SUPPLEMENTARY MATERIAL

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

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Differential IgG Recognition of Desmoglein 3 by Paraneoplastic Pemphigus and Pemphigus Vulgaris Sera

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TO THE EDITOR

Paraneoplastic pemphigus (PNP) is a frequently lethal autoimmune bullous disease characterized by severe polymorphous mucocutaneous lesions and is commonly associated with hematologic malignancies, particularly non-Hodgkin lymphomas, chronic lymphatic leukemia, and Castleman's disease (Anhalt, 2004). The autoantibody profile of PNP is less restricted and includes recognition of the autoantigens of pemphigus vulgaris (PV), pemphigus foliaceus (PF), and bullous pemphigoid (BP). Whereas the sera of patients with PV, PF, and BP nearly selectively recognize the desmogleins (Dsg) or bullous pemphigoid antigens 1 (BP230) and 2 (BP180), respectively, PNP sera exhibit IgG (and IgA) reactivity against an array of intracellular and transmembrane adhesion proteins including plakins (desmoplakin I and II, BP230, envoplakin, periplakin), desmosomal cadherins such as Dsg3, Dsg1, desmocollins (Dsc) 1–3, as well as plakophilin 3, and a 170-kDa protein, which was recently identified as a α-macroglobulin-like 1 protease inhibitor (Oursler *et al.*, 1992; Nagata *et al.*, 2001; Lambert *et al.*, 2010; Schepens *et al.*, 2010).

As PNP is clinically heterogeneous and may mimic a variety of inflammatory skin disorders, such as PV, BP, graft-versus-host disease, or lichen planus (Anhalt, 2004), more detailed knowledge about the fine specificity of the autoantibody profile may help identify PNP more readily.

In this study, a total of 39 sera from 15 patients with the clinical diagnosis of PNP (Table 1) along with 24 patients with acute-onset PV were studied. The clinical diagnosis of PNP was confirmed by histopathology, direct immunofluorescence, and, additionally, proven by positive immunoreactivity with the 190-kDa antigen periplakin and the 210-kDa antigen envoplakin. The study was conducted in accordance with the Declaration of Helsinki Principles, approved by the local ethics committees of the participating institutions, and all patients gave written informed consent.

Initially, total IgG and IgA reactivites of the PNP and PV sera were tested by ELISA against Dsg3, Dsg1, and Dsc1–3 proteins as described previously (Muller *et al.*, 2008, 2009). As expected, all PNP and PV sera showed IgG reactivity against Dsg3, which is in line with previous findings (Amagai *et al.*, 1998; Futei *et al.*, 2003; Mentink *et al.*, 2007). In contrast, only 2 out of 15 (13.3%) PNP sera exhibited IgG reactivity against Dsg1 (Figure 1a), whereas 13 out of 24 (54.2%) PV sera reacted with Dsg1 (Figure 1b). In addition, PNP (Figure 1a), but not PV sera (Figure 1b) contained IgG, and in one case IgA autoantibodies (Figure 1c), directed against Dsc, of which Dsc3 (8/15) was the most common antigen. In addition, five PNP sera showed IgA against Dsg3, and three PNP sera exhibited IgA reactivity against Dsg1 (Figure 1c).

In light of these findings, we sought to identify potential differences in epitope recognition of the Dsg3 ectodomain

Abbreviations: BP, bullous pemphigoid; Dsc, desmocollin; Dsg, desmoglein; EC, extracellular; PF, pemphigus foliaceus; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris

Table 1. Clinical features of investigated PNP patients

Patient no.	Age (years)	Sex	Mucosal lesions		Skin lesions			Pulmonary involvement	Underlying malignancy
			Oral	Ocular	Flaccid vesicles	Lichenoid	Multiforme		
1	43	M	+	+	+	–	–	+	Follicular lymphoma
2	56	F	+	–	–	–	–	+	Epitheloid leiomyosarkoma
3	49	F	+	–	+	+	–	–	Low-grade NHL
4	61	M	+	+	+	–	–	–	Malignant fibrohistiocytoma
5	57	M	+	–	+	–	–	–	NHL
6	86	M	+	–	+	–	–	–	Larynx-Ca
7	70	M	+	–	+	+	–	–	Follicular NHL
8	78	M	+	–	+	–	+	–	Large B-cell NHL
9	65	M	+	+	+	–	–	–	Low-grade B-cell NHL
10	57	F	+	–	+	+	–	–	Leukemia
11	42	F	+	+	–	–	–	+	Castleman tumor
12	69	M	+	+	+	–	–	–	Low-grade B-cell NHL
13	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
14	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
15	46	F	+	+	+	–	+	–	Follicular NHL

Abbreviations: F, female; M, male; NHL, non-Hodgkin lymphoma; PNP, paraneoplastic pemphigus.
Ø, clinical data not available/accessible.

by IgG autoantibodies from PV and PNP sera. Using recombinant forms of the Dsg3 extracellular (EC) subdomains 1–5, IgA and IgG autoantibodies from the PNP sera were found to preferentially recognize the COOH-terminal EC4 and EC5 domains of Dsg3 (Figures 1d and e), whereas the PV sera preferentially showed IgG reactivity directed against the NH_2 terminus of Dsg3 (EC1 and EC2; Figure 1f). These findings are in contrast to previous observations that identified the NH_2-terminal EC domains of Dsg3 (EC1 and EC2) as the primary targets for both PNP and PV sera (Futei *et al.*, 2003). The reason for this discrepancy may be that the authors of the previous study used domain-swapped molecules of Dsg3 and Dsg1 in a competition ELISA, whereas we used Dsg3 recombinants that represented only distinct Dsg3 subdomains (Muller *et al.*, 2008).

Several PNP sera also recognized the EC domain of Dsc3 (Figure 1a). This observation is of particular interest as several authors recently demonstrated the importance of the adhesive function of Dsc for the integrity of the epidermis, and reported that loss of Dsc function results in impaired cell–cell adhesion leading to intraepidermal blistering (Spindler *et al.*, 2009; Rafei *et al.*, 2011). Furthermore, these results confirm previously published data of our group and others showing that Dsc-specific IgG or IgA autoantibodies are exclusively detected in paraneoplastic, atypical, and IgA pemphigus (Hisamatsu *et al.*, 2004; Muller *et al.*, 2009).

Antibodies of the IgG isotype predominate in several autoimmune diseases; IgG1 autoantibodies have been associated with the pathogenesis of BP, whereas IgG4 is the major autoantibody isotype in pemphigus (Mihai *et al.*, 2007). In the studied PNP sera, IgG1 reactive with Dsg3 was detectable in 100% of the sera, whereas IgG2 was found in 33.3%, IgG3 in 53.3%, and IgG4 in 26.6% of the sera (Figure 1g). These results are in line with a previous study demonstrating a predominance of IgG1 in PNP and IgG4 autoantibodies in PV sera (Futei *et al.*, 2003). As IgG1 is a potent recruiter and activator of leukocytes and possesses a high blister-inducing capacity, it is tempting to speculate that the more severe course seen in PNP compared with PV may at least in part be explained by different IgG autoantibody subclasses involved.

Recently, it has been shown that autoantibodies of the IgE class are associated with acute-onset PV and have been previously also linked to the pathogenesis of BP (Fairley *et al.*, 2007). As PNP clinically shares features of PV and BP, we sought to identify IgE autoantibodies in the studied PNP sera (for details see Supplementary Material online). However, neither IgE reactivity against Dsg1/3 nor against Dsc1-3 or BP180/BP230 was detected in the PNP sera (data not shown).

Finally, we were interested in whether a correlation between the clinical manifestation of PNP (Table 1) and individual autoantibody profiles exists. However, we were unable to detect any relationship between clinical signs and defined autoantibody patterns.

In summary, the present study demonstrates that, in PNP, IgG autoantibodies show a distinct autoantibody profile that is characterized by (1) IgG reactivity against the major

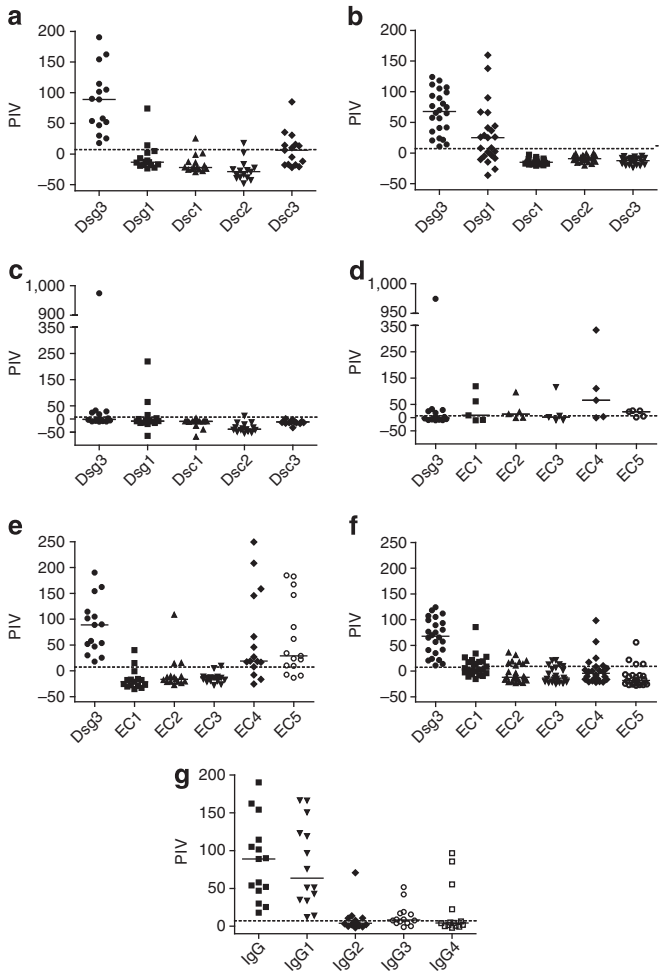

Figure 1. The autoantibody profiles of paraneoplastic pemphigus (PNP) and pemphigus vulgaris (PV) sera differ fundamentally. Sera of PNP and PV patients with acute disease were analyzed with regard to IgG and IgA reactivities against desmoglein 3 (Dsg3) and Dsg1 and desmocollins (Dsc) 1–3 (IgG: **a**, **b**; IgA: **c**, **d**), IgA and IgG reactivity against Dsg3 subdomains (**d**, **e**, **f**), and IgG autoantibody subclasses (**g**). Reactivity was measured at an optical density of 405 nm (OD = 405 nm); the cutoff value was set at a protein index value (PIV) of 7.2 (dotted line). For details see Supplementary Materials online.

target antigens of pemphigus, Dsg3 and Dsg1, (2) preferential recognition of the COOH terminus of the Dsg3 ectodomain (in contrast to PV sera which preferentially target the NH2-terminus), (3) occasional recognition of Dsc1, 2, and 3 (which were not found in PV sera), and (4) IgG1 as the dominant autoantibody subclass. Our findings may help distinguish PNP from other pemphigus variants by the autoantibody profile and may provide new insight into the relative role of IgG autoantibody profiles in the pathogenesis of PNP.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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siRNA-Mediated Allele-Specific Inhibition of Mutant Type VII Collagen in Dominant Dystrophic Epidermolysis Bullosa

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TO THE EDITOR

Dominant dystrophic epidermolysis bullosa (DDEB) is a blistering disease of the skin and mucosae, in which mutant type VII collagen monomers exert dominant-negative interference on normal α1(VII) chains upon homotrimer assembly (Burgeson, 1993). Specific inhibition of the mutant mRNA has been achieved recently in other dominant skin disorders, including epidermolysis bullosa simplex and pachyonychia congenita, using small interfering RNAs (siRNAs) targeting mutations in the keratin 5 and the keratin 6A genes, respectively (Hickerson *et al.*, 2008; Atkinson *et al.*, 2011). We have investigated allele-specific RNA interference as a new therapeutic approach for DDEB, by targeting in-frame skipping of exon 87 (Δ87) of *COL7A1* (OMIM *120120) caused by several intronic or exonic mutations, some of which are recurrent mutations, underlying DDEB pruriginosa (Supplementary Table S1 online; Sakuntabhai *et al.*, 1998; Mellerio *et al.*, 1999; Covaciu *et al.*, 2011).

We developed a fluorescence-based screen for siRNAs selectively blocking the mutant mRNA, using cultured cells transfected with a plasmid vector encoding either the mutant ($COL7A1_{\Delta 87}$) or wild-type ($COL7A1_{WT}$) mRNA upstream of an internal ribosome entry site (IRES)-firefly luciferase reporter (Figure 1a). The abnormal exon 86–exon 88 splice junction was scanned with 21 siRNAs; some were 5′-end modified to increase efficiency (Grimm, 2009; Figure 1b). Positive (siwt, targeting *COL7A1* exon 22) and negative control (NC) siRNAs were used in parallel at 1–10 nM with no observed dose effect (data not shown). Eleven siRNAs, siCOL7Δ87mut3, −9 to −11, −14 to −19, and −21, displayed over 40% inhibition of $COL7A1_{\Delta 87}$ (Figure 1c).

Best differential inhibition was observed with siCOL7Δ87mut3, 45% (1 nM), and siCOL7Δ87mut18, 55% (2 nM), versus 14% inhibition of $COL7A1_{WT}$. High specificity for the mutant mRNA was confirmed by transfecting these siRNAs into fibroblasts and keratinocytes from patients carrying a Δ87 mutation, NM_000094.3:c.6900 + 4A > G (Drera *et al.*, 2006); mRNA extinction was measured by Δ87-specific PCR amplification (Figure 2). The positive control siRNA achieved 55% inhibition of $COL7A1_{WT}$ or $COL7A1_{\Delta 87}$ mRNA (Figure 2a and b), whereas the NC siRNA had no significant effect. In patient fibroblasts, siCOL7Δ87mut3 inhibited $COL7A1_{\Delta 87}$ by 38% (1 nM), 36% (2 nM), 31% (5 nM), and 41% (10 nM), versus 2–8% $COL7A1_{WT}$ inhibition in healthy control fibroblasts (Figure 2b). In patient keratinocytes, $COL7A1_{\Delta 87}$ extinction by siCOL7Δ87mut3 was 38, 58, 45, and 54%, respectively. Conversely, no depression but enhancement of $COL7A1_{WT}$ levels was observed in healthy control keratinocytes, consistent with luciferase assay data (Figure 1). siCOL7Δ87mut18 inhibited $COL7A1_{\Delta 87}$ by 47% (1 nM), 58% (2 nM), 54% (5 nM), and 65% (10 nM) in patient fibroblasts, and by 53, 41, 35, and 52%, respectively, in patient keratinocytes. Experiments on healthy control cells showed no significant reduction of $COL7A1_{WT}$ levels by siCOL7Δ87-mut18 (Figure 2c).

siCOL7Δ87mut18, which shows the strongest specific $COL7A1_{\Delta 87}$ inhibition, carries a 5′-terminal amine modification of the sense strand to promote antisense strand incorporation into the RNA-induced silencing complex (RISC). siRNA duplex thermodynamics determine which strand enters RISC as the guide strand, and only the antisense strand can direct cleavage of the sense mRNA targets (Khvorova *et al.*, 2003). Avoidance of off-target effects (Grimm, 2009) is another potential benefit of sense-strand suppressive modification, which was not specifically addressed here.

Earlier studies of allele-specific siRNAs targeting keratin or collagen genes achieved 70–95% inhibition of the mutant allele (Hickerson *et al.*, 2008; Lindahl *et al.*, 2008; Atkinson *et al.*, 2011). The structure of the target mRNA could explain the difficulty in achieving similarly high inhibitory activity and specificity here. First, the G/C content of the Δ87 region attains 68–73%,

Abbreviations: COL7A1Δ87, COL7A1 mRNA with deleted exon 87; COL7A1WT, wild-type COL7A1 mRNA; DDEB, dominant dystrophic epidermolysis bullosa; Δ87, in-frame skipping of COL7A1 exon 87; IRES, internal ribosome entry site; NC, negative control siRNA; RISC, RNA-induced silencing complex; siRNA, small interfering RNA; siwt, positive control siRNA; WT, wild type