Anti-laminin gamma-1 pemphigoid

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Anti-p200 pemphigoid has been characterized by autoantibodies to an unidentified 200-kDa protein (p200) of the dermal–epidermal junction. The objective of this study was to identify p200. We performed 2D gel electrophoresis of dermal extracts and immunoblotting with patients' sera, followed by MS analysis of a unique protein band. The protein band corresponded to laminin γ1. Anti-laminin γ1 mAb reacted with the anti-p200 immunoprecipitates by immunoblotting. Sera from 32 patients with anti-p200 pemphigoid showed 90% reactivity to the recombinant products of laminin γ1. None of the healthy control sera reacted with laminin γ1. By immunoblotting, reactivity of a patient's serum with p200 was competitively inhibited by adding anti-laminin γ1 C-terminus mAb. Purified anti-p200 IgG also inhibited the reactivity of this mAb to dermal laminin γ1. Most laminin γ1-positive sera showed reactivity with recombinant laminin γ1 C-terminal E8 fragment. Reactivity of patients' sera and purified IgG to dermal laminin γ1 was higher than reactivity to blood vessel laminin γ1 under reducing conditions. These results suggest that laminin γ1 is the autoantigen for patients with anti-p200 pemphigoid. The autoantibodies may specifically recognize dermal laminin γ1 with unique posttranslational modifications. The epitope is localized to the 246 C-terminal amino acids within the coiled-coil domain. The 9 C-terminal residues are known to be critically involved in laminin recognition by integrins.

autoimmune disease | basement membrane | bullous pemphigoid | proteomics

Anti-p200 pemphigoid is a novel autoimmune subepidermal blistering diseases (1, 2). It is characterized by autoantibodies against a 200-kDa protein (p200) of the dermal–epidermal junction. This protein has been shown to be distinct from all other known autoantigens within the dermal–epidermal anchoring complex (3, 4), including type XVII collagen (BP180) (5), bullous pemphigoid antigen 1 (BP230) (6), $\alpha 6\beta 4$ integrin (7), laminins 332 and 311 (8, 9), and type VII collagen (10).

Clinically, most reported cases present with tense blisters and urticarial eruptions, symptoms that are different from those of any established bullous disease but closely resemble bullous pemphigoid (11). Following our first cases described in 1996 (1, 2), more than 50 cases have been published (12). So far, the identity of the protein has remained an enigma.

Previous attempts to characterize p200 localized this autoantigen to the lower lamina lucida by indirect immunogold electron microscopy (1). A polyclonal anti-laminin 1 antibody stained the similar 200-kDa band by immunoblotting with extracts of cultured human fibroblasts and dermal extracts (1, 11). However, serum from an anti-p200 pemphigoid patient could not react detectably with purified laminin 111 by immunoblot study under reducing conditions (1). Further, by indirect immunofluorescence, the anti-laminin 111 antibody reacted with both the dermal basement membrane zone and vessels, whereas patients' sera reacted only with the dermal basement membrane zone (1, 11). Indirect immunofluorescence with skin lacking laminin 332 and type VII collagen showed that p200 does not correspond to these proteins (3, 4). Biochemically, p200 is a noncollagenous *N*-glycosylated acidic protein (13) that is distinct from subunits of type VI collagen (13) and nidogen 2 (14).

Here, we report that laminin γ1 is the autoantigen in patients with anti-p200 pemphigoid.

Results

Characterization of a Unique Immunoreactive Protein Band from 2D-Separated Dermal Extracts. Dermal extracts were separated by 2D electrophoresis with an isoelectric focusing between isoelectric point (pI) 4 and 7, followed by 7.5% SDS gel electrophoresis. Each extract was run on 2 gels; 1 gel was stained with Coomassie brilliant blue (Fig. 1*A*), and the other was transferred onto a nitrocellulose membrane, followed by immunoblotting with serum from a patient with anti-p200 pemphigoid (Fig. 1*B*). The serum detected multiple bands migrating around pI 5.0 and in the 120–250-kDa region, the most reactive band of which was located at 250 kDa. The identical band on the Coomassie-stained gel was analyzed by MS (Fig. 1*D*). A database search characterized the protein band as laminin γ1, nominal mass 184,596, calculated pI value 5.01, with 28% of sequence coverage (Fig. 1*E*). To confirm the identity of the particular band, immunoblotting using anti-laminin γ1 mAb was performed on the membrane to which 2D-separated dermal extracts were transferred. The patient's serum and the mAb reacted with the same bands (Fig. 1*C*). Sera from 3 additional patients also labeled the same bands (not shown).

Anti-laminin γ1 mAb reacted with an immunoprecipitate formed of anti-p200 pemphigoid serum and dermal extract, but the antibody did not react with an immunoprecipitate of healthy control serum (Fig. 2*A*). The supernatant of immunoprecipitation of a dermal extract with patient serum was tested for immunoblotting with anti-laminin γ1 mAb: no band was detected, suggesting that all laminin γ1 was completely adsorbed by the patient serum. Inversely, on immunoblotting, immunoprecipitates with anti-laminin γ1 mAb reacted with patients' sera in a dose-dependent manner (Fig. 2*B*). The supernatant of anti-laminin γ1 immunoprecipitation showed reactivity to anti-p200 pemphigoid serum in inverse proportion to the amount of anti-laminin γ1 antibody used. The results of immunoblotting with anti-p200 pemphigoid sera suggest that the γ1 chain of laminins is the dermal component of the p200 band.

Reactivity of Patients' Sera with Laminin γ1. To investigate whether the reactivity of the patient serum with laminin γ1 is shared among patients with anti-p200 pemphigoid, the sera from 20 representative patients were examined for their reactivity toward purified laminin

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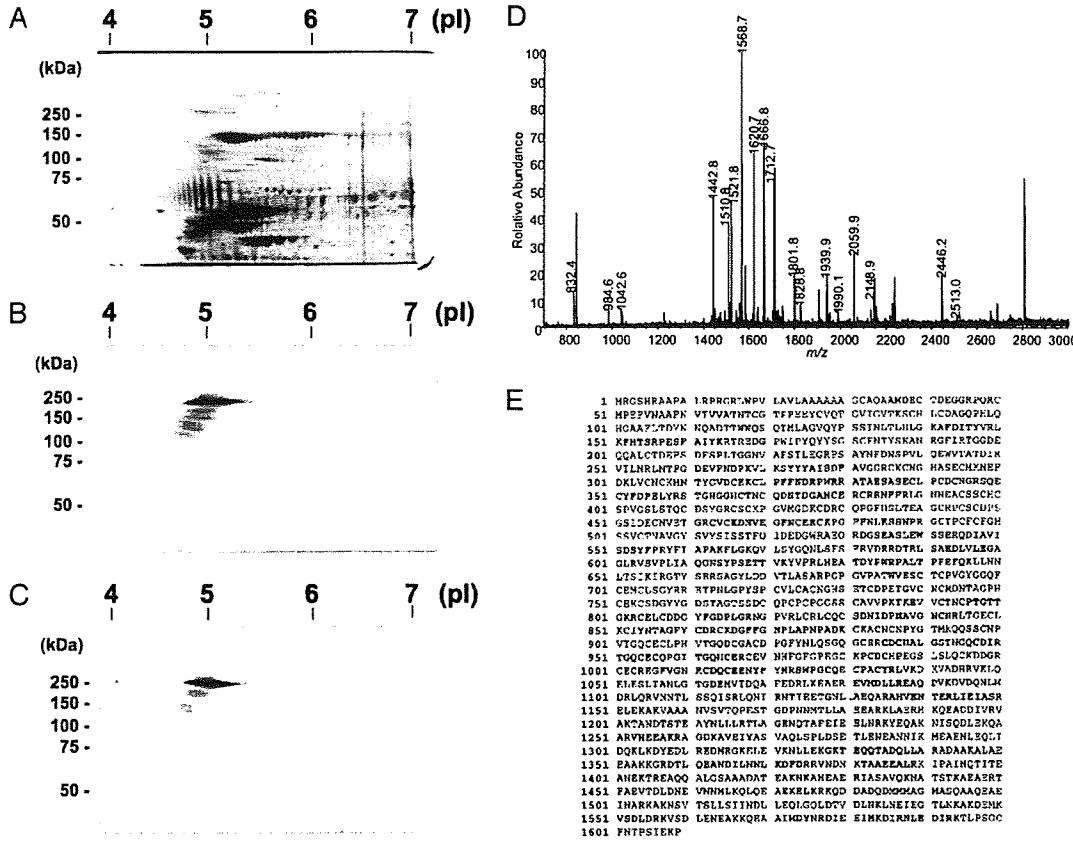

Fig. 1. 2D gel electrophoresis of dermal extracts followed by immunoblotting with anti-p200 pemphigoid serum. (*A*) A proteome pattern of dermal extracts was visualized by Coomassie brilliant blue stain on a 7.5% SDS gel. (*B*) Immunoblotting with the patient's serum showed a particular protein band with a molecular weight of about 250 kDa and a pI of 5.0. (*C*) Immunoblotting with anti-laminin γ1 mAb B-4 detected an identical band. (*D*) MALDI-TOF-MS spectrum of the particular band on the gel. The *m/z* values from 700 to 3,000 are shown. (*E*) All the remarkable tryptic peptide peaks corresponding to laminin γ1 are labeled with those *m/z* values. The matched triptic peptides (shown in red) corresponded to laminin γ1 by a database search. The sequence coverage was 28%.

111 and laminin 211/221, both of which contain the laminin γ1 chain. All 20 sera reacted with the 200-kDa protein on immunoblotting using dermal extract, and the position of the band coincided with that of the band detected with anti-laminin-γ1 mAb (Fig. 3*A*), thus supporting the possibility that the autoantigen in anti-p200 pemphigoid is laminin γ1. Consistent with this possibility, 80% and 85% of the patients' sera reacted positively with the γ1 chains of purified laminin 111 and laminin 211/221, respectively (Fig. 3, *B* and *C*, and Table 1). Sera from a total of 32 patients showed comparable reactivity (not shown). These results were confirmed further by immunoblot analysis using recombinant products of laminin 111, in which 90% of the patients' sera showed evident reactivity to the recombinant laminin γ1 (Fig. 3*D* and Table 1). Importantly, no sera from the 9 healthy controls reacted with purified or recombinant laminin γ1. Sera from patients with other bullous diseases, such as bullous pemphigoid, anti-laminin 332 mucous membrane pemphigoid, and epidermolysis bullosa acquisita, showed no reactivity to laminin γ1 (not shown). These results suggest that laminin γ1 is the

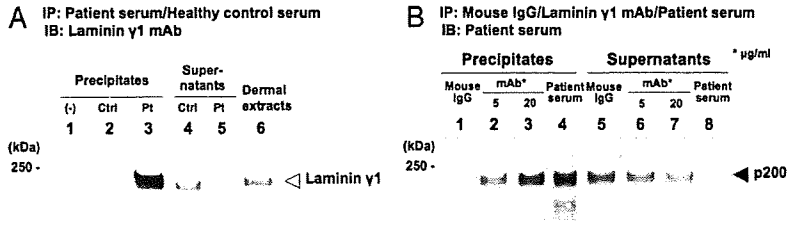

Fig. 2. Immunoprecipitation (IP) of dermal extracts followed by immunoblot (IB) analyses. (*A*) Dermal extracts were precipitated without (lane 1), with healthy (Ctrl) (lane 2), or with anti-p200 pemphigoid patient (Pt) serum (lane 3) at a 1:20 dilution. Each supernatant from immunoprecipitation with control serum (lane 4) or patient serum (lane 5) was examined also. Immunoblotting with mouse anti-laminin γ1 mAb B-4 detected bands in lanes 3 and 4 identical to the band of laminin γ1 from untreated dermal extracts (lane 6). (*B*) Dermal extracts were precipitated with 20 μg/ml of mouse control IgG (lane 1), mAb B-4 (5 μg/ml in lane 2 and 20 μg/ml in lane 3), and patient serum (1:20 dilution) (lane 4) and followed by immunoblotting with the patient serum. Each supernatant also was examined (lanes 5–8). Patient serum reacted with immunoprecipitates by anti-laminin mAb in a dose-dependent manner (lane 2, 3, and 6, 7).

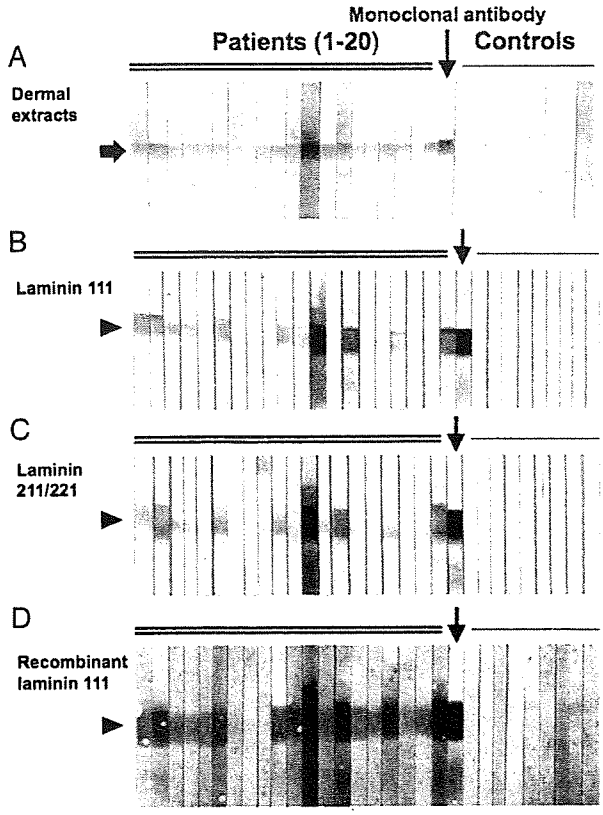

Fig. 3. Immunoblot analyses of sera from 20 patients with anti-p200 pemphigoid (*double line*), anti-laminin γ1 mAb B-4 (*long arrows*), and sera from 9 healthy controls (*single line*). (*A*) All patients' sera reacted with the 200-kDa protein in dermal extracts, whereas none of the healthy control sera reacted. The bands were identical to the band detected by anti-laminin γ1 mAb. (*B*) Most patients' sera reacted with laminin γ1 from laminin 111 purified from JAR human choriocarcinoma cells, (*C*) laminin 211/221 purified from human placenta, and (*D*) and recombinant products of laminin 111. None of the healthy control sera reacted with laminin γ1. The short arrow indicates the p200 band, and arrowheads indicate the laminin γ1 band (6% SDS).

exclusive autoantigen in anti-p200 pemphigoid, although there were a few negative cases.

Responsible Epitope on Laminin γ1. By immunoblotting, reactivity of a patient's serum to the p200 from dermal extract was competitively inhibited by adding anti-laminin γ1 *C*-terminus mAb (lanes B-4), but not by adding anti-laminin γ1 N terminus mAb (lanes C-13S) or anti-collagen VI mAb (lanes 3C4) (Fig. 4*A*). Serum and purified IgG from an anti-p200 pemphigoid patient inhibited the reactivity of the mAb B-4 to dermal laminin γ1 in a dose-dependent manner, but healthy control serum or IgG did not (Fig. 4*B*). These results indirectly showed that the reactive site on the dermal p200 protein for the patient's serum was located at the laminin γ1 *C*-terminus.

To narrow down further the responsible epitope within the laminin γ1 chain, we produced a recombinant "E8 fragment" of human laminin 111, a truncated version of laminin 111 comprising the C-terminal 25% of the coiled coil domain and 3 laminin G-like modules (i.e., LG1–3) of laminin 111 (Fig. 5*A*). Immunoblot analyses using this recombinant protein showed that most laminin γ1-positive sera showed reactivity with the laminin γ1 E8 fragment under both reduced and nonreduced conditions (Fig. 5*B*). The results of immunoblot analyses obtained with sera from 20 different patients are summarized in Table 1. These results suggest that the putative epitope targeted by patients' autoantibodies is located within the *C*-terminal 246-amino acid residues of the laminin γ1 chain.

Organ Specificity of the Autoantibodies. Indirect immunofluorescence microscopy on 1 M NaCl-split human skin sections showed that purified IgG from anti-p200 pemphigoid patients reacted with the dermal side of the split (Fig. 6*A* and *B*). IgG from a particular case also reacted with vessel walls of the capillaries in the papillary dermis (Fig. 6*B*). However, other patients' IgG showed no visible reactivity with blood vessels (Fig. 6*A*), a finding that is consistent with previously reported results (1, 11). In contrast, anti-laminin γ1 mAb C13S reacted with both the dermal side of the split skin and vessel walls (Fig. 6*C*).

In immunoblot studies under reducing conditions, the loaded amounts of extracts of dermis and the vessels were adjusted by the reactivity of anti-laminin γ1 mAb B-4 (Fig. 6*D*, *Lower*). In the same immunoblotting, reactivity of purified IgG from patient #20 to the dermal laminin γ1 was much higher than reactivity to blood vessel laminin γ1 (Fig. 6*D*, *Upper*). This observation was reproducible using sera from several other patients (not shown). However, purified IgG from patient #12, which reacted with the basement membrane zones of both dermis and vessel by indirect immunofluorescence microscopy, showed comparable immunoblot reactivity with laminin γ1 extracted from dermis and blood vessels (Fig. 6*D*, *Middle*). Except for this case, these results suggest that circulating IgG antibodies in patients with anti-p200 pemphigoid react with laminin γ1 within the dermal basement membrane zone but have much less reactivity with laminins in blood vessel walls.

Discussion

In this study, we identified laminin γ1 as the autoantigen in anti-p200 pemphigoid by MS analysis. We confirmed this result by several immunoblot approaches using anti-laminin γ1 mAb. Both anti-laminin γ1 mAb and anti-p200 pemphigoid serum reacted with an identical band in 2D-separated dermal extracts. Moreover, immunoprecipitates of anti-p200 pemphigoid serum reacted positively with anti-laminin γ1 mAb. Also, reactivity of anti-200 pemphigoid serum with dermal extracts was competitively inhibited by anti-laminin γ1 mAb, and vice versa.

Laminin γ1 is a 200-kDa *N*-linked glycoprotein (15) that is present in the cutaneous basement membrane zone. These molecular characteristics are the same as those of p200 defined previously (13, 14), so it is quite reasonable to conclude that it is the autoantigen of this autoimmune subepidermal blistering disease. Laminin γ1 is a component of different forms of laminin heterotrimers, such as laminin 311/321 and 511 (16). It contributes to dermal–epidermal adhesion outside hemidesmosomes (16). A laminin γ1 gene knockout results in embryonic lethality at day 5 because of the failure to form basement membranes, which are prerequisites for embryonic ectoderm differentiation (17). Functional inhibition of laminin γ1 by a nidogen-binding laminin γ1-chain fragment resulted in complete suppression of basement membrane formation in a 3D coculture of human skin keratinocytes and fibroblasts (18).

Our immunoblot analyses demonstrated that most patients' sera reacted with recombinant forms of laminin γ1. This result negates the possibility that patients' sera actually reacted with contaminating proteins of 200-kDa molecular weight that are associated with laminins, such as nidogens and collagen fragments. A few laminin γ1-negative sera were present, but all patients' sera reacted with p200 from dermal extracts by immunoblotting. This discrepancy could be explained by differences in sensitivity of the different assays. The laminin γ1-negative sera showed weak reactivity with p200 from dermal extracts but did not show visible reactivity to purified or recombinant laminins, perhaps because of distinct posttranslational modifications that are different from those of dermal laminins. Another, less likely but possible, explanation for the discrepancy between immunoblot results obtained using dermal

Table 1. Summary of the reactivity of sera from 20 patients with anti-p200 pemphigoid and healthy controls to laminin γ1 by immunoblot analyses in Figs. 3 and 5

Antigen	Positive in Pt/Ctrl (%)	Patients (1–20)																		mAb B-4 (21)		Healthy Controls (22–30)								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Purified materials																															
Reduced																															
JAR-laminin 111	80/0	+	+	+	+	Δ	+	Δ	−	−	+	+	+	Δ	+	+	Δ	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Placental laminin 211/221	85/0	+	+	+	+	+	+	Δ	−	Δ	+	+	+	+	+	+	Δ	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Recombinant products																															
Reduced																															
Laminin 111	90/0	+	+	+	+	+	+	Δ	−	−	+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−
Laminin 111-E8	85/0	+	+	+	+	+	+	Δ	−	−	+	+	+	+	+	+	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−
Nonreduced																															
Laminin 111-E8	85/44	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	+	−	+	−	+	−	+	−	−	−	+	+	−	+

Δ indicates weakly positive.

extracts and different laminin preparations is that p200 may be a heterogeneous protein, although laminin γ1 certainly is a major component.

A previous study to identify p200 using the same technique showed that p200 is a non-collagenous *N*-linked glycoprotein with a pI of 5.4–5.6 (13). In that study the α3 chain of type VI collagen was a candidate for p200, but patients' sera did not react with the purified α3 chain of type VI collagen by immunoblotting (13). We reproduced these results in some patients' sera and confirmed that the α3 chain of type VI collagen with a pI of 5.4–5.6 is not p200 (not shown). Although in previous studies we had suggested the possibility that p200 is laminin γ1 (1, 11), we could not detect any reactivity of sera from some previous cases of anti-p200 pemphigoid to placental laminin (11), purified laminin 111 (1, 11), or laminin 311 (11) by immunoblotting under reducing conditions. Nevertheless, by using multiple approaches with improved sensitivity and specificity, the present study strongly suggests that p200 is laminin γ1.

A few healthy control sera showed a weak reactivity with the laminin 111-E8 fragment under nonreducing conditions (Fig. 5*B*, *Lower Column*, and Table 1), but the reactivity of patients' sera was much stronger. These results may be caused by an unspecific reactivity, as has been described for other autoantigens of autoimmune bullous diseases, or by nonspecific bands derived from cell lines used for expression of the recombinant protein mimicking the molecular weight of the recombinant products (19).

In our study, most laminin γ1-positive sera showed reactivity with the recombinant *C*-terminal fragment of laminin γ1. Among laminin heterotrimers, the laminin γ1 *C*-terminus interacts with different integrins such as α3β1 and α6β4 integrins in the cutaneous basement membrane zone (20, 21). We reported previously that the glutamic acid residue at the third position from the *C*-terminus of laminin γ1 is required for integrin binding by laminin trimers (21). One therefore may speculate that anti-laminin γ1 autoantibodies modify the laminin–integrin interaction by interfering with the intrinsic binding site used for connecting these molecules in patients' skin in a direct or indirect manner. Interaction of laminin γ1 with the nidogen anchors laminin γ1 to the type IV collagen in the basement membrane zone (22, 23). Whether patients' sera react with nidogen-binding sites on the laminin γ1 molecule needs further clarification.

Anti-laminin γ1 autoantibodies from patients with anti-p200 pemphigoid are associated with skin blisters but show no pathology in other organs, although laminin γ1 is widely expressed in different basement membrane zones. The results of immunoblot studies suggest that laminin γ1 in the epidermal basement membrane zone may have different posttranslational modifications, such as glycosylation, compared with laminin γ1 expressed in blood vessels (Fig. 6*D*). Differences in posttranslational modification may allow further possible explanations for the organ specificity of the disease. Specifically, differences in posttranslational modification could result in (*i*) different 3D structures of laminin trimers within each organ, (*ii*) differences among associating molecules, such as integrin dimers, or (*iii*) organ-specific function by intermolecular associations, which can be specifically targeted by autoantibodies. Circulating anti-laminin γ1 autoantibodies in the patients may inhibit specific laminin–integrin interactions in the skin, which have an intrinsic role within the dermal–epidermal junction but are dispensable in other organs.

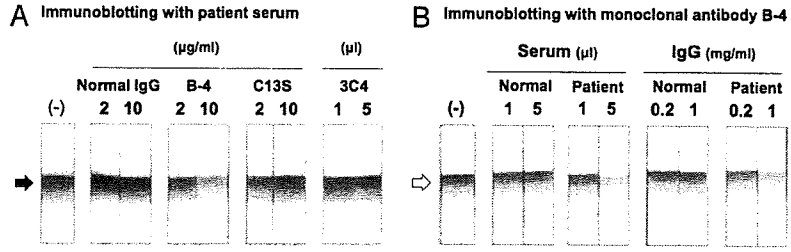

Fig. 4. Reactivity of anti-p200 pemphigoid sera to the laminin γ1 *C*-terminal portion. (*A*) In immunoblotting with dermal extracts (7.5% SDS), reactivity of a patient's serum to p200 (*closed arrow*) was reduced by adding anti-laminin γ1 mAb B-4 (B-4, 2 and 10 μg/ml), recognizing the *C*-terminus of this protein in a dose-dependent manner. Normal human IgG (Normal IgG), anti-laminin γ1 *N*-terminal mAb (C13S), or anti-collagen VI mAb (3C4, in ascites) did not inhibit binding of patient's serum to p200 from dermal extracts. The indicated doses of IgG (μg/ml), ascites, or sera (μl) were added or not (−) to each cellulose membrane during the reaction. (*B*) Serum and purified IgG from a patient with anti-p200 pemphigoid reduced binding of mAb B-4 to laminin γ1 extracted from dermis (*open arrow*), but serum or IgG from a healthy control did not.

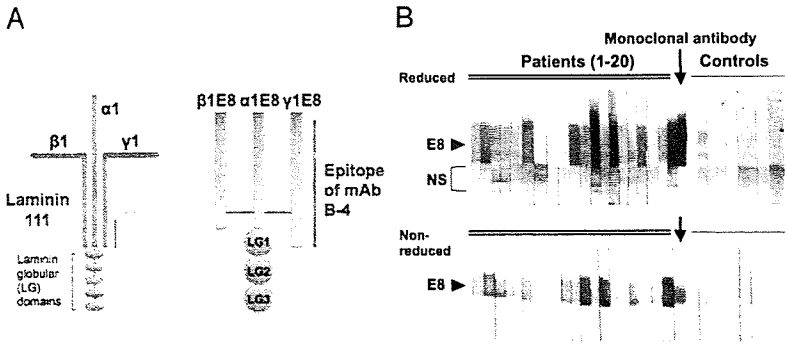

Fig. 5. Immunoblotting with the E8 fragment of laminin 111 (12% SDS). (*A*) Scheme of construction of the recombinant C-terminal E8 fragment of laminin 111. (*B, Upper*) All laminin γ1-positive sera in Fig. 2, except for serum from patient 18 (18th lane from the left), showed a positive reactivity with the E8 fragment of laminin γ1 by immunoblot analysis under reducing conditions. (*B, Lower*) All laminin γ1-positive sera in Fig. 2, except for patients 16 and 18 (16th and 18th lanes from the left), showed positive reactivity with the E8 fragment of laminin γ1 by immunoblot analysis under nonreducing conditions. Sera from 20 patients with anti-p200 pemphigoid are indicated by double lines. Arrow indicates anti-laminin γ1 mAb B-4. Single lines indicate sera from 9 healthy controls. Arrowheads indicate the E8 band. NS, nonspecific bands.

In conclusion, we have demonstrated that autoantibodies from patients with anti-p200 pemphigoid react with laminin γ1. Therefore, we propose a new name, anti-laminin γ1 pemphigoid, for this autoimmune bullous disease. Future studies will aim at providing direct evidence of the pathogenic role of anti-laminin γ1 autoantibodies. In addition, it will be of interest to investigate whether anti-laminin γ1 autoantibodies affect a specific interaction between laminin γ1 and its ligands within the cutaneous basement membrane zone.

Materials and Methods

Patients. All described studies were performed following the guidelines of the medical ethics committee of Kurume University School of Medicine. All participants in this study provided informed consent, and these studies were conducted according to the Declaration of Helsinki Principles. Thirty-two patients (10 males, 6 females, and 16 of unreported sex, 17–87 years old) were diagnosed as having anti-p200 pemphigoid by their clinical course and manifestation of blister formation, indirect immunofluorescence with 1 M NaCl-split skin demonstrating IgG reactivity with the dermal side of the basement membrane zone, and Western blotting with dermal extracts showing a 200-kDa band. Sera from 9 healthy volunteers and from patients with bullous pemphigoid, anti-laminin 332 mucous membrane pemphigoid, and epidermolysis bullosa acquisita were used as controls.

Antibodies. Mouse anti-laminin γ1 mAb B-4 was purchased from Santa Cruz Biotechnology. Mouse anti-laminin γ1 mAb C13S was produced in our laboratory (20, 24). Mouse anti-collagen VI mAb, 3C4, and mouse control IgG were purchased fromChemicon, Millipore, and Santa Cruz Biotechnology, respectively. The second antibodies, rabbit anti-human IgG or mouse Ig antisera conjugated with horseradish peroxidase and rabbit anti-human IgG or mouse Ig antisera conjugated with fluorescein isothiocyanate, were purchased from (Dako Corporation). IgG from patients' sera and healthy controls were purified on a HiTrap Protein G HP column (Amersham Biosciences).

Preparation of Dermal and Blood Vessel Extracts. Dermal extract for immunoblot analysis was prepared according to the method reported previously (11). The extract of blood vessels was prepared by a similar method with several modifications. Mesenteric arteries from which adventitia had been removed were incubated in PBS containing 2 mM EDTA and 2 mM PMSF for 2 d at 4 °C. Blood vessel proteins were extracted with 8 M urea, 0.3 M β-mercaptoethanol, and 1 mM PMSF in 25 mM Tris (pH 6.8) for 2 h at room temperature. The vessel extract was dialyzed for 2 d at 4 °C against H_2O and was precipitated by adding an equal volume of acetone.

Preparation of Laminins. Human laminin 111 was purified from the JAR human choriocarcinoma cell line, and laminin 211/221 was purified from human placenta (20). Recombinant laminin 111 was produced by transfecting human 293-F cells (Invitrogen) with expression vectors encoding human α1/β1/γ1 chains (21).

A recombinant E8 fragment of laminin 111, a heterotrimer of the truncated C-terminal portions of α1, β1, and γ1 chains, was prepared as follows. Expression vectors for the recombinant E8 fragment of human laminin β1 and γ1 (coding Leu^{1561}-Leu^{1786} and Asn^{1364}-Pro^{1609}, respectively) were prepared as described previously (21). cDNA encoding the truncated C-terminal portions (Phe^{1878}-Gln^{2700}) of the laminin α1 chain was amplified by PCR using full-length human laminin α1 expression vector as a template (25) and was inserted into the expression vector pSecTag2A (Invitrogen). A recombinant E8 fragment of laminin 111 was produced using the Free-Style™ 293 Expression system (Invitrogen) and was purified from conditioned medium as described previously (21). Briefly, the conditioned media were applied to nickel nitrilotriacetic acid affinity columns (Qiagen), and bound proteins were eluted with 200 mM imidazole. The eluted proteins were purified further on anti-FLAG columns (Sigma-Aldrich Inc.). The purified protein was dialyzed against TBS. Protein concentration was determined by the BCA™ Protein Assay Kit (Pierce Biotechnology).

Immunoblot Analyses. SDS/PAGE was performed as described previously (26). Extracts of dermis and blood vessels, human laminins, and human laminin-E8 fragment were boiled in Laemmli's sample buffer with or without 5% β-mercaptoethanol and were fractionated on 6%, 7.5%, or 12% SDS gels. Subsequently,

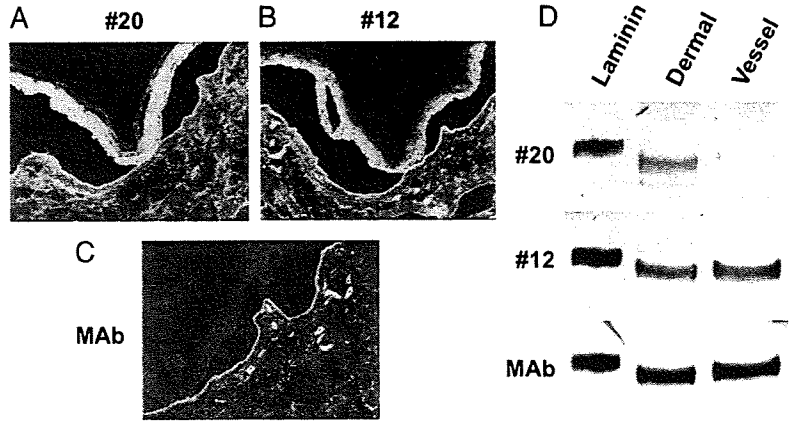

Fig. 6. Indirect immunofluorescence on 1 M NaCl-split human skin. (*A* and *B*) Purified IgG from anti-p200 pemphigoid patients reacted with the dermal side of the split. IgG from case #20 did not react with blood vessels in the upper dermis (*A*), whereas IgG from case #12 did (*B*). (*C*) Anti-laminin γ1 mAb C13S showed reactivity with both the dermal side of the basement membrane zone and blood vessel walls in the dermis. (*D*) Immunoblotting with extracts of dermis and blood vessels. The amount of extract loaded was adjusted by the reactivity of anti-laminin γ1 mAb B-4 (*Bottom*). Under reducing conditions, reactivity of the patient's purified IgG from case #20 (also used in Fig. 6*A*) to the dermal laminin γ1 was much higher than reactivity with blood vessel laminin γ1 (*Top*). Purified IgG from case #12 (also used in Fig. 6*B*) showed similar reactivity with laminin γ1 from dermal extract and blood vessel extract (*Middle*). Lane 1, purified laminin 111 from the JAR cell line; lane 2, dermal extract; lane 3, extract of vessels (7.5% SDS, under reducing conditions).

proteins were transferred to nitrocellulose sheets electrophoretically. After blocking with 3% skim milk, blots were incubated with patients' sera at 1:5–1:800 dilutions, with purified IgG at 10 μg/ml or 1 mg/ml, or with mouse anti-human laminin γ1 mAb B-4 at a 1:200 dilution. Peroxidase-conjugated anti-human IgG or anti-mouse Ig antisera were used as secondary antibodies. Color was developed using 4-chloro-1-naphthol.

Immunoprecipitation of dermal extracts was performed as follows. Lyophilized dermal extract was dissolved with immunoprecipitation buffer containing 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 1% Protein Inhibitor Mixture (Sigma-Aldrich). The solution was incubated with Protein G-Sepharose 4 Fast Flow (GE Healthcare) with or without normal control or patient serum (1:20 dilution), anti- laminin γ1 mAb B-4 (5 or 20 μg/ml), or mouse control IgG (20 μg/ml) at 4 °C overnight. After extensive washes, the collected precipitates were boiled in Laemmli's sample buffer with 5% β-mercaptoethanol. The supernatant was assessed by immunoblot analyses as described in previous sections.

In inhibition assays, serum from patient 12 (1:800) was incubated with competitors, i.e., normal human IgG, B-4, C13S (2 or 10 μg/ml), or 3C4 (1:100 or 1:20 dilution), on nitrocellulose strips containing transferred dermal extracts, followed by the development procedure with anti-human IgG antisera described in previous sections. On the other strips, B-4 (1 μg/ml) was incubated with competitors, i.e., serum (1:100 or 1:20 dilution) or IgG (0.2 or 1 mg/ml), at 4 °C overnight, followed by the development procedure with anti-mouse Ig antisera.

2D Electrophoresis. Freeze-dried dermal extracts were dissolved in the lysis buffer consisting of 7 M urea, 2 M thiourea, 40 mM Tris, 1% C7 detergent, and appropriate amounts of Complete Mini EDTA-free Protease Inhibitor Mixture Tablets (Roche Applied Science). For reduction and alkylation, 5 mM (final concentration) tributylphosphine and 10 mM (final concentration) acrylamide were added. After incubation for 90 min at room temperature, 10 mM (final concentration) DTT was added, and the mixture was incubated for 10 min at room temperature to stop the reaction. After centrifugal ultrafiltration using an Amicon Ultra-4 100 K device (Millipore), the concentrate was mixed with the isoelectric focusing (IEF) buffer composed of 7 M urea, 2 M thiourea, and 1% C7. For IEF analysis, the ReadyStrip™ IPG Strip (pH 4–7, 11 cm long, 3.3 mm wide, and 0.5 mm thick, Bio-Rad Laboratories) was rehydrated overnight with the sample solution including appropriate amounts of Bio-Lyte 3–10 Buffer (Bio-Rad Laboratories) and Orange G solution. Following completion of IEF, SDS/PAGE was performed using Criterion™ Ready Gels J (4%T stacking gel, 7.5%T resolving gel, Tris·HCl buffer type, 13.3 cm wide, 8.7 cm high, and 1.0 mm thick, Bio-Rad Laboratories) with Precision Plus Protein Standards (Bio-Rad Laboratories). The obtained 2D gel was stained with Coomassie brilliant blue or used on the subsequent immunoblot analysis.

In-Gel Digestion. The protein spot was excised and de-stained with 50% acetonitrile/100 mM ammonium bicarbonate, pH 8.0. After the excised gel piece was dried in a drier for 15 min, trypsin proteomics-grade solution (0.2 mg/ml in 0.1 mM HCl/36 mM ammonium bicarbonate in 9% acetonitrile, Sigma-Aldrich) was applied to it, followed by incubation in 2.5 mM ammonium bicarbonate in 9% acetonitrile overnight at 37 °C. The incubated solution was collected and dried (27).

Mass Spectrometry. The dried tryptic peptides were dissolved in 2–5 μl of 0.1% TFA/50% acetonitrile and then mixed with α-cyano-4-hydroxycinnamic acid (CHCA) solution (prepared by dissolving 10 mg of CHCA in 1 ml of 0.1% TFA/50% acetonitrile) as matrix, followed by analysis in an AXIMA-CFR Plus (Shimadzu/Kratos Analytical) MALDI-TOF-MS equipped with a delayed extraction mechanism and operated at a 20-kV accelerating voltage in a reflector and the positive ion mode. The monoisotopic *m/z* values of tryptic peptide peaks were entered into the Peptide Mass Fingerprint in the Mascot Search (Matrix Science Ltd., http://www.matrixscience.com/cgi/search_form.pl?FORMVER = 2&SEARCH = PMF) for a protein database search and for characterization of the protein (27).

Immunofluorescence on 1 M NaCl-Split Skin. Normal human skin was placed for 48 h at 4 °C in 100 ml of 1 M NaCl solution that contained 1 mM PMSF. Skin specimens were frozen quickly in liquid nitrogen, sectioned in a cryostat, and stained for indirect immunofluorescence with patients' sera (1:10 dilution), purified patients' IgG (0.08 mg/ml), and mouse anti-laminin γ1 mAb (C13S, 10 μg/ml) as first antibodies, followed by fluorescein isothiocyanate-conjugated rabbit anti-human IgG or anti-mouse Ig polyclonal antisera as second antibodies.

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A case of epidermolysis bullosa acquisita with clinical features of Brunsting-Perry pemphigoid showing an excellent response to colchicine

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Background: Brunsting-Perry pemphigoid is a rare subepidermal blistering disease characterized by scarring blisters on the head and neck. However, the identity of the responsible autoantigens is still unresolved.

Methods: We reported a patient with epidermolysis bullosa acquisita who had clinical features typical of Brunsting-Perry pemphigoid and investigated the involved type VII collagen epitopes. The patient was a 65-year-old Japanese woman with a 20-month history of recurrent subepidermal bullae on her head, face, and neck.

Results: Immunoblot studies revealed that the serum of this patient reacted with type VII collagen, specifically with the noncollagenous domain 1 and the triple-helical domain. The patient responded completely to colchicine monotherapy.

Limitations: This study was performed on only one case.

Conclusion: This study suggests that Brunsting-Perry pemphigoid may be a clinical variant of epidermolysis bullosa acquisita. (J Am Acad Dermatol 2009;61:715-9.)

Key words: Brunsting-Perry pemphigoid; epidermolysis bullosa acquisita; type VII collagen.

In 1957, Brunsting and Perry[1] described 7 patients with a localized form of cicatricial pemphigoid, characterized by pruritic chronic recurrent circumscribed vesiculobullous eruptions located on the head, face, and neck and leaving atrophic scarring. This disease is common in middle-aged and elderly populations. Skin lesions are usually confined to the head, neck, scalp, and upper aspects of the trunk. Mucous membranes are also affected in some patients.[2-5]

The identity of the responsible autoantigens in Brunsting-Perry pemphigoid is still controversial. Indirect immunofluorescence and immunoelectron microscopy has shown that some patients' sera react with a dermal antigen, suggesting that Brunsting-Perry pemphigoid is a variant of epidermolysis bullosa acquisita.[5,6] However, there has been no case report of this disease with immunoblot analyses showing reactivity with type VII collagen or bullous pemphigoid antigens, except for our reports in the Japanese literature showing reactivity of patients' sera with a recombinant protein of the 180-kd bullous pemphigoid antigen (BP180) noncollagenous (NC)16a domain.[7,8] These findings suggest that Brunsting-Perry pemphigoid is not a single disease entity but is heterogeneous.

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We describe a patient who had typical clinical features of Brunsting-Perry pemphigoid, whereas the results of indirect immunofluorescence on 1-mol/L salt-split human skin sections and immunoblot analyses were consistent with a diagnosis of epidermolysis bullosa acquisita. The patient responded well to colchicine therapy. In addition, we investigated the epitopes in all the 3 structural domains of type VII collagen.

METHODS

Patient

A 65-year-old Japanese woman with diabetes mellitus showed recurrent bullous skin lesions on the head, face, and neck that had first appeared 20 months previously. According to the patient, similar bullous lesions had also appeared on the oral mucosa at the onset of the skin disease. On her first visit, there were tense blisters and crusts with erythematous and slightly atrophic scars over the face, neck, and upper aspect of back (Fig 1, *A* and *B*). There were no milia, and other areas and mucous membranes were unaffected. The results of laboratory tests were almost within normal limits.

Histopathologic studies

A skin biopsy specimen was taken from a spontaneously formed blister on the face. The specimen was formalin fixed and paraffin embedded. The skin sections were stained with hematoxylin and eosin in a standard protocol.

Indirect immunofluorescence on 1-mol/L salt-split skin

Normal-appearing human skin was placed for 48 hours at 4°C in 100 mL of 1-mol/L salt solution that contained 1 mmol/L of ethylenediaminetetraacetic acid, 1 mmol/L of phenylmethanesulfonyl fluoride, and 25 mmol/L of tris (hydroxymethyl)-aminomethane-hydrochloric acid (pH 7.4). The skin specimen was quickly frozen in liquid nitrogen, sectioned in a cryostat, and stained for indirect immunofluorescence with fluorescein isothiocyanate-conjugated rabbit antihuman IgG polyclonal antiserum.[4]

Immunoblot analyses

Immunoblot analyses with extracts of normal human dermis, bacterial recombinant proteins of NC1 and NC2 domains of human type VII collagen, recombinant full-length type VII collagen, and the triple-helical domain of type VII collagen were performed as described previously.[9-11] Specifically, dermal extracts containing type VII collagen were prepared using ethylenediaminetetraacetic acid-split normal-appearing skin.[9] We prepared recombinant glutathione-S-transferase fusion proteins containing the entire 1253 residues of the NC1 domain and the entire 161 residues of the NC2 domain of type VII collagen.[9] Because the two recombinant proteins of type VII collagen were not soluble in phosphate-buffered saline containing 1% Triton X-100, the pellets were further extracted by resuspending and sonicating them in 3 mL of 2-mol/L urea solution. Subsequently, the pellets were resuspended in 1 mL of Laemmli sample buffer, boiled for 5 minutes, and centrifuged. The supernatants were used for immunoblot analyses. Recombinant expression of full-length type VII collagen was performed as described previously.[10,11] The triple-helical domain of type VII collagen was prepared by pepsinization of human keratinocyte extracts.[12] In brief, full-length type VII collagen was extracted from confluent human keratinocytes cultured in the presence of ascorbic acid (50 $\mu g\ mL^{-1}$) and native triple-helical domain was generated by pepsin digestion at 4°C.

RESULTS

Histopathologic findings

A histopathologic examination showed a subepidermal blister formation that contained numerous eosinophils and neutrophils. Distinct fibrosis with loss of elastic fibrils was detected under the blister, surrounded by a sparse mixed infiltrate of lymphocytes, histiocytes, eosinophils, and neutrophils (Fig 1, *C*).

Immunopathologic findings

Indirect immunofluorescence on normal-appearing skin sections showed circulating IgG antiepidermal basement membrane zone antibodies that reacted with the dermal side of an artificial blister on 1-mol/L salt-split human skin sections (Fig 1, *D*). In immunoblotting with extracts of normal human dermis, the patient's serum reacted with a 290-kd antigen that was identical to that detected by control epidermolysis bullosa acquisita serum, showing IgG antibodies reactive with type VII collagen (Fig 2, *A*). Immunoblotting using recombinant protein confirmed the reactivity of the patient's serum with the full-length type VII collagen (Fig 2, *C*). In immunoblot analyses using recombinant proteins of NC1 and NC2 domains of type VII collagen, the IgG antibodies from the patient's serum showed a clear reactivity with the NC1 domain but not with the NC2 domain (Fig 2, *B*). In immunoblotting using pepsin-treated procollagen VII, the patient's serum reacted with the central triple-helical collagenous domain,[13] although the reactivity was relatively mild (Fig 2, *C*). The patient's serum (both IgG and IgA) did not show any positive reactivity with the recombinant proteins of

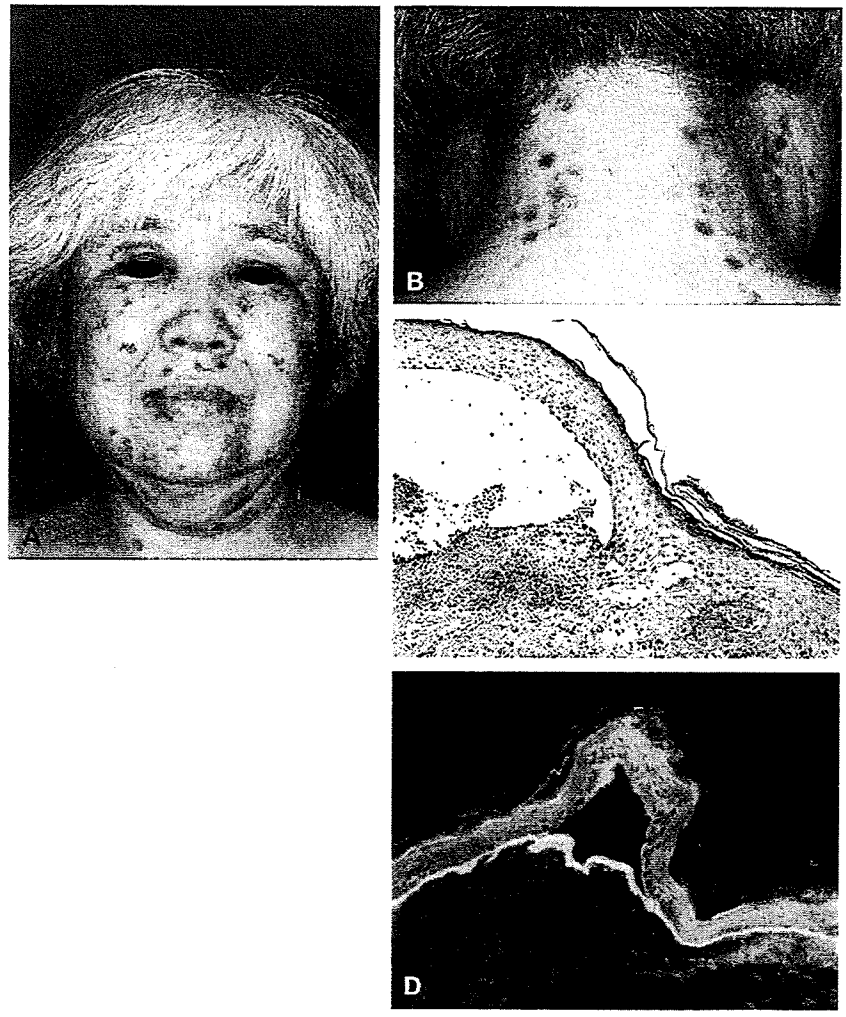

Fig 1. Clinical features: blisters and erosions with erythematous atrophic plaques on face (**A**) and neck (**B**). Histologic examination showed subepidermal blister formation with infiltration of numerous eosinophils (**C**). Indirect immunofluorescence on 1-mol/L salt-split human skin sections showed that IgG antibodies reacted with dermal side of artificial blister (**D**).

either BP180 NC16a domain or BP180 C-terminal domain (data not shown).[14,15] The IgG antibodies of the patient's serum did not react with any subunit of laminin 332 (formerly laminin 5) in immunoblotting using purified laminin 332 (data not shown).[16]

Clinical response

Systemic steroid administration was avoided because of the possible exacerbation of diabetes mellitus. Topical corticosteroids and an administration of dapsone (50 mg/d) showed no significant effect. Because of the results of the immunoblot analyses, an administration of colchicine (1 mg/d), which has been shown to be effective in epidermolysis bullosa acquisita,[17-19] was initiated. Blister formation quickly ceased, leaving mild scarring within a month, and the patient remained free from skin lesions on this regimen.

DISCUSSION

To our knowledge, this is the first reported case of epidermolysis bullosa acquisita with clinical features of Brunsting-Perry pemphigoid, in which reactivity with type VII collagen was confirmed by immunoblot analyses. Since the original description by Brunsting and Perry[1] in 1957, 57 cases have been described with vesiculobullous lesions located on the head and neck that left atrophic scarring.[4,5,12,20] Among these cases, only one previous report has confirmed reactivity with type VII collagen by immunoblotting.[4] However, clinical features of this case were not typical of

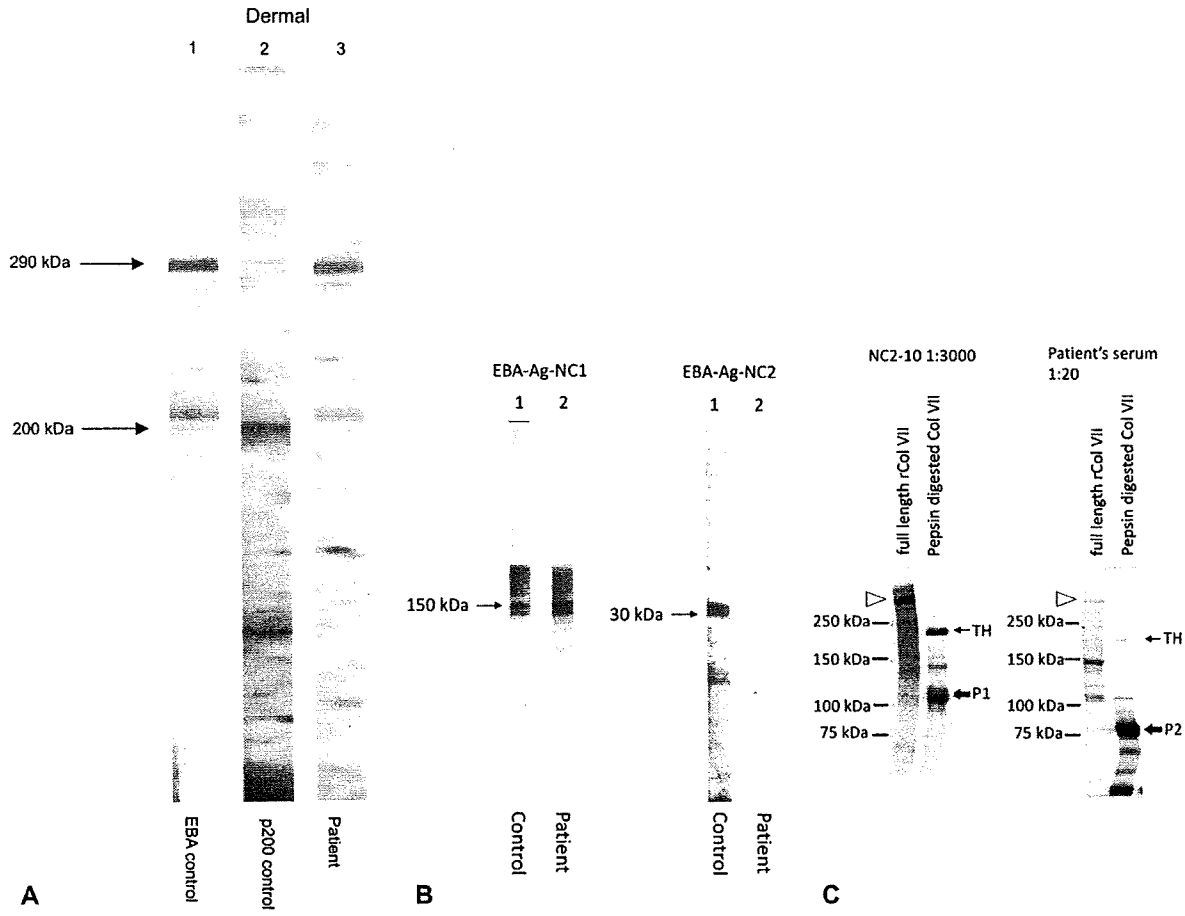

Fig 2. **A**, Immunoblotting using normal human dermal extracts. Both control epidermolysis bullosa acquisita (*EBA*) serum (*lane 1*) and patient serum (*lane 3*) reacted with 290-kd EBA antigen (*Ag*), ie, type VII collagen (*Col*) (*arrow*), whereas control anti-p200 pemphigoid serum reacted only with 200-kd Ag (*lane 2*). **B**, Immunoblot analyses using recombinant proteins of noncollagenous (*NC*)1 and NC2 domains. Control EBA serum (*lanes 1*) reacted with both NC1 (*left*) and NC2 (*right*) domains of type VII Col. IgG antibodies in our patient's serum (*lanes 2*) showed clear reactivity with NC1 domain, but not with NC2 domain. **C**, Immunoblotting using pepsin-treated pro-Col VII (7% sodium dodecyl sulfate [SDS]). Control polyclonal antibody (NC2-10) (*left*), which recognizes C-terminus of type VII Col, reacted with recombinant protein of full-length type VII Col (*arrowhead*). Also, control antibody mainly recognized full triple helix (*TH*) (*arrow*) of pepsin-digested Col VII and peptide 1 (P1)-fragment of TH.[13] Patient's serum (*right*) mainly recognized full-length type VII Col, TH, and shorter peptide 2 (P2)-fragment, although intensities of bands of full-length type VII Col and TH were relatively weak. For technical reasons, bands do not exactly correspond to one another.

Brunsting-Perry pemphigoid. In addition, this case reacted not only with type VII collagen but also with laminin 332.

Immunoblotting using bacterial recombinant proteins and cell-derived fragments of type VII collagen revealed that the patient's IgG antibodies reacted with the NC1 domain and the triple-helical collagenous domain of type VII collagen. The former reaction is common in typical cases of epidermolysis bullosa acquisita. Whether this set of antigenic sites is specific for the clinical features of Brunsting-Perry pemphigoid remains to be determined in more patients in the future.

In the Japanese literature, there are 3 cases of Brunsting-Perry pemphigoid showing reactivity with a recombinant protein of BP180 NC16a domain by immunoblot analyses.[7,8] These results, in conjunction with the results in our study, suggest that Brunsting-Perry pemphigoid is a heterogeneous disease in regard to antigenic features.

Historically, autoimmune bullous diseases, including epidermolysis bullosa acquisita and

Brunsting-Perry pemphigoid, were diagnosed by their clinical manifestations.[21] However, current molecular biological techniques have changed the diagnostic approach and, as a result, some confusion exists concerning definitive diagnosis of some cases. Several reports suggest that Brunsting-Perry pemphigoid may be a clinical variant of epidermolysis bullosa acquisita.[2-6] Generally, the localization of lesions varies in patients with bullous dermatoses and might also be affected by subclass switching and intermolecular epitope spreading during long-term disease.[22] The current case showed typical clinical features of Brunsting-Perry pemphigoid. However, we ultimately diagnosed this case as epidermolysis bullosa acquisita with a Brunsting-Perry pemphigoid-like presentation because of the results of the molecular analyses.

It might then be asked whether application of current molecular biological techniques in the diagnoses of autoimmune bullous disease makes Brunsting-Perry pemphigoid an illusion? We do not think so. Rather, molecular dermatology has never fully revealed the basis for the localized skin manifestations of this disease, which was determined originally by traditional descriptive dermatology. Our careful observation and scientific exploration based on our predecessors' description have elucidated the pathogenesis of this unique disease at the molecular level.

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Autoantibodies against desmocollins in European patients with pemphigus

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Summary

Background. Autoimmune bullous disorders of the pemphigus group are characterized by autoantibodies targeting desmoglein (Dsg)1, Dsg3 and Dsg4 and occasionally, desmocollin (Dsc)1, Dsc2 and Dsc3. Both Dsg and Dsc are components of desmosomal adhesion complexes.

Aim. To investigate the presence of IgG and IgA autoantibodies against Dsc1, Dsc2 and Dsc3 in a cohort of patients with bullous disorders.

Methods. IgG and IgA autoantibodies against Dsc1, Dsc2 and Dsc3 were investigated by ELISA and immunoblotting analysis in a cohort of European patients with pemphigus vulgaris (PV; $n = 74$), IgA pemphigus ($n = 3$), paraneoplastic pemphigus (PNP; $n = 3$) and two cases of atypical pemphigus ($n = 2$).

Results. Of the two cases with atypical pemphigus, one showed IgA reactivity against Dsc1 and Dsc3 and weak reactivity against Dsc2, and the other showed both IgG and IgA reactivity against Dsc1. One patient with IgA pemphigus had IgA autoantibodies against Dsc1, Dsc2 and Dsg1, and one patient with PNP had IgG reactivity against with Dsc3. In contrast, all the PV sera showed IgG reactivity against Dsg3 but not against Dsc1–3. Thus, IgG and IgA reactivity against Dsc was restricted to cases of PNP, IgA pemphigus and atypical pemphigus.

Conclusions. These findings support the concept that desmocollins are not important autoantigens in PV.

Introduction

Two types of desmosomal cadherins are currently known: desmogleins (Dsg) and desmocollins (Dsc). These are critical transmembranous components of the desmosomal adhesion complex of epidermal keratinocytes. There are four different isoforms of Dsg (Dsg 1–4) and three of Dsc (Dsc1–3).[1–3] Autoimmune blistering disorders of the pemphigus group are characterized by autoantibodies that preferentially target Dsg1, Dsg3 (and Dsg4) and rarely, Dsc1–3.[4–8] Binding of autoantibodies to their target antigens induces loss of epidermal adhesion, leading to blister formation and chronic painful erosions of the mucosal surfaces and skin.[9–11]

Patients with pemphigus vulgaris (PV), the most common clinical variant, show mucosal lesions that are associated with IgG autoantibodies against Dsg3, whereas patients with PV and mucocutaneous lesions have IgG autoantibodies against Dsg3 and Dsg1.[9,12] In patients with active PV, IgA and IgE autoantibodies against Dsg3 have also been found.[13] Paraneoplastic pemphigus (PNP) is a pemphigus variant associated mainly with haematological neoplasms, which is associated with IgG reactivity against transmembranous components of desmosomes (i.e., Dsg1, Dsg3, Dsc1–3)

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and intracellular desmosomal plaque proteins (i.e., plakins).[14–16] Previously, it had also been suggested that autoantibodies of defined pemphigus sera show IgG and IgA reactivity against bovine epidermal extracts or recombinant bovine Dsc1–3 proteins, although reactivity with human Dsc1–3 was not clearly shown.[17,18] Chorzelski *et al.* reported that the serum of a patient with atypical pemphigus who had shown IgA and IgG reactivity to bovine Dsc1 and Dsc2 in an earlier study[18] also had IgG and IgA reactivity against baculovirus-derived recombinant human Dsc1.[7] In addition, a patient with PNP showed both IgG and IgA reactivity against Dsc3 and IgG reactivity against Dsc2.[19] IgG and IgA autoantibodies against Dsc1–3 have also been found in several cases of atypical pemphigus, which presents clinically with a polymorphic, rarely blistering phenotype and is associated with circulating and tissue-bound autoantibodies against desmosomal components such as Dsg and Dsc[7,20] Finally, single cases of IgA pemphigus with IgA autoantibodies against Dsc1 have been reported recently.[7,21]

The prevalence of IgG (or IgA) autoantibodies against Dsc1–3 in the various pemphigus variants is largely unknown in European patients with pemphigus. Using baculovirus-derived recombinants of Dsc1, Dsc2 and Dsc3 we investigated sera from patients with PV, PNP, IgA pemphigus, and atypical pemphigus, using ELISA and immunoblotting analysis.

Methods

All patients gave written consent to participate in this study, which adhered to the Declaration of Helsinki Guidelines and was approved by the local ethics committees.

Study population

Sera from patients with PV (n = 74), PNP (n = 3), IgA pemphigus (n = 3) and atypical pemphigus (n = 2) were studied. Sera from 22 healthy volunteers served as controls.

Diagnosis of pemphigus was based on the following criteria: (i) clinical presentation with fragile mucosal and cutaneous blisters/erosions, (ii) histopathological evidence of intraepidermal loss of adhesion, (iii) IgG/IgA deposits on the surface of epidermal keratinocytes shown by immunofluorescence, (iv) cell surface IgG or IgA reactivity on monkey oesophagus epithelium shown by indirect immunofluorescence and (v) detection of anti-Dsg3 and/or anti-Dsg1 IgG autoantibodies shown by ELISA. Patients with IgA pemphigus showed pustules with a tendency to confluence, forming annular and circinate patterns. Patients with PNP had a more severe, chronic, progressive oral involvement that was associated with haematological neoplasms, which were related to IgG reactivity against Dsg3 and various components of the desmosomal plaque (not shown). One patient with atypical pemphigus showed urticarial plaques on the trunk reminiscent of eosinophilic cellulitis without mucosal involvement, and showed intraepidermal acantholysis and cell-surface deposition of C3 (see Results). The second atypical case was previously reported by Chorzelski *et al.* and showed atypical clinical and histopathological changes associated with IgG and IgA reactivity against recombinant bovine Dsc.[18]

Expression and purification of desmocollin recombinant proteins

Recombinant Dsc1–3 baculovirus transfer vectors were cotransfected with linearized baculovirus DNA (BaculoGold™ Bright; BD Biosciences, Heidelberg, Germany) into cultured SF21 cells using the manufacturer's buffer compounds (A and B set; BD Biosciences).[22] For the production of recombinant Dsc proteins, insect cells (High Five™; Invitrogen, Karlsruhe, Germany) were incubated with recombinant baculoviruses as previously described.[22] Culture supernatants of infected cells were collected after 3 days and recombinant proteins were purified from the supernatants by affinity chromatography over nickel–nitrilotriacetic-linked agarose beads (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified proteins were gradually dialysed against phosphate-buffered saline (PBS) supplemented with 0.5 mmol/L $CaCl_2$, split into aliquots and stored at −20 °C. Using immunoblotting analysis, the purified recombinant proteins displayed the expected size and were specifically immunoreactive using appropriate monoclonal antibodies against the peptide tag (E-Tag) (Fig. 1). In addition, Dsc1 was specifically immunoreactive with a patient's serum and Dsc3 was immunoreactive with a mouse monoclonal antibody (kind gift of A. Schmidt, Marburg, Germany) directed against the extracellular domain of Dsc3 (not shown).

ELISA and immunoblotting analysis using desmoglein 3 and desmocollin 1–3 recombinants

ELISA analyses with recombinant Dsg3 and Dsc1, Dsc2 and Dsc3 proteins were performed as previously described.[22,23] Sera of patients and controls were incubated at a dilution of 1 : 50 in 96-well polystyrene plates (Maxisorb Immunoplate; Nunc, Wiesbaden, Germany)

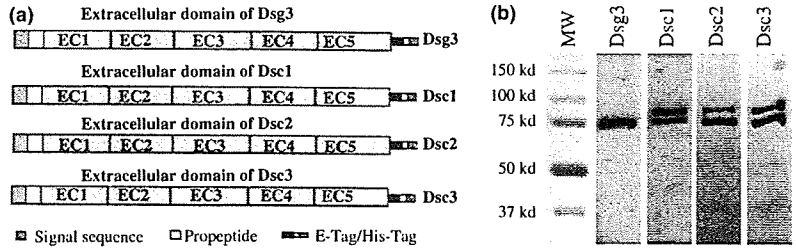

Figure 1 Baculovirus-derived recombinant proteins representing the ectodomains of desmoglein (Dsg)3 and desmocollin (Dsc)1, Dsc2 and Dsc3, respectively. (a) Recombinant proteins, containing the entire extracellular domain of Dsg3, Dsc1, Dsc2 and Dsc3 with E-Tag and His-Tag. Immunoblotting analysis using anti-E-tag monoclonal antibody shows the recombinant proteins, Dsg3, Dsc1, Dsc2 and Dsc3 at the expected molecular sizes that correspond to their glycosylated forms. (b) In the desmocollin lanes, the higher bands represent the unprocessed proteins.

overnight. The ELISA plates were then washed, and IgG and IgA binding was visualized using a horseradish peroxidase (HRP)-labelled goat antihuman IgG (1 : 5000) or IgA (1 : 1000); Dako, Hamburg, Germany) for 1 h at room temperature and 2,2′-azino-*bis*[3-ethylbenzthiazoline-6-sulphonic acid] (ABTS; Calbiochem, Schwalbach, Germany) as chromophore for 30 min at room temperature. Reactivity was measured at 405 nm in a spectrophotometer. Samples were run at least in duplicate. For evaluation, median values were used.[22]

For immunoblotting analysis, Dsc recombinants were run in a sodium dodecyl sulphate–polyacrylamide gel electrophoresis system (10% gel) and blotted onto nitrocellulose membrane. The membrane was blocked with 5% milk powder in phosphate-buffered saline with 0.05% Tween-20 (PBS-T) with 0.5 mmol/L $CaCl_2$. The sera were diluted at 1 : 200 in PBS-T with 0.5 mmol/L $CaCl_2$ and 5% milk powder, and the membrane was then incubated with the diluted sera overnight at 8 °C. After three washes with PBS-T with 0.5 mmol/L $CaCl_2$, the nitrocellulose membranes were incubated either with antihuman-IgG (1 : 5000; Dako, Hamburg, Germany) or antihuman-IgA (1 : 1000; Dako) for 1 h at room temperature. Specific immunoreactivity was finally visualized using a commercial HRP substrate (Immobilon Western Chemiluminescent HRP substrate; Millipore, Schwalbach, Germany).

Results

Baculovirus-derived Dsg3, Dsc1, Dsc2 and Dsc3 recombinant proteins

Using a baculovirus expression system, recombinant proteins representing the ectodomains of Dsg1, Dsg3, Dsc1, Dsc2 and Dsc3 were produced and affinity-purified (Fig. 1a). Dsg1 (not shown), Dsg3 and the Dsc recombinants displayed the expected size and were specifically immunoreactive using appropriate monoclonal antibodies directed against the E-Tag by immunoblotting analysis (Fig. 1b).

IgG and IgA reactivity of sera from patients with pemphigus vulgaris, IgA pemphigus and paraneoplastic pemphigus and desmocollins 1–3

Using ELISA, all of the 74 PV sera were found to have IgG reactivity with Dsg3, but not against Dsc1–3 (Fig. 2). None of the IgA pemphigus sera showed IgG or IgA reactivity against Dsg3, but one patient had IgA reactivity against Dsc1, Dsc2 and Dsg1 as shown by immunoblotting and ELISA analysis (Figs 2, 3d). All three PNP sera showed distinct IgG reactivity against Dsg3, and one PNP serum showed IgG reactivity against Dsc3 (Figs 2, 3c).

Autoantibodies against desmocollin in atypical pemphigus: case reports

An 80-year-old man presented with extremely pruritic, sharply demarcated urticarial plaques with annular configuration and central paleness, which were located on the trunk and legs (Fig. 4a). Five months later, the patient developed tense blisters on the trunk and the arms (Fig. 4b). Histological examination of a biopsy taken from lesional skin showed eosinophilic spongiosis of the epidermis (Fig. 4c), and direct immunofluorescence showed intercellular epidermal cell surface deposition of C3 but not IgA, IgG, or IgM (Fig. 4d). At first presentation, the patient's serum showed weak IgA reactivity against Dsc1 by immunoblotting (Fig. 4e). Eight months later, when bullous skin lesions were first noted, immunoblotting analysis showed strong IgA reactivity against Dsc1 and Dsc3 and weak reactivity against Dsc2 (Figs 4f, 3a). The IgA reactivity against Dsc1, Dsc2 and Dsc3 was confirmed by ELISA (Fig. 2).

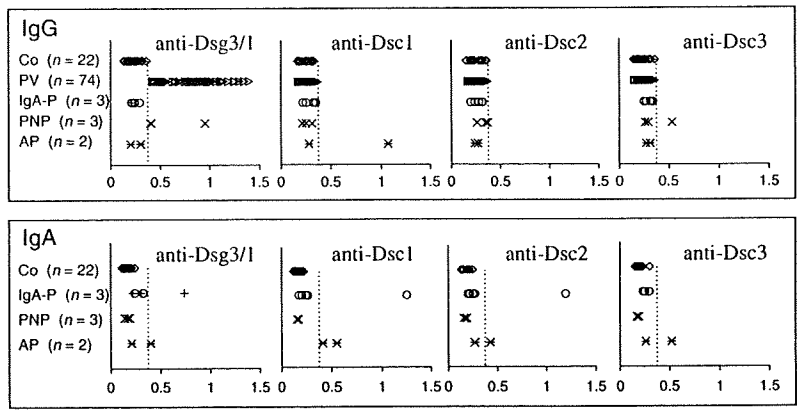

Figure 2 IgG and IgA reactivity of European patients with pemphigus against desmocollins (Dsc) 1, 2 and 3 and desmogleins (Dsg) 1 and 3 by ELISA. The patients' sera were analysed by ELISA for IgG or IgA reactivity against the ectodomains of Dsc1–3 and Dsg1 and Dsg3, respectively, and the optical densities (OD values) are shown as dots. The line indicates the cut-off value of the ELISA (OD > 0.376 nm). IgG (and IgA) reactivities against Dsg3 and Dsc1, Dsc2 and Dsc3 are shown for sera from patients with pemphigus vulgaris (PV; n = 74), IgA pemphigus (IgA-P; n = 3), paraneoplastic pemphigus (PNP; n = 3), atypical pemphigus (AP; n = 2) and healthy controls (n = 22). Note that one IgA pemphigus serum showed IgA reactivity against Dsg1 (+) but no IgA pemphigus serum showed IgA reactivity against Dsg3 (O), whereas an atypical pemphigus serum was IgA reactive with Dsg3 (⊗).

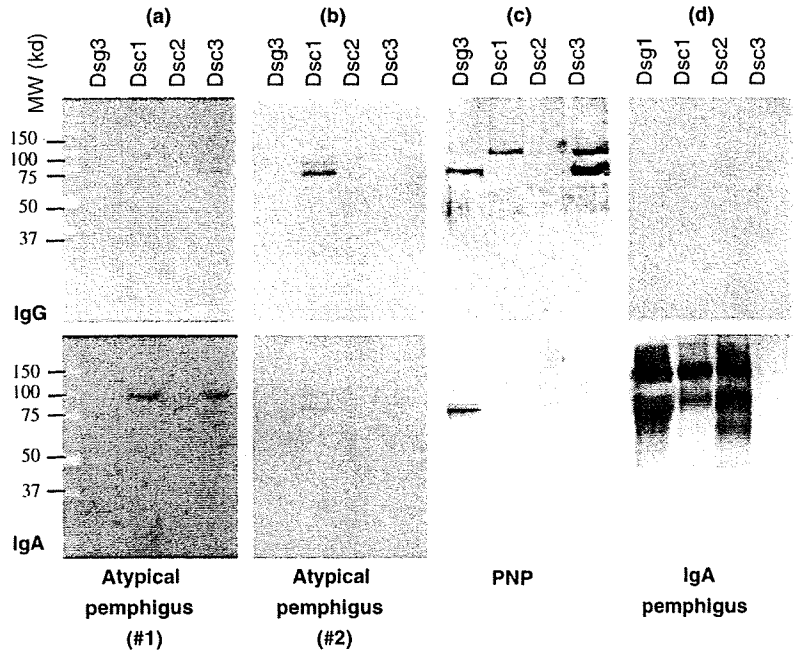

Figure 3 IgG and IgA reactivity against desmocollins (Dsc) of pemphigus sera by immunoblotting analysis. The serum of a European patient with atypical pemphigus (no. 1) showed only weak IgG reactivity against Dsc1, Dsc2 and Dsc3, but strong IgA reactivity against Dsc1 and Dsc3 (and weak IgA reactivity against Dsc2). The serum of a second patient with atypical pemhigus (no. 2) showed clear IgG and IgA reactivity against Dsc1. The serum from a patient with PNP showed IgG and IgA reactivities against Dsg3 and IgG reactivity against Dsc3. The patient with IgA pemphigus showed IgA reactivity but no IgG reactivity against Dsg1, Dsc1 and Dsc2.

In addition, the later serum showed also weak IgG reactivity against Dsc1 and Dsc3 and very weak IgG reactivity against Dsc2 (Fig. 3a).

The serum of a second patient with atypical pemphigus showed neither IgG nor IgA reactivity against Dsg3 by immunoblotting or ELISA (Figs 3b, 4) but strong IgG and IgA reactivity against Dsc1 by immunoblotting analysis (Fig. 3b) and ELISA (Fig. 2). None of the healthy control donors showed IgG or IgA reactivity against Dsg3 or Dsc1–3 (data not shown).

Discussion

Our results strongly suggest that autoantibodies of the IgG and IgA classes against Dsc are restricted to rare cases of PNP, atypical pemphigus and IgA pemphigus.

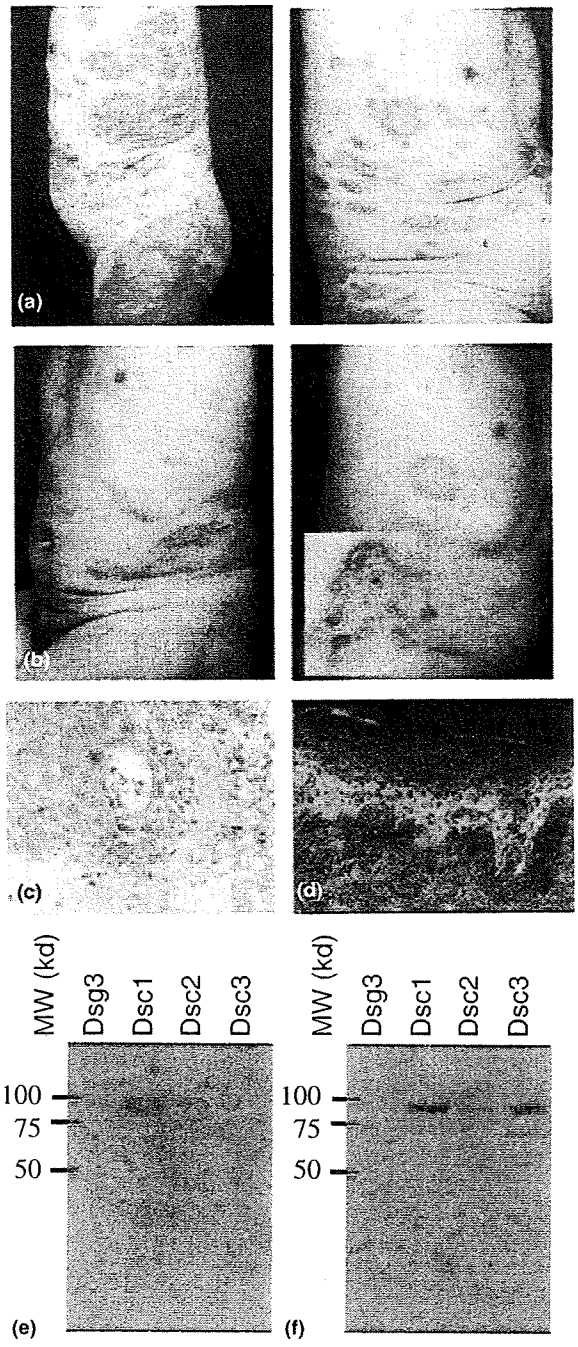

Figure 4 A patient with atypical pemphigus shows IgA reactivity against desmocollins (Dsc). The patient presented initially with sharply demarcated, urticarial plaques on (a) the trunk that transformed into (b) tense bullae 8 months later. Histological examination of skin lesions showed (c) eosinophilic spongiosis, and direct immunofluorescence showed (d) epidermal cell surface deposition of C3. (e) At first presentation, there was only weak IgA reactivity against Dsc1 by immunoblotting, but 8 months later, when bullae were first noted, immunoblotting analysis found (f) IgA reactivity against Dsc1, Dsc2 and Dsc3.

Autoantibodies reactive against Dsc were virtually undetectable in a larger cohort of patients with PV. Neither patients with acute nor those with chronic PV showed IgG reactivity against Dsc1, Dsc2 or Dsc3. Thus, it appears that anti-Dsc IgG or IgA autoantibodies are rarely seen in patients with pemphigus.

Support for the concept that Dsc-specific autoantibodies may arise from epitope spreading against desmosomal autoantigens comes from the observation that patients with PNP often have autoantibodies against several intracellular components of the desmosomal plaque in addition to IgG reactivity against Dsg3 (and Dsg1). In fact, we observed IgG reactivity against Dsc3 in a patient with PNP who also had IgG reactivity against Dsg3. In contrast, as shown here in the larger cohort, patients with PV with anti-Dsg3 IgG do not develop autoantibodies against any Dsc. Notably, one patient with IgA pemphigus and two patients with 'atypical' (i.e., clinically polymorphous) pemphigus showed IgA reactivity against desmocollins. As the more recently identified pemphigus variants are poorly characterized with regard to their immune pathogenesis and may represent heterogeneous disorders, the pathogenicity of Dsc-specific autoantibodies is difficult to estimate and needs to be further elucidated.

In this study, pemphigus sera were screened for anti-Dsc reactivity by ELISA and immunoblotting analysis, using recombinant Dsc baculovirus proteins. In previous studies by Preisz *et al.*[19] and Hisamatsu *et al.*,[7] IgA and IgG reactivity against Dsc1–3 was also found by indirect immunofluorescence techniques using COS-7 cells transfected with Dsc cDNA. Both studies found IgG or IgA reactivity against Dsc only in a few pemphigus sera. We thus conclude that Dsc reactivity of pemphigus patients is limited to a subset of cases with PNP, IgA pemphigus and atypical pemphigus, regardless of the detection assay system used.

It is still unclear whether autoantibodies against desmocollins are pathogenic. At present, only autoantibodies of the IgG class against Dsg3 or Dsg1 have been shown to induce blister formation *in vitro* or in mouse models.[10,11] A recently established $Dsc3^{-/-}$ mouse model established by Koch *et al.* shows an impressive phenotype with extensive blisters and mucosal erosions, confirming that Dsc is important for epidermal integrity.[24]

Indirect evidence for pathogenicity of anti-Dsc antibodies stems from the results reported here of the single European patient with atypical pemphigus with erythematous plaques and weak IgA reactivity against Dsc1. At a later stage of the disease, when the patient developed cutaneous blisters with intraepidermal loss of adhesion, we were able to detect strong IgA reactivity against Dsc1 and Dsc3.

In summary, our findings strongly suggest that desmocollins are rarely recognized by sera from patients with pemphigus. Evidence for a pathogenic role of anti-Dsc IgA or IgG is at best circumstantial and needs to be verified by future studies. Detection of anti-Dsc IgG/IgA may help to define rare or atypical variants of pemphigus.

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ORIGINAL PAPER

Autoantibodies to sweat glands detected by different methods in serum and in tissue from patients affected by a new variant of endemic pemphigus foliaceus

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Abstract Examining the patients with a new variant of endemic pemphigus foliaceus (EPF) in El Bagre, Colombia, (El Bagre-EPF), we noted several polymorphic clinical lesions around their axillary areas. Based on our clinical findings and on previous histopathological studies on the skin of these patients that showed abnormalities in their sweat glands, and the presence of mercuric selenides and iodines by autometallography assay, we decided to investigate immunoreactivity to the sweat glands in these patients. We tested for autoreactivity utilizing direct and indirect immunofluorescence (DIF, IIF). To be able to distinguish between non-specific immune deposits and real autoimmune response, and knowing that sweat glands have some intrinsic autofluorescence for the presence of lipofuscin granules (that naturally fluoresce under the UV light microscope), as well as by the presence of secretory IgA, we used simultaneously immunohistochemistry (IHC). We tested ten El Bagre-EPF patients, ten healthy controls from the endemic area and ten healthy controls from the United States. We were able to visualize a specific autoreactivity to sweat glands in 8/10 cases of El Bagre-EPF by DIF, IIF and by IHC. In addition when using anti-human monoclonal antibodies to CD3, CD68, and CD20, we confirmed the presence of several specific immune responses in situ, an around the sweat glands. No healthy control cases yielded positive findings. In some chronic cases, decrease and sometimes a complete absence of sweat glands and other skin appendices was found. In addition to this, sclerodermoid changes or early sclerodermatous changes sometimes extending into the adipose tissue as a membranous lipodystrophy were observed. Autoreactivity to the neurovascular components around the sweat glands were also observed. Our data demonstrate for the first time that there is immunoreactivity toward sweat glands in El Bagre-EPF patients that seems to destroy some of these structures.

Keywords Sweat glands · Autoimmunity · Pemphigus · Immunofluorescence · Immunohistochemistry

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Introduction

We described a new variant of endemic pemphigus named El Bagre-EPF (A.K.A. pemphigus Abreu-Manu). Patients affected by El Bagre-EPF, live in an area that is environmentally polluted with mercury, which is used to facilitate the mining of gold ore [1–5]. We decided to study the possible autoreactivity toward sweat glands based on the following facts: (1) multiple clinical lesions were seen in the axillary areas of most El Bagre-EPF patients, (2) we have previously reported alterations in patient sweat glands as determined by histopathology, (3) mercuric selenide, iodine and undetermined materials were detected by autometallographic analysis inside and around the sweat glands of El Bagre-EPF patients [1–5], and (4) a "painful burning" sensation upon perspiration was commonly

described in patients affected by El Bagre-EPF. Therefore, we focused on possible autoimmune reactivity within the sweat glands by using direct and indirect immunofluorescence (DIF, IIF), and by immunohistochemistry (IHC) (where neither lipofuscin nor secretory IgA autofluorescence is detected using this method) to differentiate between background and specific autoimmune responses to sweat glands in these patients. IIF was performed also to determine if the serum of the patients recognize the sweet gland components. This is an important step to differentiate between immune deposits and autoimmunity, since immune deposits can result from non-specific adherence of antibodies to these glands.

Materials and methods

Subjects of study

A case–control study was performed. We studied ten patients who fulfilled the diagnosis of El Bagre-EPF [1–5]. The El Bagre-EPF diagnosis was based on clinical, epidemiological, histopathological and immunological criteria previously reported by us and others [1–5]. Skin biopsies were taken from the patients and examined histopathologically [1–5]. H&E revealed that around one-fourth of the patients showed and several alterations on the sweat glands. The typical acantholysis was detected in new and active lesions. Positive intercellular staining on the cell junctions between keratinocytes (ICS) as well as BMZ was shown by DIF and IIF [1–5, 8]. In addition, sera used to test immunoreactivity to sweat glands in this study were properly characterized by immunoprecipitation using the Concanavalin-A affinity purified bovine tryptic fragment (45 kDa ectodomain of the mature form of Dsg1) as described by us previously [1–5]. Sera from all patients and controls from the endemic area were also tested by immunoblotting for reactivity against skin extracts showing positivity to desmoglein 1, desmoplakin, periplakin and other antigens. The samples were also tested positive as determined by an enzyme-linked immunosorbent assay (ELISA) that we developed and reported elsewhere [5]. For all of the above determinations, sera from a sporadic pemphigus foliaceus (PF) and a paraneoplastic pemphigus patient (PNP) were used as positive controls. We also tested ten healthy control sera from the endemic area and ten normal controls from the United States as negative controls. We obtained informed consent from all patients, and this study was performed. All samples were tested anonymously to comply with Institutional Review Board (IRB) requirements. The samples were imported to the USA in compliance with the Center for Disease Control (CDC) regulations in Atlanta.

DIF and IIF

Following local anesthesia without epinephrine, skin biopsies were taken from the dominantly affected areas of the axillae and kept in Michel's medium for DIF. In addition, the sera from the patients and controls were obtained. For IIF, the sera were tested using both normal human skin obtained from esthetic reduction procedures and by monkey esophagus (Oregon Primate Center, Portland, Oregon, USA) as substrates. For IIF, we used the serum at 1:25 and 1:40 dilutions in phosphate buffered saline (PBS). For DIF, in brief, 4 μm thick skin cryosections partially fixed with paraformaldehyde and then rinsed in PBS and incubated in a solubilization buffer (PBS, 0.5% Triton X-100, pH 6.8) and rinsed. After incubation with the sera, the slides were rinsed and blocked with PBS-0.01% Tween and 0.5% bovine serum albumin (BSA), and further incubated with the secondary antibodies. For DIF, we used FITC-conjugated rabbit antisera to human IgG, IgA, IgM, C3, C1q, fibrinogen and albumin. FITC-conjugated rabbit anti-human IgG (γ chain) (1:20 to 1:40), IgA (α chains) (1:20 to 1:40) and IgM (μ chain) (1:20 to 1:40) were used. For the anti-human fibrinogen and anti-albumin FITCI conjugate antibodies, we used 1:40 dilutions. All of these antisera were purchased from Dako (Carpenteria, California, USA). Goat anti-human IgE antiserum conjugated with FITC from Vector Laboratories (Bridgeport, NJ, USA) and anti-human IgD FITC-conjugated antibodies (Southern Biotechnology, Birmingham, Alabama) were also used. The slides were counterstained with DAPI (Pierce, Rockford, Illinois, USA), washed, cover slipped and dried overnight at 4°C. Other antibodies used in addition to those outlined above included mouse anti-collagen IV monoclonal antibody (CIV), Clone CIV (Zymed®; Invitrogen, Carlsbad, California, USA) and its secondary donkey anti-mouse IgG (H + L) antiserum conjugated with Alexa Fluor 555 (Invitrogen, USA). These experiments were performing in order to determine co-localization of the autoantibodies from the patients. In addition to this, to differentiate between specific immune deposits on sweet glands we performed IIF with titration of those found positive. This assay was performing to be able to distinguish between non specific immune deposits and autoimmunity, since immune deposits can result from non-specific adherence of antibody. Finally, the sections were examined with a Nikon Eclipse 50i microscope (Japan), using a Xenon arc light (XBO 75W) as the light source and a PL Apo × 40/0.80 dry objective. The slides were then examined using FITC alone, as well with a Nikon filter, i.e., DAPI/ FITC/TEXAS RED EX 395–410/490–505/560–585 nm, EM 450–490/515–545/600–652 nm.

To distinguish between genuine immunoreactivity and autofluorescence of tissue, ten skin biopsies from patients

and controls were also tested utilizing a Dako immunoperoxidase staining kit for IHC by using goat anti-human total IgG/A/M/C3-horse-radish peroxidase (HRP) (Zymed®; Invitrogen), following the manufacturer's instructions.

IHC

We performed IHC to differentiate between specific autoreactivity and non-specific intrinsic autofluorescence produced by the physiological presence of lipofuscin granules in the sweat glands, as well as the natural secretion of IgA. The rationale for these experiments was that these two molecules (lipofuscin and IgA) would not be detected by using anti-human IgG conjugated with HRP-labeled secondary antibodies. We also performed special stains using several monoclonal antibodies to try to determine the nature of the inflammatory cells located around the sweat glands. For this purpose, we used IHC staining using mouse anti-human CD3 monoclonal antibody or mouse anti-human CD20 monoclonal antibody. In addition, to verify the nature of the sweat glands, we used monoclonal mouse anti-human carcinoembryonic antigen (CEA) isotype IgG1 kappa and mouse anti-human monoclonal CD 68 isotype IgG3 kappa (ready to use solution) also from Dako. For all these IHC tests, we used dual endogenous peroxidase blockage, according to the Dako package insert, but with the addition of Envision dual link (to assist in chromogen attachment). Furthermore, we applied 3,3-diaminobenzidine and counterstained with hematoxylin. The samples were run in a Dako Autostainer Universal Staining System.

Immunoblotting (IB) procedure

The sera were evaluated by IB using dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and human skin samples, which were extracted in 62.5 mM Tris–HCl buffer (pH 6.0). In brief, SDS extractions were performed and rinsed with ice-cold TBS (10 mM Tris–HCl, 150 mM NaCl, pH 8.0). The skin was lysed in 1% SDS in TBS with 2 mM 1 PMSF. An equal volume from each sample was centrifuged at 4°C, and after the addition of 1% SDS in TBS, the sample was mixed, boiled and vortexed. Protein concentration was determined using a Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA, USA), and protein content of homogenates was adjusted to equal concentrations. Equal amounts of protein were resolved using NuPage® Novex® Bis–Tris Midi Gel system (Invitrogen, Carlsbad, California) for large protein transfers; we loaded samples onto a 4–12% Bis–Tris gel and a 3–8% Tris–Acetate gel and then transferred with the iBlot™ dry blotting device and an Invitrogen XCell II™ Blot Module (Invitrogen, USA). Molecular weight standards were used in each gel (Invitrogen). We used as controls anti-Dsg1 antibody from Progen Heidelberg (1:50 dilution) and anti-desmoplakin multi-epitope antibody from the same company at 1:100 dilution. Western lightning chemiluminescence (Perkin Elmer Life Sciences, Inc., USA) was used and exposed to X-ray film (Kodak, Rochester, New York). The membranes were washed twice for 10 min with PBS-Tween-20, and the first antibody (serum of either patient or control) was incubated and diluted at 1:75 dilutions in 1% BSA/PBS-Tween-20 and incubated for 2 h. We then washed the membranes several times with PBS-Tween-20, and then the HRP-labeled secondary antibodies, diluted in 1% BSA/PBS-Tween-20, were incubated with the membranes for two hours. Finally, we washed the membranes several times with PBS-Tween-20, mixed equal volumes of the enhanced luminol reagent and the oxidizing reagent, and incubated the membranes for about 1–4 min while shaking. When the proteins were visualized, the excess chemiluminescence reagent was drained, and we placed the membrane within a plastic protector.

Results

Figure 1 shows several of the most representative clinical findings around the axillae in patients affected by El Bagre EPF. Figure 1a–e shows small excoriations, fine scaling, hyperpigmented macules and plaques within the axillae. The clinical lesions in the axillae followed patterns similar to the lesions seen throughout the rest of the patient body, and they seemed to vary according to the clinical severity of each patient. Figure 1 shows some representative H&E stains displaying the presence of peri-inflammatory infiltrate around some sweat glands, mostly due to lymphohistocytes (Fig. 1f, g).

By DIF, 8/10 El Bagre-EPF skin samples displayed positive immunoreactivity within the sweat glands at the level of the eccrine coil, the eccrine duct, the acrosyringium and/or the secretory epidermal segment. To our surprise, by IIF, the main responses were to fibrinogen (8/10), with about of 1:80 median titers, IgG (8/10), 1:120 median titers, albumin (6/8), 1:80 median titers, C3, (6/8), 1:40 median titers, C1q (6/8), 1:80 median titers, and IgM (6/10), 1:80 median titers, all of which displayed moderately to brightly positive specific fluorescence. By IHC, positive autoreactivity showed similar results as performed by DIF or IIF, however, we also observed autoreactivity to the coiled portion of the sweat glands from (2/10) El Bagre EPF using anti-human IgE. None of the controls were positive by either IIF or DIF. Figures 1 and 2 showed some of the most representative immunological findings in the sweat glands. In addition, of the biopsies studied by IHC using anti-human CD3, anti-CEA, anti-CD20 and anti-CD68 antibodies, all of them showed strong positivity around the sweat glands (CD3,