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Mucous Membrane Pemphigoid with Antibodies to the β_3 Subunit of Laminin 332 in a Patient with Acute Myeloblastic Leukemia and Graft-versus-Host Disease

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Key Words
Laminin 332 · Mucous membrane pemphigoid · Leukemia · Graft-versus-host disease

Introduction

Mucous membrane pemphigoid (MMP), formerly termed cicatricial pemphigoid, is a heterogeneous group of autoimmune subepithelial blistering diseases that mainly affect mucous membranes, such as the oral, conjunctival, nasal, pharyngeal, laryngeal, esophageal and anogenital mucosae, leading to scarring of the affected tissue [1]. Laminin 332 (previously termed laminin 5, epiligrin, kalinin or nicein), located in the epidermal basement membrane zone, has been characterized as a heterotrimeric glycoprotein consisting of α_3, β_3 and γ_2 subunits that are covalently linked by disulfide bonds [2]. Patients with one form of MMP have pathogenic autoantibodies against laminin 332. Immunoblotting studies using extracts of cultured keratinocytes revealed that a majority of anti-laminin-332 MMP sera reacted with the α_3 subunit [3, 4] and some with the γ_2 subunit [5]. In this report we describe an unusual case of MMP with antibodies to only the β_3 subunit of laminin 332, who had acute myeloblastic leukemia (AML) and graft-versus-host disease (GVHD).

Case Report

A 35-year-old female visited our hospital for treatment of AML and received chemotherapy and a bone marrow transplantation (BMT) from a human-leukocyte-antigen-matched unrelated donor in May 2005. She developed red papules and small erythemas on the face and extremities 2 weeks after the BMT. A biopsy was performed, and a diagnosis of GVHD was made. GVHD was well controlled by treatment with oral tacrolimus. In November 2005, when relapse of AML was detected by PCR, multiple blisters appeared in the perioral region, and tacrolimus was discontinued. She received antibiotics and acyclovir under the diagnosis of impetigo and/or herpes virus infection, while blisters and erosions developed in the oral mucosa and over the face and neck (fig. 1). There was no erosion or blister in the conjunctival, nasal or pharyngeal mucosae. She did not have any other apparent skin lesion caused by GVHD. A biopsy specimen revealed a subepidermal blister containing eosinophils and neutrophils (fig. 2a). Direct immunofluorescence demonstrated a linear deposition of IgG and C3 in the basement membrane zone of the epidermis (fig. 2b). Indirect immunofluorescence revealed weak IgG anti-basement-membrane-zone antibodies which reacted with the dermal side of 1 M NaCl-split skin.

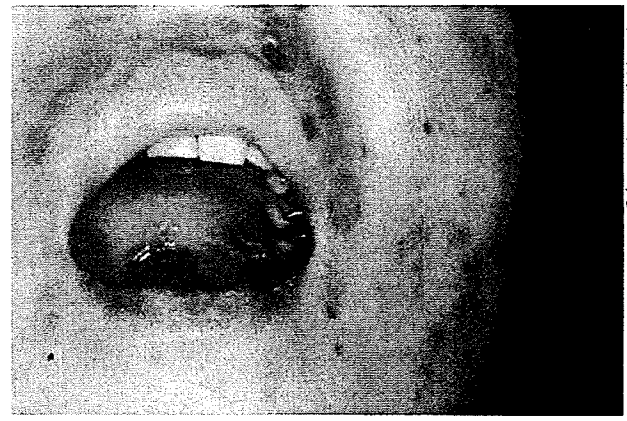

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Fig. 1. Clinical features of the patient. Blisters and erosions were seen on the face and oral mucosa.

By immunoblotting of normal human epidermal extracts, the serum of this patient reacted weakly with the 230-kDa bullous pemphigoid (BP) antigen (BP230) and the 190-kDa periplakin. The significance of these reactivities remains unclear. Immunoblotting of normal human dermal extracts showed no apparent reactivity with the serum of this patient. Immunoblotting using laminin 332 purified from human cultured keratinocytes showed that this patient's serum reacted strongly with the 140-kDa β_3 subunit, but not with the α_3 or γ_2 subunit (fig. 3). Based on these findings, she was diagnosed as having anti-laminin-332 MMP. Based on a tentative diagnosis of BP, treatment had been initiated with oral minocycline 200 mg and topical steroid before the final diagnosis of MMP was made. Although the number of blisters decreased, new blister formation persisted. Three weeks later, the number of blood leukemic cells started to increase rapidly, and she decided to receive cord blood transplantation. However, she died of disease progression after early relapse.

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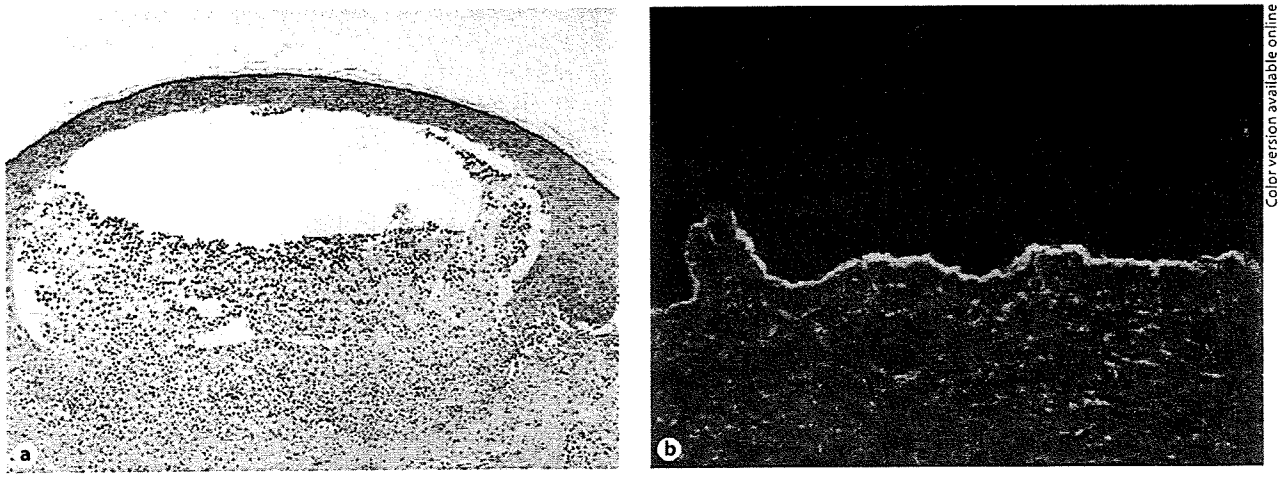

Fig. 2. a A biopsy specimen revealed a subepidermal blister containing eosinophils and neutrophils. **b** Direct immunofluorescence demonstrated a linear deposition of IgG on the basement membrane zone of the epidermis.

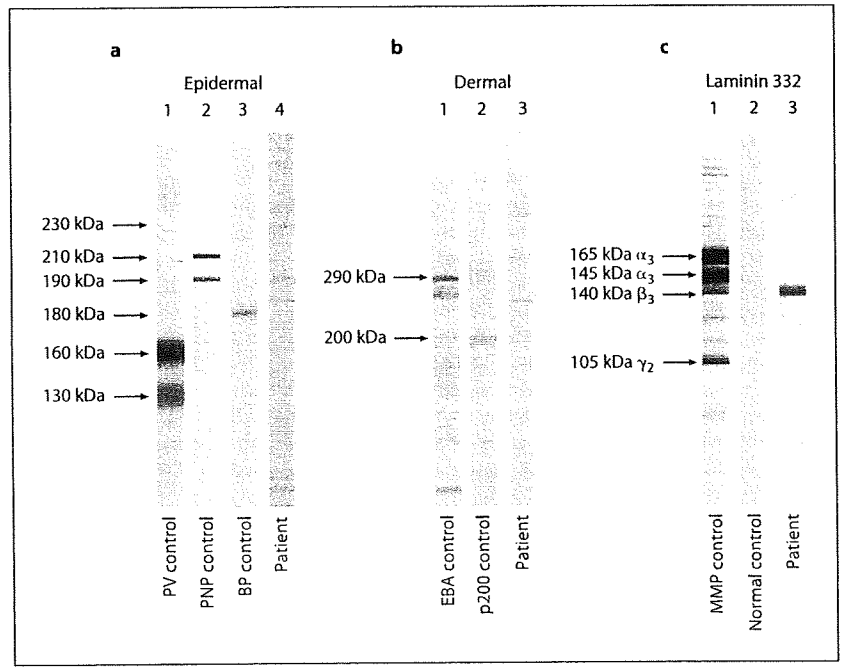

Fig. 3. a By immunoblotting of normal human epidermal extracts, the control pemphigus vulgaris (PV) serum reacted with the 160-kDa desmoglein 1 and the 130-kDa desmoglein 3 (lane 1), the control paraneoplastic pemphigus (PNP) serum reacted with the 210-kDa envoplakin and the 190-kDa periplakin (lane 2), and the control BP serum reacted with BP230 and BP180 (lane 3). The serum of our patient reacted relatively weakly with BP230 and the 190-kDa periplakin (lane 4). **b** By immunoblotting of normal human dermal extracts, the control epidermolysis bullosa acquisita (EBA) serum reacted with the 290-kDa type VII collagen (lane 1), and the anti-p200 pemphigoid serum (p200) reacted with the 200-kDa antigen (lane 2). The serum of our patient showed only a faint reactivity with a protein of about 250 kDa (lane 3). **c** By immunoblotting of purified human laminin 332, control anti-laminin-332 MMP serum reacted with the 165-kDa α_3, the 145-kDa α_3, the 140-kDa β_3 and the 105-kDa γ_2 subunits (lane 1). A normal control serum showed no reactivity (lane 2). Our patient's serum reacted strongly and exclusively with the 140-kDa β_3 subunit (lane 3).

Discussion

The present case possessed autoantibodies to only the β_3 subunit of laminin 332, which is unusual, but reported in a few cases [6–10]. The previous report includes the case of a 72-year-old Caucasian man with blisters and erythema all over the skin, and erosions in the conjunctiva, mouth and larynx [9]. He had metastatic adenocarcinoma of unknown origin in the lymph nodes of the neck. He died of respiratory failure due to laryngeal obstruction as a result of blister formation. Seo et al. [7] reported the case of a 67-year-old Korean woman with erosions in the oral and conjunctival mucosae with laryngeal and esophageal involvement. She also had cutaneous blisters and erosions on the trunk. She was successfully treated with a combination of prednisolone 50 mg/day and cyclophosphamide 50 mg/day. There was no evidence of associated neoplasm. Fukushima et al. [6] reported the case of a 90-year-old Japanese man with erosions on the oral, nasal, conjunctival mucosae, trunk and extremities. He had prostate carcinoma (poorly differentiated adenocarcinoma). The other two reports have no clinical information [8, 10]. Therefore, the differences between this form and the other forms of MMP in the clinical features, prognosis and complications are not apparent at the present time.

Initially, a diagnosis of BP was also considered because the patient had cutaneous bullae and erythemas. However, the presence of head and neck involvement and the presence of mucosal involvement do not generally support the diagnosis of BP [11]. Furthermore, autoantibodies to laminin 332 had been confirmed previously to be a specific marker of MMP [12]. Therefore, the diagnosis of anti-laminin-332 MMP was considered appropriate, based on the results of the immunoblotting studies. A reactivity against 210- and 190-kDa epidermal proteins corresponding to envoplakin and periplakin, respectively, is found in patients with paraneoplastic pemphigus. However, the histological and immunopathological findings were not consistent with this diagnosis.

It has been shown that autoimmune blistering diseases such as BP may be associated with internal malignancies [13]. Thus, anti-laminin-332 MMP has been shown to have a much higher risk for solid cancers, such as lung, gastric and colon cancers [14, 15], although the clinical features are similar to those of the other forms of MMP. Egan et al. [14] reported that 10 of 35 patients had solid cancers in a United States National Cancer Institute Surveillance, and Matsushima et al. [15] reviewed Japanese cases and showed that 5 of 16 cases of anti-laminin-332 MMP were complicated with solid cancers. However, a possible association of anti-laminin-332 MMP with a hematological neoplasm such as AML has not been clarified. Aisa et al. [16] reported a case of anti-BP230 MMP with follicular lymphoma treated with peripheral blood stem cell transplantation without any obvious chronic GVHD.

GVHD is one of the major complications after hematopoietic stem cell transplantations and critically induced and maintained by donor immunocompetent cells that attack epithelia of fast proliferating tissues [17]. The present case did not have any clinical findings indicating chronic GVHD at the onset of MMP. However, it might be possible that undiscovered slight attacks of GVHD triggered these cases of MMP, since more than 10 cases of autoimmune blistering diseases, mostly BP, after allogeneic BMT have been reported to date [18, 19]. Indeed, various autoimmune diseases, including vitiligo, have been shown to develop in patients with GVHD [20, 21], although the mechanism is unknown. It is possible that some cases may stem from donor-related transfer of pathogenic lymphocytes (autoreactive B cells against laminin 332 in our case) or their progenitors, while most of the cases can be attributed to the immunological imbalances after BMT [20].

Thus, the diagnosis of autoimmune blistering disease, including MMP, should be considered when bullous or erosive lesions are found on the skin and mucosa in patients with leukemia and/or GVHD.

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OBSERVATION

Subcorneal Pustular Dermatosis–Type IgA Pemphigus With Autoantibodies to Desmocollins 1, 2, and 3

Imke Düker, MD; Jörg Schaller, MD; Christian Rose, MD; Detlef Zillikens, MD; Takashi Hashimoto, MD; Johannes Kunze, MD

Background: IgA pemphigus is a rare neutrophilic acantholytic autoimmune disease that is characterized by IgA deposits on keratinocyte cell surfaces. Clinically and histologically, IgA pemphigus is divided into 2 major subtypes: subcorneal pustular dermatosis and intraepidermal neutrophilic IgA dermatosis. We report the first case of subcorneal pustular dermatosis–type IgA pemphigus that showed reactivity to all 3 isoforms of the desmocollin family by indirect immunofluorescence microscopy of COS7 cells transfected with desmocollin 1, 2, or 3.

Observations: We describe a 94-year-old woman with IgA pemphigus with a unique immunopathologic pattern. Direct immunofluorescence microscopy revealed IgA deposits throughout the entire epidermis, with stronger staining in the upper epidermis. The autoantibodies from this patient did not show IgA or IgG reactivity with desmogleins via immunoblotting or enzyme-linked immunosorbent assay. By indirect immunofluorescence by the use of COS7 cells transfected with desmocollin 1, 2, or 3, IgA autoantibodies in a serum sample from our patient clearly reacted with all of them.

Conclusions: The pathophysiology and autoantigen profile of bullous autoimmune diseases, especially pemphigus and its subforms, are more complex than previously believed. Because pemphigus seems to be a heterogeneous disorder, further studies are needed to evaluate the complexity of the disease.

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IGA PEMPHIGUS IS A RARE AUTOIMmune blistering disease characterized by epidermal IgA immunoglobulin deposits. The presence of IgA in the epidermis was first reported by Varigos.[1] In the past, various terms have been used to describe this condition, such as *intraepidermal neutrophilic IgA dermatosis*,[2] *intercellular IgA vesiculopustular dermatosis*,[3] and *IgA pemphigus foliaceus*.[4] Currently, IgA pemphigus is considered to be a distinct clinical entity that includes 2 subtypes with different histologic features and different IgA deposition patterns in the epidermis: intraepidermal neutrophilic IgA dermatosis (IEN) and subcorneal pustular dermatosis (SPD).[5-8]

The SPD type shows subcorneal acantholysis and pustules with intercellular IgA deposits in the upper epidermis. The IEN type is characterized by pustules located deeper in the epidermis and by intercellular IgA deposits throughout the entire epidermis.[9] Indirect immunofluorescence microscopy reveals circulating IgA antibodies to intraepidermal structures in only half the cases.[10] In the SPD type, desmocollin 1, one of the desmosomal cadherins, has been identified as a target autoantigen.[7,11,12] In most cases of the IEN type, the autoantigen remains to be fully characterized,[13] whereas, in single cases, desmoglein 1 and desmoglein 3 have been demonstrated to be targets of the autoantibodies in this variant.[6,14] Clinically, patients commonly present with flat pustules, often on a slightly erythematous base, which tend to coalesce to form annular patterns. The regions mainly affected are the trunk, axillae, and groin. Mucosal involvement is usually lacking.

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REPORT OF A CASE

A 94-year-old woman presented with a 2-week history of bullous dermatosis. Results of physical examination revealed a disseminated vesiculopustular eruption on an erythematous base and erosions with yellow crusts. The sites of predilection were the axillae, groin, and proximal portions of the extremities (**Figure 1**). There was no mucosal involvement. Bacterial cultures of the pustules were negative. The

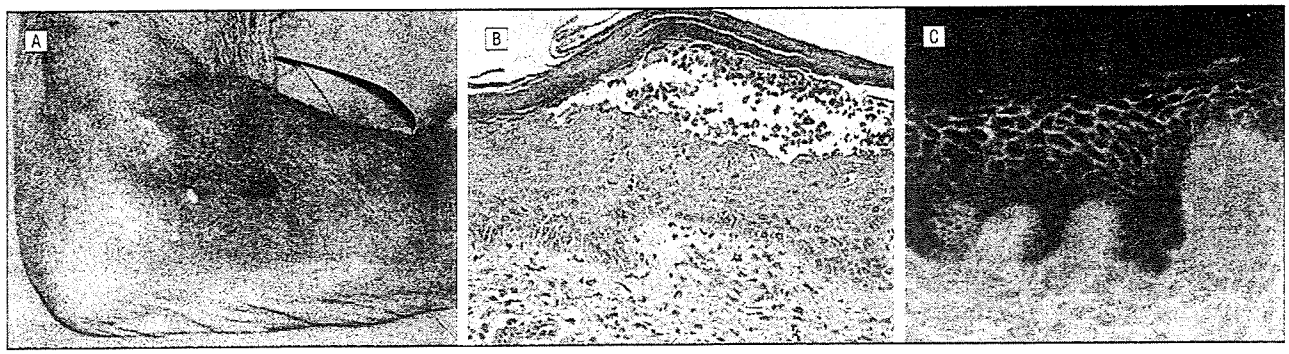

Figure 1. Bullous dermatosis in a 94-year-old woman. A, Right arm with pustules and crusts on an erythematous background. B, Histopathologic analysis of lesional skin that shows a subcorneal blister filled with neutrophilic granulocytes and erythrocytes (hematoxylin-eosin, original magnification ×100). C, Direct immunofluorescence microscopy of perilesional skin showing intercellular IgA deposits in the entire epidermis with stronger staining in the upper layers of the epidermis (original magnification ×200).

results of laboratory investigations, such as serum immunofixation of IgG, IgA, and IgM, were within normal ranges.

HISTOPATHOLOGIC ANALYSIS

Histopathologic examination of a lesional skin biopsy specimen showed a subcorneal cleft with acantholysis. The blister was filled by fibrin, erythrocytes, and numerous neutrophilic granulocytes (Figure 1). In the upper dermis, there were also neutrophils that infiltrated the epidermis.

DIRECT IMMUNOFLUORESCENCE MICROSCOPY

Direct immunofluorescence microscopy of a perilesional skin biopsy specimen revealed intercellular deposits of IgA in the entire epidermis with a stronger staining of the superficial layers (Figure 1). Fluorescein isothiocyanate–conjugated goat anti-human IgA (specific to α chains) and IgG (specific to γ chains) were used as secondary antibodies (DiaMed Inc, Windham, Maine).

INDIRECT IMMUNOFLUORESCENCE MICROSCOPY

The results of indirect immunofluorescence microscopy performed on monkey esophagus and healthy human skin were negative for circulating IgA or IgG autoantibodies. Interestingly, when performed on sodium chloride split human skin, IgA autoantibodies that stained the interface of the stratum corneum and stratum granulosum were detected at a titer of 1:320.

IMMUNOBLOT ANALYSIS AND ENZYME-LINKED IMMUNOSORBENT ASSAY

Immunoblot analysis of healthy human epidermal extracts showed no specific IgA or IgG reactivity in the serum of the patient. Enzyme-linked immunosorbent assay (ELISA) that used recombinant desmoglein 1 and 3, expressed in the baculovirus system, showed no IgA or IgG reactivity.

INDIRECT IMMUNOFLUORESCENCE MICROSCOPY ON COS7 CELLS

By indirect immunofluorescence that used COS7 cells transfected with desmocollin 1, 2, or 3, IgA autoantibodies in the serum of our patient clearly reacted with all of them (**Figure 2**). On the basis of these results, the patient was diagnosed as having the SPD-type IgA pemphigus, and oral therapy with dapsone, 50 mg twice daily, was started. This course resulted in complete clearing of skin lesions within 3 weeks.

COMMENT

We present a case of SPD-type IgA pemphigus with a unique immunopathologic pattern. Direct immunofluorescence microscopy revealed IgA deposits on keratinocyte cell surfaces, but unexpectedly the staining was throughout the epidermis, which showed stronger intensity in the superficial and weaker staining in the basal layers. Usually, autoantibodies from patients with SPD-type IgA pemphigus do not react with the basal layers of the epidermis because of the epidermal distribution of the autoantigen (ie, desmocollin 1). Staining of the entire epidermis in the skin of our patient, which resembled the pattern of the IEN type, suggested desmoglein 3 or desmocollin 3 as potential additional autoantigens. In fact, in addition to reactivity with desmocollin 1, the serum of our patient bound to both desmocollins 2 and 3 by indirect immunofluorescence microscopy of COS7 cells transfected with the different desmocollin isoforms. In contrast, no IgA or IgG reactivity was found with desmogleins 1 and 3.

Desmocollins, together with the desmogleins, are major desmosomal glycoproteins and are members of the cadherin superfamily of Ca^{2+}-dependent cell adhesion molecules.[15] Desmocollins show at least 3 isoforms[16] and contribute to the adhesive core of the desmosome, whose basic function is to guarantee the epidermal integrity by the mediation of cell-cell adhesion. The isoforms of desmocollins are expressed in a differentiation-specific manner: desmocollin 1 expression is weak in suprabasal layers and increases further upward; it shows strong

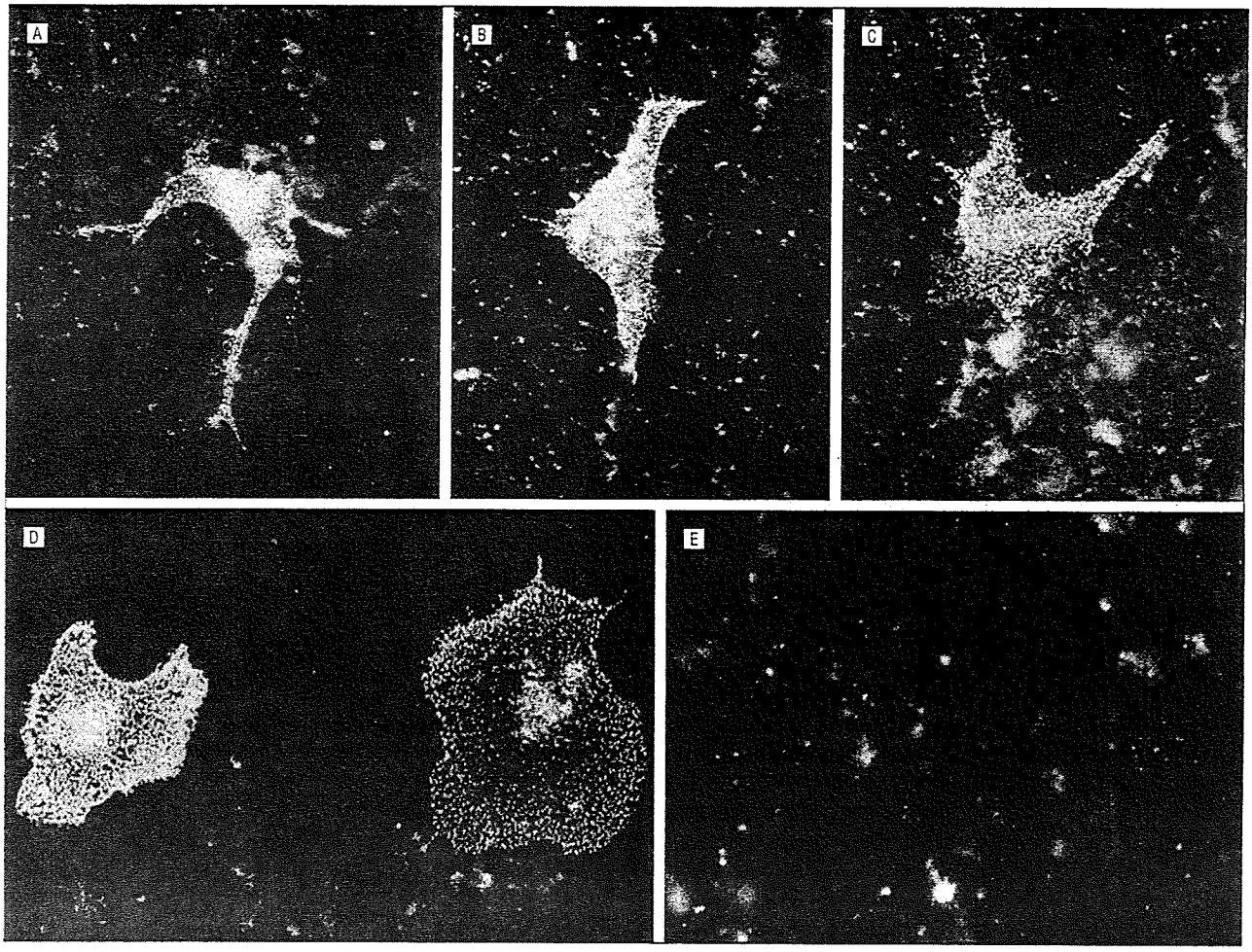

Figure 2. Indirect immunofluorescence microscopy of COS7 cells transfected with desmocollins 1, 2, and 3. The IgA antibodies of the serum of our patient reacted with the transfected cells that express desmocollin 1 (A), desmocollin 2 (B), and desmocollin 3 (C). IgA antibodies of a control individual with subcorneal pustular dermatosis–type IgA pemphigus reacted with COS7 cells transfected with desmocollin 1 (D) but not IgA antibodies of a healthy control serum (E).

expression in the upper spinous layers. Desmocollin 2 shows similar expression to desmocollin 1 but is most strongly expressed at the base of the rete ridges. Desmocollin 3 is most strongly expressed in the basal layers and weaker in the suprabasal layers.[15,17,18]

In our case, the detection of IgA autoantibodies not only to desmocollin 1 but also to the 2 other desmocollin isoforms may explain the finding of the staining throughout the epidermis by direct immunofluorescence microscopy. Kopp et al[19] recently described another case of SPD-type IgA pemphigus in which the autoantibodies reacted not only with the uppermost layers but throughout the entire epidermis. In this previous case, antibodies to both desmocollin 1 and desmoglein 1 were detected.

A staining pattern throughout the epidermis by direct and indirect immunofluorescence microscopy along with a histologic characteristic of the SPD-type IgA pemphigus was also seen by Niimi et al.[20] However, target antigens of IgA antibodies in this case remained unknown: they did not react with desmogleins or desmocollins as determined by immunoblotting, ELISA, or immunofluorescence microscopy of complementary DNA–transfected cells. In our case, via immunoblotting, we could also not detect any IgA reactivity in the serum of our patient. As reported, a possible explanation for the failure to detect desmocollins by this method is that the effect of conformation-dependent epitopes on desmocollins may have been altered by the extraction procedure or sodium dodecyl sulfate–polyacrylamide gel electrophoresis.[11,21,22] Not only immunoblotting but also ELISA may fail to detect reactivity in some cases in which the results of indirect immunofluorescence microscopy on complementary DNA–transfected cells are positive; this factor suggests that there may be differences in conformation between desmocollin 1 produced by baculovirus-infected cells and protein produced by COS7-transfected cells.[23]

Ebihara et al[6] described 3 patients with SPD-type IgA pemphigus that reacted with desmocollin 1 and 2, and Hisamatsu et al[21] detected IgA antibodies to both desmocollin 2 and 3 in one case of so-called atypical pemphigus. We report the unique detection of IgA antibodies to desmocollins 1, 2, and 3 in a patient with SPD-type IgA pemphigus.

In recent years, it has become evident that the different subtypes of IgA pemphigus may be associated with a number of different autoantibody specificities. It is becoming more obvious that certain autoantibodies are not restricted to just one form of pemphigus.[24-26] Recently, IgA autoantibodies to desmogleins 1 and 3 could also be found

in patients with pemphigus vulgaris and pemphigus foliaceus, in addition to IgG antibodies to these desmogleins.[24] Furthermore, a few patients with IgA pemphigus have been shown to react with desmoglein 1 and 3 via IgA ELISA.[22] These cases, in which IgA antibodies react exclusively with desmogleins 1 or 3, should be named *IgA pemphigus foliaceus* and *IgA pemphigus vulgaris*, respectively. In a few cases, neutrophilic infiltration can be lacking.[27] Autoantibodies to desmocollins have also been described in certain pemphigus serum samples,[18,21] which may be owing to the epitope spreading concept.[28] However, as in mucocutaneous-type pemphigus vulgaris with IgG antibodies to both desmoglein 1 and 3, individual cases may show antibodies to multiple antigens. Autoantibodies to these different antigens are not necessarily considered to be produced by epitope spreading. It is therefore feasible, in our case, that IgA antibodies to desmocollins 1, 2, and 3 may be produced independently. Because pemphigus seems to be a heterogeneous disorder, further studies are needed to evaluate the complexity of the disease.

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Author Contributions: Drs Düker, Schaller, Rose, Zillikens, Hashimoto, and Kunze had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Düker, Schaller, and Kunze. *Aquisition of data:* Düker, Schaller, Rose, Zillikens, Hashimoto, and Kunze. *Analysis and interpretation of data:* Düker, Schaller, Rose, Zillikens, Hashimoto, and Kunze. *Drafting of the manuscript:* Düker. *Critical revision of the manuscript for important intellectual content:* Düker, Schaller, Rose, Zillikens, Hashimoto, and Kunze. *Administrative, technical, or material support:* Düker, Schaller, Rose, Zillikens, Hashimoto, and Kunze. *Study supervision:* Düker, Schaller, and Kunze.
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mg/d, was initiated, leukonychia totalis of all fingernails was noted. No follow-up information is available.

All 3 patients denied a personal or family history of abnormal nails or trauma or chemical exposures to the nails. None of the other medications the patients were taking have been associated with leukonychia. On examination, all 3 patients lacked onychodystrophy, onycholysis, nail thickening, or pitting of the nails, and no obvious peripheral vasoconstriction was present. Toenails were not involved. None of the 3 patients had hypocalcemia, hypoalbuminemia, or anemia. Creatinine levels had slightly increased in 2 patients who were taking vorinostat.

Comment. Leukonychia can be classified into "true" or "apparent" subtypes.[2,3] In the true subtype, a defect in the nail matrix causes an abnormal keratinization of the nail plate. In contrast, apparent leukonychia involves a pathologic process in the subungual tissue or nail bed and includes changes in adherence of the nail plate to the nail bed or changes in the translucence of the nail bed epithelium or its vasculature status.[4] The present cases best represent apparent leukonychia as manifested by (1) persistence of opacity as the nail grew; (2) rapid improvement once treatment with the presumed causative agent was stopped; and (3) resolution starting distally and moving proximally (the opposite from what is generally seen with true leukonychia). Diffuse or total apparent leukonychia has been seen with medical illnesses such as liver cirrhosis (Terry's nails), anemia, hypocalcemia, hypoalbuminemia, and zinc deficiency.

To our knowledge, this is the first report of diffuse leukonychia secondary to treatment with an HDAC inhibitor, a condition that resolved once treatment with the drug was halted. Although the arrest of cell growth and apoptosis in a wide range of cells is the primary effect of HDAC inhibitors in cancer cells, it may be their effect on repression of endothelial nitric oxide synthase and the resultant compromise of endothelial cell function in vasorelaxation and angiogenesis that best explains the mechanism in apparent leukonychia.[5]

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An Association of Idiopathic Chronic Eosinophilic Pneumonia With Pemphigoid Nodularis: A Rare Variant of Bullous Pemphigoid

Report of a Case. In October 2006, a 70-year-old Japanese woman came to our hospital for evaluation of her severe itching accompanied by nodular skin lesions on the trunk and extremities (**Figure 1** A), symptoms that clinically suggested prurigo nodularis. There were no blister formations at the time of initial presentation, but several small blisters sometimes appeared on the extremities during the course of the disease (Figure 1B).

Her medical history included (1) idiopathic chronic eosinophilic pneumonia (ICEP) confirmed by chest radiography and computed tomography, (2) pulmonary eosinophilia in bronchoalveolar lavage fluid (BALF), and (3) a transbronchial lung biopsy 6 years previously. She had no atopic diathesis and was taking prednisolone, 2 mg/d, to control ICEP.

Laboratory investigations revealed an elevated white blood cell count (15 700/μL) and serum IgE titer (1160 μg/L). (To convert white blood cells to number of cells $\times 10^9$ per liter, multiply by 0.001; to convert IgE to milligrams per liter, multiply by 0.001.) Other results of blood tests were within the normal range. Histopathologic examination of a nodular lesion specimen revealed irregular acanthosis with pseudoepitheliomatous hyperplasia and infiltration of many lymphocytes and a few eosinophils (**Figure 2**A).

Direct immunofluorescence of perilesional skin specimens demonstrated linear deposits of IgG and C3 at the basement membrane zone (BMZ) of the epidermis (Figure 2B). On indirect immunofluorescence with normal human skin as substrate, circulating IgG anti-BMZ autoantibodies were detected. Immunoblot analysis using BP180-NC16a domain recombinant protein demonstrated that the patient had circulating IgG but not IgE autoantibodies. Enzyme-linked immunosorbent assay detected IgG anti-BP230 (49.45 index) and anti-BP180 (75.60 index) autoantibodies. These immunologic pro-

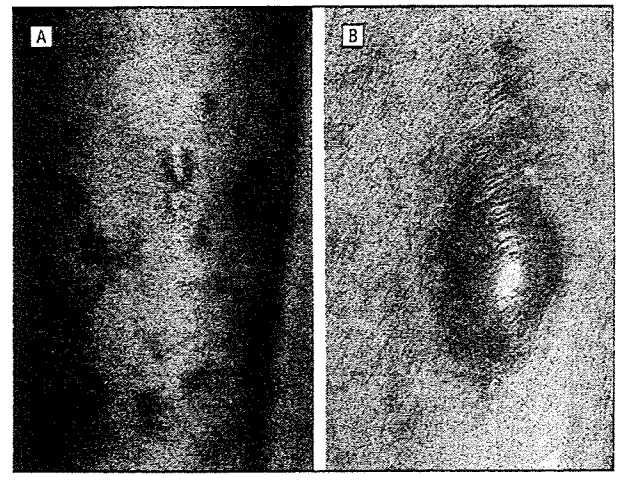

Figure 1. Prurigo nodularis–like lesions are present on the lower limb (A), and a bulla and erosion are found on the forearm (B).

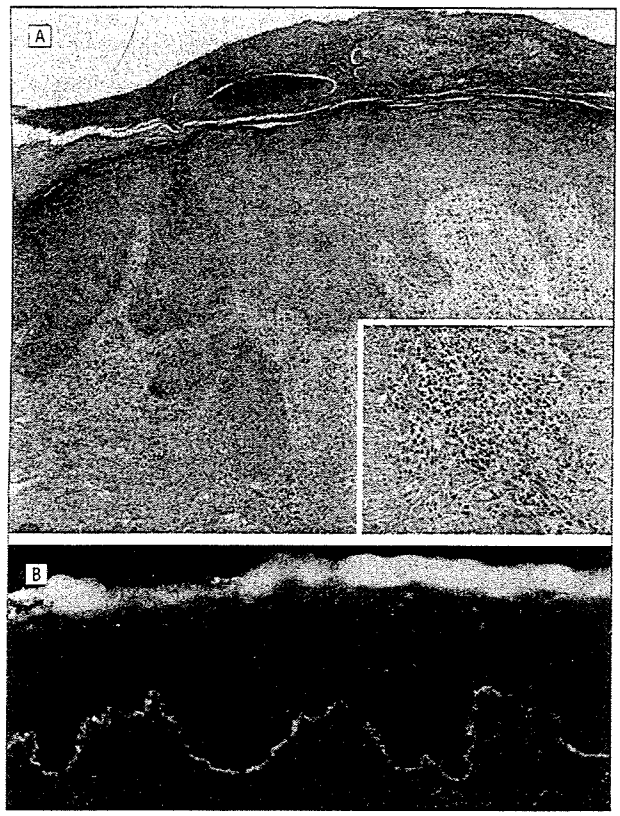

Figure 2. Immunohistopathologic studies. A, Hematoxylin-eosin staining under light microscopy reveals irregular acanthosis with pseudoepithelioma-tous hyperplasia (original magnification ×40); the inset shows an infiltration of many lymphocytes and a few eosinophils in the upper dermis (original magnification ×200). B, Direct immunofluorescence demonstrates linear deposits of IgG at the basement membrane zone of the epidermis (original magnification ×200).

files were identical to those of bullous pemphigoid (BP). Tetracycline and nicotinamide were administered for 6 months without apparent benefits.

Comment. Bullous pemphigoid is a common acquired subepidermal autoimmune bullous disease in elderly patients. Pemphigoid nodularis is a rare clinical variant of BP characterized by overlapping prurigo nodularis–like lesions and blisters and immunologic findings identical to those seen in BP.[1] This condition is very difficult to diagnose unless the blisters are present. The diagnosis is confirmed by the presence of anti-BMZ autoantibodies under direct and indirect immunofluorescence. Therefore, it is important to perform immunohistopathologic analyses in patients with persistent prurigo nodularis–like lesions.

Bullous pemphigoid shows dermal infiltrate containing numerous eosinophils in the subepidermal clefts of skin specimens as well as marked peripheral eosinophilia. Ultrastructural examination reveals direct adherence of eosinophils with degranulation into basal keratinocytes.[2] These eosinophils may directly damage the dermoepidermal junction by releasing their cytotoxic proteins such as eosinophilic cationic protein and major basic protein. Similarly, eosinophils with degranulation are also observed around necrotic pneumocytes and along the BMZ at the destroyed alveolar structures in ICEP.[3] The chemokine eotaxin, associated with helper T cell subtype 2, has an important role in eosinophil recruitment in tissue. Elevated levels of eotaxin in blister fluid from BP and BALF of ICEP specimens have been reported.[4,5] Thus, eosinophils are suggested to be involved in the pathogenesis of both BP and ICEP.

To our knowledge, BP complicated by ICEP has never been described before. Our patient developed pemphigoid nodularis during follow-up of ICEP, and while it is possible that she only incidentally had both diseases, this study provides us with an opportunity to assess the relationship between the 2 diseases.

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Erosive Pustular Dermatosis of the Scalp Following Treatment With Topical Imiquimod for Actinic Keratosis

Erosive pustular dermatosis of the scalp (EPDS) is a rare chronic disease described first in 1977 by Burton[1] and then in 1979 by Pye et al.[2] It manifests as extensive pustular lesions, erosions, and crusting of the scalp in elderly individuals and leads to scarring alopecia. The cause remains unknown, although local trauma such as that caused by topical treatment of actinic keratosis with fluorouracil, 5%, tretinoin, surgery,

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Desmoglein1 and BP 180 ELISA indexes correlating with disease activity in a patient with coexisting pemphigus foliaceus and bullous pemphigoid

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An 88-year-old Japanese woman presented with erosions in the oral cavity. On physical examination, oral erosions, tense vesicles and erythema with partially crusted erosions were seen on the trunk. Acantholysis (Fig. 1a) and subepidermal blisters (Fig. 1b) were seen in skin biopsy specimens taken from the trunk. Direct immunofluorescence of the skin revealed *in vivo* deposition of IgG in both the cell surface and the basement membrane zone (BMZ) of the epidermis (Fig. 1c). Using indirect immunofluorescence using human skin section as substrate, IgG autoantibodies against cell surface and BMZ were detected at a titre of > 1 : 160 (Fig. 1d). Immunoblotting assays using normal human epidermal extracts and BP180 NC16a domain

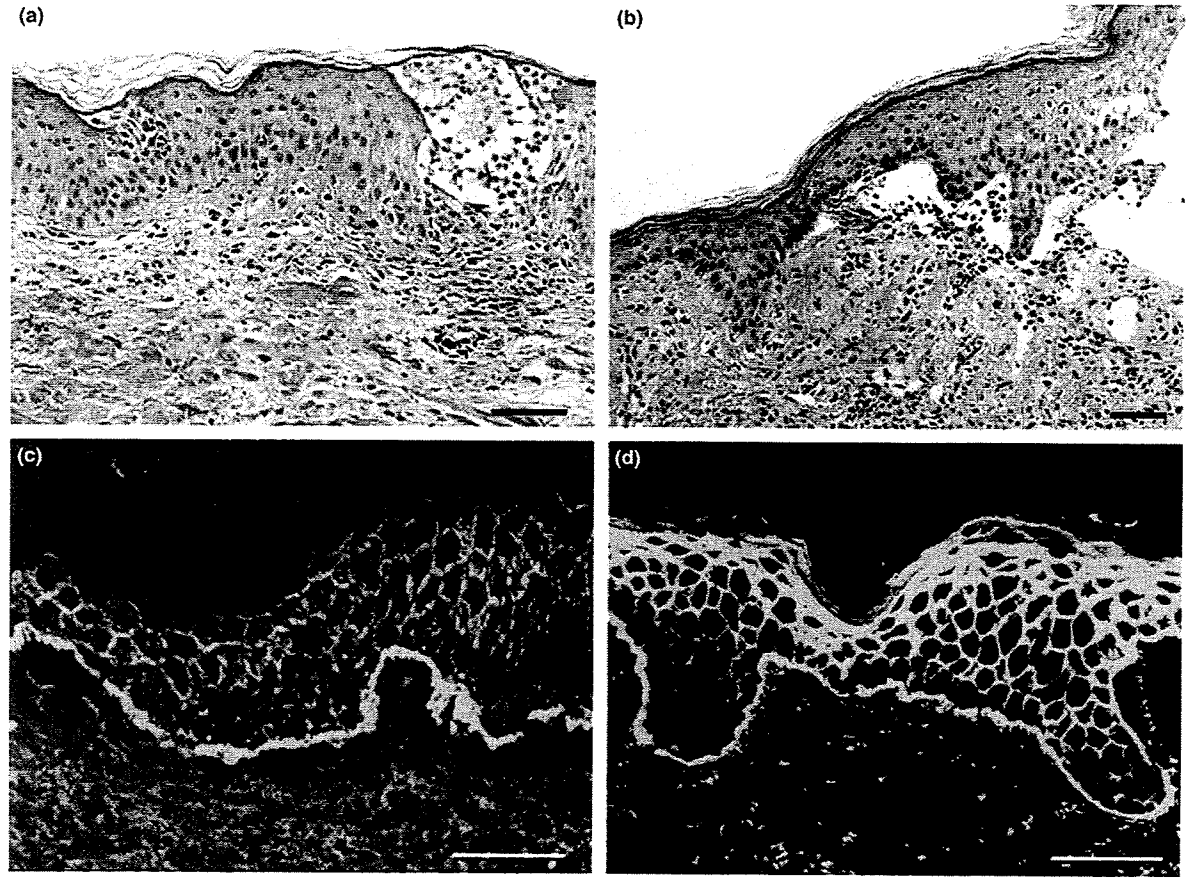

Figure 1 (a) Acantholysis and (b) subepidermal blisters (haematoxylin and eosin). Both (c) direct immunofluorescence of the skin and (d) indirect immunofluorescence using human skin sections confirmed IgG antibodies to the cell surface and basement membrane zone of the epidermis. Scale bar, 100 μm.

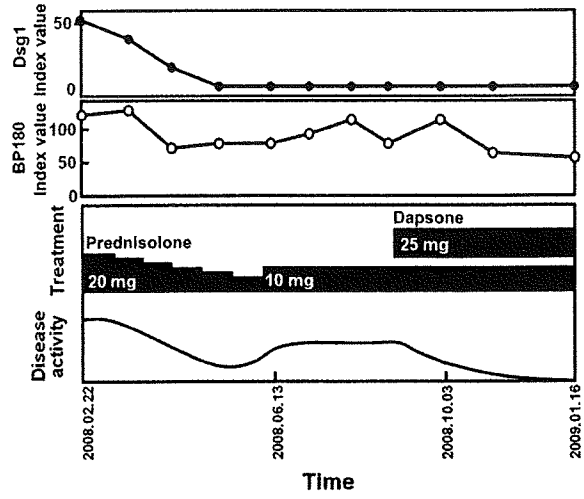

Figure 2 Desmoglein (Dsg)1 and BP180 ELISA indexes correlated with disease activity. With oral prednisolone 20 mg/day, the erosions and blisters disappeared, and the Dsg1 ELISA index became negative. Additional treatment with dapsone 25 mg/day, resulted in resolution of the bullous lesions and the BP180 ELISA index gradually decreased.

recombinant protein confirmed the presence of serum IgG antibodies against BP180 and the BP180 NC16a domain, respectively. The desmogein (Dsg)1 and BP180 ELISA indexes were 54 (normal range < 20) and 125 (normal range < 8), respectively. Oral prednisolone 20 mg/day and topical steroid application were effective, and the Dsg1 ELISA index decreased to 10. However, the BP180 ELISA index did not decrease, and it was still 88 when the patient had a flare while on oral prednisolone 5 mg/day. Oral dapsone 25 mg/day plus prednisolone 10 mg/day cleared the bullous skin lesions, and the BP180 ELISA index gradually decreased (Fig. 2).

In this case, the clinical features, histological findings, immunofluorescence and ELISA indexes confirmed the simultaneous coexistence of PF and BP. Previous reports[1–3] reported the simultaneous coexistence of PF and BP based on histological or immunofluorescence findings. None of the three cases had coexistence of anti-Dsg1 antibodies and anti-BP180 antibodies by ELISA. To our knowledge, our patient is the first case of coexistence of PF and BP confirmed by positive ELISA results for both anti-Dsg1 and anti-BP180 antibodies.

It was previously reported that ELISAs for Dsg1, Dsg3 and BP180 are more sensitive and specific than indirect immunofluorescence and that ELISA indexes tend to correlate with the disease activity.[4,5] In our case, Dsg1 and BP180 ELISA indexes correlated with disease activity along the time course. ELISA is a valuable tool for monitoring disease activity and provides the important information for determining treatments for various immunobullous diseases.

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Some epidermolysis bullosa acquisita sera react with epitopes within the triple-helical collagenous domain as indicated by immunoelectron microscopy

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Summary

Background Epidermolysis bullosa acquisita (EBA) autoantibodies recognize epitopes predominantly within the N-terminal noncollagenous (NC)-1 domain of type VII collagen. Recently, some EBA cases with reactivity to other domains, i.e. the triple-helical (T-H) collagenous domain and the NC-2 domain, have been reported.
Objectives To investigate the ultrastructural localization of epitopes for sera from five patients with EBA that were unreactive by immunoblotting with the NC-1, NC-2 and collagenous domains of type VII collagen.
Methods Immunogold postembedding indirect immunoelectron microscopy was performed using normal human skin and type VII collagen-deficient skin as substrates.
Results Postembedding indirect immunoelectron microscopy revealed that the five EBA sera showed immunoreactivity in the dermis, mainly located 0–400 nm below the lamina densa. IgG labelling was not observed in type VII collagen-deficient skin from a patient with recessive dystrophic epidermolysis bullosa. The distribution histogram found in this study was different from those of sera that reacted with the NC-1 and/or NC-2 domains, and was similar to those of sera reacting with the T-H collagenous domain.
Conclusions Our results suggest that epitopes within the T-H collagenous domain of type VII collagen are recognized by IgG antibodies from some EBA sera. These antibodies appear to be found in patients with inflammatory-type EBA.

Epidermolysis bullosa acquisita (EBA) is an autoimmune subepidermal blistering skin disease characterized by IgG autoantibodies against a 290-kDa dermal protein, type VII collagen, a major component of anchoring fibrils.[1,2] The major epitopes have been shown to be present in the N-terminal noncollagenous (NC)-1 domain of type VII collagen.[3] The blistering process in EBA is determined by the production of autoantibodies directed against the NC-1 domain of type VII collagen which are clearly pathogenic, as demonstrated in murine models.[4,5]

In addition to these EBA IgG autoantibodies, however, previous reports have demonstrated that some patients with EBA have autoantibodies to other regions, i.e. the triple-helical (T-H) collagenous domain and the NC-2 domain.[6–9] We have recently reported that most EBA sera reacted with the NC-1 domain, while a few sera reacted with the NC-2 domain by immunoblot analysis using bacterial recombinant proteins of type VII collagen.[10] In addition, immunoelectron microscopy using these sera indicated that the NC-1 domain is present in the lamina densa and the NC-2 domain is present in the dermis 300–360 nm below the lamina densa.

In the present study, using postembedding indirect immunoelectron microscopy, we have characterized the localization of target epitopes for EBA sera which do not react with either the NC-1 or NC-2 domain and found that the localization was different from those of NC-1 and NC-2 positive sera in the previous report. We further elucidated that these EBA sera did not show any reactivity with recessive dystrophic epidermolysis bullosa skin sections by immunoelectron microscopy, confirming that type VII collagen is the antigen in these cases.

Patients and methods

We collected serum samples from five patients with EBA, prior to the initiation of therapy. The patients were all adults: three

men and two women. All the patients showed: (i) blisters and erosions on the skin; (ii) subepidermal blister formation by histopathology; (iii) linear deposits of IgG and C3 along the dermal–epidermal junction by direct immunofluorescence of perilesional biopsy skin; (iv) circulating IgG autoantibodies reactive with the dermal side of 1 mol L^{-1} NaCl-split normal human skin by indirect immunofluorescence; and (v) no reactivity with the 200-kDa protein as detected by immunoblot analysis using dermal extracts, and no reactivity with any subunits of laminin 5 by immunoblot analysis using purified laminin 5. We also obtained skin sections from a patient with recessive dystrophic epidermolysis bullosa, which were completely negative for the monoclonal antitype VII collagen antibody (LH7.2) (data not shown). Immunoblot analyses using normal human dermal extracts and recombinant proteins of the NC-1 and NC-2 domains of type VII collagen,[10] as well as pepsin-digested type VII collagen extracted from cultured keratinocytes,[7] were performed as described previously. Immunogold labelling for postembedding immunoelectron microscopy was performed using normal human skin and recessive dystrophic epidermolysis bullosa skin, as described previously.[10,11]

Results

The immunoblot analysis using dermal extracts demonstrated that two sera reacted with the 290-kDa type VII collagen, whereas three sera did not. None of the five patient sera recognized either the NC-1 domain or the NC-2 domain. Furthermore, three of the five patient sera did not react with the T-H collagenous domain of type VII collagen. The clinical forms and the results of immunoblot analyses are summarized in Table 1.

By immunoelectron microscopy using normal human skin, all five EBA sera used in this study showed gold particles in the dermis, mainly located at 0–400 nm below the lamina densa (Fig. 1a,b). The labelling was rarely observed within the lamina densa. In a representative case (EBA1), quantitative analysis revealed that the gold particles were mainly distributed in the dermis below the lamina densa, without an apparent peak at the lamina densa region (Fig. 1c). The other four cases also showed similar distribution patterns to that of this representative case (data not shown). To confirm that the IgG labelling in the dermis by patient sera (EBA1, EBA2 and EBA3) was actually to type VII collagen, we performed immunoelectron microscopy using recessive dystrophic epidermolysis bullosa skin. None of the patient sera showed reactivity in recessive dystrophic epidermolysis bullosa skin, which lacked type VII collagen (Fig. 1d).

Table 1 Summary of immunoblot (IB) analysis in this study

Patient	Clinical form	IB (kDa)[b]	IB[a] NC-1	NC-2	T-H
EBA1	Inflammatory	–	–	–	–
EBA2	Inflammatory	–	–	–	–
EBA3	Inflammatory	–	–	–	ND
EBA4	Inflammatory	290	–	–	ND
EBA5	Unknown	290	–	–	–

[a]Results of IB for recombinant proteins of the noncollagenous (NC)-1, NC-2 and triple-helical (T-H) collagenous domains. [b]Results of IB using normal human dermal extracts. ND, not done.

Discussion

In the present study, we first observed that reaction products of immunoelectron microscopy in all five EBA sera without reaction to either the NC-1 domain or the NC-2 domain were seen in the dermis, located at 0–400 nm below the lamina densa. Quantitative analysis revealed that more than 20% of the labelling was distributed with a peak at 90–150 nm below the lamina densa. The histogram for the immunoelectron microscopic results for these sera confirmed a different distribution of epitopes when compared with those of sera reactive with the NC-1 and/or NC-2 domains.[10] The distribution of the IgG antibodies in this study was consistent with the binding pattern of antibody to the T-H collagenous domain in a previous study.[11] This study also confirmed the previous reports that some EBA sera contain autoantibodies against the T-H collagenous domain.[6–9]

Although type VII collagen is the most abundant component protein of anchoring fibrils, there are other component proteins in anchoring fibrils, e.g. a part of type IV collagen and others. The results of immunoelectron microscopy, that the sera without reactivity to the NC-1 and NC-2 domains showed no positive reaction in the skin sections of recessive dystrophic epidermolysis bullosa, confirmed that these sera reacted with some parts of type VII collagen, but not to other anchoring fibril proteins.

Clinically, two distinct phenotypes of EBA have been described: the classical noninflammatory type and the inflammatory type. Clinically, four of our patients were considered to have the inflammatory type and the remaining patient could not be classified into either phenotype. This may suggest that patients with EBA with autoantibodies to the T-H collagenous domain show clinical features of the inflammatory phenotype. Interestingly, several previous reports described that some patients with childhood EBA with reactivity to the T-H collagenous domain, as well as to the NC-1 and NC-2 domains, had the inflammatory type.[6–9]

Although the relationship between the epitope profile and the clinical features remains to be elucidated, the results of the present study suggest that reactivity to different epitopes, such as the NC-1 domain and other domains, leads to different clinical phenotypes. One possible mechanism is that reaction of patient serum with regions other than the NC-1 domain results in an inflammatory phenotype, further suggesting the possible causative role of such autoantibodies on complement activation and inflammatory infiltrates, which in turn leads to

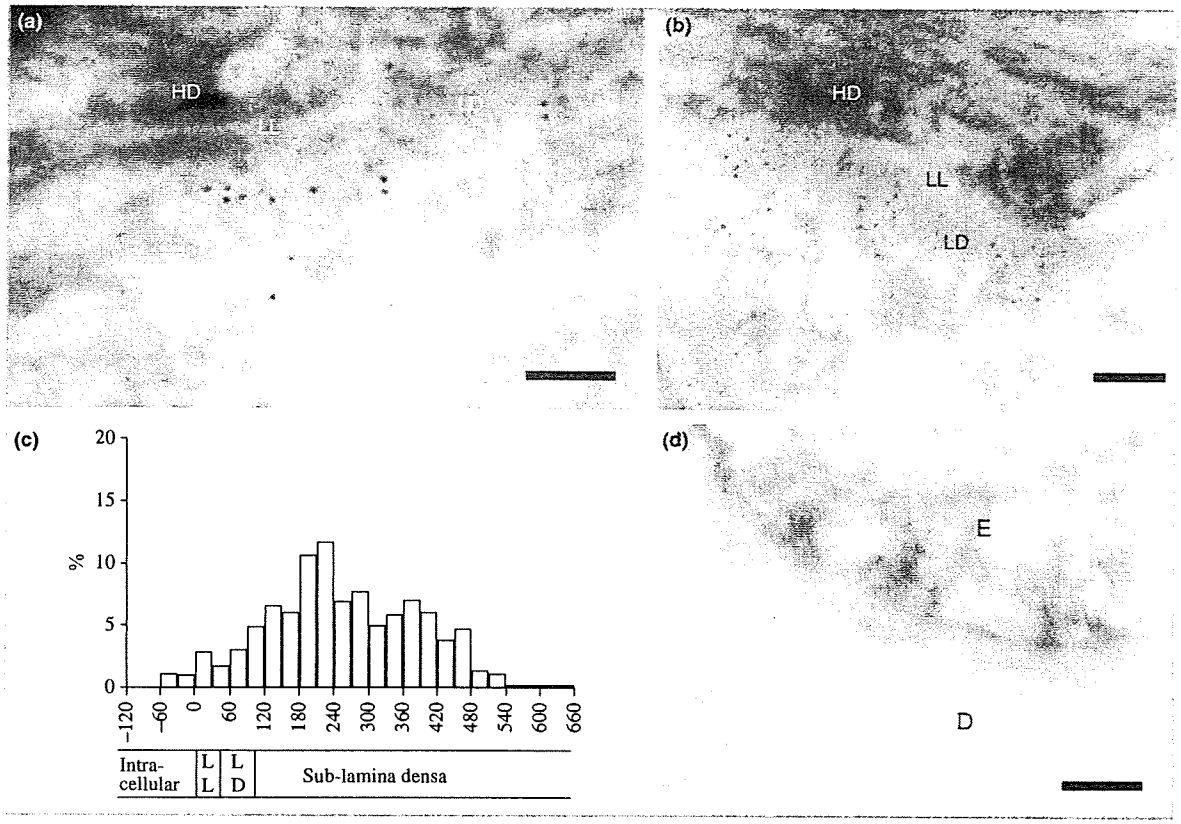

Fig 1. (a, b) The results of immunoelectron microscopy. Gold particles for two representative epidermolysis bullosa acquisita (EBA) sera localized in the dermis, 0–400 nm below the lamina densa in normal skin section. (c) For assessment of immunogold distribution, the distances from the lamina densa to the gold particles were measured (patient EBA1). Approximately 90% of IgG labelling was distributed throughout the dermis 0–400 nm below the lamina densa. (d) In recessive dystrophic epidermolysis bullosa skin, the representative three EBA sera showed no reactivity with the anchoring fibrils. LD, lamina densa; LL, lamina lucida; HD, hemidesmosome; D, dermis; E, epidermis. Bars = 200 nm.

the inflammatory type of EBA. Further studies on a large number of patients with EBA should characterize the target antigens and their correlation with clinical features, such as age at disease onset, extent of skin lesions and clinical course.

In the present study, unexpectedly, immunoblot analysis using pepsin-digested type VII collagen extracted from cultured keratinocytes showed no reactivity with the T-H collagenous domain in three cases of EBA, while the IgG deposit profile in immunoelectron microscopy strongly suggested binding with the T-H collagenous domain. One reason for this discrepancy may be the lower sensitivity of the immunoblot analysis compared with immunoelectron microscopy, suggesting that the epitopes are denatured by the strong detergent treatment during the immunoblotting procedure.

In conclusion, we have demonstrated that IgG autoantibodies exclusively to the T-H collagenous domain of type VII collagen are present in a minor group of patients with EBA. We suggest that the IgG antibodies to this domain may lead to development of an inflammatory phenotype, although the pathogenicity of these antibodies remains to be elucidated. To clarify these suggested findings, further studies will address the pathogenic relevance of autoantibodies to the T-H collagenous domain.

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Letter to the Editor

Lesional Th17 cells in pemphigus vulgaris and pemphigus foliaceus

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To the Editor,

T helper 17 (Th17) plays a pivotal role in the pathogenesis of autoimmune diseases, such as autoimmune encephalitis and inflammatory bowel diseases, in which Th1 previously were thought essential [1–3]. Also, previous reports have suggested that interleukin (IL)-17 is a crucial factor in the homing of granulocytes and tissue injury in bronchial asthma [4,5], which is believed to be a Th2 type disease. However, it has never been investigated whether Th17 is involved in autoimmune bullous diseases. We have here, for the first time, evaluated the status of lesional Th17 and T regulatory (Treg) cells immunohistochemically in pemphigus vulgaris and pemphigus foliaceus.

Patient backgrounds: Eight men and 12 women, 30–80 years old (average age: 60 years), eleven cases of pemphigus vulgaris, seven cases of pemphigus foliaceus, one case of pemphigus foliaceus with bullous pemphigoid and one case of paraneoplastic pemphigus. The severity index, which was scored according to the diagnostic criterion of the Specified Disease Treatment Research Program by the Japanese Ministry of Health, Labour and Welfare, was mild in eight cases, moderate in two cases, severe in three cases and unknown in seven cases.

The lesional biopsy specimens from the patients' skin were obtained at their first visit in order to evaluate the acute state before treatment. Specimens were stored at −80 °C until use. Immunohistochemistry of cryosections from those specimens were performed with the following first antibodies: Goat anti-human IL-17 antibody (R&D Systems, Inc., MN, USA) [6], mouse monoclonal antibody [236A/E7] to Foxp3 (Abcam, Cambridge, UK) [7] and anti-CD4 mouse monoclonal antibody (Nichirei Corporation, Tokyo, Japan). For negative control preparations, the primary antibodies were replaced with an irrelevant isotype-matched control immunoglobulins. All positively stained lymphocytes in two sections from each specimen were counted. The IL-17+ (Th17) and Foxp3+ cell counts (Treg) relative to the CD4+ cell count (%) were quantitatively evaluated.

The specimens from skin lesions of pemphigus vulgaris (Fig. 1a and b), pemphigus foliaceus (Fig. 1d and e) and paraneoplastic pemphigus (not shown) contained both IL-17 producing cells and Foxp3-expressing cells. Both IL-17-positive cells and Foxp3-positive cells were undetectable in healthy control skin (not shown). The ratio of IL-17+ cell count to CD4+ cell count was significantly higher in pemphigus vulgaris (5.2%) than in pemphigus foliaceus (1.8%) (Fig. 1g). The ratios of Foxp3+ cell count to CD4+ cell count were comparable (about 20%) in pemphigus vulgaris and pemphigus foliaceus (Fig. 1h). Although there were more infiltrating CD4+ cells in the specimens of pemphigus vulgaris than in those of pemphigus foliaceus, there was no correlation between infiltrating CD4+ cell count and the percentage of IL-17+ or Foxp3+ cells (Fig. 2a and b). The each cell count per specimen was as follows. IL-17 positive cells: 1–251 (average: 83) in pemphigus vulgaris and 0–72 (average: 14) in pemphigus foliaceus. Foxp3-positive cells: 1–638 (average: 275) in pemphigus vulgaris and 20–637 (average: 153) in pemphigus foliaceus. There were no significant positive or negative correlations between the percentage of IL-17+ or Foxp3+ cells and the severity score (Fig. 2c and d). There were also no significant correlations between the percentage of IL-17+ or Foxp3+ cells and the titers of anti-desmoglein (Dsg) 1 or Dsg3 antibodies (Fig. 2e and f).

In autoimmune diseases, a relative decrease in Treg function may be involved in the pathogenesis. Inductions of both Th17 and Treg require participation of transforming growth factor (TGF)-β. In addition, the presence of IL-6 induces Th17 differentiation and the absence of IL-6 results in Treg differentiation [3]. Therefore, Th17 and Treg seem to have opposite

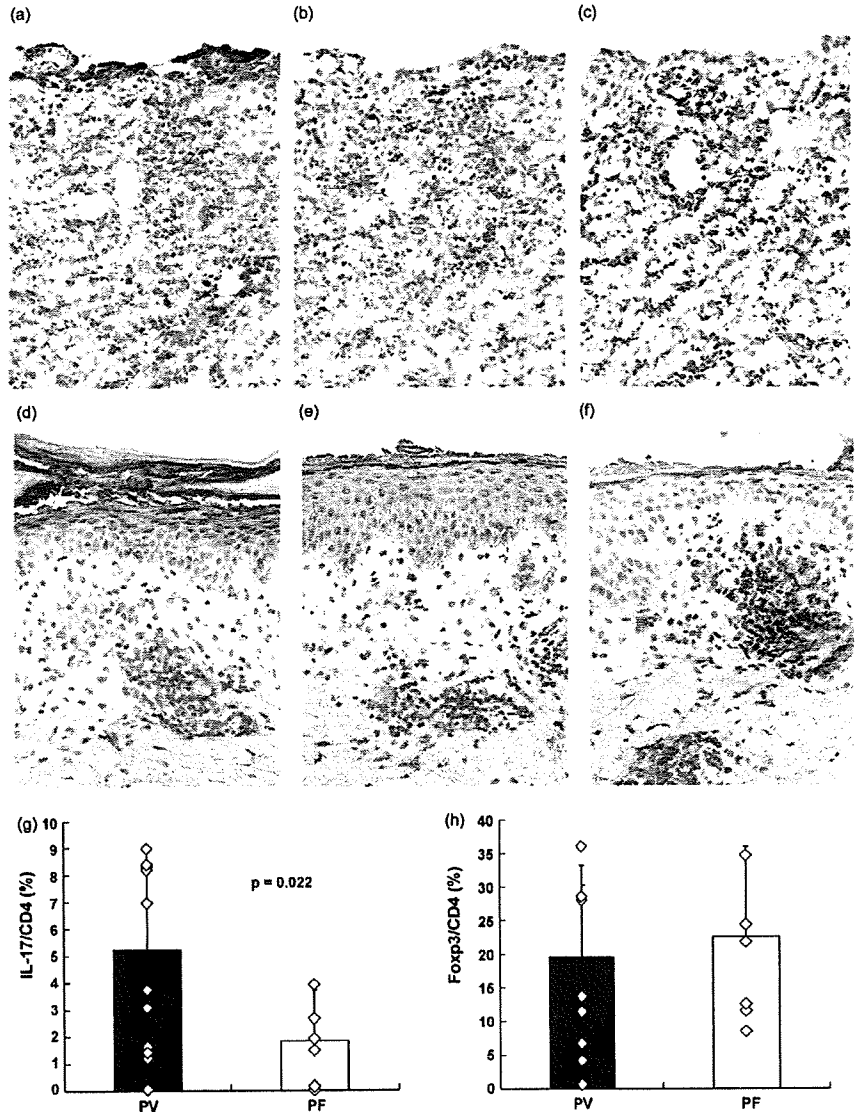

Fig. 1. Immunohistochemistry of specimens from lesions of pemphigus vulgaris (a–c) and pemphigus foliaceus (d–f). Positively stained lymphocytes were detected by antibodies to IL-17 (a and d), Foxp3 (b and e) and CD4 (c and f), respectively. Th17 in pemphigus vulgaris is significantly predominant compared to its presence in pemphigus foliaceus (g), while the Treg distribution is comparable in the two diseases (h). The significance of the difference was assessed by an unpaired *t*-test. Open rhombuses show plots of the cases and error bars show standard deviations.

roles in autoimmunity although they do not suppress each other as do Th1 and Th2.

An earlier report suggested that the number of Treg cells is markedly decreased in blood of patients with pemphigus vulgaris [8]. However, the present study showed that Treg did not disappear from the skin lesions. Therefore, a decrease of Treg in peripheral blood is not necessarily accompanied by a decrease of Treg in the lesions, so this decrease cannot explain the pathogenicity of pemphigus vulgaris. However, the decrease of Treg in peripheral blood may perhaps be a result of the accumulation of Treg in the lesion and draining lymph nodes.

After evaluating the results of the present study, we conclude that the rate of Th17 accumulation could not reflect the severity of pemphigus vulgaris and pemphigus foliaceus. The question then is: What is the role of Th17 in the lesional skin in pemphigus? The mechanism of the Th17 effect in pemphigus may be similar to that in several autoimmune diseases, such as autoimmune encephalitis and inflammatory bowel diseases, in which Th17 has been reported to have a pivotal role. These diseases are cellular autoimmune diseases and different from pemphigus, a humoral autoimmune disease, and it is not known how Th17 is involved in the breakdown of the immune tolerance in either of them.

The reason why results do not show a correlation between Th17 cells and disease activity or antibody titers is not certain. We suggest two possibilities: first, lesional Th17 cells may be an initiator of the disease via granulocytes or macrophages in a non-dose-dependent manner *in vivo*, although autoantibodies alone can induce experimental pemphigus [9]. Another, lesional Th17 cells may not be a cause but a result of the disease, i.e., protective response that maintain epithelial barrier homeostasis [10].

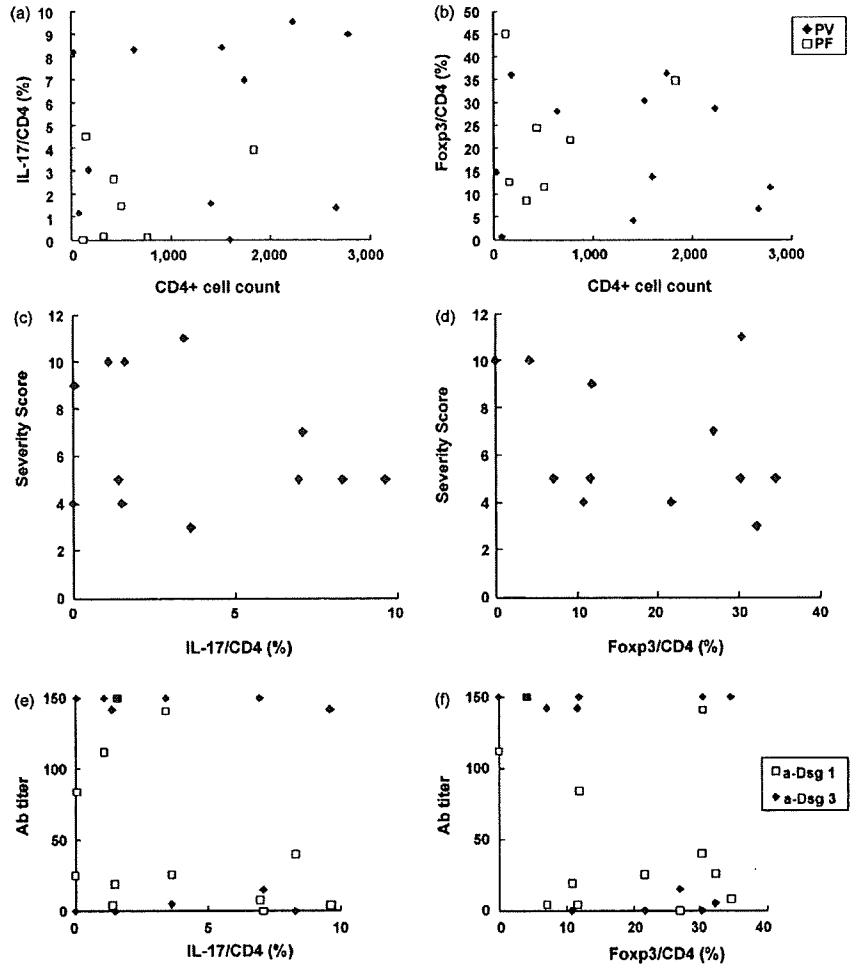

Fig. 2. Correlation of lesional Th17 (a, c and e) or Treg (b, d and f) with infiltrating CD4+ cell count, the severity of the diseases and autoantibodies. Correlation with infiltrating CD4+ cell count (a and b). Closed rhombuses are plots for pemphigus vulgaris and open squares are for pemphigus foliaceus. Correlation with disease severity score (c and d) and with titers of autoantibodies (e and f). Significance of the correlation was assessed by Spearman's rank correlation coefficient; no significant correlations between each parameter was detected. Open squares show plots for anti-Dsg1 antibodies and closed rhombuses show plots for anti-Dsg3 antibodies.

Some problems in the present study remain to be solved. Thus it is unclear why more Th17 cells were present in lesional specimens from pemphigus vulgaris than in those from pemphigus foliaceus, and we could not show that IL-17-producing lymphocytes in the skin were exclusively CD4+ lymphocytes. Also, the timing of the Th17 migration and the cells' autoreactivity in pemphigus should be further investigated.

In conclusion, the present study suggests the possibility that Th17 may be involved in the pathogenesis of autoimmune bullous diseases. In order to develop new therapeutic approaches in autoimmune bullous diseases, it would be valuable to elucidate the precise mode of function of Th17.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jdermsci.2008.09.008.

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Letter to the Editor

Bath-PUVA therapy induces circulating regulatory T cells in patients with psoriasis

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FoxP3
Psoriasis
Psoriasis area and severity index scores (PASI)

To the Editor,

$CD4^+CD25^+$ regulatory T cells (Treg) comprise a T cell subset that has immunoregulatory function and inhibits the development of autoimmune diseases [1]. Circulating Treg in the peripheral blood maintain peripheral immune tolerance against self and foreign antigens [2]. FoxP3 is considered to be a specific Treg marker. $FoxP3^+$ Treg are present in the skin lesions of psoriasis [3]. Sugiyama et al. [4] recently reported functional defects in Treg suppressor activity in psoriasis patients that were not associated with a decrease in the number of $CD25^+$ Treg in the peripheral blood. These reports indicate that Treg may be involved in the pathogenesis of psoriasis. Photochemotherapy with psoralen and UVA (PUVA) is commonly used to treat psoriasis. Bath water delivery of 8-methoxypsoralen and subsequent UVA-irradiation (bath-PUVA therapy) for the treatment of psoriasis is an effective alternative to systemic application. Brazzelli et al. recently reported that PUVA induces a longer remission period than narrow band (NB)-UVB [5]. Therefore, we evaluated whether $FoxP3^+$ Treg contribute to the clinical efficacy of bath-PUVA therapy.

To examine whether bath-PUVA affects circulating Treg in the peripheral blood of psoriasis patients, 10 healthy controls and 10 psoriasis patients who had not received previous phototherapy were enrolled in this study (Table 1; age 27–76 years, Psoriasis Area and Severity Index [PASI] score 6.6–23.5). All procedures received prior approval from the Ethics Committee of Nagoya City University Graduate School of Medical Science, Nagoya, Japan, and all subjects provided written informed consent. Patients were treated with 0.0001% psoralen baths for 15 min preceding treatment with UVA radiation 5 times weekly (starting UVA dose, 0.5 J/cm^2; dose increment, 0.5 J/cm^2). Peripheral blood was obtained from patients before bath-PUVA therapy and 24 h after last exposure. Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare Bio-Sciences, Tokyo, Japan) and stained with phycoerythrin-conjugated antibodies against human CD4 (clone: MT310, DAKO, Tokyo, Japan) and fluorescein isothiocyanate conjugated anti-human CD25 (clone: ACT-1, DAKO), followed by intracellular staining with allophycocyanin-conjugated anti-human FoxP3 (clone: PCH101, eBiosciences, San Diego, CA). Fluorescence intensity of the cells was analyzed by fluorescence-activated cell sorting analysis (FACSCalibur™ Flow Cytometry System, Becton Dickinson, Tokyo, Japan). To determine the effect of bath-PUVA therapy on peripheral blood Treg, we assessed the percentages of $CD4^+CD25^+FoxP3^+$ cells in $CD4^+$ cells. The percentage of $CD4^+CD25^+FoxP3^+$ Treg in peripheral blood mononuclear cells isolated from psoriasis patients before bath-PUVA therapy ($2.86 \pm 1.52\%$) was slightly lower than that in cells from healthy volunteers ($3.69 \pm 0.86\%$), but the difference was not significant (Fig. 1A and B). In contrast, the percentage of Treg in peripheral blood $CD4^+$ T cells in the psoriasis patients was significantly higher after bath-PUVA therapy ($5.40 \pm 1.43\%$); Fig. 1B). PASI scores were assessed before and after treatment. The PASI scores concomitantly improved in all 10 psoriasis patients after bath-PUVA therapy (Table 1 and Fig. 1C; PASI before bath-PUVA = 16.2 ± 5.3, PASI after bath-PUVA = 3.5 ± 2.1). The correlations between the PASI scores and the percentage of Treg in blood $CD4^+$ cells are shown in Fig. 2. The ratio of Treg in $CD4^+$ cells obtained from psoriasis patients before and after bath-PUVA therapy was inversely proportional to the PASI score (Fig. 2, $r = -0.559$), indicating that $FoxP3^+$ Treg have an important role in the beneficial outcome of bath-PUVA therapy.

Treg are a T cell subset that is distinct from Th1 and Th2 cells, comprising a heterogeneous group of T cells that actively inhibit immune responses [6]. FoxP3 is considered to be a specific Treg marker because overexpression of the *FoxP3* gene in T cells induces a suppressor phenotype and an expansion of $CD4^+CD25^+$ T cells [7]. Exposure to UV radiation induces Treg production. We previously demonstrated that NB-UVB induces peripheral immune tolerance and Treg [8]. Brazzelli et al. [5] recently demonstrated that PUVA induces a longer remission period than NB-UVB. Furthermore, extracorporeal photopheresis also induces $CD4^+CD25^+$ Treg [9] and a very long remission period. Therefore, we hypothesized that PUVA may induce a long remission period via Treg induction.

In our study, the percentage of Treg in the peripheral blood was not significantly different between healthy donors and psoriasis patients prior to bath-PUVA. This finding is consistent with