TABLE 2: Comparison of granular and fibrillar deposition groups.

	Granular ($N = 50$) N or age/N of data available		Fibrillar ($N = 33$) N or age/N of data available		t-test
Gender					
Male	33/50	(66.0%)	22/33	(66.7%)	
Female	17/50	(34.0%)	11/33	(33.3%)	
Age at the initial visit, mean ± SD (range), years	47.2 ± 20.1/50	(1–86)	43.8 ± 18.0/33	(16–78)	$P = 0.436$
Male	53.1 ± 19.5/33	(1–86)	45.9 ± 20.2/22	(16–78)	$P = 0.071$
Female	35.9 ± 15.9/17	(18–72)	39.6 ± 11.4/11	(22–68)	$P = 0.780$
Age at onset, mean ± SD (range), years	44.4 ± 18.2/42	(1–73)	39.7 ± 16.0/25	(12–74)	$P = 0.295$
Male	50.3 ± 17.0/26	(1–73)	40.9 ± 19.4/16	(12–74)	$P = 0.114$
Female	34.9 ± 15.9/16	(14–72)	37.7 ± 5.7/9	(29–50)	$P = 0.630$
Site of lesion					
Elbow	23/46	(50.0%)	5/30	(16.7%)	
Knee	20/46	(43.5%)	5/30	(16.7%)	
Buttock	25/46	(54.3%)	4/30	(13.3%)	
Elbow and/or knee and/or buttock	30/46	(65.2%)	7/30	(23.3%)	
Face	8/46	(17.4%)	3/30	(10.0%)	
Ear	7/46	(15.2%)	2/30	(6.7%)	
Neck	3/46	(6.5%)	5/30	(16.7%)	
Scalp	5/46	(10.9%)	1/30	(3.3%)	
Groin	4/46	(8.7%)	0/30	(0.0%)	
At least one predilection site	36/46	(78.3%)	12/30	(40.0%)	
Extremities*	20/46	(43.5%)	17/30	(56.7%)	
Trunk**	27/46	(58.7%)	22/30	(73.3%)	
Whole body***	3/46	(6.5%)	3/30	(10.0%)	
Other deposition in the papillary dermis					
C3	13/50	(26.0%)	4/33	(12.1%)	
IgG	5/50	(10.0%)	4/33	(12.1%)	
IgM	3/50	(6.0%)	1/33	(3.0%)	
Fibrinogen	2/50	(4.0%)	0/33	(0.0%)	
Small bowel disease	3/50	(6.0%)	1/33	(3.0%)	
Associated diseases					
Diabetes mellitus	5/50	(10.0%)	2/33	(6.1%)	
Lymphoma	3/50	(6.0%)	0/33	(0.0%)	
Thyroid disease	0/50	(0.0%)	1/33	(3.0%)	
Sjögren syndrome	1/50	(2.0%)	0/33	(0.0%)	
HLA antigen§					
DR4	4/14	(28.6%)#	8/13	(61.5%)##	
DR9	9/14	(64.3%)¶	3/13	(23.1%)¶¶	

*Not including cases limited only to elbow or knee; **not including cases limited only to buttock, neck, or groin; ***not including cases limited to combination of predilection sites; §frequency in HLA antigens of control was depicted from [90]. #$P = 0.3$ (versus controls), $P = 0.09$ (versus Fibrillar); ##$P = 0.2$ (versus controls), ¶$P = 0.002$ (versus controls, corrected $P = 0.02$), $P = 0.03$ (versus Fibrillar), ¶¶$P = 0.8$ (versus controls).

GSE [87, 89]. Although most of Caucasian DH patients have clinically no or mild gastrointestinal symptoms, they are treated with strict GFD [89]. The maintenance of GFD is important because gluten challenge leads to a flare of the cutaneous symptoms in Caucasian DH [88]. However, this is not the case in Japanese DH. Only 2 Japanese DH patients were treated with strict GFD [47, 59], while most patients continued to take a normal diet and were successfully controlled by dapsone or topical steroid. In addition, the lesions of some Japanese DH patients were completely cleared by short-term administration of dapsone or topical steroid while taking a normal diet. Moreover, except for the 3 patients with GSE, no patients developed clinical symptoms of gluten sensitivity throughout the course on a normal diet. Taken together, Japanese DH may not be closely associated with gluten sensitivity, in contrast to Caucasian DH.

The final diagnosis of CD is made by the results of jejunum biopsy, but not by clinical symptoms. In fact, the jejunum biopsy in 2 Japanese DH patients with no clinical symptoms of gluten sensitivity revealed villous atrophy [14, 39]. However, the fact that the jejunum biopsy of 3 patients with no clinical symptoms of gluten sensitivity revealed no pathological changes may raise the possibility that some Japanese DH patients did not have GSE. To confirm the exact association of GSE in Japanese DH, jejunum biopsies are necessary. However, most Japanese DH patients refused jejunum biopsy and GFD because of no clinical symptoms [84]. Accordingly, it may be difficult to clarify the exact association of GSE in Japanese DH based on the histopathological changes of the jejunum. Nevertheless, GSE in Japanese DH seems rare, considering the good response to the dapsone or topical steroid therapy during taking a normal diet. The rarity of GSE in Japanese DH patients is considered to be well correlated with the extreme rarity of CD in Japan [98].

Regarding genetic testing for Caucasian DH, the absence of HLA-DQ2 or DQ8 has a high negative predictive value because patients lacking these alleles are very unlikely to have DH [87, 99]. However, because the prevalence of these alleles among the Caucasian population is rather high, positive results in the HLA test are not sufficient to diagnose DH. In contrast, our study disclosed that Japanese DH patients never had HLA-DQ2 or -DQ8. Although no frequencies of HLA class I antigens in Japanese DH patients were increased in comparison with the controls among Japanese [90], there was a slightly increased frequency of HLA-DR9 in all the Japanese DH patients examined for HLA. Moreover, patients in the granular group showed a statistically significant frequency of HLA-DR9 when compared to Japanese normal controls. Since the number of data available was small, further analyses are needed to conclude whether Japanese DH is associated with specific HLA alleles.

Serologic tests of IgA antigliadin and antireticulin antibodies are no longer considered to be sensitive and specific markers of DH [87]. Instead, tests for IgA antibodies to eTG, tTG, and endomysium are considered to be useful diagnostic tools for Caucasian DH [87, 88]. Intestinal damage caused by exposure to gluten was suggested to produce IgA anti-tTG and anti-eTG antibodies [100]. Among them, eTG, rather than tTG is considered as the domain autoantigen in DH [101]. Recent studies reported a high sensitivity and specificity of eTG ELISA for DH [102, 103]. In a few Japanese DH patients who were tested for DH-related autoantibodies, no autoantibodies except for IgA anti-eTG antibodies were detected. A recent report suggested that the absence of GSE in Japanese DH may be due to an absence of anti-tTG antibodies, and that anti-eTG antibodies may be a diagnostic marker for Japanese DH [84]. These serologic tests, particularly for IgA anti-eTG antibodies should be performed in the future studies of Japanese DH.

Caucasian DH is known to be associated with a number of autoimmune conditions, including thyroid disease, type I DM, and autoimmune connective tissue diseases such as Sjögren syndrome, rheumatoid arthritis, and lupus erythematosus [89]. However, in Japanese DH, these diseases were relatively rare. Most Japanese DH patients with DM had type II DM. A higher risk of non-Hodgkin lymphoma was also reported in Caucasian DH [89], but this relation was not found in Japanese DH.

In summary, the absence of HLA-DQ2/DQ8, the inability to identify CD in most cases, the predominance of fibrillar IgA, and the unusual distribution of clinical lesions in Japanese patients suggest that Japanese DH may be a subset of DH patients and have a pathogenesis which is different from that currently proposed in Caucasian DH patients.

5. Conclusions

In conclusion, we reported the differences between Caucasian DH and Japanese DH. The characteristics of Japanese DH are (1) a high frequency of fibrillar IgA deposition in the papillary dermis, (2) a rare occurrence of GSE, (3) the absence of HLA-DQ2 or -DQ8, and (4) a rare association with autoimmune diseases or lymphomas. Although a previous Japanese DH review reported the prevalence of fibrillar IgA deposition, our study revealed that Japanese DH patients showed granular IgA deposition more frequently than fibrillar IgA deposition. In addition, we found that HLA-DR9 was frequently detected in Japanese DH, particularly in the granular group. These data suggest distinct characteristics of Japanese DH and raise the possibility that Japanese DH has a different pathogenesis from Caucasian DH. Serological tests for IgA anti-eTG antibodies and HLA genotyping should be performed in the future because Japanese DH may frequently have anti-eTG antibodies and HLA-DR9.

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ORIGINAL ARTICLE

Epitope Spreading Is Rarely Found in Pemphigus Vulgaris by Large-Scale Longitudinal Study Using Desmoglein 2–Based Swapped Molecules

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Epitope spreading is involved in inducing and maintaining self-reactivity. Epitope spreading in pemphigus vulgaris (PV), caused by IgG autoantibodies to desmoglein 3 (Dsg3) and Dsg1, was previously analyzed using Dsg3/Dsg1 extracellular domain–swapped molecules. However, precise identification of the responsible epitopes in each molecule by using only this method was problematic. In this study, we studied epitope spreading in PV by a novel immunoprecipitation–immunoblot method using Dsg3 (or Dsg1)/Dsg2 domain–swapped molecules, which overcomes the problems associated with the previous approaches. We analyzed the antigenic epitopes recognized by 212 sera collected from 53 PV patients at multiple disease stages. The major epitopes were present at the N-terminal region of Dsgs and were unchanged over the course of the disease in both anti-Dsg3 mucosal dominant-type PV and anti-Dsg3/Dsg1 mucocutaneous-type PV. These N-terminal epitopes were calcium dependent. Circulating antibodies in paraneoplastic pemphigus and pemphigus herpetiformis had unique epitope distributions, although the Dsg N-termini still contained the major epitopes. These results suggest that, after onset, intramolecular and intermolecular epitope spreading among extracellular domains on Dsg3 and Dsg1 is rare in PV and has no correlation with disease course.

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INTRODUCTION

Epitope spreading is a phenomenon in which immune responses can spread over the disease course to recognize epitopes that are different from the original target. If it occurs in the same molecule, this is termed "intramolecular epitope spreading" (Lehmann *et al.*, 1992). When responses begin to target epitopes on other proteins, this is termed "intermolecular epitope spreading" (Steinman and Conlon, 1997). Accumulating evidence supports the epitope-spreading hypothesis (McRae *et al.*, 1995; Robinson *et al.*, 2003; McMahon *et al.*, 2005; Chen *et al.*, 2006). However, a pathological role for epitope spreading was not demonstrated in human diseases. One reason may be that it is difficult to evaluate the responsible epitopes (Vanderlugt and Miller, 2002). In addition, published studies were small in scale (Tuohy *et al.*, 1997, 1999; Goebels *et al.*, 2000; Jones *et al.*, 2003; O'Connor *et al.*, 2005).

Pemphigus is caused by IgG autoantibodies against desmogleins (Dsgs) (Amagai, 2003). Four isoforms of Dsgs were described. Dsg1 is the autoantigen in pemphigus foliaceus (PF) and mucocutaneous-type pemphigus vulgaris (PV; Amagai *et al.*, 1995; Stanley and Amagai, 2006). Dsg3 is the autoantigen in mucocutaneous-type PV and mucosal dominant-type PV (Amagai *et al.*, 1991, 1994; Koch *et al.*, 1997; Mahoney *et al.*, 1999). Pemphigus sera occasionally contain Dsg4/Dsg1 cross-reacting IgG autoantibodies (Kljuic *et al.*, 2003; Whittock and Bower, 2003; Nagasaka *et al.*, 2004). No reactivity against Dsg2 was found in PF or PV sera (Ota *et al.*, 2003).

There were no definitive investigations elucidating intramolecular epitope spreading in tissue-specific autoimmune diseases, although some attempts were made for epitopes within each domain of Dsg3 and Dsg1 in PV. These relied on molecules constructed by combining the extracellular (EC) domains of Dsg1 and Dsg3 (Futei *et al.*, 2000; Sekiguchi *et al.*, 2001; Li *et al.*, 2003; Salato *et al.*, 2005). However, these analyses had a limitation. Although most of

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Abbreviations: Dsg, desmoglein; EC, extracellular; HP, herpetiform pemphigus; IP–IB, immunoprecipitation–immunoblotting; PF, pemphigus foliaceus; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris

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the anti-Dsg3 IgG autoantibodies in PV did not cross-react with Dsg1, one could not analyze anti-Dsg3 and anti-Dsg1 antibodies simultaneously in mucocutaneous-type PV by using this method. We therefore generated domain-swapped molecules comprising Dsg3 (or Dsg1) with Dsg2 as the backbone, taking advantage of the fact that PV sera show no reactivity with Dsg2. We defined epitope distribution and intramolecular epitope spreading by immunoprecipitation–immunoblotting (IP–IB) using 212 sera collected from 53 PV patients, and showed that epitope spreading is rare in PV.

RESULTS

Generation of new domain-swapped molecules

We first generated Dsg1, Dsg2, and Dsg3 molecules with full-length EC domains (Figure 1a). By replacing the five EC domains of Dsg2 with the corresponding Dsg3, we obtained a set of five new Dsg3/Dsg2 domain-swapped molecules as secreted proteins, using the previously reported technique (Chan *et al.*, 2010; Figure 1a). The primers used in this study are shown in Supplementary Table S1 online. Characterization of Dsg1/Dsg2 domain-swapped molecules has been detailed previously (Chan *et al.*, 2010).

Immunoblotting of culture supernatants by anti-E tag mAb

Culture supernatants containing full-length EC domains of Dsg1, Dsg2, and Dsg3, as well as the five Dsg3/Dsg2 and the five Dsg1/Dsg2 domain-swapped molecules, were fractionated by SDS-PAGE and immunoblotted with anti-E tag mAb. Protein bands of the expected sizes were observed for the unmodified Dsg1–3 and all 10 Dsg3/Dsg2 and Dsg1/Dsg2 domain-swapped molecules (Figure 1b and c).

Domain-swapped molecules of the N-terminal (EC1, EC2, and EC3) but not C-terminal regions (EC4 and EC5) of Dsg1 and Dsg3 are calcium dependent

We investigated whether domain-swapped molecules were recognized by anti-Dsg1 or anti-Dsg3 IgG autoantibodies in a calcium-dependent manner using EDTA treatment. Domain-swapped molecules were left untreated or were treated with EDTA, and then immunoprecipitated with anti-Dsg1 or anti-Dsg3 IgG antibodies recognizing each of the five EC domains of Dsg1 or Dsg3. EDTA treatment abolished the reactivity of both anti-Dsg1 and anti-Dsg3 IgG to the respective EC1–3, but not to the EC4–5 domains of Dsg1 and Dsg3. (Figure 1d. Note the diminishment of the EC1-swapped molecule without prosequence). Thus, anti-Dsg1 and anti-Dsg3 IgG antibodies recognize calcium-dependent epitopes on the EC1–3 domains of Dsg1 and Dsg3, whereas recognition of epitopes on the EC4–5 domains is calcium independent.

Most anti-Dsg3 IgG autoantibodies recognize N-terminal regions on the Dsg3 EC domains

We analyzed the Dsg3 epitopes recognized by 212 serum samples from 53 cases of mucosal dominant-type PV and mucocutaneous-type PV over the disease course. All 212 sera reacted with the full-length EC domain of Dsg3, but none with that of Dsg2 (Figure 2a). Of these 212 PV sera, 193 (91.0%) reacted with EC1, 151 (71.2%) with EC2, 107 (50.5%) with EC3, 40 (18.9%) with EC4, and 26 (12.3%) with the EC5 domain of Dsg3. The reactivity of PV sera to the EC1 domain of Dsg3 was significantly higher than that to the EC2–EC5 domains ($P<0.0001$). The reactivity of PV sera to the EC2 domain of Dsg3 was also significantly higher than that to the EC3–5 domains ($P<0.0001$).

Further, we determined the epitope profiles recognized by these 212 PV sera obtained at different disease stages: active, moderate, and remission (Table 1a). The results revealed that the major epitope remained on the N-terminal domain of Dsg3 at every clinical stage. A total of 164 sera (77.4%) recognized multiple EC domains of Dsg3 (Table 1b). Of these, 63 (29.7%) reacted with two, 64 (30.2%) with three, 34 (16.0%) with four, and only 3 (1.4%) with all five EC domains. In contrast, the remaining 48 sera (22.6%) reacted with only one of the EC domains of Dsg3; specifically, 40 (18.9%) reacted with the EC1 domain, 5 (2.4%) only with EC2, and 3 (1.4%) only with EC3 (Table 1c). No sera reacted only with the EC4 or EC5 domain of Dsg3. The number of epitopes recognized by each anti-Dsg3 IgG autoantibody correlated with an increasing ELISA index score ($P=0.0011$), but not with clinical disease activity ($P=0.2730$; Supplementary Tables S2–S4 online).

Most anti-Dsg1 IgG autoantibodies in mucocutaneous-type PV sera recognize the N-terminal region of Dsg1 EC domains

Next, we analyzed the recognition of Dsg1 epitopes by autoantibodies in 46 serum samples from 15 cases of mucocutaneous-type PV with different clinical disease activity levels (Supplementary Table S3 online). All 46 serum samples reacted with the full-length EC domain of Dsg1, but not with Dsg2 (Figure 2b). Of these PV sera, 45 (97.8%) reacted with the EC1 domain of Dsg1, 12 (26.1%) with EC2, 4 (8.7%) with EC3, 2 (4.3%) with EC4, and 3 (6.5%) with EC5. The reactivity of mucocutaneous-type PV sera to the EC1 domain of Dsg1 was significantly higher than that to the EC2–5 domains ($P<0.0001$), whereas the reactivity to the EC2 domain of Dsg1 was not. Thus, the EC1 domain of Dsg1 was the major epitope recognized by anti-Dsg1 IgG autoantibodies. We also established the Dsg1 epitope profiles for these 46 PV sera obtained at different stages of clinical disease activity (Table 1d). Again, the major epitopes always resided on the N-terminal domain (EC1 domain) of Dsg1 at every stage of clinical disease activity, and their distribution was almost the same no matter which clinical disease activity level was tested. We found that 14 sera (30.4%) recognized epitopes on the multiple EC domains of Dsg1, of which 10 (21.7%) reacted with two; 2 (4.3%) with three, and 2 (4.3%) with four EC domains (Table 1e). No sera were found to react with all five EC domains. A total of 32 sera (69.6%) reacted with only one of the EC domains of Dsg1, 31 (67.4%) reacted only with the EC1 domain, and 1 (2.2%) only with the EC2 domain. No sera reacted only with the Dsg1 EC3, EC4, or EC5 domain (Table 1f).

Epitope profiles of mucocutaneous-type PV

We compared the epitope profiles of anti-Dsg1 and anti-Dsg3 IgG autoantibodies in 15 cases (46 sera) of mucocutaneous-

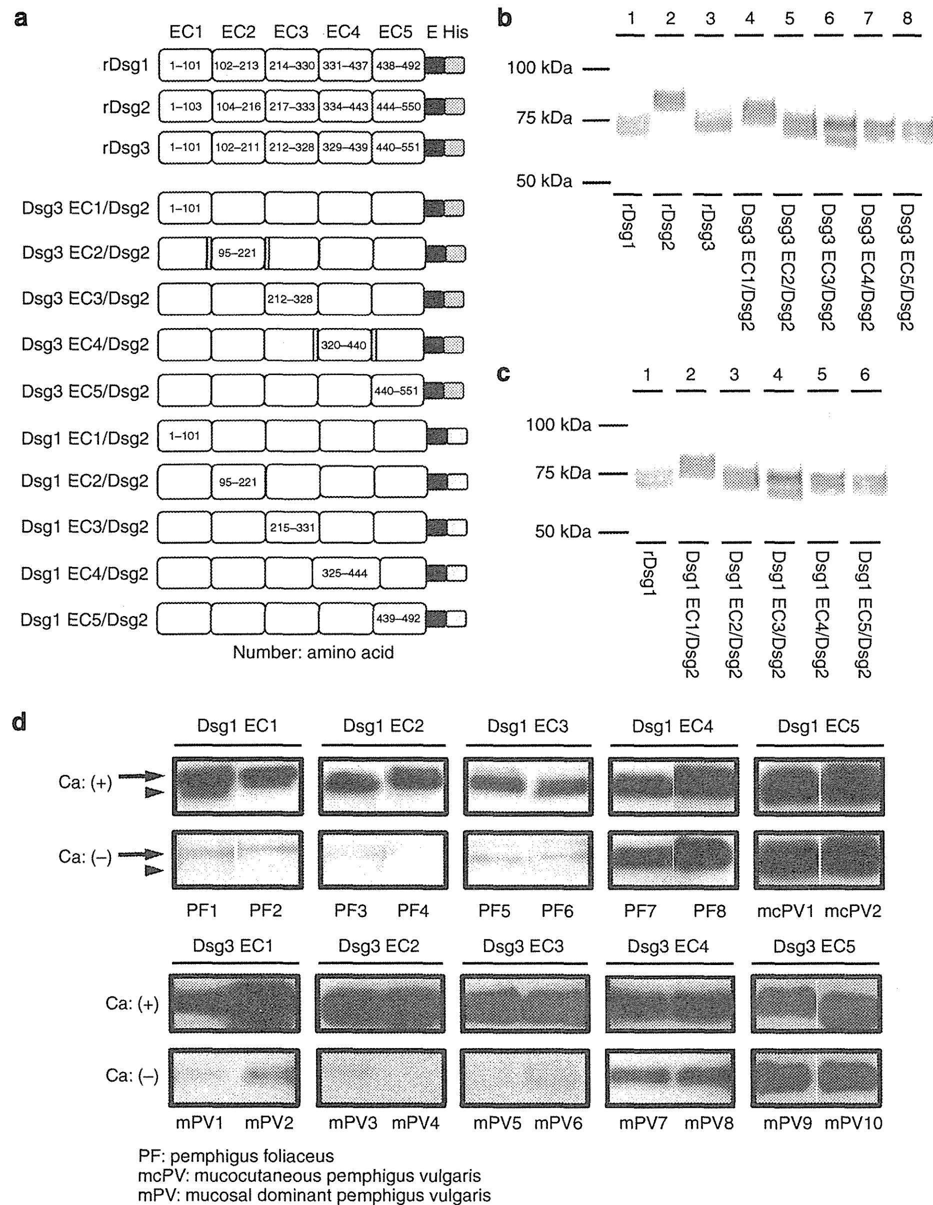

Figure 1. Calcium-dependent pemphigus foliaceus (PF) and pemphigus vulgaris (PV) epitopes analyzed by new domain-swapped molecules. (**a**) Full-length recombinant proteins for the extracellular (EC) domains of human desmoglein 1 (Dsg1) (rDsg1), Dsg2 (rDsg2), Dsg3 (rDsg3), and 10 domain-swapped molecules (Dsg3 EC1/Dsg2–Dsg3 EC5/Dsg2 and Dsg1 EC1/Dsg2–Dsg1 EC5/Dsg2). (**b**) Culture supernatants stained with anti-E tag mAb: rDsg1 (lane 1), rDsg2 (lane 2), rDsg3 (lane 3), Dsg3 EC1/Dsg2 (lane 4), Dsg3 EC2/Dsg2 (lane 5), Dsg3 EC3/Dsg2 (lane 6), Dsg3 EC4/Dsg2 (lane 7), and Dsg3 EC5/Dsg2 (lane 8). (**c**) Culture supernatants stained with anti-E tag mAb: rDsg1 (lane 1), Dsg1 EC1/Dsg2 (lane 2), Dsg1 EC2/Dsg2 (lane 3), Dsg1 EC3/Dsg2 (lane 4), Dsg1 EC4/Dsg2 (lane 5), and Dsg1 EC5/Dsg2 (lane 6). (**d**) Dsg1/Dsg2 and Dsg3/Dsg2 domain-swapped molecules pretreated with calcium (Ca: (+)) or EDTA (Ca: (–)) detected by immunoprecipitation–immunoblotting. EC1-swapped molecules with (arrows) and without (arrowheads) prosequence are marked.

type PV (Supplementary Table S3 online). Both anti-Dsg1 and anti-Dsg3 IgG autoantibodies reacted predominantly with the EC1 domain, whereas epitope profiles for the other domains did not always show the same pattern for Dsg1 or Dsg3. Of the 46 sera, 38 reacted with the EC1 domain of both Dsg1 and Dsg3. Only five sera showed the same epitope pattern with Dsg1 and Dsg3.

Epitope spreading is rare in PV

We next analyzed whether epitope spreading occurred at any time over the disease course. At every clinical disease activity level, most anti-Dsg3 IgG autoantibodies predominantly recognized the EC1 domain, and no intramolecular epitope shift was apparent in 51 (96.2%) of 53 PV cases (Figure 3a and Supplementary Table S2 online). We found only two

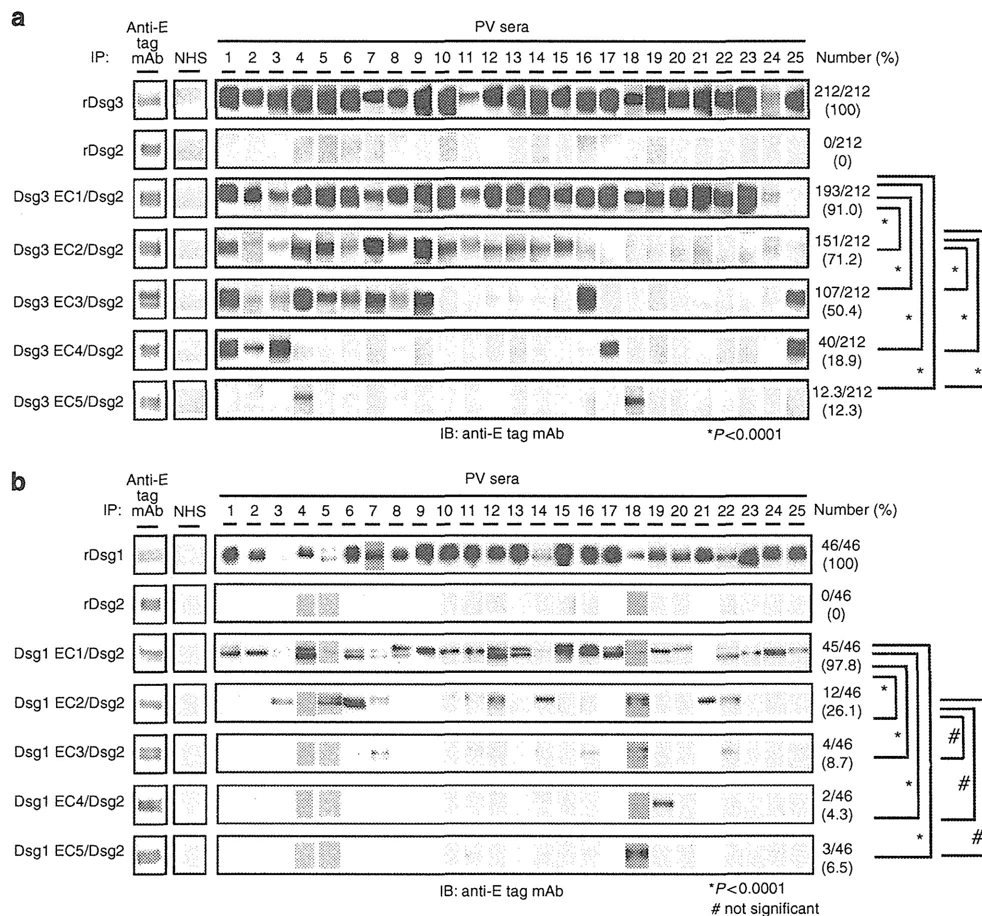

Figure 2. Epitope profiles of pemphigus vulgaris (PV). (**a**) Anti-desmoglein 3 (Dsg3) IgG antibodies preferentially bind to the N-terminal extracellular (EC) domains of Dsg3. All 212 pemphigus vulgaris (PV) sera reacted with the full-length EC domain of Dsg3, but none with the full-length Dsg2 EC domain. Of the 212 PV sera, 193 reacted with the Dsg3 EC1 domain, 151 with the EC2 domain, 107 with the EC3 domain, 40 with the EC4 domain, and 26 with the EC5 domain. (**b**) Anti-Dsg1 IgG antibodies preferentially bind to the N-terminal EC domains of Dsg1. Of the 46 PV sera, 45 reacted with the EC1 domain of Dsg1, 12 with the EC2 domain, 4 with the EC3 domain, 2 with the EC4 domain, and 3 with the EC5 domain. *$P<0.0001$, #not significant. IB, immunoblotting; IP, immunoprecipitation; NHS; normal human serum.

patients (3.8%) manifesting apparent Dsg3 epitope spreading during the disease course (Figure 3b), and none for Dsg1 in any of the 15 PV cases (Supplementary Tables S2 and S3 online). In 13 mucocutaneous-type PV cases (44 sera), in which both anti-Dsg3 and anti-Dsg1 IgG autoantibodies were present, no intermolecular epitope spreading between Dsg1 and Dsg3 was apparent (Supplementary Table S3 online). In addition, we found that, in some PV patients, Dsg3 ELISA reactivity and clinical activity did not correlate well (Supplementary Tables S2–S4 online). Therefore, we have analyzed the possible epitope spreading from pathogenic (i.e., EC1–3) to nonpathogenic epitopes (EC4–5) for cases in which Dsg ELISA index score did not decrease even after clinical improvement was obtained. However, statistically, it was not the case ($P=1.000$ for both anti-Dsg3 and anti-Dsg1 antibodies).

Epitope profiles of paraneoplastic pemphigus

Paraneoplastic pemphigus (PNP) is a rare autoimmune blistering disease associated with neoplasia (Amagai *et al.*, 1998; Ohyama *et al.*, 2001). All 14 PNP sera tested reacted with the EC1 domain of Dsg3. Intriguingly, 12 (85.7%) PNP sera reacted with EC4 (Figure 4a and Table 1g). The reactivity to the EC4 domain of Dsg3 in PNP was comparable to that to the EC1 domain ($P=1.00$), but was significantly higher than that to the EC5 domain ($P=0.0092$).

Epitope profiles of pemphigus herpetiformis

Herpetiform pemphigus (HP) is a pemphigus variant with clinical features of dermatitis herpetiformis and immunological features of pemphigus (Kubo *et al.*, 1997; Ishii *et al.*, 1999; Seitz *et al.*, 1999). Of the 19 HP cases, 15 possessed only anti-Dsg1 IgG autoantibodies, 4 had only anti-Dsg3 IgG autoantibodies, and 2 had both; although one serum (#15) did not react to the full-length Dsg3, it reacted to its EC1–3 domains (Figure 4b). The major epitopes in 15 Dsg1-reactive HP sera tested were present on the EC1 domain of Dsg1, whereas EC2, EC3, and EC4 domains were also recognized by one-third of these sera (Figure 4b and Table 1h). All six sera from Dsg3-reactive HP cases reacted with both EC1 and

Table 1. Epitope profiles of Dsg3 and Dsg1 in PV, PNP, and HP

(a) Epitope profile of Dsg3 at all clinical stages

	Recombinant proteins						
			EC domains of Dsg3				
Clinical activity	**Dsg3**	**Dsg2**	**EC1**	**EC2**	**EC3**	**EC4**	**EC5**
Active (%) (*n*=85)	85 (100)	0 (0)	78 (91.8)	61 (71.8)	38 (44.7)	14 (16.5)	12 (14.1)
Moderate (%) (*n*=60)	60 (100)	0 (0)	52 (86.7)	42 (70.0)	29 (48.3)	11 (18.3)	6 (10.0)
Remission (%) (*n*=67)	67 (100)	0 (0)	63 (94.0)	48 (71.6)	40 (59.7)	15 (22.4)	8 (11.9)
Total (%) (*n*=212)	212 (100)	0 (0)	193 (91.0)	151 (71.2)	107 (50.5)	40 (18.9)	26 (12.3)

(b) PV sera recognizing on multiple EC domain of Dsg3

	Number of domains recognized epitopes			
Clinical activity	**Two**	**Three**	**Four**	**Five**
Active (%) (*n*=64)	25	25	13	1
Moderate (%) (*n*=46)	22	15	8	1
Remission (%) (*n*=54)	16	24	13	1
Total (%) (*n*=164)	63	64	34	3

(c) PV sera recognizing on single EC domain of Dsg3

	Number of domains recognized epitopes				
Clinical activity	**EC1**	**EC2**	**EC3**	**EC4**	**EC5**
Active (%) (*n*=21)	18	2	1	0	0
Moderate (%) (*n*=14)	11	2	1	0	0
Remission (%) (*n*=13)	11	1	1	0	0
Total (%) (*n*=48)	40	5	3	0	0

(d) Epitope profile of Dsg1 at all clinical stages

	Recombinant proteins						
			EC domains of Dsg1				
Clinical activity	**Dsg1**	**Dsg2**	**EC1**	**EC2**	**EC3**	**EC4**	**EC5**
Active (%) (*n*=20)	20 (100)	0 (0)	19 (95.0)	4 (20.0)	1 (5.0)	0 (0)	2 (1.0)
Moderate (%) (*n*=18)	18 (100)	0 (0)	18 (100)	6 (33.0)	0 (0)	0 (0)	0 (0)
Remission (%) (*n*=8)	8 (100)	0 (0)	8 (100)	2 (25.0)	3 (37.5)	2 (25.0)	1 (12.5)
Total (%) (*n*=46)	46 (100)	0 (0)	45 (97.8)	12 (26.1)	4 (8.7)	2 (4.3)	3 (6.5)

(e) PV sera recognizing on multiple EC domain of Dsg1

	Number of domains recognized epitopes			
Clinical activity	**Two**	**Three**	**Four**	**Five**
Active (%) (*n*=4)	3	0	1	0
Moderate (%) (*n*=6)	6	0	0	0
Remission (%) (*n*=4)	1	2	1	0
Total (%) (*n*=14)	10	2	2	0

Table 1 continued on following page

Table 1. Continued

(f) PV sera recognizing on single EC domain of Dsg1

	Number of domains recognized epitopes				
Clinical activity	**EC1**	**EC2**	**EC3**	**EC4**	**EC5**
Active (%) (*n*=16)	15	1	0	0	0
Moderate (%) (*n*=12)	12	0	0	0	0
Remission (%) (*n*=4)	4	0	0	0	0
Total (%) (*n*=32)	31	1	0	0	0

(g) Epitope distributions on Dsg3 in 14 PNP cases

	Recombinant proteins						
			EC domains of Dsg3				
	Dsg3	**Dsg2**	**EC1**	**EC2**	**EC3**	**EC4**	**EC5**
PNP (%) (*n*=14)	14 (100)	0 (0)	14 (100)	8 (57.1)	10 (71.4)	12 (85.7)	3 (21.4)

(h) Epitope distributions on Dsg1 for 15 sera and Dsg3 for 6 sera in HP cases

	Recombinant proteins							
				EC domains of Dsg1				
	Dsg1		**Dsg2**	**EC1**	**EC2**	**EC3**	**EC4**	**EC5**
HP (%) (*n*=15)	15 (100)	0 (0)		12 (80.0)	5 (33.3)	6 (40.0)	5 (33.3)	1 (6.7)
		Dsg2	**Dsg3**	**Extracellular domains of Dsg3**				
				EC1	**EC2**	**EC3**	**EC4**	**EC5**
HP (%) (*n*=6)		0 (0)	5 (83.3)	6 (100)	6 (100)	5 (83.3)	1 (16.7)	0 (0)

Abbreviations: Dsg, desmoglein; EC, extracellular; HP, herpetiform pemphigus; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris.

EC2 domains, and five reacted with the EC3 domain (Figure 4b). One serum reacted with the EC1, EC2, EC3, and EC4 domains of Dsg3. The reactivity to the EC1 domain of Dsg1 (or Dsg3) in HP was comparable to that to the EC2–4 domains of Dsg1 (or Dsg3) (Dsg1 EC1 vs. EC2: $P=0.1013$, EC1 vs. EC3: $P=0.2417$, EC1 vs. EC4: $P=0.1013$; Dsg3 EC1 vs. EC2: $P=1.0000$, EC1 vs. EC3: $P=1.0000$, EC1 vs. EC4: $P=0.0606$), but was significantly higher than that to the EC5 domain of Dsg1 (or Dsg3; Dsg1 EC1 vs. EC5: $P=0.0005$, Dsg3 EC1 vs. EC5: $P=0.0087$) (Figure 4). Thus, HP sera recognized a broader range of epitopes than PV.

DISCUSSION

This study documented the rarity of epitope spreading after the onset of disease in PV. At every clinical activity level, the major Dsg3 epitopes remained in the EC1–2 domains, and the epitope profile was essentially identical in most cases. Furthermore, in mucocutaneous-type PV, dominant epitopes of Dsg1 were present in EC1 but not in the EC2–5 domains. We concluded that there was no significant alteration in epitope profiles over the disease course. In addition, we showed that PNP and HP showed broader epitope distribution compared with classical pemphigus. Moreover, we showed for the first time that calcium-dependent conformational epitopes on human Dsg1 and Dsg3 recognized by pemphigus IgG autoantibodies are located within the EC1–3 domains, whereas EC4 and EC5 are calcium stable, as had been suggested in animal models (Tsunoda *et al.*, 2003).

The new domain-swapped molecules described here are useful for epitope mapping and for analyzing epitope spreading. Our new strategy has several advantages. First, the swapped domains have similar structures but distinct epitopes. Hence, they allow precise mapping of the conformational epitopes embedded in the three-dimensional molecular structure (Müller *et al.*, 2006, 2008). Second, by using a disease-irrelevant Dsg2 as the backbone of the domain-swapped molecules, one can analyze the epitopes recognized by anti-Dsg3 IgG autoantibodies (or anti-Dsg1 IgG autoantibodies) without the influence of the presence of anti-Dsg1 IgG autoantibodies (or anti-Dsg3 IgG autoantibodies, respectively). Previously, Dsg/Dsg1-swapped molecules were used to analyze intra- and intermolecular epitope spreading (Futei *et al.*, 2000; Sekiguchi *et al.*, 2001; Li *et al.*, 2003; Salato *et al.*, 2005). However, although Dsg3 and Dsg1 do not have common structure, the Dsg3/Dsg1-swapped molecule system did not allow us to analyze intra- and

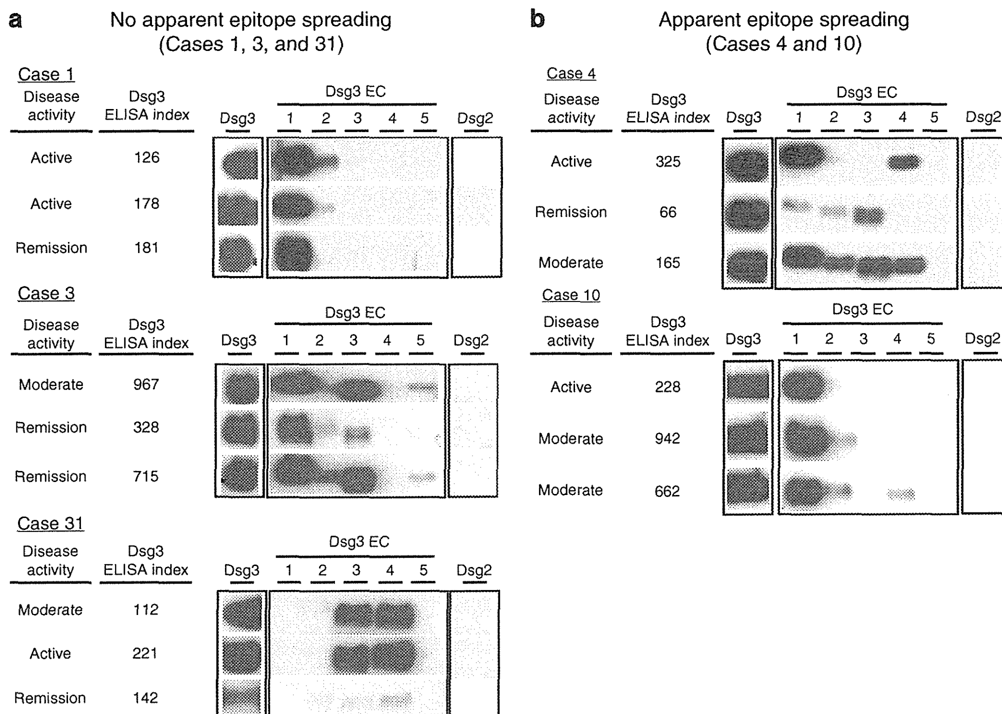

Figure 3. Epitope shift in the target antigens for anti-desmoglein 3 (Dsg3) IgG antibodies occurs rarely over the disease course. (**a**) Most cases ($n=51$) had no epitope shift. (**b**) Epitope profiles for anti-Dsg3 IgG antibodies from pemphigus vulgaris (PV) patients 4 and 10. Only these two PV sera showed apparent epitope shift for anti-Dsg3 IgG target antigens. Disease activity and anti-Dsg3 ELISA index for each serum are also shown at the left of each figure. EC, extracellular.

intermolecular epitope spreading precisely, namely in mucocutaneous-type PV, in which anti-Dsg1 and anti-Dsg3 antibodies are present simultaneously in the blood. The advantage of the method reported here is depicted in Figure 5.

Our results suggest that human autoimmunity conforms to the concept of "original antigenic sin", i.e., domination of the immune response by the initial epitope, which does not change over time. We recently also demonstrated that epitope spreading is rare in PF (Chan *et al.*, 2010). Our results are in agreement with a previous study in which epitope shift rarely occurred in PV (Futei *et al.*, 2000). Our results are not in agreement with previous studies that support epitope spreading in pemphigus (Li *et al.*, 2003; Salato *et al.*, 2005). Li *et al.* (2003) showed the possible intramolecular epitope spreading from EC5 at onset to EC1 and EC2 in active stage, and then toward EC5 in remission in endemic PF. As Chan *et al.* (2010) suggested that epitope shift did not occur in nonendemic PF, the discrepancy may be due to the difference between endemic and nonendemic PF. Salato *et al.* (2005) reported that intramolecular epitope spreading from EC2–5 to the EC1 domain of Dsg3 occurred and was a critical step for intermolecular epitope shift from Dsg3 to Dsg1. However, it is a questionable idea, because their claim was based on the result of only one representative case out of three mucosal dominant-type PV patients who transitioned to mucocutaneous-type PV, and they used the Dsg3/Dsg1-swapped system. The present study does not exclude the possibility of autoimmunity initiated by bystander activation, molecular mimicry, or release of cryptic epitopes before disease onset (Miller *et al.*, 1997; Horwitz *et al.*, 1998; Olson *et al.*, 2001).

Our present results revealed a unique Dsg3 epitope distribution in PNP and HP. The EC4 domain in PNP and EC1–3 domains in HP were frequently recognized by autoantibodies, although major Dsg3 epitopes in both PNP and HP were still found in N-terminal EC domains. The result for PNP is consistent with a previous study using competition ELISA with domain-swapped Dsg3/Dsg1 molecules (Futei *et al.*, 2003). The different autoantibody profiles between these diseases and PV may contribute to their unique clinicohistopathological characteristics.

There are limitations to our study. First, it is impossible to evaluate epitope spreading before the onset of disease. Spreading from viral to self-epitopes has been shown in a virus-induced autoimmune disease model (Miller *et al.*, 1997). However, there is no consensus that tissue-specific autoimmune diseases develop during persistent viral or other infections (Horwitz *et al.*, 1998; Zhao *et al.*, 1998; Olson *et al.*, 2001). Second, we cannot exclude the fact that treatment influenced epitope spreading, when using immunosuppressives, apheresis, or intravenous immunoglobulin, all of which could impair epitope spreading. Third, there are technical issues in quantitative evaluation of autoantibodies when using IP-IB on sera with low Dsg titers. Fourth, it is

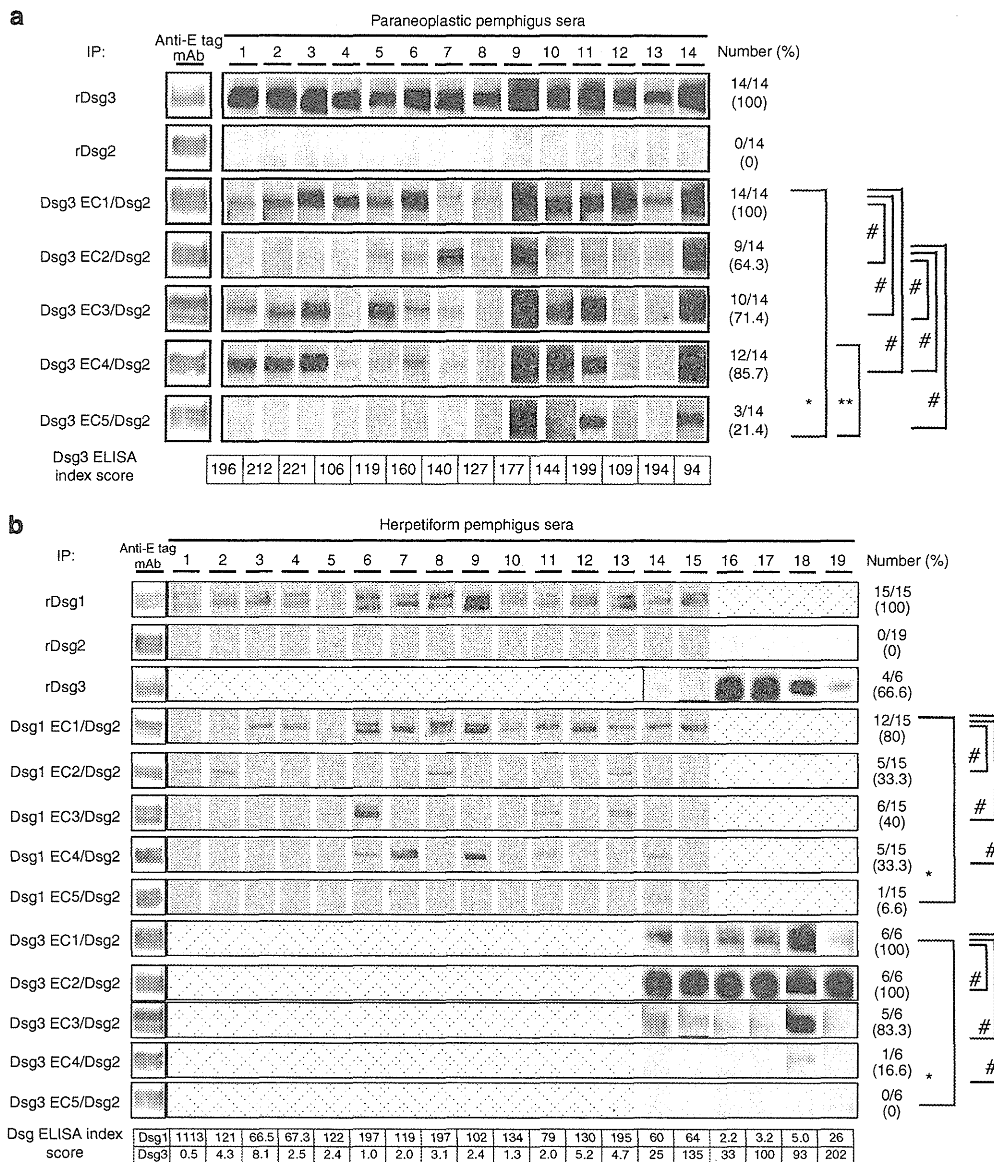

Figure 4. Epitope profiles of atypical pemphigus. (**a**) All 14 PNP sera reacted with the full-length desmoglein 3 (Dsg3) extracellular (EC) domain, but none with the full-length EC domain of Dsg2. $*P=0.0001$, $**P<0.001$, and #not significant. All 14 PNP sera reacted with the EC1 domain of Dsg3, 9 with EC2, 10 with EC3, 12 with EC4, and 3 with EC5 (**b**). In the 15 sera from herpetiform pemphigus (HP) cases, the major epitopes were present on the Dsg1 EC1 domain. However, six HP patients had anti-Dsg3 IgG autoantibodies, and all six sera reacted with EC1 and EC2 domains; five reacted with the EC3 domain. All six sera reacted most strongly with the EC2 domain of Dsg3. $*P<0.001$, #not significant. IP, immunoprecipitation.

impossible to analyze the intra-domain epitope spreading within each EC domain by this study.

In conclusion, we have documented the rarity of epitope spreading in PV. Therefore, targeting treatments to the N-terminal domains of Dsg3/Dsg1 should be promising to control PV.

MATERIALS AND METHODS

Human sera

All studies followed the guidelines of the Medical Ethics Committees of both Kurume University School of Medicine and Keio University School of Medicine, conducted according to the Declaration of Helsinki Principles. All participants provided informed consent. A total of 212 sera were obtained from 31 patients with mucocutaneous-type PV and 22 with mucosal dominant-type PV confirmed by clinical, histological, and immunological findings. Clinical disease activity was defined according to an arbitrary score as follows: active, >30% of the skin affected by lesions, and functional impairment; moderate, 10–30% skin involvement but no functional disability; remission, no active clinical skin lesions for at least 1 month under treatment with <5 mg per day prednisolone or without any treatment. The interval between measurements was 376 days on average (SD 526).

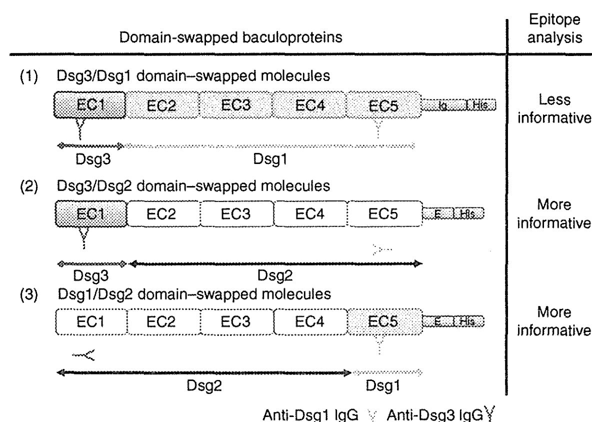

Figure 5. Schematic diagram of the advantages of new desmoglein 1 (Dsg1)/ Dsg2 and Dsg3/Dsg2 domain-swapped molecules. In mucocutaneous-type pemphigus vulgaris, it is difficult to analyze more informative epitopes using Dsg3/Dsg1 domain-swapped molecules, because both anti-Dsg1 autoantibodies and anti-Dsg3 autoantibodies bind to the same swapped molecules (1). Dsg2 backbone new domain-swapped molecules are able to analyze the epitopes without the influence of other antibodies (2) and (3).

In all, 19 HP and 14 PNP cases were diagnosed by clinical, histological, and immunological findings. All 14 PNP cases possessed IgG autoantibodies against envoplakin and periplakin as assessed by immunoblotting using human epidermal extracts (Hashimoto *et al.*, 1995). All sera were assayed by ELISA using recombinant Dsg1 and Dsg3 baculoprotein as substrates (Ishii *et al.*, 1997; Amagai *et al.*, 1999) and 1,600 dilutions of patients' sera (Cheng *et al.*, 2002).

Preparation of the plasmid constructs

Production of recombinant Dsg1 (rDsg1), Dsg2 (rDsg2), and Dsg3 (rDsg3) has been described elsewhere (Amagai *et al.*, 1994; Ishii *et al.*, 1997; Ota *et al.*, 2003). To produce the plasmid constructs, we used an overlap-extension PCR technique. For example, to prepare the Dsg3 plasmid construct with the EC3 domain swapped, first we amplified the EC1–2 domains of Dsg2, the EC3 domain of Dsg3, and the EC4–5 domains of Dsg2. The cDNAs for the different domains of Dsg2 and Dsg3 were amplified with appropriate primers (Supplementary Table S1 online) using pQE-hDsg2 and pQE-hDsg3 (Qiagen, Hilden, Germany) as templates. Next, we annealed the PCR products of EC1–2 of Dsg2 and EC3 of Dsg3 several (5 or 7) times, and amplified them with appropriate primers. We then annealed the annealed/amplified products of EC1–2 of Dsg2 and EC3 of Dsg3 and the PCR product of EC4–5 of Dsg2 several times, and amplified them with appropriate primers. Plasmid constructs of the EC1 and EC5 domains were produced by the two-step method of annealing and extension, whereas the constructs of EC2, EC3, and EC4 domains were produced by a three-step method of annealing and extension. The PCR products of Dsg3 domain-swapped molecules were digested with *NcoI/XhoI* and ligated to *NcoI/XhoI*-cut pQE-Tri expression vector (pQE-hDsg2). These constructs contained an E-tag and a His-tag at their C-terminal region. Sequences were confirmed using an ABI310 genetic analyzer (Applied Biosystems, Carlsbad, CA). Plasmid constructs were designated pQE-Dsg3 EC1/Dsg2, pQE-Dsg3 EC2/Dsg2, pQE-Dsg3 EC3/Dsg2, pQE-Dsg3 EC4/Dsg2, and pQE-Dsg3 EC5/Dsg2.

Domain-specific swapped Dsg1 constructs were prepared according to the previous report (Chan *et al.*, 2010), and designated pQE-Dsg1 EC1/Dsg2, pQE-Dsg1 EC2/Dsg2, pQE-Dsg1 EC3/Dsg2, pQE-Dsg1 EC4/Dsg2, and pQE-Dsg1 EC5/Dsg2.

Protein production by baculovirus expression

Plasmids were co-transfected with Sapphire baculovirus DNA (Orbigen, San Diego, CA) and Cellfectin reagent (Invitrogen, San Diego, CA) into cultured insect Sf9 cells. A high titer of recombinant baculovirus stock was obtained after several rounds of re-amplification. High Five cells (Invitrogen) cultured in serum-free EX Cell 405 medium (JRH Biosciences, Lenexa, KS) were infected with the recombinant viruses and incubated at 27 °C for 3 days; domain-swapped molecules were secreted into the culture supernatant and stored at −80 °C after cell debris removal by centrifugation. The Dsg3 domain-swapped baculoproteins were designated Dsg3 EC1/Dsg2, Dsg3 EC2/Dsg2, Dsg3 EC3/Dsg2, Dsg3 EC4/Dsg2, and Dsg3 EC5/Dsg2. The Dsg1 domain-swapped baculoproteins were designated Dsg1 EC1/Dsg2, Dsg1 EC2/Dsg2, Dsg1 EC3/Dsg2, Dsg1 EC4/Dsg2, and Dsg1 EC5/Dsg2.

Immunoblotting

Culture supernatants containing recombinant baculoproteins were fractionated by SDS-PAGE. Mouse anti-E tag mAb (Pharmacia Biotech, Uppsala, Sweden) was used as a primary antibody and alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Zymed Laboratories, San Francisco, CA) as the secondary antibody.

IP–IB

Culture supernatants containing recombinant baculoproteins were incubated with PV sera and then precipitated with protein G-Sepharose (Amersham Biosciences, Uppsala, Sweden) overnight. After centrifugation, the precipitates were resuspended in SDS sample buffer and boiled for 2 minutes. After centrifugation, the supernatants were applied to SDS-PAGE, and proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The precipitated recombinant baculoproteins were reacted with mouse horseradish peroxidase-conjugated anti-E tag mAb (Amersham Biosciences), and then with the chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA). Finally, the film (Kodak Biomax, Rochester, NY) was exposed to the polyvinylidene difluoride membrane.

EDTA treatment

A volume of 50 µl of 0.5 M EDTA was added to 400 µl culture supernatants containing the five domain-swapped molecules and incubated at 4 °C for 1 h. The culture supernatants with EDTA were dialyzed against TBS (−). For positive controls with calcium, 400 µl of culture supernatants was added to 50 µl of TBS (−) supplemented with 0.5 mM $CaCl_2$ and dialyzed in TBS (−) with 0.5 mM $CaCl_2$. Subsequently, culture supernatants with or without calcium treatment were analyzed by IP–IB.

Statistical analysis

The associations among distributions of reactivity of ECs were assessed by the χ^2 test with Bonferroni correction. The associations

between clinical improvement with high ELISA index score and shift from EC1–3 to EC4–5 were assessed by the Fisher exact test. Relationships of reactivity of ECs with clinical disease activity and ELISA index score were assessed by the generalized estimation equation method with AR1 working correlation matrix. Disease activity was treated as an ordinal variable. A P-value <0.01 was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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

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Pathogenicity of Autoantibodies in Anti-p200 Pemphigoid

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Abstract

Recently, the C-terminus of laminin γ1 has been identified as target antigen in anti-p200 pemphigoid and the disease was renamed as anti-laminin γ1 pemphigoid. However, the pathogenic relevance of these autoantibodies has not yet been demonstrated. Therefore, we employed an ex vivo model of autoantibody-mediated leukocyte-dependent neutrophil activation and dermal-epidermal separation (DES) using cryosections of human skin. We showed that anti-p200 pemphigoid sera (n = 7) induced DES in a time-dependent manner, in contrast to sera from healthy controls. Furthermore, laminin γ1-specific IgG and serum depleted from anti-laminin γ1 reactivity were generated using the recombinant C-terminus of laminin γ1 (LAMC1-term; amino acids 1364 to 1609). Interestingly, both fractions labeled the dermal-epidermal-junction (DEJ) by indirect immunofluorescence microscopy on human foreskin and recognized a 200 kDa protein by immunoblotting with dermal extract. Human and rabbit IgG against LAMC1-cterm failed to attract neutrophils at the DEJ and to induce DES. In contrast, patient serum depleted from LAMC1-cterm reactivity led to the same extent of DES as non-depleted IgG. Repeated injection of rabbit anti-murine LAMC1-cterm IgG into both neonatal and adult C57BL/6mice as well as repetitive immunization of various mouse strains with murine LAMC1-cterm failed to induce macro- and microscopic lesions. In all mice, circulating anti-LAMC1-cterm antibodies were present, but only in some mice, IgG deposits were seen at the DEJ. We conclude that autoantibodies in anti-p200 pemphigoid sera are pathogenic while pathogenicity is not mediated by autoantibodies against laminin γ1. Further studies are needed to identify the pathogenically relevant autoantigen in anti-p200 pemphigoid.

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Introduction

Anti-p200 pemphigoid is an autoimmune subepidermal blistering disease which was first described in 1996 [1,2]. Clinically, the disease is characterized by tense blisters and resembles bullous pemphigoid, the most frequent autoimmune blistering disease although patients with anti-p200 pemphigoid tend to be younger [3]. Autoantibodies in patients' skin localize along the dermal-epidermal junction (DEJ) by direct immunofluorescence (IF) microscopy. Serum IgG autoantibodies label the dermal side of 1 M NaCl-split human skin by indirect IF microscopy and recognize a 200 kDa protein by immunoblotting of human dermal extract [1,2]. Subsequently, the target antigen was characterized as an acidic non-collagenous N-linked glycoprotein of the lower lamina lucida [1,2,4,5]. Recently, Dainichi *et al.* showed reactivity with anti-laminin γ1 in about 90% of patients' sera and coined the term anti-laminin γ1 pemphigoid [6,7]. Furthermore, the C-terminus of laminin γ1 was identified as the immunodominant region of this protein, a finding that we recently confirmed by developing an ELISA using a recombinant monomeric C-terminal fragment of laminin γ1 [8].

So far, nothing is known about the pathogenic relevance of anti-laminin γ1 autoantibodies. We and others have previously developed various experimental models that demonstrated the pathogenic relevance of autoantibodies in different subepidermal blistering autoimmune disorders using passively transferred IgG [9–15]. More specifically, we previously developed an ex vivo model in which incubation of IgG from patients with bullous pemphigoid (BP) and epidermolysis bullosa acquisita (EBA) or rabbit antibodies raised against the target antigens BP180 (type XVII collagen) and type VII collagen, respectively, induced leukocyte-dependent dermal-epidermal separation in cryosections of human skin [10,16]. Furthermore, both the injection of anti-murine type VII collagen and the immunization with recombinant murine type VII and type XVII collagen led to blistering phenotypes in adult mice closely mimicking EBA and BP, respectively [11,17,18].

In the present study, serum from patients with anti-p200 pemphigoid induced dermal-epidermal splitting in cryosections of human skin. In contrast, patient IgG affinity-purified against the recombinant C-terminus of human laminin γ1 (hLAMC1-cterm),

and a C-terminal fragment of laminin 111 [6] and the whole laminin γ1 chain, respectively, failed to induce dermal-epidermal separation in this model. In addition, total IgG and IgG affinity-purified using recombinant mLAMC1-cterm generated from mLAMC1-cterm-immunized rabbits, respectively, were ineffective in the ex vivo cryosection model and did not cause macro- and microscopic disease after injection into both neonatal and adult mice. Furthermore, immunization of mice with mLAMC1-cterm induced mLAMC1-cterm-specific autoantibodies but did not result in clinical disease. These studies indicate that autoantibodies in anti-p200 pemphigoid are pathogenic ex vivo but autoantibodies against the C-terminus of laminin γ1 do not mediated pathogenicity in this disease.

Materials and Methods

Human Sera and Anti-laminin γ1 Antibodies

Serum samples were obtained from patients with anti-p200 pemphigoid (n = 25) and characterized as described before [8]. The study was approved by the ethics committee of the University of Luebeck (11-143). Written informed consent was obtained from all patients seen in our department. The majority of sera were obtained anonymously from the routine autoimmune laboratory of our department to which they were sent from various hospitals worldwide. The anonymous use of patients' sera left over after routine diagnosis has been approved by our local ethics committee for patients that were not seen in our department (11-143). Rabbits SA6539 and SA6794 were generated against recombinant murine (m)LAMC1-cterm. IgG affinity-purified from a BP patient serum with anti-BP180 NC16A reactivity, rabbit IgG against murine BP180 NC15A, and preimmune rabbit serum were used as controls. Polyclonal rabbit anti-hLAMC1 crossreacting with mLAMC1 (H-190) and mouse monoclonal anti-hLAMC1 (clone B-4) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Mice

C57BL/6, BALB/c, and SJL mice were obtained from Charles River Laboratories (Sulzfeld, Germany). All injections and bleedings were performed on mice anesthetized by intraperitoneal administration of a mixture of ketamine (100 μg/g) and xylazine (15 μg/g). Experiments were approved by the Animal Rights Commission of the Ministry of Agriculture and Environment, Schleswig-Holstein (V312-72241.122-5(79-6/09) and V312-72241.122-5(80-6/09)).

Preparation of Dermal Extracts

Murine dermal extracts were prepared as described for human skin [1] The dermal extract was then stored in −80°C.

Cloning, Expression and Purification of Recombinant Fragments of LAMC1

Human LAMC1-cterm (hLAMC1-cterm; amino acids 1364 to 1609) was expressed in *E.coli* as described previously [8] and in the human cell line HEK293T (Cell Lines Service, Eppelheim, Germany). For latter expression, the sequence included a signal peptide from Ig kappa for secretion into the culture medium. HEK293T cells cultured in DMEM (Invitrogen, Karlsruhe, Germany) with 10% fetal calf serum were transfected with hLAMC1-cterm-pEE14.4 (Lonza, Cologne, Germany) After 48 h, hLAMC1-cterm was purified by immobilized metal affinity chromatography on TALON superflow (Clontech, Palo Alto, CA, USA). Furthermore, 5 overlapping fragments of hLAMC1 comprising the portion not covered by hLAMC1-cterm were expressed as His-fusion proteins in the *E.coli* strain Rosetta DE3 (Fig. 1). DNA sequence data for human LAMC1 was retrieved from GenBank (accession number: NM-002293). Primer pairs used in standard PCR reaction are shown in table 1 (VBC-Biotech, Vienna, Austria). The amplification products were subcloned into pQE40, using a BamH*I*/Hind*III* cutting site. Expression and purification were performed as described above as described previously [8]. Previous study showed that anti-p200 pemphigoid patients' sera are not reactive with murine skin [19]. Therefore we generated the C-terminal fragment (mRNA: 4330-5069 bp, 740 bp, accession number NM- 010683) of murine laminin γ1 (mLAMC1-cterm) which was optimised for expression in *E. coli* (Mr. Gene, Regensburg, Germany). Murine LAMC1-cterm was subcloned from the obtained vector mLAMC1-cterm-pMA into the expression vector pQE-40 as described for hLAMC1-cterm [8].

The recombinant E8 fragment of laminin 111, a heterotrimer of the truncated C-terminal portions of α1, β1, and γ1 chains was kindly provided by Dr. Sekiguchi (Institute for Protein Research Osaka University) [6]. The plasmid pTriEx-1 containing full length human laminin γ1 (hLAMC1-FL) was kindly provided by Euroimmun AG. Transfection of HEK293T cells and purification of hLAMC1-FL was performed as for hLAMC1-cterm described above.

Immunoblotting

Recombinant proteins and dermal extracts were fractionated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted as reported [8]. Human (1:50), mouse (1:50), and rabbit sera (1:1,000), polyclonal rabbit anti-hLAMC1 crossreacting with mLAMC1 (clone H-190) and mouse monoclonal anti-hLAMC1 (clone B-4; both 1:200) were diluted in TBST containing 5% skimmed milk powder plus 1% BSA. As secondary antibody horseradish peroxidase (HRP)-conjugated monoclonal mouse anti-human IgG4 antibody (Southern Biotech, Birmingham, Alabama, USA), polyclonal rabbit anti-mouse IgG antibody (DAKO, Hamburg, Germany) and polyclonal goat anti-rabbit antibody (DAKO) were used.

Affinity Purification of Rabbit and Human IgG

IgG from rabbit sera was isolated using Protein G Sepharose Fast Flow affinity column chromatography (GE Healthcare, Munich, Germany) as described previously [20]. Concentrations of IgG were determined using BCA assay (Thermo Scientific, Rockford, USA). Antibodies to hLAMC1-cterm (prokaryotic and eukaryotic expressed forms), E8 fragment of laminin 111, as well as antibodies to hLAMC1-FL and mLAMC1-cterm were affinity-purified from sera of anti-p200 pemphigoid patients and rabbits immunized with mLAMC1-cterm, respectively, using Affi-Gel 15 (Bio-Rad, Munich, Germany) and the MicroLink™ Protein Coupling Kit (Thermo Fisher Scientific p/a Pierce Biotechnology, Rockford, USA) following the manufacturer's instructions. Autoantibodies to hLAMC1-FL were generated using the sera affinity-purified against eukaryotic expressed hLAMC1-cterm protein followed by incubation with the immobilized hLAMC1-FL protein. After both steps of affinity-purification, eluted antibodies were pooled and used for the experiments.

Cryosection Assay

Blister-inducing capacity of patients' autoantibodies and rabbit IgG was evaluated using the cryosection assay, an *ex vivo* model of autoantibody-induced dermal-epidermal separation originally described by Gammon *et al.* [21] and modified by Sitaru *et al.* [10,16]. Briefly, cryosections of neonatal human foreskin and

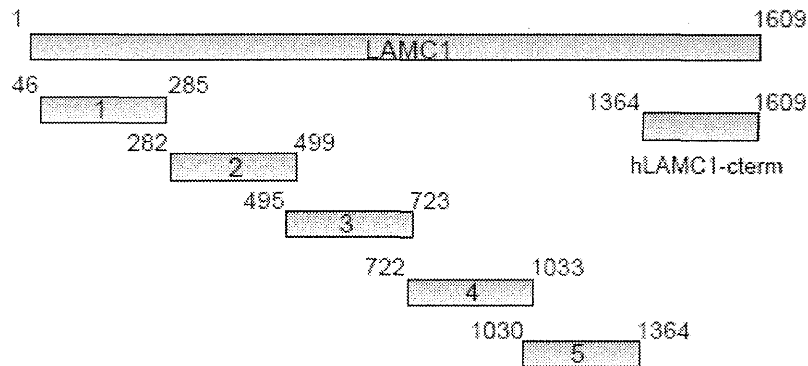

Figure 1. Schematic diagram of the 6 recombinant fragments of human laminin γ1 (hLAMC1) used in this study. The recombinant C-terminus (hLAMC1-cterm) was previously used as antigenic target in an ELISA for the diagnosis of anti-p200 pemphigoid [8]. Each recombinant fragment is fused with an N-terminal His-tag. Amino acid numbers are shown next to the fragments.
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mouse tail were incubated for one hour at 37°C with 50 µl of patients' and rabbit sera (dilution 1:3 in PBS). In addition, hLAMC1-cterm-specific IgG and sera depleted from anti-hLAMC1-cterm reactivity were used after adjusting to serum IgG concentrations. Then, leukocyte suspension from healthy volunteers isolated by dextran 500 (ROTH, Karlsruhe, Germany) *sedimentation mixed with medium* (RPMI, LONZA, Cologne, Germany) was incubated with the skin sections for 3 hours at 37°C. After washing with PBS slides were fixed in formalin and stained with haematoxylin and eosin. Sections were examined by two blinded independent investigators at x200 magnification. Analogous experiments were performed using the E8 fragment of laminin111 and hLAMC1-FL.

Mouse Experiments

In the passive transfer model, purified rabbit IgG generated against mLAMC1 and preimmune rabbit IgG (15 mg per adult mouse and 10 mg/g per neonatal mouse) as well as affinity-purified rabbit antibodies specific to mLAMC1-cterm (1 mg/g per neonatal mouse) were injected subcutaneously in abdominal skin every second day for 12 days. On day 14, mice were sacrificed, and samples (ears, tails, blood) were taken for further analysis. In the immunization-induced model, mice were immunized subcutaneously into footpads 4 times (with 3 weeks interval) with 60 µg of purified mLAMC1-cterm emulsified in adjuvant (TiterMax®, Alexis, Lörrach, Germany). Mice were examined every second week for evidence of cutaneous lesions (i.e., erythema, blisters, erosions, or crusts). Control mice were immunized with PBS and TiterMax®. All mice were observed for at least 16 weeks. From *every mouse, serum and tissues samples were obtained at weeks* 2, 4, 6, 8, and 16 for further analysis.

Table 1. Primer sequences for PCR amplification of cDNA fragments of human.

	Size (bp)	Primer sequence (5'-3')
1	740	F: ATGGATCCCGGCCGCAGCGCTGCA
		R:AGCGGTACCTCATCTGCCACCTACAGCAAAATCAGAGA
2	670	F: ATGGATCCACTGTAGGTGGCAGATGTAAATGTAATG
		R: ATGGTACCTCAGCAGAAGCAGGGTGTGCAACC
3	706	F: ATGGATCCTGCTTCTGCTTTGGGCATTCT
		R: ATGGTACCTCAAAGCACACATGGACTGTATGGTC
4	958	F: ATGGATCCAGTGTGCTTTGCGCCTGCAATG
		R: ATGGTACCTCAGGCTGGACATTCCTGGCAGCCAG
5	1021	F:ATGGATCCGAATGTCCAGCTTGTTACCGG
		R: ATGGTACCTCAAGCTTCTTGTAAGGTATCCCG

F, forward primer; R, reverse primer.
doi:10.1371/journal.pone.0041769.t001

Immunofluorescence Microscopy

For direct IF microscopy, 6 µm sections of mouse skin were incubated with FITC-labelled polyclonal rabbit anti-mouse IgG (1:100, DAKO) and anti-murine C3 IgG (1:50, Cappel Organon-Teknika, Durham, NC). For indirect IF microscopy, 6 µm sections of human and mouse skin were incubated with human, rabbit, and mouse sera (diluted 1:50 in PBS) and for detection, FITC-labelled monoclonal anti-human IgG4 (1:50, Sigma Aldrich, Munich, Germany) and polyclonal swine anti-rabbit IgG (1:100, DAKO) were employed.

Anti-mLAMC1-cterm ELISA

Each well of 96-well microtiter plates (MaxiSorp, Nunc, Roskilde, Denmark) was coated with 4 µg/ml mLAMC1-cterm in PBS at 4°C over night. After blocking with PBST containing 5% skimmed milk, wells were incubated with a 100-fold dilution of mouse sera for 1 h. Bound antibodies were detected using an HRP-conjugated polyclonal rabbit anti-mouse IgG antibody (DAKO) and polyclonal goat anti-rabbit antibody (DAKO) diluted 1:2,000 in blocking buffer followed by addition of 1-Step Turbo TMB-ELISA solution (Fisher Scientific, Schwerte, Germany) for 1–3 minutes. The OD_{450nm} was measured using a VICTOR3 Wallac 1420 microplate reader (Perkin-Elmer LAS, Rodgau, Germany). All steps were carried out at room temperature. All sera were tested in duplicates. From the mean OD value for each serum sample, the mean OD value of the blank (PBST) was subtracted.

Results

Sera from anti-p200 Pemphigoid Patients Induce Subepidermal Splitting in Cryosections of Human Skin

Serum samples from anti-p200 pemphigoid patients (n = 7) as well as sera from a patient with BP and from healthy volunteers were incubated with cryosections of human skin in the presence of

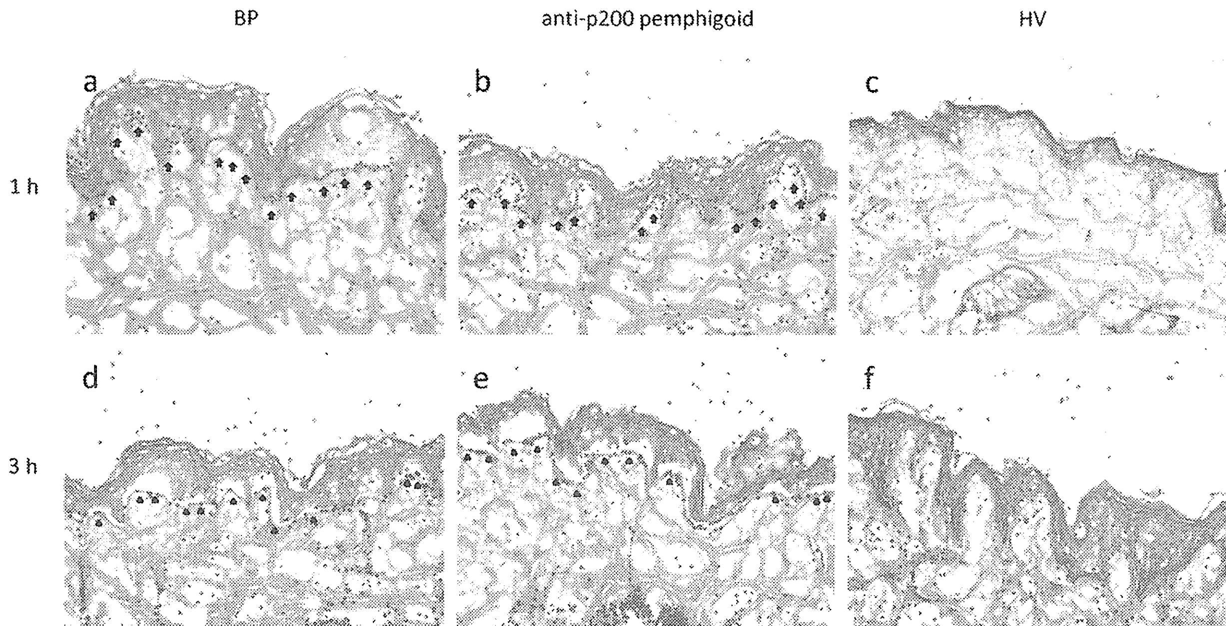

Figure 2. Anti-p200 pemphigoid patients' sera induce dermal-epidermal separation ex vivo. Sera from anti-p200 pemphigoid patients recruit neutrophils to the dermal-epidermal junction (DEJ) and induce dermal-epidermal separation (DES) in cryosections of human skin. Anti-p200 pemphigoid sera (anti-p200 pemphigoid; b, e), but not from sera of healthy volunteers (HV; c, f), recruited leukocytes to the DEJ after 1 h of incubation (b, c) and induced DES after 3 h (e, f). As positive control for DES, IgG from a patient with bullous pemphigoid was used (BP a, d). Recruited neutrophils are marked by arrows, base of the split is marked by black triangles. All sections were stained with hematoxylin and eosin. Magnification, x200.
doi:10.1371/journal.pone.0041769.g002

leukocytes purified from healthy donors. Autoantibodies from all tested patients bound to the DEJ and led to recruitment of leukocytes (Fig. 2a, b), followed by dermal-epidermal separation (DES) after a 3 h incubation with leukocytes (Fig. 2d, e). Sera from healthy volunteers served as controls (Fig. 2c, f).

Anti-hLAMC1-specific IgG from Patients with anti-p200 Pemphigoid does not Induce Split Formation in Cryosections of Human Skin

To explore the pathogenic effect of anti-laminin γ1 antibodies, IgG from anti-p200 pemphigoid patients was affinity-purified using four different recombinant antigens: (i) hLAMC1-cterm expressed in *E.coli*, (ii) hLAMC1-cterm expressed in HEK293 cells, (iii) E8 fragment covering the C-terminus of laminin 111, and (iv) the entire hLAMC1-FL molecule. hLAMC1-cterm-specific IgG of all 5 tested sera as well as the monoclonal anti-LAMC1 antibody (B-4) recognized both recombinant hLAMC1-cterm and the p200 antigen by immunoblotting (Fig. 3a, c) and labelled the DEJ by indirect IF microscopy on human skin (Fig. 3h, d), but did not induce splitting in cryosections of human skin (Fig. 3i, e). In contrast, all 5 sera depleted from anti-hLAMC1-cterm reactivity labelled the p200 antigen by immunoblotting and the DEJ by indirect IF microscopy of human skin, respectively (Fig. 3a, c, j), but induced DES in the cryosection model (Fig. 3k). While in addition, anti-p200 pemphigoid serum depleted of total IgG did not induce DES in the cryosection model (data not shown). To exclude suboptimal dosing, hLAMC1-cterm-specific IgG was applied at a 5-fold higher concentration compared to the serum. The concentrated hLAMC1-cterm-specific IgG did also not induce DES.

Since laminin γ1 is known to be N-glycosylated [5], in the next set of experiments, IgG from anti-p200 pemphigoid patients (n = 3) was affinity-purified using recombinant hLAMC1-cterm expressed in HEK293 cells. The same set of experiments was also performed using the recombinant E8 fragment (n = 3). In both cases the obtained hLAMC1-cterm-specific IgG reacted with the p200 protein in dermal extract but did not induce DES in the cryosection model. In contrast, incubation of serum depleted from reactivity with eukaryotic expressed hLAMC1-cterm still resulted in DES (data not shown). In a final approach, the entire protein hLAMC1-FL was employed in 3 patient sera. Again, the affinity-purified IgG reacted with the p200 protein in dermal extract (Fig. 3c) but did not induce DES (Fig. 3m) while serum depleted from hLAMC1-FL reactivity induced splitting (Fig. 3o).

By indirect IF microscopy on human skin, in addition to a linear binding pattern, staining of the basal keratinocytes was noticed with (i) the monoclonal anti-LAMC1 antibody (Fig. 3d, insert), (ii) patient hLAMC1-cterm-specific IgG (Fig. 3h, insert), and (iii) patient hLAMC1-FL-specific IgG (Fig. 3l). In contrast, patient sera and sera depleted from anti-hLAMC1-cterm and hLAMC1-FL antibodies, respectively, revealed a linear staining throughout the specimen (Fig. 3f, h, n; inserts).

We concluded that autoantibodies to laminin γ1 did not mediate DES induced by anti-p200 pemphigoid sera in the cryosection model.

Epitope Mapping of the Entire hLAMC1

To test the hypothesis that major antigenic sites are present on laminin γ1 outside hLAMC1-cterm, 5 overlapping recombinant fragments covering the entire laminin γ1 molecule outside hLAMC1-cterm were expressed in *E. coli* (Fig. 1). Twenty-five anti-200