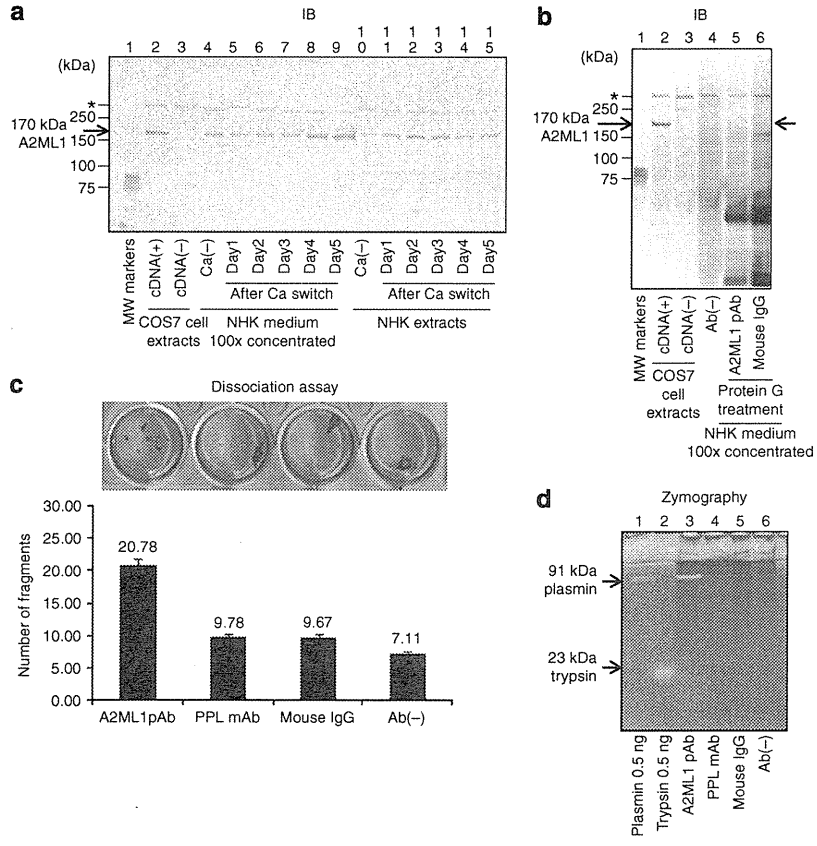

Figure 5. Anti-alpha-2-macroglobulin-like-1 (A2ML1) polyclonal Ab (pAb) caused cell–cell dissociation and plasmin activation in cultured normal human keratinocytes (NHKs). (**a**) A2ML1 expression in cultured NHKs. Immunoblotting (IB) using anti-A2ML1 mouse polyclonal antibody (A2ML1 pAb) was performed for either 100 × concentrated culture media or cell extracts of NHKs cultured in low calcium (at day 1) and in high calcium (at days 1–5). The position of A2ML1 recombinant protein (RP) is shown by an arrow in the left. (**b**) Anti-A2ML1 pAb bound to A2ML1 in NHK culture medium. IB using anti-A2ML1 pAb was performed for either 100 × concentrated culture media of NHKs. Lane 4 was for culture medium without treatments of antibody and protein G, lane 5 for culture medium incubated with anti-A2ML1 pAb and precipitated with protein G, and lane 6 for culture medium incubated with negative control mouse IgG and precipitated with protein G. Although the reactivity was weak, A2ML1 disappeared in culture medium incubated with anti-A2ML1 pAb after precipitation with protein G agarose (lane 5). The position of A2ML1 RP is shown by arrows in both sides. Extracts of COS7 cells with or without transfection of A2ML1 complementary DNA (cDNA) were used as positive and negative controls, respectively, in both panels (**a**) and (**b**). The positions of molecular weight (MW) markers and a nonspecific band are indicated by bars and asterisks (*), respectively, in the left of both panels (**a**) and (**b**). (**c**) Anti-A2ML1 pAb decreased cell–cell adhesion of NHKs. Upper panel shows culture dishes after the process of dissociation assay, and lower panel shows numbers of fragments by histograms, in which each bar corresponds to the dish in the upper panel. NHKs were cultured with anti-A2ML1 pAb, anti-periplakin (PPL) mAb, or normal mouse IgG at a concentration of 1 $\mu g\,ml^{-1}$, or without antibody (Ab(−)), for one day after calcium shift. After treatment with dispase II, detached cell sheets were pipetted and fragments were counted. (**d**) Anti-A2ML1 pAb increased plasmin activity. Casein zymography was performed using culture media of NHKs cultured with anti-A2ML1 pAb, anti-PPL mAb, or normal mouse IgG at a concentration of 1 $\mu g\,ml^{-1}$, or without antibody (Ab(−)), for one day after calcium shift. The arrows in the left indicate the position of the 91 kDa plasmin and the 23 kDa trypsin.

The results in our previous study suggested that intramolecular disulfide bonds within A2ML1 molecule are important for conformational epitopes for autoantibodies in PNP sera (Schepens *et al.*, 2010). This assumption was further confirmed by the results in the present study that 22 (37.9%) PNP sera reacted with A2ML1 in IB of NHKs under non-reducing, but not reducing condition. To the best of our knowledge, A2ML1 is the antigen, which has such unique epitopes that have not been reported previously.

However, the positive rate in non-reducing IB of NHK extracts was much lower than that in IP–IB using A2ML1-expressing COS7 cells. Noteworthy, all PNP sera with positive A2ML1 reactivity in non-reducing IB were also positive in IP–IB, whereas none of IP–IB–negative sera showed positive reactivity by non-reducing IB (Supplementary Table S1 online), concluding that IP–IB using A2ML1 RP is more sensitive than non-reducing IB of NHK extracts.

In the second part of this study, we presented the evidence of the pathogenic role of anti-A2ML1 antibodies in PNP by functional studies, which to our knowledge is previously unreported. We first confirmed the presence of A2ML1 in NHK culture media by IB of concentrated samples. Then, we also confirmed actual binding of anti-A2ML1 pAb by IB of culture media immunoprecipitated with protein G agarose.

NHKs incubated with A2ML1 pAb showed decreased cell adhesion by dissociation assay, and increased activity of plasmin was detected by casein zymography. The results of these functional studies suggested that anti-A2ML1 pAb

decreased NHK cell adhesion through plasmin activation by inhibition of A2ML1.

Relationship between proteases and acantholysis was previously described (Morioka *et al.*, 1981), and plasmin and plasminogen activator (PA) were reported to be involved in the pathogenesis of pemphigus (Hashimoto *et al.*, 1983; Seishima *et al.*, 1997). However, this hypothesis was later negated by a study using PA-knockout mice (Mahoney *et al.*, 1999).

Plasmin activation is controlled by at least two different systems, i.e., inhibition of processing from plasminogen to plasmin by PA inhibitor and direct inhibition of plasmin by anti-plasmin and alpha-2-macroglobulin family inhibitors, including A2ML1. In addition, A2ML1 may inhibit kallikrein, which also processes from plasminogen to plasmin, although kallikrein activation was not found in our study. We suggest that A2ML1 has strong anti-plasmin activity and anti-A2ML1 autoantibodies may have a role in PNP lesions, probably through decrease in cell adhesion. However, new approaches using anti-A2ML1 autoantibodies in PNP sera are needed to unravel this hypothesis.

In the preliminary IF studies using anti-A2ML1 pAb, A2ML1 was confirmed to be present in uppermost layers of the normal human epidermis. By IF of rat bladder, which is one of the diagnostic criteria for PNP, although PNP sera clearly reacted with transitional epithelia, no positive reactivity was found by anti-A2ML1 pAb, indicating that A2ML1 is not responsible for the positive reactivity with rat bladder by PNP sera.

As PNP sera react with multiple antigens, their expression patterns in various tissues should be important to speculate the pathogenicity of each antigen. Expressed sequence tags (ESTs) profile for A2ML1 (Hs.620532, NCBI Unigene, EST profile viewer) suggests high A2ML1 transcript levels in the esophagus, mouth, pharynx, intestine, and muscle. At protein level, A2ML1 is also expressed in non-epithelial tissues, including thymus and testis. Therefore, future studies of the precise expression of A2ML1 in various tissues should be required to understand the role of anti-A2ML1 antibodies in the development of extra-cutaneous lesions.

In this study, the relationship of the presence of anti-A2ML1 antibodies with various clinical and immunological findings in PNP patients was also statistically examined. In our analyses, the presence of anti-A2ML1 autoantibodies was more frequently associated with early onset ($P<0.05$) and absence of ocular lesion ($P=0.05$), while no significant difference was found in any other parameters, including associated malignancies and bronchiolitis obliterans.

In previous IP studies, antibodies to the 170 kDa antigen appeared to be detected in young PNP patients (Williams *et al.*, 2000; Inaoki *et al.*, 2001; Fujimoto *et al.*, 2002; Mimouni *et al.*, 2002; Niimi *et al.*, 2010), which is in line with our results. The reason why the presence of anti-A2ML1 antibodies correlates with absence of ocular involvement is unknown, because the expression of A2ML1 in conjunctivae has not been studied.

In conclusion, the pathogenic role of anti-A2ML1 antibodies is still unclear. Anti-A2ML1 antibodies are already detectable in the initial stage of disease (Reich *et al.*, 1999; Borradori *et al.*, 2001; Heizmann *et al.*, 2001; Schepens *et al.*, 2010), and preferentially react with the N-terminal domain of A2ML1 where target proteases bind (Schepens *et al.*, 2010). These findings raise the possibility that anti-A2ML1 autoantibodies are involved in the development of PNP lesions, through various effects, including inhibition of keratinocyte cell adhesion by activating plasmin, which was suggested in this study.

MATERIALS AND METHODS

All studies followed the guidelines of the Medical Ethics Committees of Kurume University School of Medicine, and were conducted according to the Declaration of Helsinki Principles. Written informed consents were obtained from all patients and the controls.

Patients and antibodies

Serum samples were obtained from 58 patients with PNP, none of which were used in our previous study (Schepens *et al.*, 2010). The diagnosis of PNP was made by the characteristic clinical and histopathological features, direct IF, indirect IF of normal human skin sections and rat bladder sections, enzyme-linked immunosorbent assays of Dsg1 and Dsg3, and recognition of autoantibodies against the EPL and PPL by IB of normal human epidermal extracts. All clinical data were collected from clinical chart of each patient, which are shown in Figure 4, Table 1, and Supplementary Table S1 online. All sera were taken at the time before therapy or on the incipient stage of PNP. Age at onset ranged 11–80 years (average, 57.4 years). Disease control sera were obtained from 10 PV, 10 PF, and 10 BP patients, and normal control sera were obtained from 30 healthy volunteers. See Supplementary Materials and Methods for details for antibodies used in this study.

Indirect IF of normal human skin and rat bladder sections for A2ML1 protein expression

See Supplementary Materials and Methods for technical details.

IF of A2ML1 cDNA-transfected COS7 cells

The cDNA coding entire human A2ML1 was cloned into pcDNA3 eukaryotic expression vector (Invitrogen, Palo Alto, CA) with c-Myc tagged in carboxy-terminus, as described previously (Ruhrberg *et al.*, 1996; Schepens *et al.*, 2010). A2ML1-harboring plasmids were transfected into COS7 cells using Lipofectamine (Invitrogen). After 48 hours, the cells were fixed with cold 100% methanol and blocked with 1% BSA/PBS. Cells were double-stained with 1:20 diluted patient serum, together with either 1:100 diluted anti-c-Myc pAb or 1:50 diluted anti-A2ML1 pAb. Then, the cells were incubated with appropriate secondary antibodies (1:100 diluted FITC-conjugated anti-human IgG, 1:1,000 diluted Texas Red-conjugated anti-rabbit IgG, or 1:1,000 diluted Alexa Fluor 568-conjugaed anti-mouse IgG) and 4′,6-diamidino-2-phenylindole (Nacalai, Kyoto, Japan).

IB of normal human epidermal extracts and transfected COS7 cell extracts

See Supplementary Materials and Methods for technical details (Hashimoto *et al.*, 1990).

Preparation of fractions of A2ML1 protein

Forty eight hours after transfection with A2ML1 construct, the culture medium was harvested. After washing the cells with cold PBS, COS7

cells were scraped in washing buffer (cold PBS) and centrifuged. After supernatant was stored, PBS containing 1% protease inhibitor cocktail (PIC, Sigma-Aldrich, St Louis, MO) and 1% polyoxyethylene-9-octylphenyl ether (NP40, Sigma-Aldrich) (IP buffer) was added to pellet, mixed gently, and incubated for 20 minutes on ice. After centrifugation, both supernatant and pellet resuspended with IP buffer were stored. All the four materials were separated by SDS-PAGE on 5–20% gel (ATTO, Tokyo, Japan) and transferred onto nitrocellulose membrane by semi-dry transfer method using iBlot (Invitrogen). Membrane was blocked with 3% skim milk for 1 hour at room temperature and incubated over night at 4 °C with 1:100 diluted anti-c-Myc pAb or 1:100 diluted anti-A2ML1 pAb, washed, and probed with 1:100 diluted horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or 1:2,000 diluted alkaline phosphatase (AP)-conjugated anti-mouse IgG. Protein bands were visualized using AP and HRP detection systems.

IP–IB of A2ML1-transfected COS7 cell extracts

IP–IB was performed with transfected COS7 cell extracts as substrate for PNP, PV, PF, BP, and normal control sera. In this study, A2ML1 immunoprecipitated with these sera was detected by IB using anti-c-Myc pAb. IP–IB for eukaryotic recombinant A2ML1 protein was performed as described previously (Ruhrberg *et al.*, 1996; Schepens *et al.*, 2010). From preliminary IB results, cell extracts in IP buffer were used for IP–IB. For preclearance, cell extracts were incubated with prewashed protein A agarose (Bio-Rad Laboratories, Hercules, CA) in IP buffer for 30 minutes at 4 °C. After centrifugation, patient serum or anti-c-Myc pAb (positive control) was added to supernatant and incubated with rotation overnight at 4 °C. Then, prewashed protein A agarose was added and incubated for 3 minutes at 4 °C. After centrifugation, the sepharose were washed with wash buffer (PBS containing PIC) with rotation for 3 minutes at 4 °C. Proteins bound to the protein A agarose were eluted by boiling in SDS sample buffer and processed for IB using 1:100 diluted anti-c-Myc pAb as described above, except that protein-A/G–HRP conjugate (Pierce, Rockford, IL) diluted 1:5,000 was used.

IB of NHK extracts under reducing and non-reducing conditions

See Supplementary Materials and Methods for technical details.

Statistical analysis

Differences among qualitative results were compared using the χ^2 test and Bonferroni adjustment. Differences among quantitative parameters between groups were assessed using the Mann–Whitney test. All data are expressed as means ± SD. All analyses were performed using SPSS (SPSS, Chicago, IL).

Detection of A2ML1 protein in cultured NHKs

A2ML1 expression in cultured NHKs was examined by IB. NHKs were cultured in EpiLife medium (Invitrogen) with NHK growth supplement (Invitrogen). After NHKs were reaching confluency, medium was changed to the medium containing 1.2 mM calcium without growth factor and phenol red. We harvested both culture media and cell extracts of NHKs in low calcium (at day 1) and in high calcium (at days 1–5). Culture media were concentrated 100 × using Amicon Ultra-0.5–30K (Millipore, Billerica, MA), and cell extracts were prepared by suspending the NHKs in 1 × SDS sample buffer. IB for A2ML1 was performed as described above.

IB of protein G immunoprecipitated NHK culture media to confirm binding of anti-A2ML1 pAb to A2ML1

After NHKs were cultured as described above, antibodies were added to the concentration of 1 $\mu g\,ml^{-1}$ and incubated at 37 °C for 24 hours. Protein G agarose (Millipore) and binding buffer (20 mM $NaPO_4$) were added to NHK culture media, and incubated for 30 minutes at 4 °C. After centrifugation, the supernatant was concentrated as above. IB for A2ML1 was performed by the same method described above.

Dissociation assay

Dissociation assay was performed as described previously (Ishii *et al.*, 2005; Saleh *et al.*, 2012). NHKs were cultured as described above. After washing with PBS with calcium and magnesium (PBS (+)), the cells were incubated with 2.4 $U\,ml^{-1}$ dispase II (Roche Diagnostics, Mannheim, Germany). Released monolayer was washed with PBS (+) and pipetted five times in 1 ml PBS (−) using 1 ml pipette. The cells were fixed for 10 minutes with 5% formaldehyde. Twenty microliter of 0.02% crystal violet was added and incubated overnight to stain completely. Three pictures were taken and number of fragments was counted manually.

Protease assays

Protease assays using Pierce Colorimetric Protease Assay Kit (Thermo, Rockford, IL) and Serine-Protease detection zymo-electrophoresis Kit (Cosmo Bio, Tokyo, Japan) were performed according to the instruction from manufacturers. Culture medium was prepared and concentrated as above. In zymography, areas of caseinolytic activity appeared as clear zones against a dark blue background. Plasmin and trypsin (0.5 ng each) were also loaded as controls.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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

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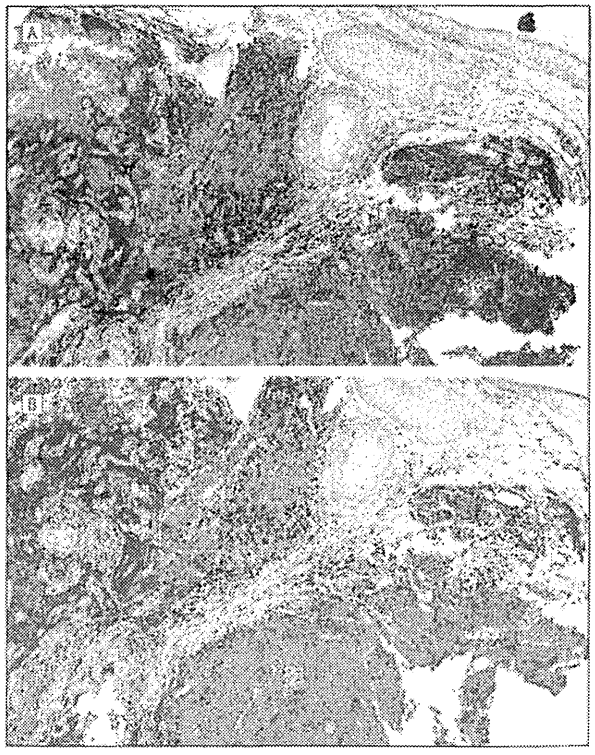

Figure 2. Immunohistochemical studies with anti-CD4 staining (A) and anti-CD8 staining (B) (original magnification ×10 for both).

roid transcription factor 1 (TTF-1). A moderate lymphocytic infiltrate was observed on the biopsy specimens, with $CD4^+$ lymphocytes arranged mainly around the tumor and fewer $CD8^+$ lymphocytes located mainly within the tumor (**Figure 2**). One month later, the patient presented with both a complete regression of the tumor on the nose and enlargement of cervical bilateral lymph nodes (Figure 1B). The patient reported that the tumor had spontaneously regressed within the week following the biopsies. Hypermetabolic uptake was observed on multiple right and left cervical enlarged nodes, but the nose area was found to be normal (Figure 1C and D) on positron-emission tomography.

The patient had stage IIIB disease and refused any surgical treatment for the cervical lymph nodes. Radiation therapy was performed on bilateral cervical areas at a dose of 50 Gy with 2-Gy fractionation for 5 weeks. The patient was free of recurrence at 12-month follow-up.

Comment. To our knowledge, 27 cases of regression in stages I and II MCC have been reported in the literature, including 16 patients with complete regression and 11 with partial regression. Regarding lesions with complete regression, all but 1 were located on the head and the nose area. The MCC lasted for 1 to 16 months prior to spontaneous regression. No recurrence was noted in all 16 cases, the longest follow-up being 15 years. The regression occurred after a tumor biopsy in all cases and was complete within 1 to 5 months.[1] In contrast, in our patient, the nasal tumor regressed within 1 week, while cervical lymph node metastases developed simultaneously.

The spontaneous regression phenomenon has been approximately estimated at 1 in 60 000 to 1 in 100 000 MCC cases.[2] This phenomenon is not specific for this neoplasm; it has also been reported to occur in melanoma, basal cell carcinoma, and other noncutaneous malignant neoplasms. The mechanisms leading to this regression are poorly understood. Biopsy-driven skin disruption may provoke an antitumor inflammatory response by stimulating the local adaptive and/or innate immune system. Some authors have identified $CD4^+$ and $CD8^+$ cells in the lymphocytic tumor infiltrate, as seen in the present case (Figure 2). Others have found clusters of $CD3^+$ cells around tumor nests, suggesting that T-cell–mediated immunity is an important event in tumor regression.[3]

Our patient experienced complete regression of the primary tumor and concomitant development of nodal MCC involvement. This may argue for a strong local antitumoral effect (mediated by adaptive and/or innate defenses) but without sufficient antitumoral regional effect. A massive lymphocytic infiltrate may explain the lymph node enlargement driven by the regional tumoral progression in our patient.

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Conflict of Interest Disclosures: None reported.

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Coexistence of Pemphigus Herpetiformis With IgG Antibodies to Desmocollin 1 and Pemphigoid With IgG Antibodies to BP180 C-Terminal Domain and Laminin γ2

Pemphigus herpetiformis (PH) cases with IgG antibodies to desmocollin 1 (Dsc1) and pemphigoid cases without mucosal involvement reactive with epitopes on various antigens of mucous

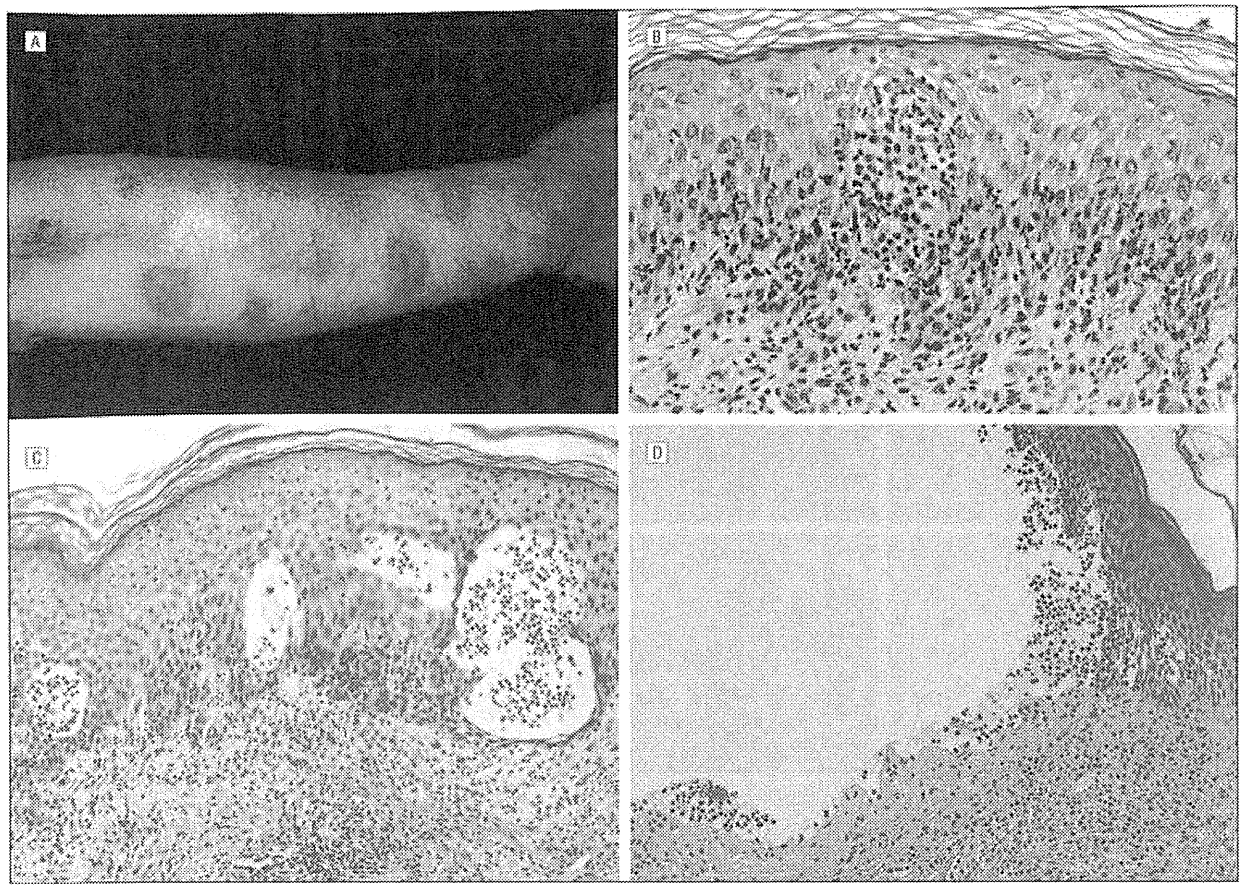

Figure 1. Clinical image and hematoxylin-eosin–stained histopathologic specimens from the subject case. A, Skin lesions on the upper extremity. B and C, Biopsy specimen from the right forearm, showing eosinophilic spongiosis (B) (original magnification ×200) and intraepidermal bullae with eosinophils (C) (original magnification ×100). D, Biopsy specimen from the left forearm, showing subepidermal blister with eosinophils (original magnification ×40).

membrane pemphigoid (MMP) are rare.[1-3] We report a case of coexistence of these 2 bullous diseases.

Report of a Case. A 63-year-old Japanese man with no relevant medical history developed pruritic small urticarial papules and erythemas with vesicles on the neck and upper extremities, which were exacerbated 1 month later. Physical examination revealed coalescing erythemas with peripheral vesicles on the trunk and extremities (**Figure 1**A). Mucous membranes were not involved. Laboratory examination revealed hypereosinophilia (differential eosinophil count, 37.8%) with normal leukocyte count. Biopsy specimens from the right forearm revealed eosinophilic spongiosis together with intraepidermal bullae with eosinophils; specimens from the left forearm revealed subepidermal blister with eosinophils (Figure 1B-D).

Direct immunofluorescence revealed strong IgG deposition and weak IgA deposition on keratinocyte cell surfaces (CS) in the entire epidermis as well as C3 deposition on epidermal basement membrane zone (BMZ) and CS in the lower epidermis (**Figure 2**A-C). Indirect immunofluorescence revealed IgA antibodies to BMZ of human skin, and IgG antibodies to CS and IgA antibodies to BMZ of monkey esophagus, as well as IgG antibodies to transitional epithelia of rat bladder (Figure 2D). Indirect immunofluorescence of 1M sodium chloride–split skin demonstrated IgG reactivity on both the epidermal and dermal sides and IgA reactivity only on the epidermal side (Figure 2E and F).

Immunoblotting detected IgG and IgA antibodies to periplakin in normal human epidermal extract, strong IgG and weak IgA antibodies to BP180 C-terminal recombinant protein, and IgG antibodies to the γ2 subunit of purified human laminin-332 (Figure 2G and H). The reactivity with periplakin might explain positive immunofluorescence of rat bladder findings. No positive reactivity was detected by immunoblotting of normal human dermal extract, HaCaT cell culture supernatant, and BP180 NC16a domain recombinant protein. By enzyme-linked immunosorbent assays, IgG and IgA antibodies to desmoglein 1 (Dsg1), Dsg3, BP230, and BP180 were not detected, while strong IgG reactivity to newly developed eukaryotic recombinant protein of human Dsc1 (optical density, 1.179; cutoff, ≤0.200), but not Dsc2 or Dsc3, was detected (unpublished data, Norito Ishii, MD, February 15, 2013). We made a diagnosis of coexistence of PH reactive with Dsc1 and pemphigoid reactive with BP180 C-terminus and laminin-332 γ2 subunit. Treatment with prednisolone, 60 mg/d, rapidly cleared the lesions, and the dose was tapered subsequently.

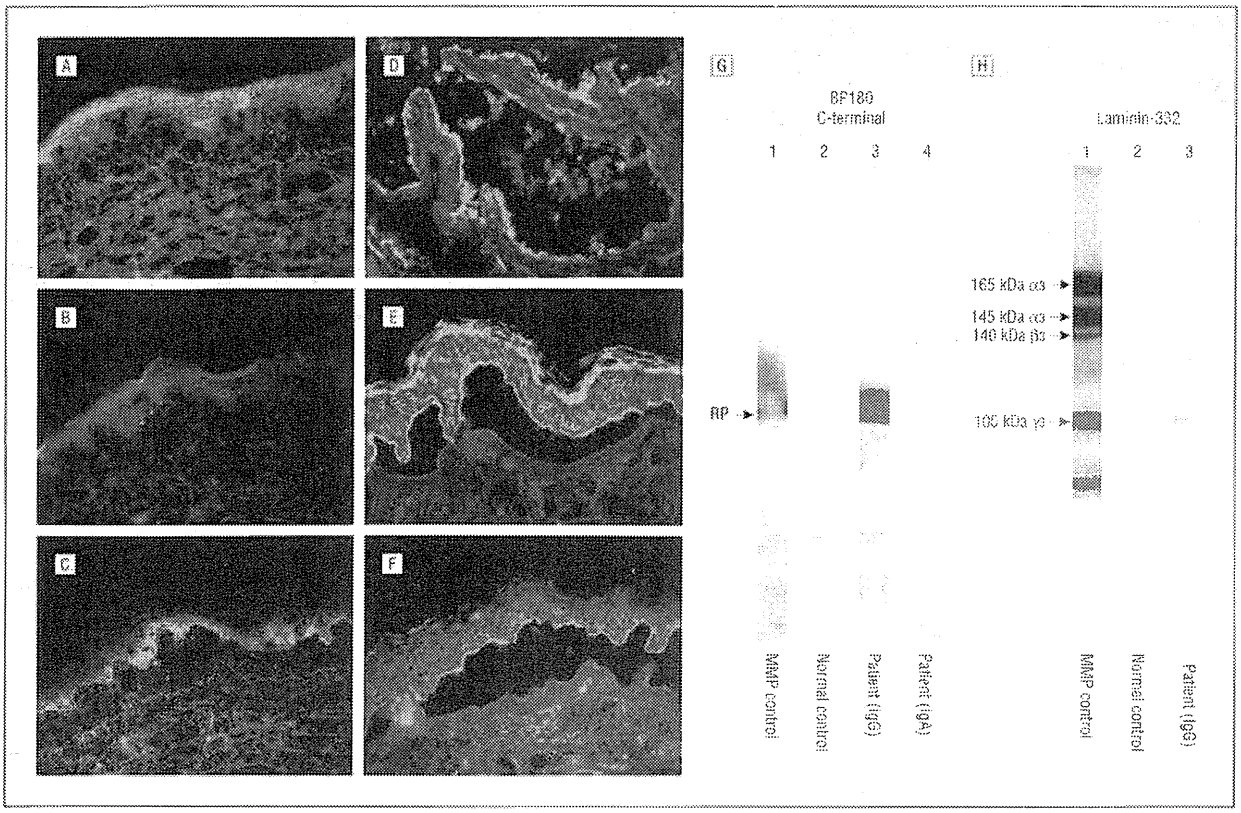

Figure 2. Immunofluorescence and immunoblotting studies of the subject case. A-C, Direct immunofluorescence images for IgG (A), IgA (B), and C3 (C). D, Indirect immunofluorescence of rat bladder for IgG antibodies. E and F, Indirect immunofluorescence of 1M sodium chloride–split skin sections for IgG antibodies (E) and IgA antibodies (F). G, Immunoblotting of recombinant protein of BP180 C-terminal domain, showing positive reactivity by IgG antibodies in control anti-BP180 type mucous membrane pemphigoid (MMP) serum (lane 1) and patient IgG (lane 3) and IgA (weak) (lane 4) antibodies in this case. H, IgG immunoblotting of purified human laminin-332, showing strong reactivity with the α3, β3, and γ2 subunits of laminin-332 for control anti–laminin-332 type MMP (lane 1), and weak reactivity with only the γ2 subunit for this case (lane 3).

Comment. Although obvious acantholysis was not found in this case, eosinophilic spongiosis, intraepidermal bullae, clinical manifestations, and serologic findings were consonant with PH. This case and a recently reported PH case[1] in which IgG anti-Dsc1 antibodies were detected without Dsg1 or Dsg3 reactivity suggest that PH can be associated with anti-Dsc1 antibodies.

Our previously described patient with pemphigoid without mucosal involvement[3] showed autoantibodies to the laminin-332 γ2 subunit, and IgG anti-BP180 C-terminus antibodies are occasionally found in bullous pemphigoid without mucosal involvement.[4] Therefore, IgG antibodies to MMP-specific epitopes may not cause mucosal lesion in some cases. In this case, epitope spreading from Dsc1 to BMZ antigens might cause the coexistence of PH and pemphigoid, although such epitope spreading has not been confirmed.

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Concurrence of bullous pemphigoid and herpetiform pemphigus with IgG antibodies to desmogleins 1/3 and desmocollins 1–3

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MADAM, Autoantibodies in pemphigus preferentially target desmoglein 1 (Dsg1) and Dsg3, and rarely desmocollins 1–3 (Dsc1–3).[1] Pemphigus herpetiformis (PH) is a subtype of pemphigus and is characterized by pruritic annular erythemas with vesicles in the periphery, rarity of mucosal involvement and histopathologically determined eosinophilic spongiosis. Recently, it was suggested that immunoglobulin G (IgG) anti-Dsc3 autoantibodies caused skin lesion in a case of pemphigus vulgaris (PV).[2] In this study, we report the first case of concurrent bullous pemphigoid (BP) and PH with IgG antibodies to both Dsgs and Dscs.

An 83-year-old woman presented with a 3-month history of painful oral erosions and ulcers. The patient's medical history was unremarkable. Physical examination revealed multiple blisters, erosions and ulcers on the tongue, soft palate and buccal mucosa (Fig. 1a). Ten days later, pruritic urticarial annular erythemas developed on the abdomen and lower extremities, some of which showed small vesicles at the periphery (Fig. 1b). Laboratory examination revealed slight hypereosinophilia. A biopsy specimen from the right lower extremity revealed eosinophilic spongiosis and many intraepidermal vesicles without apparent acantholysis in the epidermis (Fig. 1c), and also a few subepidermal vesicles with many eosinophilic infiltrates (Fig. 1d).

Direct immunofluorescence showed IgG deposition on the cell surfaces of keratinocytes in the whole epidermis (Fig. 2a), and C3 deposition to the epidermal basement membrane zone (BMZ) and cell surfaces in the lower epidermis (Fig. 2b). Indirect immunofluorescence of normal human skin sections revealed IgG antikeratinocyte cell surface antibodies at a titre of 1:10. Indirect immunofluorescence of monkey oesophagus sections also revealed anticell surface antibodies (Fig. 2c). Indirect immunofluorescence of 1 mol L^{-1} NaCl split skin sections showed negative results. Enzyme-linked immunosorbent assays (ELISAs) revealed elevated indices of anti-BP180 antibodies (97, normal < 20), anti-Dsg1 antibodies (47, normal < 20) and anti-Dsg3 antibodies (96, normal < 20). Anti-BP230 antibodies were not detected by ELISA. The patient's IgG reacted with Dsc1–3 expressed on the cell surfaces of COS-7 cells, which were transfected with cDNAs of human Dsc1–3 (Fig. 2d–f).

By immunoblotting, IgG autoantibodies showed no positive reactivity in either normal human epidermal and dermal extracts, HaCaT cell culture supernatant or purified human laminin 332, while they reacted with a recombinant protein of BP180 NC16a domain (Fig. 2g), but not of BP180 C-terminal domain. Our new IgG ELISA using eukaryotic recombinant proteins of human Dsc1–3 (Ishii *et al.*, manuscript in preparation) revealed a high titre of anti-Dsc2 antibodies and slightly elevated anti-Dsc3 antibodies, but the optical density value of anti-Dsc1 antibodies was lower than the cut-off value. Based on these findings, we diagnosed this case as concurrent BP with anti-BP180 antibodies and PH with IgG antibodies to Dsg1/3 and Dsc1–3.

Systemic corticosteroids, 20 mg daily, improved all lesions, and the ELISA indices of BP180, Dsg1 and Dsg3 decreased to normal levels along with an improvement in the disease.

In our case, a diagnosis of PH was suggested by all clinical, histopathological and immunofluorescence findings. While our previous studies using Dsg1 and Dsg3 ELISA showed that the target antigens of PH were mainly Dsg1 and occasionally Dsg3,[3] anti-Dsc antibodies were also detected in some cases of

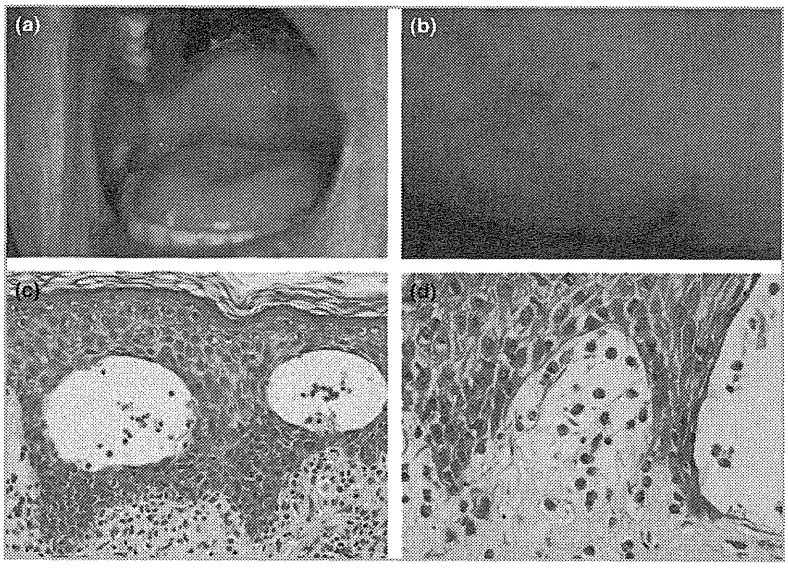

Fig 1. Clinical and histopathological features in our patient. (a) Oral mucosal lesions. (b) Skin lesions on the lower extremity. (c) Eosinophilic spongiosis without apparent acantholysis [haematoxylin and eosin (HE) staining, original magnification ×200]. (d) Subepidermal vesicles with many eosinophilic infiltrates (HE staining, original magnification ×400).

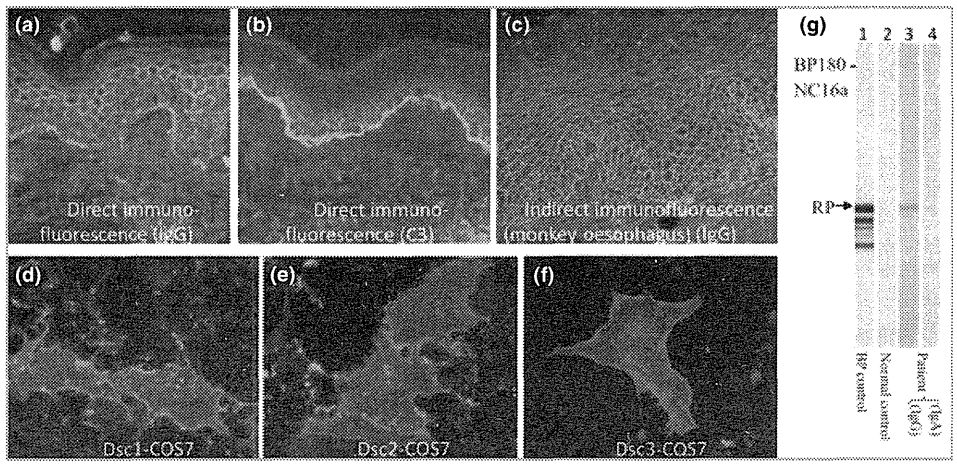

Fig 2. The results of immunological studies. (a) Direct immunofluorescence for immunoglobulin G (IgG) deposition. (b) Direct immunofluorescence for C3 deposition. (c) Indirect immunofluorescence of monkey oesophagus sections for IgG antibodies. (d–f) IgG antibodies reacted with desmocollins 1–3 (Dsc1–3) expressed on COS-7 cells. (g) Immunoblotting of the recombinant protein of the bullous pemphigoid (BP) 180 NC16a domain. IgG antibodies (lane 3), but not IgA antibodies (lane 4), were positive. Lane 1: positive BP control serum. Lane 2: negative normal control serum.

PH.[4,5] Therefore, the serological results for autoantigens in our patient were consistent with PH.

The histopathological change of subepidermal vesicles with eosinophilic infiltrate, immunofluorescence detection of IgG anti-BMZ antibodies, and the presence of circulating antibodies to the BP180 NC16a domain by ELISA and immunoblotting, clearly signified the concurrence of BP in our case. One biopsy specimen showed both eosinophilic spongiosis and subepidermal vesicles, suggesting that BP and pemphigus lesions were admixed throughout the whole body. However, it was difficult to differentiate BP lesions from pemphigus lesions clinically. In our case, oral lesions may have been induced by antibodies to either Dsg3 and Dsc3 or BP180. However, anti-BP180 antibodies may not be responsible for oral lesions, because we could not detect antibodies to the BP180 C-terminal domain, which are considered to be pathogenic in anti-BP180-type mucous membrane pemphigoid.[6]

Our case can also be considered a type of atypical pemphigus because of the rare involvement of oral lesions in PH. We have reported that IgG and IgA anti-Dsc1–3 antibodies were found mainly in atypical pemphigus.[7] A European cohort study also disclosed that IgG and IgA anti-Dsc autoantibodies were present in paraneoplastic pemphigus sera, and that IgA anti-Dsc antibodies were present in atypical pemphigus sera, while PV did not show these antibodies.[8] The findings from our patient also confirmed this close relationship between anti-Dsc1–3 antibodies and various types of atypical pemphigus.

We have recently reported a case with IgG antibodies to Dsc1–3 determined by cDNA transfection.[1] Similar to the previous case, the present case also showed anti-Dsc1–3 antibodies by cDNA transfection. However, our new ELISAs using eukaryotic recombinant proteins of human Dsc1–3 detected antibodies to Dsc2 and Dsc3, but not Dsc1. The identical results from cDNA transfection and our new ELISA might not have been obtained due to the different examination systems. However, at least IgG anti-Dsc3 antibodies seemed to play an important role in our case, which is consistent with the finding in the previous study.[2]

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CD8$^+$ Sézary syndrome with interleukin-22 production modulated by bacterial sepsis

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MADAM, Patients with Sézary syndrome are susceptible to bacterial sepsis, as cutaneous T-cell lymphoma (CTCL) can be driven by a superantigen-stimulated clonal proliferation of T cells.[1,2] Recently, it has been reported that interleukin (IL)-22 plays an important role in innate immunity to regulate early defence mechanisms against attaching and effacing bacterial pathogens.[3] Here, we report a case of Sézary syndrome characterized by a high frequency of circulating IL-22-producing CD8$^+$ tumour cells under a condition of bacterial sepsis.

A 54-year-old Japanese woman was referred to us for evaluation of a 13-year history of a pruritic, erythrodermic eruption with eosinophilia, which was resistant to oral corticosteroids and ciclosporin. Notably, for the past 2 years, she had had several episodes of sepsis caused by long-standing pyogenic spondylitis or pneumonia. On examination, she had pigmented erythroderma, which was poikilodermic in some parts (Fig. 1a), with lymphoadenopathy on her groins. Peripheral blood examination showed normal counts of leukocytes ($7{\cdot}1 \times 10^9\ L^{-1}$) with 83·0% neutrophils, 11·0% eosinophils, 5·0% lymphocytes, and 0·5% of lymphocytes exhibiting

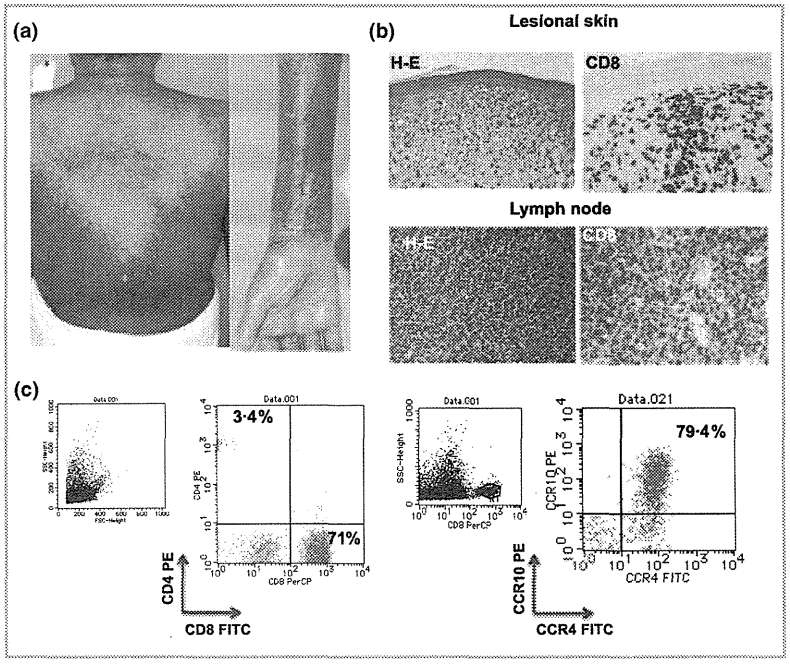

Fig 1. Clinical features, histopathological findings and flow cytometric analysis. (a) Erythroderma with hyperpigmentation and poikiloderma. (b) Haematoxylin and eosin (H-E) and immunohistochemical stainings, showing that CD8$^+$ atypical lymphoid cells infiltrated into the epidermis and lymph node (original magnification × 200). (c) Flow cytometric analysis, showing predominant expansion of CD8$^+$ T cells expressing CCR4 and CCR10.

Case Report/Case Series

Two Cases of Pemphigus Vegetans With IgG Anti-Desmocollin 3 Antibodies

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IMPORTANCE Pemphigus vegetans shows clinically vegetating and/or pustular skin lesions mainly on the intertriginous areas and histopathologically neutrophilic and eosinophilic pustules in the epidermis. Pemphigus vegetans shows IgG reactivity mainly with desmoglein (Dsg) 3, but also with other autoantigens, including Dsg1 and desmocollins (Dscs).

OBSERVATIONS We examined antigen profiles in 2 cases of pemphigus vegetans. (1) A women in her 80s presented with typical vegetating skin lesions on the right inguinal region with typical histopathological features. Immunoblotting using normal human epidermal extracts detected IgG antibodies to Dsg1 and Dscs. Enzyme-linked immunosorbent assays (ELISAs) revealed IgG antibodies to Dsg1 but not to Dsg3. Complementary DNA (cDNA) transfection method to COS-7 cells and novel ELISAs using eukaryotic recombinant proteins of human Dsc1, Dsc2, and Dsc3 confirmed specific IgG reactivity with Dsc3. (2) A women in her 70s presented with pustular skin lesions on the left fingers with typical histopathological features. Immunoblotting and ELISAs did not detect antibodies to either Dsg1 or Dsg3. Conversely, immunoblotting detected IgG antibodies to Dscs, cDNA transfection method revealed IgG reactivity only with Dsc3, and findings from ELISAs showed that IgG reacted weakly with Dsc2 and strongly with Dsc3.

CONCLUSIONS AND RELEVANCE Autoantibodies to Dscs, particularly to Dsc3, may play a pathogenic role in some cases of pemphigus vegetans.

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Pemphigus is an autoimmune bullous disorder characterized by autoantibodies to keratinocyte cell surface antigens and is divided into 2 major forms, pemphigus foliaceus and pemphigus vulgaris.[1] Pemphigus vegetans, a variant of pemphigus vulgaris, is characterized clinically by hypertrophic vegetating skin lesions and/or pustules mainly on the intertriginous areas and histopathologically by neutrophilic and eosinophilic pustule formation in the epidermis.[2] Pemphigus vegetans is classified as Neumann-type and Hallopeau-type according to different clinical features and disease courses.[2] However, some pemphigus vegetans cases simultaneously showed features of both types, indicating common pathomechanisms between the 2 variants.[2]

Major autoantigens for pemphigus are desmogleins (Dsgs); transmembrane cell-cell adhesion proteins belonging to cadherin family; and Dsg1 and Dsg3, which are autoantigens for pemphigus foliaceus and pemphigus vulgaris, respectively.[1] Major autoantigen for pemphigus vegetans is Dsg3,[3] although a few cases were reported to show autoantibodies to Dsg1 or desmocollins (Dscs), another group of desmosomal cadherin.[4-7] We have identified Dsc1 as an autoantigen for subcorneal pustular dermatosis-type IgA pemphigus.[8] In addition, our previous study of enzyme-linked immunosorbent assays (ELISAs) using baculovirus-expressed human Dsc recombinant proteins demonstrated that Dscs reacted with serum samples of patients with atypical pemphigus but not with those of patients with classic pemphigus.[9]

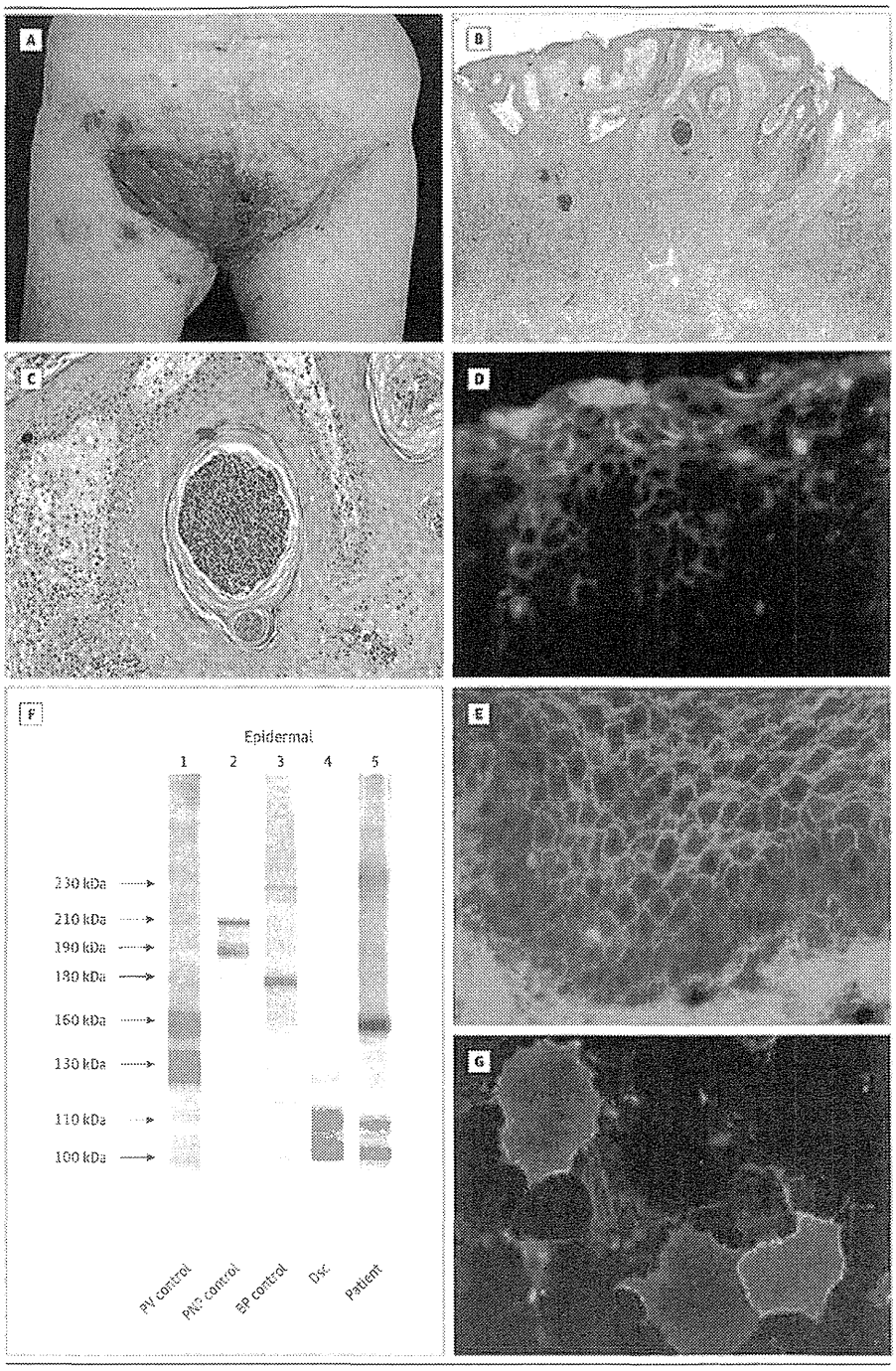
In the present study, we report 2 cases of pemphigus vegetans in which IgG autoantibodies to Dscs, particularly Dsc1, were detected by immunoblotting, complementary DNA (cDNA) transfection method, and novel ELISAs using mammalian recombinant proteins of human Dsc1, Dsc2, and Dsc3.

Report of Cases

Case 1

A woman in her 80s presented with elevated skin lesions on the right inguinal region, which first developed as accumulated small pustules. The patient had no remarkable family history. One member of the patient's family noticed the discharging tumorous skin lesion 2 weeks prior to presentation,

Figure 1. Case 1



A, Clinical appearance. B and C, Histopathological features (hematoxylin-eosin, original magnification ×100 [B], ×400 [C]). D, Direct immunofluorescence for C3. E, Indirect immunofluorescence of monkey esophagus sections for IgG antibodies. F, The results of immunoblotting of normal human epidermal extracts for IgG antibodies. A control pemphigus vulgaris serum sample reacted with both the 160-kDa Dsg1 and 130-kDa Dsg3 (lane 1, PV control); a control paraneoplastic pemphigus serum sample reacted with both the 210-kDa envoplakin and the 190-kDa periplakin (lane 2, PNP control); a control bullous pemphigoid serum sample reacted with both the 230-kDa BP230 and the 180-kDa BP180 (lane 3, BP control); anti-desmocollin monoclonal antibody 52-3B reacted with the 110-kDa a-form and the 100-kDa b-form of desmocollins (lane 4, Dsc); and IgG antibodies of case 1 reacted clearly with Dsg1 and Dsc3 (lane 5, Patient). G, Complementary DNA transfection method for IgG antibodies to Dsc3.

although the patient did not remember the onset of the lesion owing to dementia.

Physical examination revealed a slightly gray-colored vegetating plaque, with several surrounding erythemas and small pustules, on the right inguinal area (Figure 1A). Laboratory tests revealed elevations in erythrocyte sedimentation rate (29 mm/h [reference range, <15 mm/h]), presence of antihuman T-cell lymphoma/leukemia virus-1 antibodies (2048 s/co [signal to cutoff] [reference range, <16 s/co]), carcinoembryonic antigen level (7.3 ng/mL [reference range, <5.0 ng/mL]), and squamous cell carcinoma antigen level (6.5 ng/mL [reference range, <1.5]). ELISAs detected anti-Dsg1 antibodies (ELISA index value, 101 [reference range, <14]) but not anti-Dsg3 antibodies (index, <5 [reference range <14]).

Chest radiography, abdominal ultrasonography, gastrointestinal endoscopy, electrocardiogram, and vaginal examination revealed no abnormal findings. Enhanced computed tomography of the abdomen and pelvis revealed hypertrophic

figures, measuring 6 × 6 × 1 cm, for the skin lesion on the right inguinal area. In addition, edema and increased amounts of subcutaneous fat to the depth of the long adductor muscle were observed around the skin lesions. Gallium scintigraphy also revealed a strong abnormal accumulation on the right inguinal region.

Histopathological analysis of a biopsy specimen of the skin lesion revealed a reticularly arranged acanthotic epidermis with minimum acantholysis and eosinophilic spongiosis, which contained keratinous cystlike structures with eosinophilic and neutrophilic pustules (Figure 1B and C). Extensive inflammatory infiltration of eosinophils, neutrophils, and plasma cells was found in the superficial and mid-dermis (Figure 1B).

Direct immunofluorescence detected C3 deposit to keratinocyte cell surfaces (Figure 1D), without any deposits of IgG, IgA, or IgM. Findings from indirect immunofluorescence of normal human skin sections were negative for both IgG and IgA antibodies, whereas indirect immunofluorescence of monkey esophagus sections detected IgG (Figure 1E), but not IgA, antiepithelial cell surface antibodies.

Immunoblotting of normal human epidermal cell extracts revealed that IgG antibodies in a serum sample from the patient reacted with the 110-kDa and 100-kDa doublet proteins, which comigrated with a-form and b-form of Dscs, in addition to the 160-kDa Dsg1 (Figure 1F). cDNA transfection method using cDNAs of human Dsc1, Dsc2, and Dsc3 and cultured COS-7 cells was performed as described previously.[8] This study showed that IgG, but not IgA, antibodies reacted with Dsc3 but not with either Dsc1 or Dsc2 (Figure 1G). By novel ELISAs using eukaryotic recombinant proteins of human Dsc1 through Dsc3 (Ishii et al, unpublished data; 2012), IgG antibodies reacted strongly with Dsc3 (optical density [OD], 1.803 [cutoff, 0.120]), but not with either Dsc1 (OD, 0.069 [cutoff, 0.200]) or Dsc2 (OD, 0.040 [cutoff, 0.070]).

From the typical clinical and histopathological findings, the diagnosis of pemphigus vegetans was made. We treated the patient with oral prednisolone, 20 mg/d, which successfully controlled the skin lesion, and the mass disappeared almost completely.

Case 2

A woman in her 70s presented with erosive skin lesions on the left fingers. The patient had no remarkable family history. She first noticed an erosion on the left third finger web 3 months prior to presentation. Antifungal cream, which was prescribed by an internist under the putative diagnosis of tinea interdigitale, was ineffective. The lesion increased in number and size.

Physical examination revealed erosive skin lesions with pustules and scaly erythemas on the left third and fourth fingers (Figure 2A). Laboratory tests revealed an elevated numbers of eosinophils (580.5/μL) and presence of antinuclear antibodies with speckled pattern (×40), with no abnormality in other test results. ELISAs did not detect antibodies to either Dsg3 or Dsg1.

Histopathological analysis of a biopsy specimen of the skin lesion showed acanthotic epidermis with no apparent acantholysis and extensive inflammatory infiltrates in the dermis (Figure 2B). Typical large eosinophilic pustules with a few neutrophils were also present in the epidermis (Figure 2C).

Direct immunofluorescence detected no deposits of IgG, IgA, IgM, or C3. Results of indirect immunofluorescence of normal human skin sections were negative for both IgG and IgA antibodies, while indirect immunofluorescence of monkey esophagus sections detected IgG (Figure 2D), but not IgA, antiepithelial cell surface antibodies.

Immunoblotting of normal human epidermal cell extracts revealed that IgG antibodies in a serum sample from the patient reacted with the 110-kDa a-form and the 100-kDa b-form of Dscs, as well as the 230-kDa BP230-like band and the190-kDa periplakin-like band, but did not react with Dsgs (Figure 2E). cDNA transfection method revealed that IgG, but not IgA, antibodies reacted only with Dsc3 (Figure 2F). Novel Dsc ELISAs revealed that IgG antibodies reacted with Dsc2 (OD, 0.142 [cutoff, 0.070]) and strongly with Dsc3 (OD, 1.324 [cutoff, 0.120]), but not with Dsc1 (OD, 0.103 [cutoff, 0.200]).

From the typical clinical and histopathological findings, the diagnosis of pemphigus vegetans was made. We treated the patient first with a topical corticosteroid because the lesions were limited to the fingers. Although the treatment was effective, oral mucosal lesions and pustular skin lesions on the left inguinal area subsequently appeared. Oral prednisolone, 20 mg/d, completely controlled the lesions. We tapered the prednisolone dose to 5 mg/d without any recurrence. The clinical course was well correlated with serum eosinophil numbers.

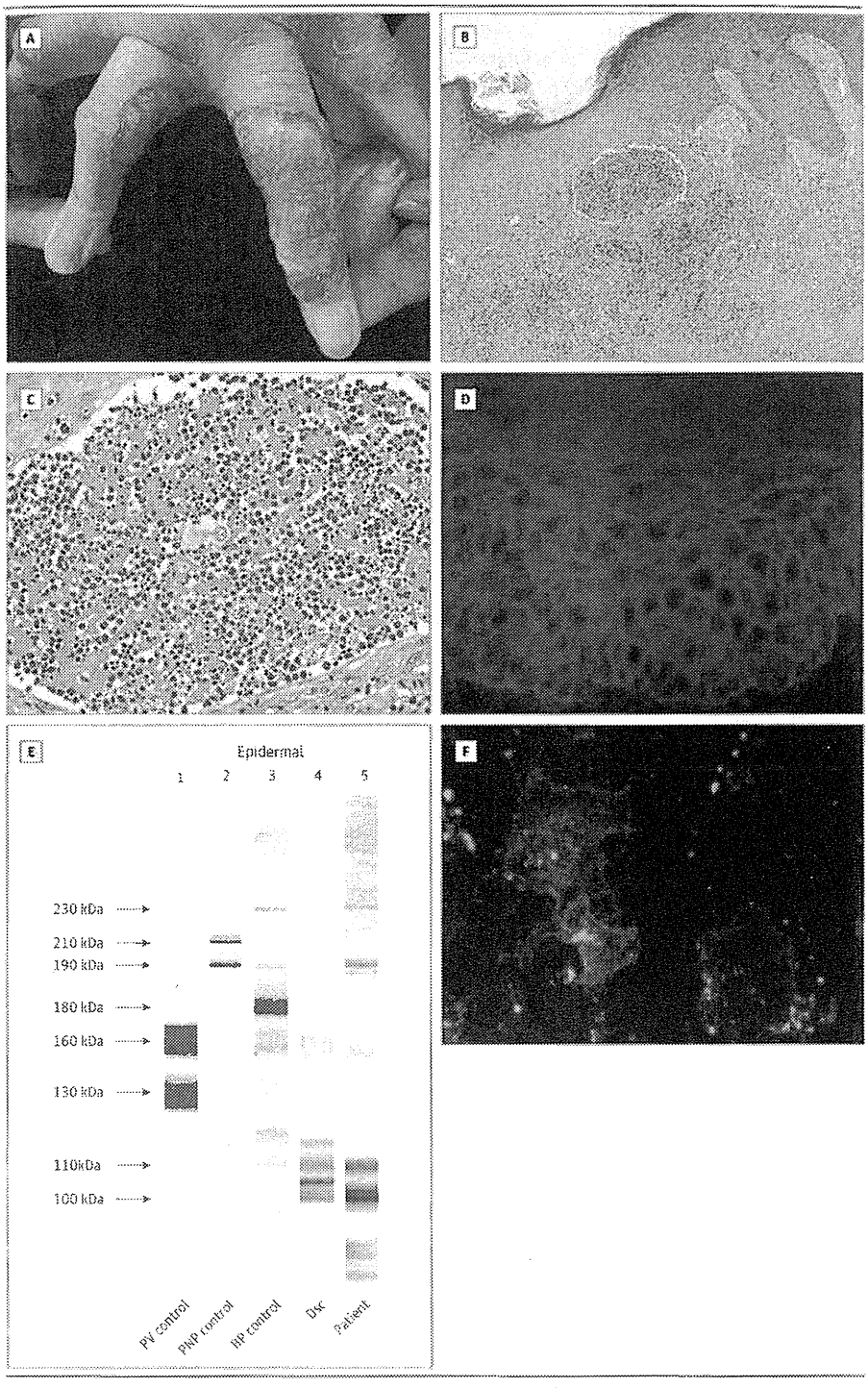
Discussion

We extensively analyzed autoantigens for 2 cases of clinically and histopathologically typical pemphigus vegetans. Three serological tests, including immunoblotting, cDNA transfection method, and novel ELISAs for Dsc1, Dsc2, and Dsc3, indicated that case 1 had IgG anti-Dsc3 antibodies, in addition to IgG anti-Dsg1 antibodies. Case 2 also showed strong IgG reactivity with Dsc3, although lower reactivity with Dsc2 was detected by ELISA but not cDNA transfection method. Intriguingly, case 2 showed no antibodies to either Dsg3 or Dsg1.

Immunoblotting of normal human epidermal extracts is known to detect anti-Dsc antibodies in only few cases, probably because epitopes on Dscs are conformation dependent and cannot be detected by immunoblotting.[4,5] Because the results of reactivity with Dsc1, Dsc2, and Dsc3 in this study were almost identical between cDNA transfection method and novel Dsc ELISAs, combination of these tests should be a reliable method to detect autoantibodies to Dscs in various types of pemphigus in the future.

In case 2, immunoblotting of normal human epidermal cell extracts showed the 190-kDa periplakin-like and 230-kDa BP230-like bands. We considered the periplakin-like band as a nonspecific reaction because it occasionally occurs even in normal control serum samples. The BP230-like band was also considered as nonspecific reaction because the patient's serum sample did not show anti-basement membrane zone an-

Figure 2. Case 2



A, Clinical appearance, B and C, Histopathological features (hematoxylin-eosin, original magnification, ×100 [B] and ×400 [C]). D, Indirect immunofluorescence of monkey esophagus sections for IgG antibodies. E, The results of immunoblotting of normal human epidermal extracts for IgG antibodies. The explanation for each lane is the same as that in Figure 1, F, Complementary DNA transfection method for IgG antibodies to Dsc3.

tibodies in indirect immunofluorescence, and the 230-kDa band is occasionally shown in serum samples from patients with nonbullous pemphigoid.

Clinically, both case 1 and case 2 showed Hallopeau-type clinical features. Both cases showed almost identical histopathological features, characteristic of pemphigus vegetans. In addition, both cases showed strong IgG reactivity with Dsc3. Considering that clinical features of both Neuman- and Hallopeau-types concur in some cases of pemphigus vegetans,[2] the results of the present study may suggest a common pathomechanism in the Hallopeau-type of pemphigus vegetans.

To date, autoantibodies to Dscs were identified only occasionally in patients with nonclassic types of pemphigus, in-

cluding pemphigus vegetans, pemphigus herpetiformis, paraneoplastic pemphigus, and atypical pemphigus.[4-9] However, precise prevalence of IgG anti-Dsc autoantibodies in various types of pemphigus, including pemphigus vegetans, has not been fully elucidated. To answer this question, extensive studies using Dsc ELISAs in large numbers of patients with pemphigus should be performed.

The pathogenic role of anti-Dsc antibodies is not well understood in any types of pemphigus. In our study, case 2 is particularly interesting because this case showed typical clinical features of pemphigus vegetans but no anti-Dsg antibodies, supporting our previous speculation of pathogenic role of anti-Dsc autoantibodies in pemphigus vegetans.[4]

The studies of knockout mice of Dsc1 and Dsc3 showed pemphigus-like skin fragility with defective epidermal barrier function.[10,11] In addition, the pathogenic role of anti-Dsc3 antibodies were demonstrated in a single pemphigus case with anti-Dsc3 antibodies[12] and by an in vitro cell culture study.[13] These previous studies suggested that anti-Dsc antibodies play a pathogenic role by influencing keratinocyte cell adhesion. Further functional studies should be required to elucidate the pathogenic relevance of anti-Dsc antibodies in pemphigus.

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Study concept and design: Hashimoto.
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Analysis and interpretation of data: Teye, Ohyama, Ohata, Furumura, Tsuruta.
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CONCISE COMMUNICATION

Novel *IL36RN* mutation in a Japanese case of early onset generalized pustular psoriasis

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ABSTRACT

Generalized pustular psoriasis is a distinct type of psoriasis characterized by recurrent febrile attacks with disseminated subcorneal pustules on generalized skin rashes. Recently, homozygous and compound heterozygous mutations of the *IL36RN* gene, which encodes the anti-inflammatory cytokine interleukin (IL)-36 receptor antagonist, were identified in familial and sporadic cases of various ethnicities with generalized pustular psoriasis. Here we report a 39-year-old Japanese male patient who had suffered from repeated attacks of generalized pustular psoriasis since infancy with intervals of several years. At presentation, erythematous lesions with a few pustules were found only on some parts of the body and controlled with topical corticosteroids. An analysis of the *IL36RN* gene revealed compound heterozygous mutations of c.28C>T and c.368C>T. While the former mutation causing the premature termination p.Arg10X is recurrent in Japanese cases, the latter missense mutation causing p.Thr123Met substitution is novel, but another mutation in the same position has been reported in one Japanese case. Our report further supports the presence of the Japanese-specific hot spots in the *IL36RN* gene, 28C and 368C, and suggests the functional significance of Thr123. This special type of generalized pustular psoriasis caused by *IL36RN* mutations has been designated as deficiency for IL-36 receptor antagonist, a new hereditary autoinflammatory disease, and its phenotypes have emerged to include other related pustular disorders, palmoplantar pustulosis, acrodermatitis continua of Hallopeau, and acute generalized exanthematous pustulosis. The genetic analysis of the cases with these diseases would be important for establishment and application of the specific treatments targeting the IL-36 signaling.

Key words: generalized pustular psoriasis, hereditary autoinflammatory disease, *IL36RN*, interleukin-36 receptor antagonist, mutation.

INTRODUCTION

Generalized pustular psoriasis (GPP) is a distinct type of psoriasis characterized by recurrent febrile attacks with disseminated subcorneal pustules on generalized skin rashes. Similar to psoriasis vulgaris, patients with GPP are mostly sporadic with an adult onset, but some of them are familial and/or have an early onset.[1] In 2011, homozygous mutations were identified in the *IL36RN* gene which encodes the interleukin (IL)-36 receptor antagonist (IL-36Ra), by homozygosity mapping of the genome of Tunisian cases with familial GPP and by exome sequencing of the genome of sporadic European GPP cases.[2,3] IL-36Ra is an anti-inflammatory cytokine antagonizing IL-36 signaling. IL-36Ra and IL-36α, -β and -γ belong to the IL-1 family, and were formerly termed IL-1F5 and -F6, -F8 and -F9, respectively. They are predominantly expressed in keratinocytes, and play a role in the induction and regulation of skin inflammation.[4,5] GPP-associated *IL36RN* mutations cause the defective expression and/or impaired antagonistic effect of IL36Ra, leading to dysregulated skin inflammation. Recently, the identification of other homozygous and compound heterozygous *IL36RN* mutations has been reported in Japanese sporadic GPP cases.[6,7] Thus, it has now been proven that these GPP-associated *IL36RN* mutations are present globally, and that different mutations are present in different ethnicities.

METHODS

The case we present here is a 39-year-old Japanese man who had suffered from repeated GPP attacks since infancy with intervals of several years. He was born to non-consanguineous parents without any troubles during or after pregnancy. No remarkable skin and/or immunological disorders had been reported in his relatives. Skin rashes with segregated pustules first developed on his head and abdomen at approximately 2.5 years of age, and they gradually enlarged to be generalized at 3 years of age with high fever. Once the attack had diminished, the skin rashes with some pustules remained without palmoplantar involvement. Skin lesions with plaque-type

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psoriasis were also observed between GPP attacks. Although the skin lesions were controlled with topical corticosteroids, similar attacks occurred at 9, 12, 22 and 37 years of age, without any remarkable preceding symptoms. A photograph of the generalized erythematous plaques with disseminated subcorneal pustules when he was 12 years old is shown in Figure 1(a). At presentation, erythematous lesions with a few pustules were found only on some parts of the body, especially in the intertriginous areas including the neck, axilla and under the breast (Fig. 1b,c).

As the involvement of genetic variations was suspected in his disease, an analysis of the *IL36RN* gene was performed according to the protocol approved by the Ethical Committee of Wakayama Medical University, which followed the Declaration of Helsinki. After written informed consent was obtained, genomic DNA was extracted from his peripheral blood, and all exons of the *IL36RN* gene with adjacent exon–intron boundaries were amplified by polymerase chain reaction and directly sequenced using the gene-specific primers as described elsewhere.[7] To analyze each allele, the amplified DNA fragments containing the whole exons were subcloned and the inserted plasmids extracted from selected clones were directly sequenced.

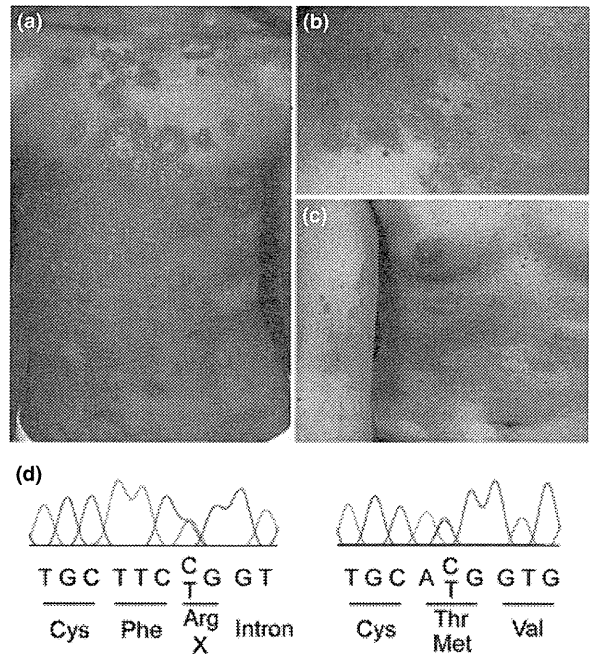

Figure 1. Clinical pictures and genetic abnormalities of the patient. (a) Various sizes of erythematous plaques lined with multiple pustules and crusts on the trunk at 12 years of age. (b) Erythematous lesions with a few pustules at 39 years of age on the right neck and (c) on the right arm and abdomen. (d) Electropherograms of the *IL36RN* gene sequence showing a heterozygous c.28C>T transition causing p.Arg10X (left) and a c.368C>T causing p.Thr123Met (right).

RESULTS

Compound heterozygous mutations of c.28C>T causing the premature termination p.Arg10X and c.368C>T causing p.Thr123Met substitution were identified, as shown in Figure 1(d). These mutations were revealed to be located on the different alleles (data not shown). Genetic analysis of the patient's relatives was not performed, because informed consent was not obtained. As shown in Table 1, which summarizes the *IL36RN* mutant alleles reported thus far associated with GPP and related pustular diseases, the c.28C>T transition is recurrent in Japanese cases. In contrast, the c.368C>T transition is novel, but another mutation in the same position, the c.368C>G causing p.Thr123Arg, has been reported in one Japanese case. Because it has been shown that IL-36Ra possessing the p.Thr123Arg substitution has impaired antagonistic effects in *in vitro* experiments, a similar defect can be expected in the case of p.Thr123Met.[7] Notably, the c.368C>T transition has never been identified by direct sequencing in 200 control alleles (data not shown). This result is compatible with the previous report that the

Table 1. Summary of the reported *IL36RN* mutant alleles associated with generalized pustular psoriasis and related pustular disorders

Location	Genomic mutation	Amino acid change	No. of alleles	Disorders	Racial origin	References
Exon 2	28C>T	Arg10X	4	GPP	Japanese	6,7, this study
Exon 3	80T>C	Leu27Pro	34	GPP, AGEP	African	2,10
Exon 3	104A>G	Lys35Arg	1	GPP	European	1,9
Intron 3	115+6T>C	Arg10ArgfsX (Exon 3 skipping)	16	GPP	Asian (Japanese, Chinese, Malay)	7,9
Exon 4	142C>T	Arg48Trp	2	GPP, AGEP	European	3,10
Exon 5	304C>T	Arg102Trp	1	ACH	European	1,9
Exon 5	338C>T	Ser113Leu	25	GPP, PPP, ACH, AGEP	European	3,9,10
Exon 5	368C>G	Thr123Arg	1	GPP	Japanese	7
Exon 5	368C>T	Thr123Met	1	GPP	Japanese	This study

ACH, acrodermatitis continua of Hallopeau; AGEP, acute generalized exanthematous pustulosis; GPP, generalized pustular psoriasis; PPP, palmoplantar pustulosis.

BaeGI restriction site containing 368C is intact in 200 control alleles.[7]

DISCUSSION

We have reported a Japanese case with an early onset GPP, who showed compound heterozygous *IL36RN* mutations including the recurrent c.28C>T (p.Arg10X) and the novel c.368C>T (p.Thr123Met). Our report further supports the presence of the Japanese-specific hot spots in the *IL36RN* gene, 28C and 368C, and suggests the functional significance of Thr123. As early onset of the disease, as well as its familial occurrence, suggests genetic involvement, a greater number of early onset GPP cases with any *IL36RN* mutations would be discovered, if genetically analyzed more extensively.

This special type of GPP caused by *IL36RN* mutations is considered a novel hereditary systemic autoinflammatory disease distinguishable from other GPP cases, and has been designated as deficiency for IL-36 receptor antagonist (DITRA).[2,8] As it has more recently been reported that the same mutations were detected in the patients with GPP-related pustular disorders, including palmoplantar pustulosis (PPP), acrodermatitis continua of Hallopeau (ACH) and a GPP-like drug eruption acute generalized exanthematous pustulosis (AGEP), the phenotypes of DITRA cases have emerged to include GPP, PPP, ACH and AGEP, as summarized in Table 1.[9,10] Because no apparent phenotypical differences have been reported between the *IL36RN* mutation-negative versus positive cases, the genetic analysis of any cases with these diseases would be important for establishment and application of the specific treatments targeting the IL-36 signaling.

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Case Report

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A New Infant Case of Nakajo-Nishimura Syndrome with a Genetic Mutation in the Immunoproteasome Subunit: An Overlapping Entity with JMP and CANDLE Syndrome Related to *PSMB8* Mutations

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Key Words
Nakajo-Nishimura syndrome · *PSMB8* mutation · Immunoproteasome · Partial lipodystrophy

Abstract
Nakajo-Nishimura syndrome (NNS) is a very rare hereditary autoinflammatory disorder that generally has its onset in infancy with pernio-like rashes and gradually develops into partial lipodystrophy. A distinct homozygous *PSMB8* mutation encoding an immunoproteasome subunit has recently been identified as its genetic cause. Here, we report a new case of a patient with NNS who developed exudative erythemas on his face and extremities at 2 months of age, along with high fever, elevated serum hepatic aminotransferase levels and hepatosplenomegaly. Massive infiltration of inflammatory cells was observed histologically in the dermis and subcutis without apparent leukocytoclastic vasculitis. These symptoms improved with oral corticosteroids but recurred periodically, and a thin angular face with long clubbed fingers gradually developed. Identification of the *PSMB8* mutation finalized the diagnosis of NNS at 5 years of age. Understanding a variety of clinicopathological features at the developmental stages is necessary to make an early diagnosis of NNS.

Introduction

Nakajo-Nishimura syndrome (NNS; OMIM #256040, ORPHA2615) is an autosomal, recessively inherited disorder which has been reported uniquely in Japan [1]. Patients with this disease show pernio-like skin rashes since infancy, and they gradually develop partial lipodystrophy mainly in the face and upper extremities, as well as characteristic long clubbed fingers with contracture of the interphalangeal joints accompanied by remittent fever and nodular erythema-like skin eruptions. Although a diagnosis of NNS is not difficult when the characteristic features are fully developed, it can be difficult soon after disease onset in infancy. Currently, more than 20 cases of NNS have been reported, but none of the patients were younger than 5 years when reported, except for the 8-month-old sister of an affected 10-year-old patient [2–6]. Recently, a homozygous missense mutation of the *PSMB8* gene, encoding the β5i subunit of immunoproteasome, has been identified to be responsible for NNS, and thus genetic analysis is expected to be the most reliable method for diagnosis [7, 8].

Here, we describe the detailed clinical observations of a new NNS case whose correct diagnosis was finally made at 5 years of age by genetic analysis. He was born more than 20 years after the birth of the last patient with NNS, and currently is the only living infant case in Japan.

Case Report

A 5-year-old Japanese boy, without parental consanguinity or any remarkable family history, presented with occasional periorbital pernio-like erythemas. He was delivered by cesarean section and received phototherapy for severe newborn jaundice, but no other remarkable abnormalities were observed around the time of his birth. Disease onset was at the age of 2 months, in the summertime, when exudative erythe-

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mas appeared on his fingers and toes, accompanied by a high fever reaching 40°C. As the symptoms worsened despite the administration of oral antibiotics, he was referred to the Departments of Dermatology and Pediatrics of Wakayama Rosai Hospital at the age of 3 months. On examination, multiple swollen erythemas with central necrosis and ulcerations with diameters of several centimeters were observed not only on his fingers, toes, palms and soles, but also on his face and trunk (fig. 1). Laboratory investigations showed a high white blood cell count (13,400/mm^3) with lymphocytosis (neutrophils 18%, monocytes 7%, eosinophils 3%, lymphocytes 74%), slight anemia (hemoglobin 11.3 g/dl), a normal platelet count (326,000/mm^3), elevated levels of serum aspartate aminotransferase (141 IU/l; normal: 22–73 IU/l), alanine aminotransferase (215 IU/l; normal: 13–63 IU/l) and lactate dehydrogenase (424 IU/l; normal: 156–362 IU/l). In contrast, his serum alkaline phosphatase, γ-glutamyl transferase, creatinine, blood urea nitrogen, uric acid, creatine kinase and C-reactive protein levels were within normal ranges. Although neonatal lupus erythematosus was suspected, his serum antinuclear antibody, anti-Sm, anti-SS-A, anti-SS-B, anticardiolipin antibodies and cryoglobulin were all negative. Notably, his first infection with cytomegalovirus (CMV) was obvious, because both IgM and IgG antibodies were positive against CMV. Hepatosplenomegaly was observed by computed tomography (CT). A histopathologic examination of an erythematous lesion in his left palm at the age of 4 months revealed a massive infiltration of inflammatory cells from just below the epidermis to the subcutaneous adipose and deep striated muscle tissues (fig. 2a). In the dermis, the inflammatory cell infiltration was mainly observed around the vessels and adnexa, without any apparent leukocytoclastic vasculitis by neutrophilic infiltration (fig. 2b). Immunohistochemistry showed that the infiltrating cells were partly immunopositive for CD45RO and CD68, but immunonegative for CD1a, CD20, CD56 and S-100. Some of the mononuclear cells showed nuclear atypia but were considered to be reactive histiocytes without mitosis (fig. 2c). Although CMV antigen was immunohistochemically detected, neither apparent hemorrhage suggestive of acute hemorrhagic edema nor hemophagocytosis suggestive of cytophagic histiocytic panniculitis were found in the lesional skin. Bone

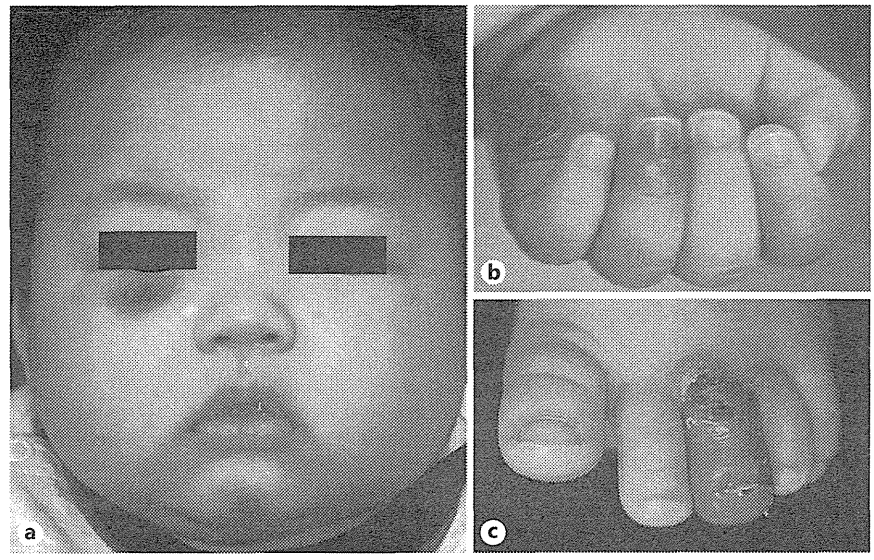

Fig. 1. Clinical photographs of the patient at 3 months of age. Multiple swollen erythemas with central necrosis or ulcerations on his face (**a**), hand (**b**) and foot (**c**) were observed.

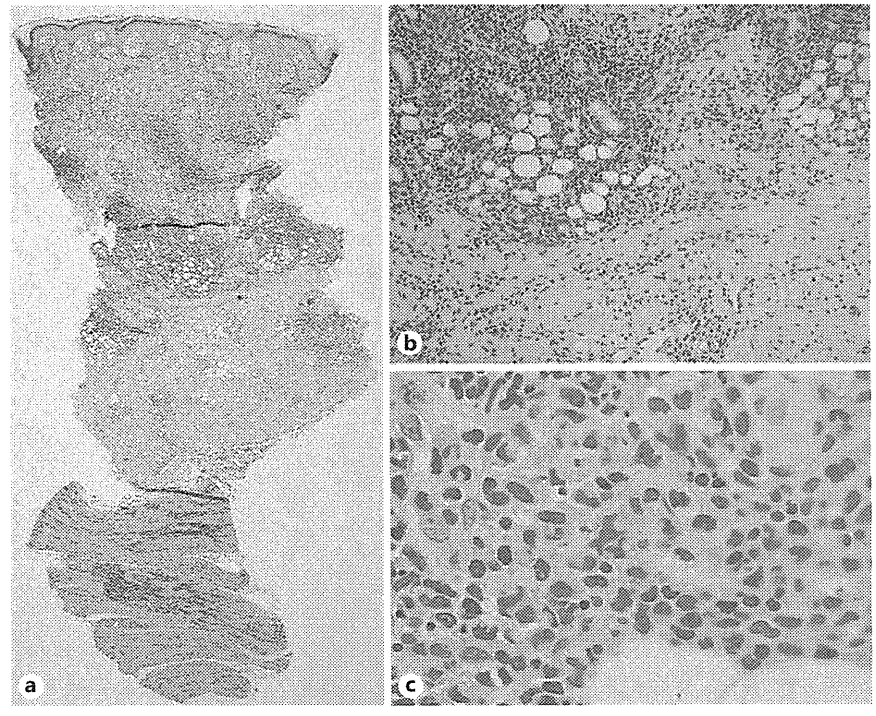

Fig. 2. Histopathological findings of erythematous lesions. The samples were obtained when the patient was 4 months (**a**, **b**) and 1 year (**c**) old. HE. Original magnifications: **a** ×20, **b** ×100, **c** ×400.