membrane. This study enrolled 104 patients, who showed mostly typical clinical, histopathological and immunological features of PNP. All sera were stored at −30 °C or −80 °C, and aliquots with 0·1% sodium azide as a preservative were kept at 4 °C during the experiments.

Firstly, we clinically analysed the patients in terms of age, symptoms, clinical course, associated neoplasms, complications, treatments and prognosis. Then we examined their histopathological and DIF features. For serological tests, we performed IIF of normal skin, 1 mol L^{-1} NaCl-split normal human skin and rat bladder using standard methods. IB analyses of normal human epidermal extract were performed as described previously.[9] IgG enzyme-linked immunosorbent assays (ELISAs) for Dsg1 and Dsg3 were performed using commercially available kits (MBL, Nagoya, Japan).[10] The cut-off index values were 14·0 for Dsg1 and 7·0 for Dsg3. In addition, recently developed ELISAs of mammalian recombinant proteins of human desmocollin (Dsc)1, Dsc2 and Dsc3 were also performed.[11] In this study, our cut-off values were calculated as the mean + 3 SD, and were 0·2 for Dsc1, 0·07 for Dsc2 and 0·12 for Dsc3.

We statistically compared differences in various clinical features and immunological results using the Mann–Whitney rank sum test, Student *t*-test, χ^2-test and Pearson's correlation using SigmaPlot 12.0 software (Hulinks, Inc., Tokyo, Japan). *P*-values < 0·05 were considered statistically significant.

Results

The age, sex, presence of BO, clinical outcome and immunological results for all 104 patients with PNP are shown in Table S1 (see Supporting Information).

Patient background

Among the 104 patients, 34 (32·7%) were male and 59 (56·7%) female, with the sex not reported for the remaining 11 (10·6%) patients. The patient's age was available in 92 cases (88·5%), and the range was 11–83 years (average 56·7). Regarding complications, seven patients had myasthenia gravis, six had hypertension, three had type 2 diabetes mellitus and one each had hypothyroidism, pulmonary emphysema, optic neuritis and lung tuberculosis.

Mucocutaneous lesions

All 104 patients with PNP were reported to have severe lesions on at least one mucosa. Reliable information for mucocutaneous lesions was obtained for 88 (84·6%) of the 104 patients. Eighty-two (93%) of 88 patients described had oral lesions, while the remaining six (7%) were free from oral lesions (Table 1). Twenty-four (27%) of 88 patients had only mucosal lesions. Thirty-three of 81 (41%), nine of 77 (12%) and 28 of 79 patients (35%) had ocular, nasal and genital lesions, respectively (Table 1).

Fifty-nine (67%) of 88 patients showed cutaneous lesions in addition to mucosal lesions. Fifty-two (60%) and 46 (53%) of 87 patients had skin lesions on the trunk and extremities, respectively. Thirty-seven (61%) of 61 and 24 (41%) of 59 patients showed erythemas and blisters, respectively. In the 32 cases in which adequate clinical manifestations were described, 18 (56%), nine (28%), four (13%) and one (3%) showed erythema exsudativum multiforme-like, pemphigus vulgaris-like, lichen planus-like and bullous pemphigoid-like skin lesions, respectively (Table 1).

Associated neoplasms

Associated neoplasms are summarized in Figure 1. Of 104 patients with PNP, some kind of associated neoplasm was found in 88 (84·6%); malignant lymphoma was suspected in four (3·8%); and in 12 patients (11·5%) no tumour was found. Eight patients (7·7%) had double cancers. Therefore, among the patients in whom some kind of associated neoplasm was found, the total number of associated neoplasms was 96. Regarding haematological tumours, 43 (45%) of 96 were various types of malignant lymphomas, including follicular lymphoma. Fourteen (15%) of 96 were Castleman disease and seven (7%) were various types of leukaemia. Five (5%) of 96 were chronic lymphocytic leukaemia, although classification was not available in two tumours. One neoplasm (1%) was a primary macroglobulinaemia.

Table 1 Summary of mucosal lesions and clinical phenotype in patients with paraneoplastic pemphigus

	Patients, n/N (%)
Site of mucosal lesions	
Oral	82/88 (93)
Ocular	33/81 (41)
Nasal	9/77 (12)
Genital	28/79 (35)
Clinical phenotype	
Erythema exsudativum multiforme-like	18/32 (56)
Pemphigus vulgaris-like	9/32 (28)
Lichen planus-like	4/32 (13)
Bullous pemphigoid-like	1/32 (3)

Malignant solid tumours represented 16 (17%) out of 96. The malignant solid tumours were two different types of lung cancer, gastric cancer, uterine cancer, uterine cervix cancer, laryngeal cancer, gall bladder cancer, renal cancer, colon cancer, ovarian cancer, breast cancer, thyroid cancer, oesophageal carcinoma, gastrointestinal stromal tumour, malignant thymoma and basal cell carcinoma.

Seven (7%) of 96 were thymoma, six (6%) were various types of sarcoma, and one each (1%) was fibrous histiocytoma and myofibroblastoma.

Treatments

Regarding treatment of the associated tumours, eight and four patients underwent tumour resection and radiotherapy, respectively. Regarding the treatments for PNP, 49 and 21 patients underwent oral corticosteroids and steroid pulse therapy, respectively. In addition to the steroid treatments, four and two patients were given immunosuppressive drugs and diaminodiphenylsulfone, respectively. In addition, 11, six and three patients underwent intravenous immunoglobulin, plasmapheresis and rituximab treatment, respectively.

Prognosis

In this study, 40 patients (38·5%) died and 36 (34·6%) survived, while no information for prognosis was available for the remaining 28 (26·9%) (Table S1; see Supporting Information). BO occurred in 20 (19·2%) of 104 patients. Sixteen (40%) of 40 patients with fatal outcome died of BO. Thus, four (20%) of the 20 patients with BO survived, according to the available information. The other 24 (60%) of these 40 patients died either of infection (mainly pneumonia) or of associated tumours.

Histopathological features

Histopathological findings were obtained from 61 patients. Thirty-two (52%) of 61 patients showed intraepidermal bullae and nine (15%) showed subepidermal bullae. Additionally, 35 (57%), 28 (46%) and 11 (18%) of these 61 patients showed acantholysis, epidermal cells necrosis and liquefaction degeneration, respectively.

Direct immunofluorescence

DIF was performed on 51 patients. Twenty-two (43%) showed both keratinocyte cell-surface and epidermal BMZ deposits of immunoglobulins and/or C3, while 19 (37%) showed only keratinocyte cell-surface deposits and 10 (20%) showed only epidermal BMZ deposits.

Specifically, deposits of IgG, C3, IgA and IgM to epidermal keratinocytes were detected in 34 (67%), 18 (35%), six (12%) and three (6%) patients, respectively, whereas deposits of IgG, C3, IgA and IgM to the epidermal BMZ were detected

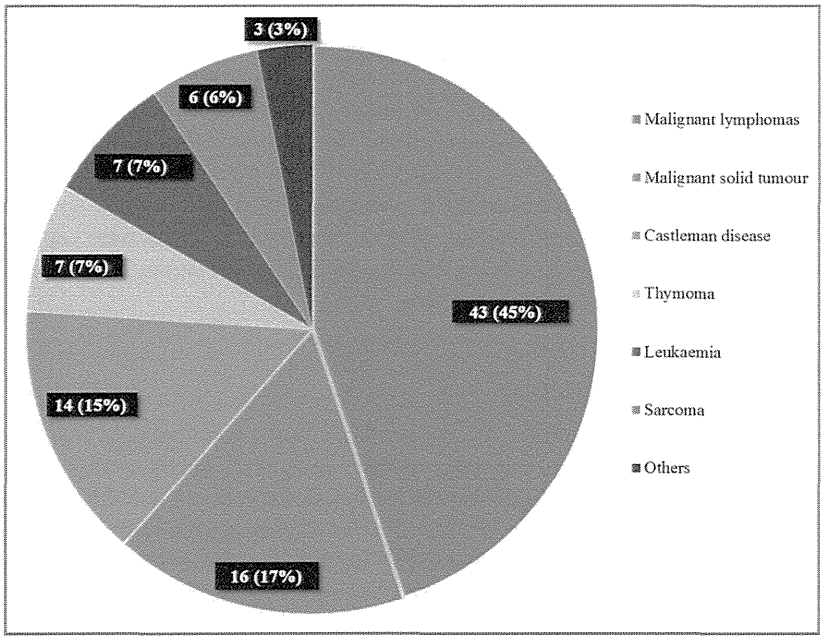


Fig 1. Graphical representation of the numbers of associated neoplasms in patients with paraneoplastic pemphigus. Eight patients had double cancers. The total number of associated neoplasms was 96.

in 17 (33%), 26 (51%), five (10%) and two (4%) patients, respectively.

Indirect immunofluorescence

IIF of normal human skin and rat bladder was performed on all 104 patients (Table 2). With regard to IIF of normal human skin, circulating IgG anticell-surface autoantibodies were found in 69 patients (66·3%), IgG anti-BMZ autoantibodies were found in four (3·8%), and occurrence of both antibodies was found in one patient (1·0%). IIF of rat bladder showed positive reactivity in 83 (79·8%) of the 104 patients.

Immunoblotting

In this study, we selected patients with PNP who satisfied the two inclusion criteria that envoplakin and periplakin were positive by IB. Therefore, all 104 patients showed a doublet of the 210-kDa envoplakin and the 190-kDa periplakin by IB of normal human epidermal extract. In addition, nine patients (8·7%) each reacted with the 230-kDa BP230 and the 130-kDa Dsg3, four (3·8%) reacted with the 180-kDa BP180 and one patient (1·0%) each reacted with the 250-kDa desmoplakin I and 160-kDa Dsg1 (Table 2).

Enzyme-linked immunosorbent assays for desmogleins 1 and 3

ELISAs for Dsg1 and Dsg3 were performed on all 104 patients (Table 2). Thirty-four (32·7%) and 82 (78·8%) patients had antibodies to Dsg1 and Dsg3, respectively. Twenty-seven patients (26·0%) were positive for both Dsg1 and Dsg3, whereas 15 patients (14·4%) were negative for both Dsg1 and Dsg3.

Enzyme-linked immunosorbent assays for desmocollins 1–3

We have previously reported the results of novel ELISAs for Dsc1, Dsc2 and Dsc3 for 79 patients with PNP.[11] In the present study, the ELISAs were performed on 102 patients (Table 2). Nineteen (18·6%), 42 (41·2%) and 62 (60·8%) patients revealed positive reactivity with Dsc1, Dsc2 and Dsc3, respectively. Ten patients (9·8%) were positive for all Dscs, while 29 (28·4%) were negative for all Dscs.

Antialpha-2-macroglobulin-like protein 1 antibodies

Fifty-three of the 104 patients with PNP in this study had already been examined for anti-A2ML1 antibodies in our previous study.[12] Thirty-two (60%) of the 53 patients had antibodies to A2ML1.

Statistical analyses

Correlations between various clinical features and the results of all ELISAs were first analysed statistically (Table S2; see Supporting Information). Correlations with statistically significant differences between clinical features and all ELISAs are shown. Nasal lesions were found only in patients with PNP without anti-Dsg1 antibodies ($P = 0·043$). Higher frequencies of genital lesions ($P = 0·015$) and BO ($P = 0·049$) were found in patients with anti-Dsg3 antibodies. A higher frequency of oral lesions was found in patients with PNP without anti-Dsc1 antibodies ($P = 0·007$). Patients without anti-Dsc2 antibodies showed a lower frequency of ocular lesions ($P = 0·003$), while patients without anti-Dsc3 antibodies showed a lower frequency of skin lesions on the trunk ($P = 0·038$) and a lower frequency of ocular lesions ($P = 0·012$). Table 3 shows

Table 2 Summary of indirect immunofluorescence, immunoblotting and enzyme-linked immunosorbent assays (ELISAs) in patients with paraneoplastic pemphigus

	Patients, n (%)
Indirect immunofluorescence (n = 104)	
Positive to keratinocyte cell surface (IgG)	69 (66·3)
Positive to basement membrane zone (IgG)	4 (3·8)
Positive to keratinocyte cell surface and basement membrane zone (IgG)	1 (1·0)
Positive to rat bladder (IgG)	83 (79·8)
Immunoblotting (n = 104)	
230-kDa BP230	9 (8·7)
130-kDa Dsg3	9 (8·7)
180-kDa BP180	4 (3·8)
160-kDa Dsg1	1 (1·0)
250-kDa desmoplakin I	1 (1·0)
ELISA for Dsg1 and Dsg3 (n = 104)	
Positive to Dsg1	34 (32·7)
Positive to Dsg3	82 (78·8)
Positive to Dsg1 and Dsg3	27 (26·0)
Negative to Dsg1 and Dsg3	15 (14·4)
ELISA for Dsc1–3 (n = 102)	
Positive to Dsc1	19 (18·6)
Positive to Dsc2	42 (41·2)
Positive to Dsc3	62 (60·8)
Positive to all of Dsc1–3	10 (9·8)
Negative to all of Dsc1–3	29 (28·4)

BP, bullous pemphigoid antigen; Dsc, desmocollin; Dsg, desmoglein. All 104 patients with paraneoplastic pemphigus reacted with a doublet of the 210-kDa envoplakin and the 190-kDa periplakin by immunoblotting.

Table 3 The relevant results with statistically significant difference between clinical features and enzyme-linked immunosorbent assay results

			P-value
	Dsg1 positive	Dsg1 negative	
Nasal lesions (n = 77)	0/22 (0)	9/55 (16)	0·043
	Dsg3 positive	Dsg3 negative	
Genital lesions (n = 79)	27/65 (42)	1/14 (7)	0·015
Bronchiolitis obliterans (n = 104)	19/82 (23)	1/22 (5)	0·049
	Dsc1 positive	Dsc1 negative	
Oral lesions (n = 88)	9/12 (75)	73/76 (96)	0·007

Values are n/N (%). Dsg, desmoglein; Dsc, desmocollin.
[a]P-values < 0·05 were considered statistically significant.

the relevant results with statistically significant differences between the clinical features and ELISA results.

Correlations with statistically significant differences among ELISAs were also examined. Patients with anti-Dsg1 antibodies had anti-Dsc1 antibodies more frequently ($P = 0·036$), whereas patients with anti-Dsg3 antibodies had anti-Dsc3 antibodies more frequently ($P = 0·025$). Patients positive for anti-Dsc1 antibodies had anti-Dsg1 antibodies more frequently ($P = 0·048$). Anti-Dsc2 antibody-positive patients had anti-Dsg3 antibodies ($P = 0·032$) and anti-Dsc3 antibodies ($P < 0·001$) more frequently. Patients with anti-Dsc3 antibodies had anti-Dsg3 antibodies ($P = 0·008$) and anti-Dsc2 antibodies ($P < 0·001$) more frequently.

Discussion

The clinical and histopathological findings in this study were generally similar to those in previous reports. Regarding the mucocutaneous lesions, 24 of 88 patients (27%) with PNP had only mucosal lesions. Thus, patients with PNP with only oral mucosal lesions should be carefully differentiated from those with mucosal dominant-type pemphigus vulgaris.

Regarding associated neoplasms, similarly to previous reports, the most frequently associated neoplasms in our patients were malignant lymphomas, followed by malignant solid tumours and Castleman disease. Thus, although most patients with PNP were considered to have haematological tumours, association of malignant solid tumours is not rare. Furthermore, no associated neoplasms were detected in 12 (11·5%) of 104 patients with PNP in our study. The reason may be that limited scrutiny of diagnostic imaging overlooked small tumour lesions in early stages.

In this study, we also confirmed that 82 (78·8%) of 104 patients with PNP showed anti-Dsg3 antibodies, and about one-third of the 104 patients showed anti-Dsg1 antibodies in ELISA. However, 15 (14·4%) of the 104 patients with PNP were negative for both anti-Dsg1 and anti-Dsg3 antibodies. Therefore, we speculated that although anti-Dsg antibodies are important in PNP, autoantibodies to non-Dsg antigens can produce PNP mucocutaneous lesions.

Autoantibodies to Dsc1–3 were occasionally identified in patients with atypical pemphigus by either cDNA transfection using cultured COS-7 cells or by ELISAs using baculovirus recombinant proteins of Dsc1, Dsc2 and Dsc3.[13] We have recently developed sensitive ELISAs using mammalian recombinant proteins of human Dsc1, Dsc2 and Dsc3, which were shown to be highly specific and sensitive.[11] The rates of detection of antibodies to Dsc1–3 in our study were similar to those in our previous report. Intriguingly, in the studies for 102 patients with PNP, positive rates of antibodies to Dsc2 and Dsc3 were higher than those of anti-Dsg1 antibodies. A previous report also found antibodies to Dsc2 and Dsc3 in patients with PNP with eosinophilic spongiosis.[14] The Dsc3 null mouse model established by Chen *et al.*[15] showed an impressive phenotype with intraepidermal blistering and telogen hair loss in the Dsc3 null mouse, which resembled findings in pemphigus vulgaris. Therefore, the results of our study may suggest that anti-Dsc antibodies are pathogenic in PNP. At least, detection of antibodies to Dscs, particularly Dsc2 and Dsc3, are useful for the diagnosis of PNP.

In this study, 32 (60%) of 53 patients had antibodies to A2ML1, which was greater than the rate of anti-Dsg1 antibodies (32·7%). Regarding the pathogenic role of anti-A2ML1 antibodies, we have shown that they decrease adhesion of cultured normal human keratinocytes through plasmin activation by inhibition of A2ML1 activity.[12] The pathogenic role of anti-A2ML1 antibodies in PNP is still unclear. However, if PNP is suspected, anti-A2ML1 antibodies should be studied.

With regard to the statistical analyses of the correlations between anti-Dsg antibodies and clinical parameters, the positive correlation between anti-Dsg3 antibodies and BO was important, because the recent mouse PNP model suggested involvement of Dsg3 in lung disease in BO.[16] Therefore, we thought that the Dsg3-positive patients should be carefully followed up with regard to respiratory symptoms.

Considering the statistical analyses of the correlations between anti-Dsc antibodies and clinical parameters in this study, the reasons for the significant statistical correlations between antibodies to Dsg1, Dsg3 and Dscs and various clinical features are still unclear.

This study accumulated cases of PNP from all over the world. Therefore, this study may be biased geographically and racially. We collected all possible information, but were not able to define all the clinical examinations that we wanted to understand clearly.

As specified in the original criteria for the diagnosis of PNP proposed by Anhalt *et al.*, many patients who did not satisfy the criteria were reported as having PNP. In line with the progress in pathophysiology in PNP, many new findings and diagnostic technologies have emerged. We consider that antibodies to both Dscs and A2ML1 are useful in the diagnosis of PNP.

Acknowledgments

We greatly appreciate the technical assistance of Ms Kyoko Hiromatsu and Ms Michiru Kubo and the secretarial work of Ms Tomoko Tashima. We are very grateful to the dermatologists at other hospitals in Japan, Korea, the U.S.A. and Europe for providing the PNP sera used in this study and for answering our questionnaire.

Author contribution

T.K. contributed to the statistical analysis of this study as a part-time lecturer at the Department of Dermatology, Kurume University School of Medicine.

References

1 Anhalt GJ, Kim SC, Stanley JR *et al.* Paraneoplastic pemphigus. An autoimmune mucocutaneous disease associated with neoplasia. *N Engl J Med* 1990; **323**:1729–35.

2 Anhalt GJ. Paraneoplastic pemphigus. *J Investig Dermatol Symp Proc* 2004; **9**:29–33.

3 Kim SC, Kwon YD, Lee IJ *et al.* cDNA cloning of the 210-kDa paraneoplastic pemphigus antigen reveals that envoplakin is a component of the antigen complex. *J Invest Dermatol* 1997; **109**:365–9.

4 Mahoney MG, Aho S, Uitto J, Stanley JR. The members of the plakin family of proteins recognized by paraneoplastic pemphigus antibodies include periplakin. *J Invest Dermatol* 1998; **111**: 308–13.

5 Amagai M, Nishikawa T, Nousari HC *et al.* Antibodies against desmoglein 3 (pemphigus vulgaris antigen) are present in sera from patients with paraneoplastic pemphigus and cause acantholysis *in vivo* in neonatal mice. *J Clin Invest* 1998; **102**: 775–82.

6 Schepens I, Jaunin F, Begre N *et al.* The protease inhibitor alpha-2-macroglobulin-like-1 is the p170 antigen recognized by paraneoplastic pemphigus autoantibodies in human. *PLOS ONE* 2010; **5**: e12250.

7 Poot AM, Diercks GF, Kramer D *et al.* Laboratory diagnosis of paraneoplastic pemphigus. *Br J Dermatol* 2013; **169**:1016–24.

8 Nagata Y, Karashima T, Watt FM *et al.* Paraneoplastic pemphigus sera react strongly with multiple epitopes on the various regions of envoplakin and periplakin, except for the C-terminal homologous domain of periplakin. *J Invest Dermatol* 2001; **116**:556–63.

9 Hashimoto T, Ogawa MM, Konohana A, Nishikawa T. Detection of pemphigus vulgaris and pemphigus foliaceus antigens by immunoblot analysis using different antigen sources. *J Invest Dermatol* 1990; **94**:327–31.

10 Ishii K, Amagai M, Hall RP *et al.* Characterization of autoantibodies in pemphigus using antigen-specific enzyme-linked immunosorbent assays with baculovirus-expressed recombinant desmogleins. *J Immunol* 1997; **159**:2010–17.

11 Ishii N, Teye K, Fukuda S *et al.* Anti-desmocollin autoantibodies in nonclassical pemphigus. *Br J Dermatol* 2015; **173**:59–68.

12 Numata S, Teye K, Tsuruta D *et al.* Anti-alpha-2-macroglobulin-like-1 autoantibodies are detected frequently and may be pathogenic in paraneoplastic pemphigus. *J Invest Dermatol* 2013; **133**:1785–93.

13 Müller R, Heber B, Hashimoto T *et al.* Autoantibodies against desmocollins in European patients with pemphigus. *Clin Exp Dermatol* 2009; **34**:898–903.

14 Gallo E, García-Martín P, Fraga J *et al.* Paraneoplastic pemphigus with eosinophilic spongiosis and autoantibodies against desmocollins 2 and 3. *Clin Exp Dermatol* 2014; **39**:323–6.

15 Chen J, Den Z, Koch PJ. Loss of desmocollin 3 in mice leads to epidermal blistering. *J Cell Sci* 2008; **121**:2844–9.

16 Hata T, Nishimoto S, Nagao K *et al.* Ectopic expression of epidermal antigens renders the lung a target organ in paraneoplastic pemphigus. *J Immunol* 2013; **191**:83–90.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Summary of the clinical and immunological features in all 104 patients with paraneoplastic pemphigus.

Table S2. Summary of the relationship between various parameters and the results of the enzyme-linked immunosorbent assays.

Correspondence

Nomenclature for diseases with IgA antikeratinocyte cell surface autoantibodies

DOI: 10.1111/bjd.13813

Dear Editor, We read with great interest the article by Geller *et al.*,[1] who reviewed 20 previous reports of patients with IgA antikeratinocyte cell surface antibodies, found limitations in the current classification for this condition and proposed a new term for it, namely 'IgA pemphigus spectrum'. Although we agree with most of the assumptions made in their article, we have a different opinion with regard to the new term proposed.

The condition has variable clinical, histopathological and immunological features[1–5]; accordingly, it has been reported under various names,[2–5] including intercellular IgA dermatosis[6], intraepidermal neutrophilic IgA dermatosis (IEN), intercellular IgA vesiculopustular dermatosis[7] and IgA pemphigus. Thus, we agree with the conclusion of Geller *et al.*[1] that the disease is miscellaneous and includes considerably different subtypes, although all cases have in common antikeratinocyte cell surface antibodies against epidermal cell surface components.

However, based on > 50 case reports and on > 30 years of basic research efforts, it is now well known that there are two major subtypes in this disease entity: subcorneal pustular dermatosis (SPD) and IEN.[3–5,8,9] In addition, various antigen detection studies have revealed that the autoantigen for SPD is desmocollin 1.[8] Although the autoantigen for IEN has not been identified, immunoelectron microscopic studies suggest that it is an unknown nondesmosomal protein.[9] Furthermore, IgA enzyme-linked immunosorbent assays of desmoglein (Dsg)1 and Dsg3 suggest that there are minor subsets, namely IgA pemphigus foliaceus and IgA pemphigus vulgaris, which have IgA antibodies reactive to Dsg1 and Dsg3, respectively.[10]

Thus, in addition to the two major subtypes, SPD and IEN, this condition includes a number of minor subsets, including IgA herpetiform pemphigus and intraepidermal IgA pustulosis.

Pemphigus with IgG anticell surface autoantibodies also has a complex classification. Thus, in IgG pemphigus, in addition to the two major types, namely pemphigus vulgaris and pemphigus foliaceus, there are many other minor subsets, including pemphigus vegetans, pemphigus erythematosus, paraneoplastic pemphigus, pemphigus herpetiformis and drug-induced pemphigus. Nevertheless, the term 'IgG pemphigus spectrum' is never used for the classification of IgG pemphigus. For these reasons, we consider the term 'IgA pemphigus spectrum' to be unsuitable for use with regard to diseases with IgA anticell surface autoantibodies.

However, we also agree with the conclusion by Geller *et al.*[1] that several patients with IgA anticell surface autoantibodies may not show the clinical and histopathological features characteristic of pemphigus. Clinically, many patients show only erythemas and pustules without apparent blisters.[1,2] In most patients, histopathology does not show acantholytic intraepidermal blisters;[1,2] thus, the term 'IgA pemphigus' may not be suitable for diseases with IgA anticell surface autoantibodies.

To best explain all diseases with IgA anticell surface autoantibodies by all clinical, histopathological and immunological findings, we would like to propose the term 'intercellular IgA dermatosis'. This proposal might also be justified by the fact that this term was used in *Rook's Textbook of Dermatology*.[5] Thus, two major diseases would tentatively be called 'SPD-type intercellular IgA dermatosis' and 'IEN-type intercellular IgA dermatosis'. We should collect all cases with IgA cell surface antibodies under the general term 'intercellular IgA dermatoses'. Future extensive studies of the pathogenesis in accumulated patients should provide us with more precise classification for this condition.

T. Hashimoto[1]
T. Nishikawa[2,3]

[1]*Kurume University Institute of Cutaneous Cell Biology, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan*
[2]*Samoncho Dermatological Clinic, Shinjuku, Tokyo, Japan*
[3]*Department of Dermatology, Keio University School of Medicine, Tokyo, Japan*
E-mail: hashimot@med.kurume-u.ac.jp

References

1 Geller S, Gat A, Zeeli T *et al.* The expanding spectrum of IgA pemphigus: a case report and review of the literature. Br J Dermatol 2014; **171**:650–6.
2 Hashimoto T. Immunopathology of IgA pemphigus. Clin Dermatol 2001; **19**:683–9.
3 Nishikawa T, Hashimoto T, Teraki Y *et al.* The clinical and histopathological spectrum of IgA pemphigus. Clin Exp Dermatol 1991; **16**:401–2.
4 Tsuruta D, Ishii N, Hamada T *et al.* IgA pemphigus. Clin Dermatol 2011; **29**:437–42.
5 Wojnarowska F, Venning VA. Immunobullous diseases. In: Rook's Textbook of Dermatology (Burns T, Breathnach S, Cox N, Griffiths C. eds), 8th edn. Oxford: Wiley-Blackwell, 2010; 40.
6 Hashimoto T, Inamoto N, Nakamura K *et al.* Intercellular IgA dermatosis with clinical features of subcorneal pustular dermatosis. Arch Dermatol 1987; **123**:1062–5.

7 Ebihara T, Hashimoto T, Iwatsuki K *et al.* Autoantigens for IgA anti-intercellular antibodies of intercellular IgA vesiculopustular dermatosis. *J Invest Dermatol* 1991; **97**:742–5.
8 Hashimoto T, Kiyokawa C, Mori O *et al.* Human desmocollin 1 (Dsc1) is an autoantigen for the subcorneal pustular dermatosis type of IgA pemphigus. *J Invest Dermatol* 1997; **109**:127–31.
9 Ishii N, Ishida-Yamamoto A, Hashimoto T. Immunolocalization of target autoantigens in IgA pemphigus. *Clin Exp Dermatol* 2004; **29**:62–6.
10 Hashimoto T, Komai A, Futei Y *et al.* Detection of IgA autoantibodies to desmogleins by an enzyme-linked immunosorbent assay: the presence of new minor subtypes of IgA pemphigus. *Arch Dermatol* 2001; **137**:735–8.

Funding sources: none.

Conflicts of interest: none declared.

Research letter

Presence of autoimmune regulator and absence of desmoglein 1 in a thymoma in a patient with pemphigus foliaceus

DOI: 10.1111/bjd.13617

Dear Editor, Desmoglein (Dsg)1 and Dsg3 are autoantigens for pemphigus foliaceus (PF) and pemphigus vulgaris (PV), respectively.[1,2] Thymomas are frequently associated with various autoimmune diseases, particularly myasthenia gravis (MG).[3] Thymomas are divided histopathologically into types A, AB, B1, B2, B3 and C (renamed thymic carcinoma).[4]

Autoimmune regulator (AIRE) is a causative molecule in autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED), and is expressed in thymic medulla in fetal thymus.[5,6] AIRE regulates negative selection of self-reactive T cells by expressing tissue-specific antigens (TSAs). Thus, AIRE is involved in central immune tolerance and the development of autoimmune diseases.[7] AIRE is also expressed in nonthymus tissues, including lymph nodes and spleen, but is absent in all thymomas, except for a few type B1 thymomas.[8,9]

β5t, a subunit of thymus-specific proteasome S28, is involved in antigen processing for positive selection in thymus, and is expressed in type B, but not type A, thymomas.[10,11]

In this study, we examined the expression of various skin autoantigens and AIRE in thymoma in a patient with PF, as well as in other thymic tumours. PF thymoma showed unique antigen expression, indicating a different function of AIRE to that in fetal thymus.

The materials and methods are described in Appendix S1 (see Supporting Information). All specific antibodies are summarized in Table S1 (see Supporting Information). Seven thymomas, one thymic carcinoma and two persistent thymi were obtained from individuals without pemphigus (Table S2; see Supporting Information), and were classified histopathologically (Fig. S1; see Supporting Information).

An 82-year-old Japanese woman developed widespread blistering erythematous skin lesions without mucous membrane involvement (Fig. 1a), and showed, on histopathology, acantholysis at the uppermost epidermis (Fig. 1b). Enzyme-linked immunosorbent assays detected anti-Dsg1 antibodies (index > 3000), but not anti-Dsg3 antibodies (index < 5), confirming the diagnosis of PF. Computed tomography demonstrated a low-density mass in the anterior mediastinum (Fig. 1c). Although antiacetylcholine receptor antibodies were positive, MG was ruled out by neurological examination.

Various treatments could not suppress skin lesions, and the patient died 2 months later. At postmortem, the tumour mass and persistent thymus were excised (Fig. 1d), and pieces of them either fixed or frozen. Histopathology of the tumour showed short spindle epithelial cells with few lymphocytes (Fig. 1e,f); epithelial cells and lymphocytes were positive for AE1/AE3 and CD45, respectively (Fig. 1g,h), suggesting a diagnosis of type A thymoma.

During immunoblotting (IB), PF thymoma strongly expressed Dsg2 and Dsg3, and weakly expressed desmocollin (Dsc)2 and Dsc3, but not Dsg1, while persistent thymus showed no positive reactivity (Fig. 1i). Immunofluorescence confirmed the reactivity in PF thymoma, while persistent thymus expressed these proteins to a lesser extent (Fig. 1j).

Using IB, Dsg2/Dsg3, Dsc2/Dsc3, E-cadherin, epiplakin and laminin γ1 were detected in PF thymoma and several non-PF thymomas [Fig. 2a; Table S3 (see Supporting Information)]. PF thymoma showed much stronger reactivity with these proteins, particularly Dsg2, Dsg3, Dsc2 and laminin γ1. Plectin, periplakin and α6 integrin were detected only in PF thymoma. Dsg1, Dsc2 and Dsc3 were weakly detected in thymi. Thymic carcinoma showed an expression pattern similar to that in PF thymoma. In contrast, bullous pemphigoid 230, desmoplakin, envoplakin, β4 integrin, laminin α3 and type VII collagen were not expressed in any thymomas.

In addition, Dsg1 and laminin γ1 were weakly detected in commercially available lysates of both fetal and adult thymi, and E-cadherin, plectin and epiplakin were detected in adult thymus (Fig. 2b).

Immunohistochemistry (IHC) for selected samples under favourable conditions showed positive reactivity with AE1/AE3 (Fig. S2; see Supporting Information), confirming the presence of epithelial cells in all samples (Fig. 2c). Similar to the results from IB, Dsg2, Dsg3 and Dsc2 were detected.

We next analysed the expression of AIRE in all tumours by IB, IHC and reverse transcription polymerase chain reaction (RT-PCR). Using IB, the 58-kDa AIRE was clearly detected in extracts of human squamous cell carcinoma cell line (DJM)-1 cells and PF thymoma, but not in other thymic tumours and persistent thymi (Fig. 2d). AIRE was also expressed in various keratinocyte cell lines, including KU-8, DJM-1 and HaCaT cells, and in commercially available fetal and adult thymi (Fig. S3a; see Supporting Information). IHC showed positive staining of AIRE in epithelial cell nuclei in PF thymoma, but not in other tumours (Fig. 2f). In addition, RT-PCR detected AIRE mRNA strongly in fetal and adult thymi, and weakly in DJM-1

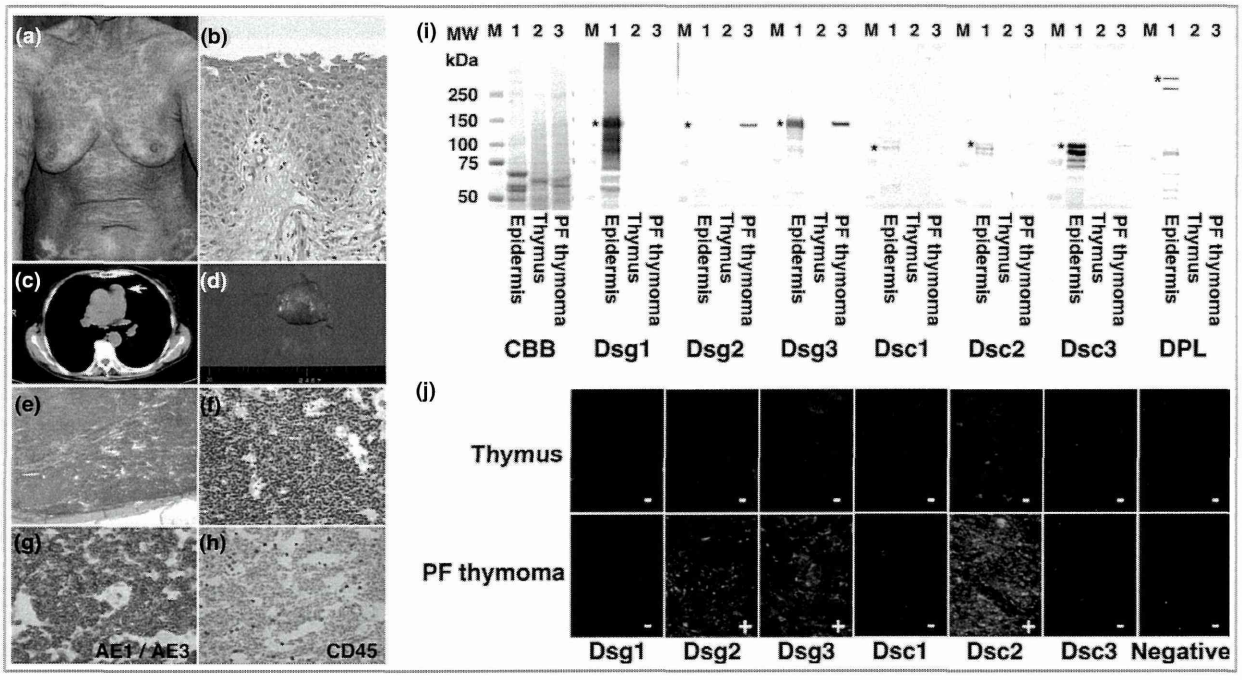


Fig 1. Clinical and histopathological features of a patient with pemphigus foliaceus (PF), and immunoblotting (IB) and immunofluorescence (IF) results of desmoglein (Dsg)1, Dsg2 Dsg3, desmocollin (Dsc)1, Dsc2, Dsc3 and desmoplakin (DPL) in PF thymoma. (a) Clinical features. (b) Histopathological features (haematoxylin and eosin staining, 400×). (c) Image of chest computed tomography. Tumour mass is indicated by an arrow. (d) Resected thymoma. (e, f) Histopathological finding of thymoma [haematoxylin and eosin staining (e, 100×; f, 400×)]. (g, h) Immunohistochemistry findings for (g) AE1/AE3 and (h) CD45 (400×). (i) The results of IB studies of epidermal extracts (lanes 1), extract of PF persisting thymus (lanes 2) and extract of PF thymoma (lanes 3). An equal amount (3 μg) of total protein was loaded onto each lane, and separated on 5–20% polyacrylamide gel. The positions of molecular weight (MW) markers (lanes labelled 'M') are shown on the left. The asterisks indicate a positive reaction. (j) The results of IF of PF persisting thymus (upper panel) and PF thymoma (lower panel) for Dsg1, Dsg2, Dsg3, Dsc1, Dsc2 and Dsc3 (400×). A plus sign indicates a positive reaction; a minus sign a negative one. CBB, Coomassie brilliant blue staining.

cells and PF thymoma, but not in another thymoma (Fig. S3b; see Supporting Information).

Using IB, the 32-kDa β5t protein bands were in type B or type AB thymomas, but not in PF thymoma, thymus and thymic carcinoma (Fig. 2e). These results were confirmed by IHC (Fig. 2g).

In this study, PF thymoma expressed Dsg2, Dsg3, Dsc2 and Dsc3, but not Dsg1 (PF autoantigen) in all IB, immunofluorescence and IHC experiments. Some thymic tumours also showed a similar expression profile, but PF thymoma showed the strongest reactivity. In addition, several other epidermal antigens were detected in several thymic samples, with the most prominent expression in PF thymoma. Two adult persistent thymi also expressed some of these autoantigens, but to a lesser extent. Thus, this is the first study of the expression of various skin antigens in human thymic tissue, although we used only thymomas and adult thymi.

All IB, IHC and RT-PCR experiments showed expression of AIRE in PF thymoma, but not in any other thymomas, persistent thymi or thymic carcinoma. The presence of AIRE in PF thymoma was inconsistent with previously published results.[8,9]

Similar to previous studies,[10,11] β5t was expressed in type B and type AB, but not type A, thymomas, suggesting that the absence of β5t in PF thymoma was merely a tumour type-specific event.

The strong expression of various skin autoantigens and AIRE in PF thymoma led us to speculate that the expression of autoantigens and AIRE in PF thymoma might cause PF-specific autoimmunity. However, the mechanism for induction of autoimmunity to Dsg1 in our patient with PF is currently unknown.

While the role of AIRE in transcriptional activation of TSAs in medullary thymic epithelial cells, thereby ensuring elimination of autoreactive T cells, is widely accepted,[7] in a murine study the expression of autoantigens was preserved in AIRE-deficient medullary thymic epithelial cells.[12] Furthermore, a recent study reported that levels of several TSAs, particularly autoantigens, were paradoxically increased in some thymomas found in patients with MG, which parallels APECED,[13] suggesting some relationship between the expression levels of AIRE and/or TSAs (including autoantigens) within thymoma tissues and the development of autoantibodies to autoantigens.

Although thymoma in our patient expressed AIRE but not Dsg1 (autoantigen), PF thymoma showed the strongest expression of Dsg3, a PV autoantigen, without the production of autoantibodies to Dsg3. Thus, we speculate that AIRE in PF thymoma might induce strong expression of Dsg3 and other

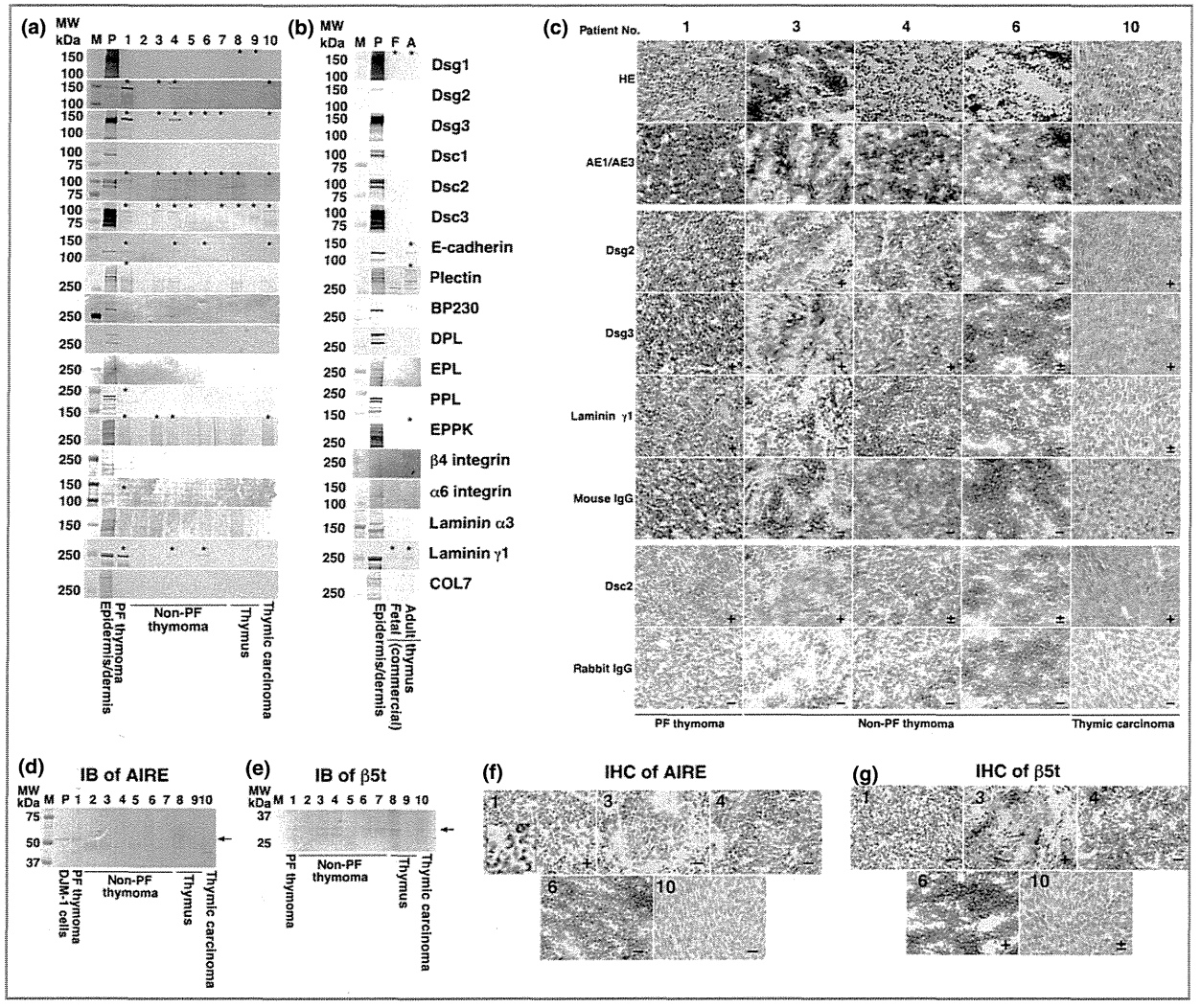


Fig 2. The results of immunoblotting (IB) and histopathological studies of various thymic tissues. (a) The IB results of positive controls (epidermal or dermal extracts) (lane P), thymomas (lanes 1–7), persisting thymi (lanes 8 and 9) and thymic carcinoma (lane 10) for various skin antigens. Each number corresponds to the patient number shown in Table S2 (see Supporting Information). (b) The IB results of positive controls (epidermal or dermal extracts) (lane P), commercially available protein lysates of fetal (lane F) and adult (lane A) thymi. An equal amount (3 μg) of total protein was loaded onto each lane and separated on 5–20% polyacrylamide gel. The positions of molecular weight (MW) markers are shown on the left. The asterisks indicate a positive reaction. All specific antibodies showed a positive reaction in positive controls. (c) Histopathological findings and the results of immunohistochemistry (IHC) for various skin autoantigens for four representative thymomas and thymic carcinoma (400×). Each number corresponds to the patient number shown in Table S2 (see Supporting Information). A plus sign indicates a positive reaction; a plus–minus sign a weakly positive reaction; and a minus sign a negative reaction. Laminin γ1 was detected clearly in pemphigus foliaceus (PF) thymoma (patient 1), but not in non-PF thymomas (patients 3, 4 and 6) in both IB and IHC. In contrast, laminin γ1 was detected by IHC but not by IB in thymic carcinoma (patient 10). (d, e) The results of IB studies for the expression of (d) autoimmune regulator (AIRE) and (e) β5t in extracts of human squamous cell carcinoma cell line (DJM-1) cells as positive control (lane P), thymomas (lanes 1–7), persistent thymi (lanes 8 and 9) and thymic carcinoma (lane 10). Arrows indicate positive reaction. The positions of MW markers are shown on the left. An equal amount (3 μg) of total protein was loaded onto each lane, and separated on 5–20% polyacrylamide gel. (f, g) IHC for the expression of (f) AIRE and (g) β5t in four thymomas and one thymic carcinoma (400×; inset, 1000×). A plus sign indicates a positive reaction; a plus–minus sign a weakly positive reaction; and a minus sign a negative reaction. Each number corresponds to the patient number shown in Table S2 (see Supporting Information). BP230, bullous pemphigoid 230; DPL, desmoplakin; EPL, envoplakin; PPL, periplakin; EPPK, epiplakin; COL7, type VII collagen; HE, haematoxylin and eosin; Dsg, desmoglein; Dsc, desmocollin.

skin autoantigens, which, in turn, lead to the induction of an autoimmune response to Dsg1 through an as yet unidentified pathway in our case, although this speculation is against the dogma of the induction of central immune tolerance.

Lastly, this and a previous study demonstrated that keratinocytes express AIRE.[14] Therefore, AIRE might increase the expression of Dsg1 in epidermis and lead to the production of anti-Dsg1 antibodies.

Acknowledgments

We gratefully appreciate the critical reading of this manuscript by Professor Seiho Nagafuchi, Kyushu University; Professor Mitsuru Matsumoto, Tokushima University; and Professor Jun Kudoh, Keio University. We also appreciate the technical assistance of Ms Michiru Kubo and Ms Kyoko Hiromatsu, and the secretarial work of Ms Tomoko Tashima and Ms Mami Nishida. We thank the patient for her participation.

[1]Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, Kurume 830-0011, Fukuoka, Japan
[2]Department of Dermatology and
[3]Department of Respiratory Surgery, Shimane University Faculty of Medicine, Izumo, Shimane, Japan
[4]Department of Pathology, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido, Japan
[5]Department of Surgery, Kurume University School of Medicine, Kurume, Fukuoka, Japan
Correspondence: Takashi Hashimoto.
E-mail: hashimot@med.kurume-u.ac.jp

A. Tsuchisaka[1]
S. Kaneko[2]
K. Imaoka[2]
M. Ota[2]
K. Kishimoto[3]
U. Tomaru[4]
M. Kasahara[4]
C. Ohata[1]
M. Furumura[1]
S. Takamori[5]
E. Morita[2]
T. Hashimoto[1]

References

1 Amagai M, Klaus-Kovtun V, Stanley JR. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell* 1991; **67**:869–77.
2 Hashimoto T. Treatment strategies for pemphigus vulgaris in Japan. *Expert Opin Pharmacother* 2008; **9**:1519–30.
3 Thomas CR, Wright CD, Loehrer PJ. Thymoma state of the art. *J Clin Oncol* 1999; **17**:2280–9.
4 Rosai J. *Histological Typing of Tumors of the Thymus*, 2nd edn. New York: Springer, 1999.
5 Nagamine K, Peterson P, Scott HS *et al.* Positional cloning of the APECED gene. *Nat Genet* 1997; **17**:393–8.
6 The Finnish-German APECED Consortium. An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy. *Nat Genet* 1997; **17**: 399–403.
7 Mathis D, Benoist C. Aire. *Annu Rev Immunol* 2009; **27**:287–312.
8 Anderson MS, Venanzi ES, Klein L *et al.* Projection of an immunological self shadow within the thymus by the AIRE protein. *Science* 2002; **298**:1395–401.
9 Ströbel P, Murumägi A, Klein R *et al.* Deficiency of the autoimmune regulator AIRE in thymomas is insufficient to elicit autoimmune polyendocrinooathy syndrome type I (APS-I). *J Pathol* 2007; **211**:563–71.
10 Takahama Y, Nitta T, Ripen AM *et al.* Role of thymic cortex-specific self-peptides in positive selection of T cells. *Semin Immunol* 2010; **22**:287–93.
11 Yamada Y, Tomaru U, Ishizu A *et al.* Expression of proteasome subunit β5t in thymic epithelial tumors. *Am J Surg Pathol* 2011; **35**:1296–304.
12 Niki S, Oshikawa K, Mouri Y *et al.* Alteration of intra-pancreatic target-organ specificity by abrogation of Aire in NOD mice. *J Clin Invest* 2006; **116**:1292–301.
13 Wolff AS, Kärner J, Owe JF *et al.* Clinical and serologic parallels to APS-I in patients with thymomas and autoantigen transcripts in their tumors. *J Immunol* 2014; **193**:3880–90.
14 Kumar V, Pedroza LA, Mace EM *et al.* The autoimmune regulator (AIRE), which is defective in autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy patients, is expressed in human epidermal and follicular keratinocytes and associates with the intermediate filament protein cytokeratin 17. *Am J Pathol* 2011; **178**:983–8.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Appendix S1. Materials and methods.

Fig S1. Histopathological findings of thymomas and thymic carcinoma.

Fig S2. Histopathological findings of frozen sections of thymomas, persisting thymi and thymic carcinoma.

Fig S3. Immunoblotting and reverse transcription polymerase chain reaction studies of autoimmune regulator expression.

Fig S4. Coomassie blue gel staining for sodium dodecyl sulfate polyacrylamide gel electrophoresis of total protein extracts from all samples for using immunoblotting studies (figure included in Appendix S1).

Table S1. Monoclonal and polyclonal antibodies used in this study.

Table S2. Summary of clinical parameters for the patients associated with thymomas.

Table S3. Summary of the results of immunoblotting for various skin autoantigens in all thymic tumours.

Funding sources: This study was supported by Grants-in-Aid for Scientific Research (Nos 20390308, 20591331, 21659271, 23591634, 23791298, 23791299, 23791300, 23791301, 24659534, 24591672, 24591640 and 24791185); Supported Program for the Strategic Research Foundation at Private Universities 2009-2013 from the Ministry of Education, Culture, Sports, Science and Technology; and by the 'Research on Measures for Intractable Diseases' project, matching fund subsidy (H23-028 to K.I. and H24-038 to T.H.), from the Ministry of Health, Labour and Welfare. The study was also supported by grants from the Kaibara Morikazu Medical Science Promotion Foundation, Ishibashi Foundation, Kanae Foundation for the Promotion of Medical Science, Takeda Science Foundation, Chuo Mitsui Trust and Banking Company, Limited, and Nakatomi Foundation.

Conflicts of interest: none declared.

INVESTIGATIVE REPORT

Granular C3 Dermatosis

Takashi HASHIMOTO[1#], Daisuke TSURUTA[1,2#], Atsushi YASUKOCHI[1**], Hisayoshi IMANISHI[2], Hideharu SEKINE[3], Teizo FUJITA[4], Hideki WANIBUCHI[5], Min GI[5], Sarolta KÁRPÁTI[6], Cassian SITARU[7], John J. ZONE[8], Daisuke ENDO[9], Shinichi ABE[9], Tomoya NISHINO[9], Takehiko KOJI[9] and Norito ISHII[1]

Departments of Dermatology, [1]Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, Fukuoka, [2]Osaka City University Graduate School of Medicine, Osaka, [3]Department of Immunology, Fukushima Medical University School of Medicine, [4]Fukushima Prefectural General Hygiene Institute, Fukushima, [5]Department of Molecular Pathology, Osaka City University Graduate School of Medicine, Osaka, Japan, Departments of Dermatology, [6]Semmelweis University, Budapest, Hungary, [7]University of Freiburg, Freiburg, Germany, [8]University of Utah School of Medicine, Salt Lake City, Utah, USA, and [9]Department of Histology and Cell Biology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan.

***Present address: Section of Oral and Maxillofacial Oncology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, Fukuoka, Japan*

[#]These 2 authors contributed equally to this work.

[EQ1] **There has been no previous systematic study of bullous skin diseases with granular basement membrane zone deposition exclusively of C3. In this study we collected 20 such patients, none of whom showed cutaneous vasculitis histopathologically. Oral dapsone and topical steroids were effective. Various serological tests detected no autoantibodies or autoantigens. Direct immunofluorescence for various complement components revealed deposition only of C3 and C5–C9, indicating that known complement pathways were involved. Studies of *in situ* hybridization and micro-dissection with quantitative RT-PCR** [AQ1] **revealed a slight reduction in expression of C3 in patient epidermis. These patients may represent a new disease entity, for which we propose the term "granular C3 dermatosis". The mechanism for granular C3 deposition in these patients is unknown, but it is possible that the condition is caused by autoantibodies to skin or aberrant C3 expression in epidermal keratinocytes. *Key words: basement membrane zone; bullous disease; C3; direct immunofluorescence; granular.***

Accepted Feb 24, 2016; Epub ahead of print Feb 26, 2016

Acta Derm Venereol 2016; 96: XX–XX.

Takashi Hashimoto, Department of Dermatology, Kurume University School of Medicine and Kurume University Institute of Cutaneous Cell Biology, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan. E-mail: hashimot@med.kurume-u.ac.jp

As one of the centres for diagnosis of autoimmune bullous diseases (AIBDs) in Japan, we have collected sera, frozen skin samples and data for more than 5,000 patients for whom there was difficulty in diagnosis over a period of 20 years. In this large cohort, we found that 20 patients, some of whom showed dermatitis herpetiformis (DH)-like clinical features, showed granular deposition of C3, but not immunoglobulin (Ig)A, IgG or IgM, in the epidermal basement membrane zone (BMZ) by direct immunofluorescence (IF).

Clinically, DH shows pruritic papulo-vesicular skin lesions preferentially on the elbows, knees and buttocks; histopathologically, neutrophilic microabscesses are present in papillary dermis; and immunologically, granular deposition of IgA with or without C3 is present in papillary dermis (1–4). DH is usually associated with coeliac disease in Caucasians (5). DH shows circulating IgA antibodies to various antigens, including gliadin and endomysium (EMA) (1). However, recent studies have shown that patients with DH have pathogenic IgA antibodies to epidermal transglutaminase (eTG, also called as TG3), as well as IgA antibodies to tissue transglutaminase (tTG) (6–10).

There are 3 distinct pathways in activation of complement cascade; i.e. the classical, alternative and lectin pathways (11, 12). The classical pathway is activated mainly by binding of IgG and IgM antibodies and involves all complement components from C1 to C5–C9. The alternative pathway is activated mainly by various microbes or IgA antibodies, and involves factor B and C5–C9. The lectin pathway is activated mainly by various bacteria, dying tissues and mannose-containing sugar chains, and involves mannose-binding lectin, ficolins and C5–C9. Thus, IgG autoantibodies in various subepidermal AIBDs may activate complement via either the classical or the alternative pathway, while IgA antibodies in either DH or linear IgA bullous dermatosis may activate complement mainly via the alternative pathway (11, 12).

In this study, we clinically, histopathologically and immunologically characterized 20 patients with granular BMZ deposition exclusively of C3, with particular comparison with DH.

The 20 patients showed several common findings in terms of clinical and histopathological features, as well as a pattern of deposition of complement components and production of C3 in epidermis. These findings were different from those found in any known AIBDs or other inflammatory skin diseases. Therefore, we propose the term granular C3 dermatosis (GCD) as a possible new disease entity for this condition.

MATERIALS AND METHODS (for complete details see Appendix SI[1])

Immunofluorescence studies

Indirect IF and complement-fixing IF studies were performed as described previously (13, 14). IF studies for various complement components were also performed.

Immunoblot and enzyme-linked immunoassay (ELISA) studies for non-dermatitis herpetiformis diseases

Immunoblot analyses of 6 antigen sources were performed as described previously (13, 15–20). ELISAs of BP180, BP230, Dsg1 and Dsg3 were performed using commercial kits (MBL, Nagoya, Japan). IgA ELISA of longer RP of BP180 ectodomain was also performed (21).

Serological tests for dermatitis herpetiformis

IgA anti-EMA antibodies were examined as described previously (22). ELISAs were performed for IgA anti-eTG and anti-tTG antibodies (6), IgA anti-eTG antibodies (23), IgA anti-tTG and anti-gliadin derived peptides (DGP) antibodies (24), IgA anti-gliadin antibodies (25) and IgA anti-F-actin antibodies (26). The result was evaluated as positive or negative using the cut-off value for each ELISA (Table SI[1]).

In situ *hybridization*

Specificity and sensitivity of probes used for *in situ* hybridization were confirmed as described previously (27). Immunohistochemical *in situ* hybridization for C3 was performed as described previously (28, 29). 28S rRNA probe was used as positive control (30).

Micro-dissection and semi-quantitative RT-PCR (qPCR)

Micro-dissection and qPCR were performed as described previously (31). Statistical analysis was performed using unpaired *t*-test.

RESULTS

Clinical and histopathological features

Mean age at onset of skin lesions for the 20 patients, 10 females and 10 males, was 61.2 years, ranging from 8 to 83 years. There were no particular or significant findings in past histories, complications and given drugs. Because there were no gastrointestinal symptoms suggesting coeliac disease, no patients underwent endoscopic studies for either upper or lower intestinal tracts.

The overall appearance of skin lesions was assessed, in particular for the presence of blisters, erythemas and eczematous changes (Table SII[1]). Approximately half of the 20 patients showed clinical features, which in general mimicked DH; i.e. annular or nummular exudative erythemas, vesicles on peripheries of erythemas and eczematous lesions (Fig. 1 a–c), However, some patients showed bullous pemphigoid (BP)-like tense bullae (Fig. 1 d, e), prurigo-like papular lesions (Fig. 1f) or annular erythemas without any blisters (Fig. 1 g, h). Seven patients showed no apparent blister formation. Seventeen patients had severe pruritus. Because of the extreme heterogeneity in the skin lesions, times when the diagnoses of AIBDs were suspected were variable.

Regarding treatments, oral administrations of various corticosteroids, dapsone and combination of minocycline and nicotinamide were used in addition to topical corticosteroids. All therapies perfectly or partially controlled skin lesions. In general, dapsone could suppress the disease completely, although discontinuation of dapsone led to recurrence. No patients died during follow-up, indicating good prognosis in this condition. However, we could not obtain final information about therapies and outcome for some patients who had consulted dermatologists in other institutes.

Histopathology and various immunofluorescence studies

Histopathologically, most patients showed either subepidermal blisters (Fig. 1 i, j) or liquefaction degeneration/oedema in papillary dermis (Fig. 1 k, l) with inflammatory infiltration of lymphocytes, eosinophils and/or neutrophils. In particular, 9 patients showed no apparent subepidermal blister, although inflammatory infiltrates were seen in dermis and around dilated blood vessels (Fig. 1 m, n). Spongiosis was seen in 12 patients. No patient showed changes suggesting the presence of cutaneous vasculitis, such as leukocytoclasis and fibrinoid deposition.

Direct IF for the 20 patients showed granular deposition of C3 in and just below the BMZ, while no depositions of IgG, IgA and IgM were detected (Fig. 2a). No patient showed C3 deposition in blood vessel walls, excluding the presence of cutaneous vasculitis. Control BP skin biopsies showed strong linear BMZ deposition of C3 and/or IgG (Fig. 2a).

No patients showed positive results in indirect IF of either normal human skin or 1M NaCl-split normal human skin (data not shown). Complement-fixing IF of both normal human skin and 1M NaCl-split normal human skin also showed negative results (data not shown).

Antigen detection studies for non-dermatitis herpetiformis autoimmune bullous diseases

There were no positive results in immunoblot analyses for either IgG or IgA antibodies (data not shown). All patients also showed negative results in IgG and IgA ELISAs for BP230 and BP180 and IgG ELISAs for Dsg1 and Dsg3. In additional IgA ELISA for larger BP180 ectodomain, none of 19 patients examined showed definitely positive results, except for borderline positive reactivity in 2 patients (Table SI[1], right-hand column).

[1]http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-2379

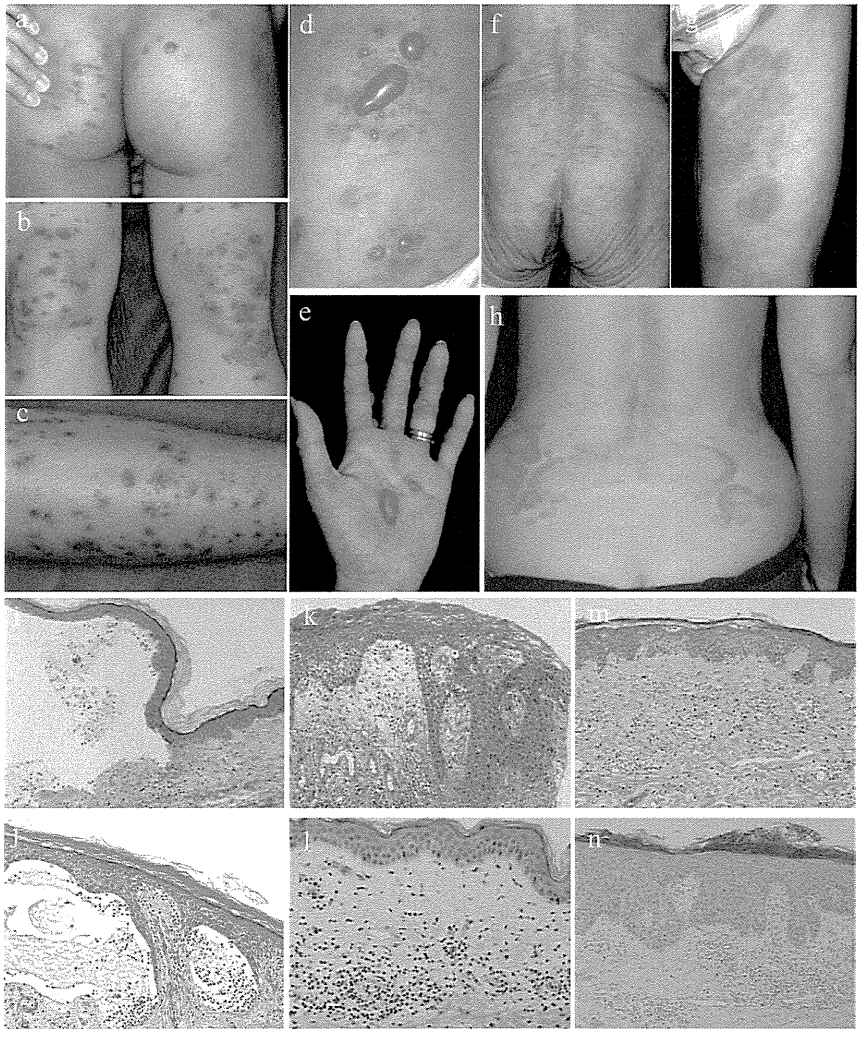


Fig. 1. Dermatitis herpetiformis-like lesions on (a) the buttock and (b) the knees. (c) Vesicles and eczematous lesions on the shins. Bullous pemphigoid-like tense blisters on (d) the abdomen and (e) palm. (f) Pruriginous lichenification on the back, and annular erythemas without apparent blisters on (g) the thigh and (h) waist. Histopathological features (haematoxylin-eosin staining). (i, j) Subepidermal blister with inflammatory infiltration. (k, l) Oedema in the upper dermis with inflammatory infiltration. (m, n) Perivascular inflammatory infiltration without apparent change in the epidermis. Original magnification: (i, m, n) ×100; (j–l) ×200.

Serological tests for dermatitis herpetiformis

To exclude the diagnosis of DH, various serological tests for DH were also performed for IgA and/or IgG antibodies for 19 of the 20 patients (Table SI[1]). In general, the 20 patients showed negative results in these studies, although a few patients showed relatively weak positive results in several tests.

Thus, both IF of monkey oesophagus and ELISA did not detect IgA anti-EMA antibodies in any patients. Commercial ELISA for IgA anti-eTG antibodies showed positive results in 3 patients. However, its significance is obscure, because all sera were negative in another home-made ELISA for eTG. IgA and IgG ELISAs for tTG, tTG/DGP and DGP and IgA ELISAs for gliadin and F-actin in general showed negative results, except for sporadic sera with positive reactivity with low titre. Although IgG ELISA for gliadin showed positive results in 4 patients, the significance was unknown.

Immunofluorescence study for various complement components

Next, to determine the complement activation pathway in the patient skin, we performed IF for various complement components using frozen skins from 6 patients. Control skin biopsies were also obtained from 6 BP patients and 6 normal volunteers. These studies used antibodies specific to IgG and C4 for the classical activation pathway, Factor B for the alternative pathway, MBL and ficolins for the lectin pathway, and C5–C9 for final stage.

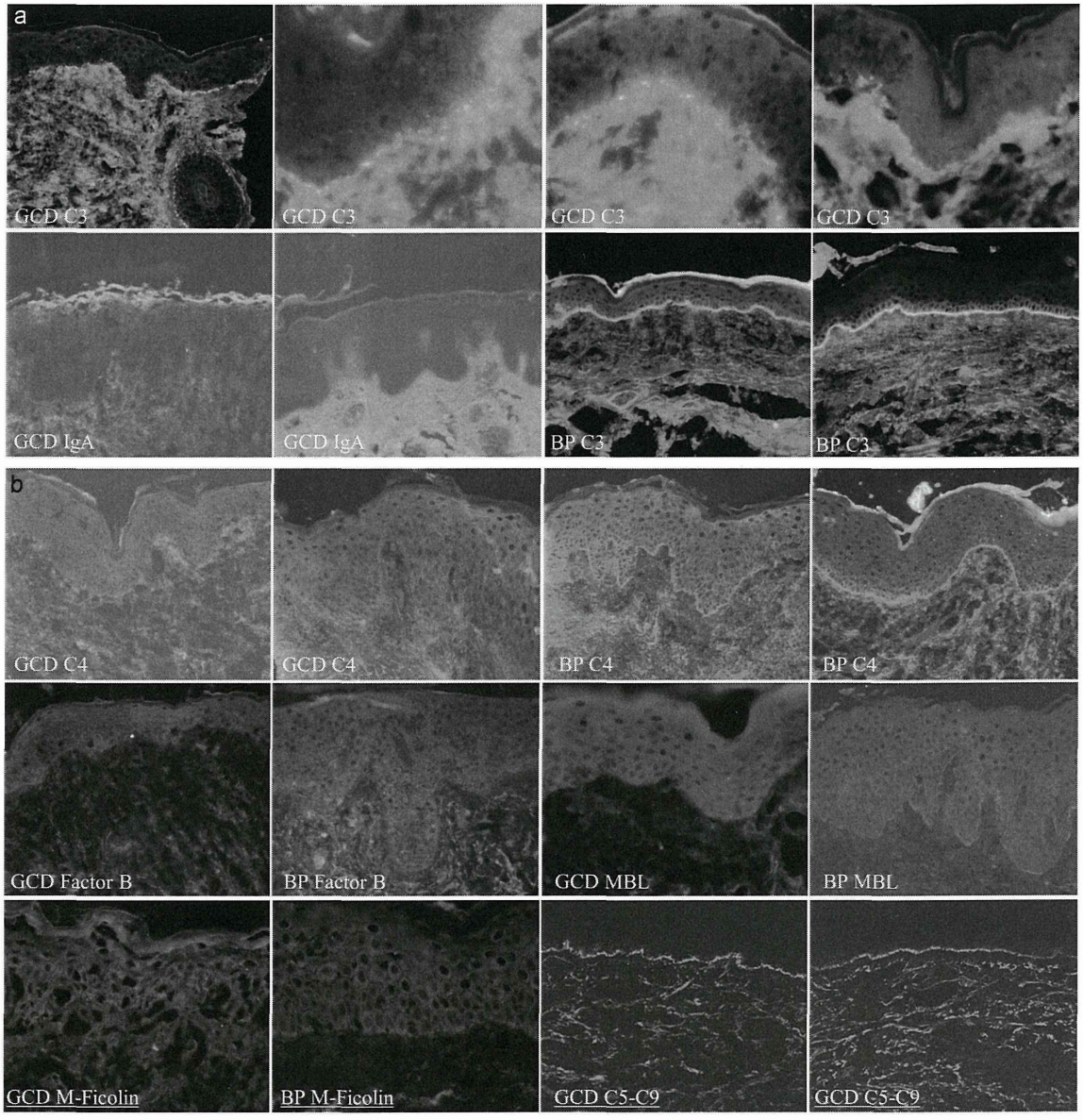
All 6 skin biopsies from the patients showed negative results for all complement components, except for positive results for C5–C9 (Fig. 2b, Table SIII[1]). In contrast, all control BP skins showed linear BMZ deposition of C4 and several BP skin showed minimum linear deposition of factor B, while neither MBL nor ficolins deposited in any BP skins. Normal skin biopsies showed no positive reactivity.

In situ *hybridization and qPCR studies for C3 production in epidermis*

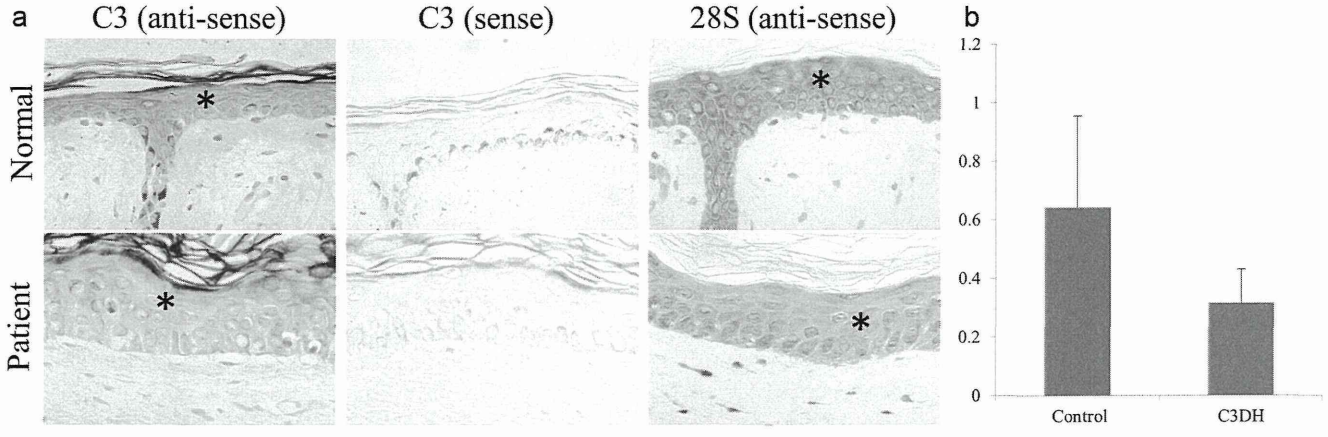
From the results of immunofluorescence for various complement components, none of classical, alternative and lectin pathways were considered to be activated in the patient skin. Therefore, we hypothesized that C3 deposition is caused by over-production of C3 in the epidermis of patient skin. In order to confirm this speculation, *in situ* hybridization and qPCR studies were performed for C3 in 4 selected patient skins, which were kept at –80°C for 1–4 years.

In situ hybridization with 28S rRNA as positive control confirmed that the procedure worked well (Fig. 3a). Lower expression of C3 mRNA was constantly seen in patient epidermis compared with normal control epidermis (Fig. 3a). Sense probe for C3 used as negative control showed no staining in adjacent sections (Fig. 3a).

In qPCR, C3 mRNA expression was also slightly lower in patient skins, although statistical significance



[EQ2] *Fig. 2.* Immunofluorescence (IF) studies. (a) Results of initial direct IF examinations. Original magnification: ×100 (upper-left); ×200 (others). (b) Results of IF studies of various complement components. Original magnification: ×200 (M-ficolin); ×100 (others).



[EQ3] *Fig. 3.* Studies of expression of C3 mRNA using skin biopsies from 4 patients. (a) The results of *in situ* hybridization using anti-sense probe, and sense probe (negative control) for C3 and positive control probe for 28S rRNA. Positive reaction: blue colour (*). (b) Result of qPCR study.

was not achieved (Fig. 3b).

DISCUSSION

This study revealed that the 20 GCD patients had several common features; i.e. (*i*) DH-like clinical features, (*ii*) histopathological features of subepidermal blister/oedema and liquefaction degeneration with infiltrations of lymphocytes, eosinophils and neutrophils in various combinations, (*iii*) benign disease course with good response to oral dapsone and topical steroids, (*iv*) granular deposition of C3 and C5–C9, but not other complement components, and (*v*) slightly reduced expression of C3 in epidermis.

Clearly, we must differentiate GCD from several other skin diseases, including various subepidermal AIBDs (DH, linear IgA bullous dermatosis, BP and epidermolysis bullosa acquisita), cutaneous leukocytoclastic vasculitis, porphyria, polymorphous light eruption, pruritic urticarial papules or plaques of pregnancy (PUPPP), insect bite and various eczematous diseases.

DH and linear IgA bullous dermatosis show positive IgA deposition in BMZ in granular and linear patterns, respectively, in direct IF (32). BP and epidermolysis bullosa acquisita show IgG autoantibodies to BP230/BP180 and type VII collagen, respectively, in various serological tests (32). Cutaneous leukocytoclastic vasculitis shows characteristic histopathological and direct IF finding in blood vessels in dermis. Porphyria and polymorphous light eruption show skin lesions on sun-exposed sites with photosensitivity (33). PUPPP should occur in pregnant females (34). However, both insect bite and various eczematous diseases may show GCD-like features and cannot easily be excluded.

In the present study, no patients showed depositions of immunoglobulins and C4, excluding involvement of classical pathway for C3 activation. Furthermore, IF studies did not show depositions of factor B, mannose-binding lectin and ficolins, suggesting either the alternative or the lectin pathway was involved. Thus, none of the known complement activation pathways seemed to play a role in deposition of granular C3.

Although the true mechanism for deposition of granular C3 is unknown, we put forward the following 5 possibilities. First, that inflammation with heavy lymphocytic infiltration in uppermost dermis induced deposition of C3 from circulation. However, in contrast to deposition of all immunoglobulins and C3 seen in lupus erythematosus and other collagen diseases known as lupus band test (35), our patients showed exclusive C3 deposition. In addition, although lupus band is induced by sun-exposure, skin lesions in our patients occurred on non-sun-exposed sites.

The 2nd possibility is that immune-complex around or inside blood vessels affected by cutaneous vasculitis in upper dermis moved to and granularly deposited in BMZ by known pathway. However, previous IF study of cutaneous vasculitis showed no C3 deposition in BMZ (36). In addition, none of our patients showed apparent cutaneous vasculitis in both histopathological and direct IF studies.

The 3rd possibility is that circulating IgG or IgA autoantibodies bound to unknown autoantigen beneath BMZ and activated complements. In this case, IgG or IgA antibodies could not be detected by direct IF, either because the amount of autoantibodies was too low or because immunoglobulin deposition was masked. However, because no deposition of C1q and C4 was shown in IF studies, it was unlikely that the classical pathway was activated by binding of immunoglobulins. In addition, although IgA antibodies can activate complements via the alternative pathway, our IF study also excluded involvement of the alternative pathway. The possibility that IgG deposition disappeared more quickly than C3 deposition also cannot be completely excluded. However, repeated direct IF performed in 3 patients always showed only C3 deposition, suggesting no preceding deposition of IgG.

A 4th possibility is that complements were activated by microbes, mannose-containing sugar moieties or dead materials via the alternative or the lectin pathway. However, as mentioned above, our IF study indicated that neither the alternative nor the lectin pathway was activated.

Epidermal keratinocytes were reported to produce C3 on stimulation by various cytokines (37, 38). Therefore, the 5th and final possibility is that inflammatory cytokines upregulated C3 production in the patient skin, and over-produced C3 was secreted and deposited in the uppermost dermis. However, unexpectedly, the results in both *in situ* hybridization and q-PCR studies for C3 expression indicated a reduced amount of C3 expression in the epidermis of our patients. These results do not support the 5th possibility.

Thus, future studies are necessary to exclude the remaining possibilities, i.e. (*i*) immunoglobulin(s) and complement components for the classical pathway, such as C1q and C4, disappeared via a so-far unknown mechanism, (*ii*) over-expression of C3 by keratinocytes led to a reduction in C3 mRNA through negative feedback, and (*iii*) dysregulation of complement activation was induced by unknown complement regulators, which are reported to activate complement systems erroneously and induce abnormal condition in several disorders, including the atypical haemolytic uraemic syndrome, systemic lupus erythematosus and glomerulonephritis (39).

GCD and classical DH with granular IgA deposition show considerably similar clinical features, but different histopathological and immunological features. Therefore, it is necessary to elucidate the pathomechanisms of how GCD and DH induce similar clinical features through different histopathological and immunological

changes. The granular pattern of C3 deposition, which is a common feature of both GCD and DH, may play a role in the same clinical features.

Finally, it is currently unknown whether C3 deposition plays a role in blister formation in GCD. Elucidation of the pathogenic role of C3 deposition in GCD should also provide a clue to understanding the pathophysiology of other subepidermal AIBDs, which show C3 deposition, in addition to IgG and/or IgA.

For confirmation of the identity of GCD and the pathogenic role of granular C3 deposition on BMZ, further clinical studies with a large sample of similar patients in addition to experimental disease model studies are required.

ACKNOWLEDGEMENTS

The authors would like to thank Ms Tomoko Tashima for secretarial work and the patients for their participation.

Funding/support. This study was supported by Grants-in-Aid for Scientific Research and Strategic Research Basis Formation Supporting Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Health and Labour Sciences Research Grants and the grants for Research on Measures for Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan. The study was also supported by grants from the Uehara Memorial Foundation, the Nakatomi Foundation, the Japan Lydia O'Leary Memorial Foundation, and the Japanese Dermatological Association (Shiseido Award).

The authors declare no conflicts of interest.

REFERENCES

(complete reference list available in electronic version)

3. Karpati S. Dermatitis herpetiformis: close to unravelling a disease. J Dermatol Sci 2004; 34: 83–90.
6. Sardy M, Karpati S, Merkl B, Paulsson M, Smyth N. Epidermal transglutaminase (TGase 3) is the autoantigen of dermatitis herpetiformis. J Exp Med 2002; 195: 747–757.
9. Zone JJ, Schmidt LA, Taylor TB, Hull CM, Sotiriou MC, Jaskowski TD, et al. Dermatitis herpetiformis sera or goat anti-transglutaminase-3 transferred to human skin-grafted mice mimics dermatitis herpetiformis immunopathology. J Immunol 2011; 186: 4474–4480.
11. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol 2010; 11: 785–797.
12. Mathern DR, Heeger PS. Molecules great and small: the complement system. Clin J Am Soc Nephrol 2015; 10: 1636–1050.
13. Sugi T, Hashimoto T, Hibi T, Nishikawa T. Production of human monoclonal anti-basement membrane zone (BMZ) antibodies from a patient with bullous pemphigoid (BP) by Epstein-Barr virus transformation. Analyses of the heterogeneity of anti-BMZ antibodies in BP sera using them. J Clin Invest 1989; 84: 1050–1055.
15. Hashimoto T, Ogawa MM, Konohana A, Nishikawa T. Detection of pemphigus vulgaris and pemphigus foliaceus antigens by immunoblot analysis using different antigen sources. J Invest Dermatol 1990; 94: 327–331.
16. Matsumura K, Amagai M, Nishikawa T, Hashimoto T. The majority of bullous pemphigoid and herpes gestationis serum samples react with the NC16a domain of the 180-kDa bullous pemphigoid antigen. Arch Dermatol Res 1996; 288: 507–509.
17. Nie Z, Hashimoto T. IgA antibodies of cicatricial pemphigoid sera specifically react with C-terminus of BP180. J Invest Dermatol 1999; 112: 254–255.
18. Ishii N, Ohyama B, Yamaguchi Z, Hashimoto T. IgA autoantibodies against the NC16a domain of BP180 but not 120-kDa LAD-1 detected in a patient with linear IgA disease. Br J Dermatol 2008; 158: 1151–1153.
19. Ishii N, Yoshida M, Hisamatsu Y, Ishida-Yamamoto A, Nakane H, Iizuka H, et al. Epidermolysis bullosa acquisita sera react with distinct epitopes on the NC1 and NC2 domains of type VII collagen: study using immunoblotting of domain-specific recombinant proteins and postembedding immunoelectron microscopy. Br J Dermatol 2004; 150: 843–851.
20. Hisamatsu Y, Nishiyama T, Amano S, Matsui C, Ghohestani R, Hashimoto T. Usefulness of immunoblotting using purified laminin 5 in the diagnosis of anti-laminin 5 cicatricial pemphigoid. J Dermatol Sci 2003; 33: 113–119.
21. Csorba K, Schmidt S, Florea F, Ishii N, Hashimoto T, Hertl M, et al. Development of an ELISA for sensitive and specific detection of IgA autoantibodies against BP180 in pemphigoid diseases. Orphanet J Rare Dis 2011; 6: 31.
22. Sardy M, Odenthal U, Karpati S, Paulsson M, Smyth N. Recombinant human tissue transglutaminase ELISA for the diagnosis of gluten-sensitive enteropathy. Clin Chem 1999; 45: 2142–2149.
23. Hull CM, Liddle M, Hansen N, Meyer LJ, Schmidt L, Taylor T, et al. Elevation of IgA anti-epidermal transglutaminase antibodies in dermatitis herpetiformis. Br J Dermatol 2008; 159: 120–124.
25. Samolitis NJ, Hull CM, Leiferman KM, Zone JJ. Dermatitis herpetiformis and partial IgA deficiency. J Am Acad Dermatol 2006; 54: S206–209.
31. Sugawara K, Tsuruta D, Kobayashi H, Ikeda K, Hopkinson SB, Jones JC, et al. Spatial and temporal control of laminin-332 (5) and -511 (10) expression during induction of anagen hair growth. J Histochem Cytochem 2007; 55: 43–55.
36. Poterucha TJ, Wetter DA, Gibson LE, Camilleri MJ, Lohse CM. Correlates of systemic disease in adult Henoch-Schonlein purpura: a retrospective study of direct immunofluorescence and skin lesion distribution in 87 patients at Mayo Clinic. J Am Acad Dermatol 2012; 67: 612–616.
37. Terui T, Ishii K, Ozawa M, Tabata N, Kato T, Tagami H. C3 production of cultured human epidermal keratinocytes is enhanced by IFNgamma and TNFalpha through different pathways. J Invest Dermatol 1997; 108: 62–67.
38. Pasch MC, Van Den Bosch NH, Daha MR, Bos JD, Asghar SS. Synthesis of complement components C3 and factor B in human keratinocytes is differentially regulated by cytokines. J Invest Dermatol 2000; 114: 78–82.