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Pemphigus Vulgaris IgG Directly Inhibit Desmoglein 3-Mediated Transinteraction

Wolfgang-Moritz Heupel,* Detlev Zillikens,† Detlev Drenckhahn,‡* and Jens Waschke‡*†

The autoimmune blistering skin disease pemphigus is caused by autoantibodies against keratinocyte surface Ags. In pemphigus vulgaris (PV), autoantibodies are primarily directed against desmosomal cadherins desmoglein (Dsg) 3 and Dsg 1, whereas pemphigus foliaceus (PF) patients only have Abs against Dsg 1. At present, it is unclear whether Dsg autoantibodies contribute to pemphigus pathogenesis by direct inhibition of Dsg transinteraction. Using atomic force microscopy, we provide evidence that PV-IgG directly interfere with homophilic Dsg 3 but, similar to PF-IgG, not with homophilic Dsg 1 transinteraction, indicating that the molecular mechanisms in PV and PF pathogenesis substantially differ. PV-IgG (containing Dsg 3 or Dsg 1 and Dsg 3 autoantibodies) as well as PV-IgG Fab reduced binding activity of Dsg 3 by ~60%, comparable to Ca²⁺ depletion. Similarly, the mouse monoclonal PV Ab AK 23 targeting the N-terminal Dsg 3 domain and AK 23 Fab reduced Dsg 3 transinteraction. In contrast, neither PV-IgG nor PF-IgG blocked Dsg 1 transinteraction. In HaCaT monolayers, however, both PV- and PF-IgG caused keratinocyte dissociation as well as loss of Dsg 1 and Dsg 3 transinteraction as revealed by laser tweezer assay. These data demonstrate that PV-IgG and PF-IgG reduce Dsg transinteraction by cell-dependent mechanisms and suggest that in addition, Abs to Dsg 3 contribute to PV by direct inhibition of Dsg transinteraction. The Journal of Immunology, 2008, 181: 1825–1834.

Pemphigus is a severe autoimmune blistering skin disease (1, 2) caused by autoantibodies against keratinocyte surface Ags (3–5). It has been demonstrated that pathogenic pemphigus autoantibodies are directed to the cadherin-type adhesion molecules desmoglein (Dsg) 3 and 1 (6–9). However, at present, there is evidence supporting the hypothesis that autoantibodies against other targets including cholinergic receptors or pemphaxin also contribute to skin blistering (10–12). Whether these different autoantibodies are pathogenic or just represent an epiphenomenon secondary to acantholysis is a matter of serious debate (13–15). It is widely accepted that there is a correlation between the clinical phenotype and the profile of Dsg autoantibodies (1, 2). Pemphigus foliaceus (PF) is usually characterized by epidermal blistering without development of mucosal erosions and an autoantibody profile including IgG directed to Dsg 1 but not to Dsg 3 (2, 16). Autoantibodies to Dsg 3, but not to Dsg 1, are usually present in patients with mucosal dominant pemphigus vulgaris (PV) (16, 17). In (PV) patients showing both mucous membrane and skin involvement, Abs to both Dsg 3 and Dsg 1 may be detected (2, 16, 17). However, there are also cases where the Abs profile against Dsg 1 and 3 in PV does not strictly correlate with these clinical phenotypes (18, 19). At present, direct evidence that the presence of Dsg 3-specific Abs in PV but not in PF may account for the more severe clinical phenotype of PV compared with PF patients is lacking.

Because Dsg 3 was found to be the target Ag of PV autoantibodies and to be a cadherin-type adhesion molecule, it was suggestive to believe that Dsg 3 Abs could directly interfere with Dsg 3 transinteraction (6). To test this hypothesis, some progress has been achieved by establishing mouse monoclonal Dsg 3 Abs, which are well characterized regarding the Dsg 3 extracellular subdomain they are directed to (20). It has been reported that AK 23, targeting the N-terminal extracellular domain 1 (EC 1) of Dsg 3, where the predicted adhesive interface is located (21, 22), is pathogenic. In contrast, Abs against the middle portion or the juxtamembrane part of the extracellular Dsg 3 domain had no effect (20). These data suggested that AK 23 could directly interfere with Dsg 3 transinteraction. This would be relevant because Abs to Dsg 1 and Dsg 3 in PV and PF patients are also primarily directed against the EC 1 subdomain (23). However, we found that PF-IgG caused keratinocyte dissociation and loss of Dsg 1 transinteraction without directly blocking Dsg 1 transinteraction, indicating that direct inhibition of Dsg transinteraction is not essential for PV pathogenesis and that Abs targeting the EC 1 subdomain do not necessarily induce direct inhibition (24). Taken together, evidence that Dsg 3 autoantibodies contribute to PV pathogenesis by directly blocking Dsg 3 transinteraction is lacking.

Therefore, the aim of the present study was to clarify whether Abs to Dsg 1 and Dsg 3 from PV patients reduced Dsg transinteraction by cellular signaling mechanisms or directly interfered with Dsg transinteraction. We used single-molecule atomic force microscopy (AFM) which allowed us to study Dsg transinteraction in a cell-free system and thus to rule out the contribution of any cellular signaling pathways. In contrast, we combined this approach with laser tweezer studies testing the binding of microbeads coated with human Dsg 1 and Dsg 3 to the surface of human HaCaT keratinocytes, thereby evaluating the contribution of cellular mechanisms to the effects of pemphigus IgG.
Pemphigus IgG were depleted of Dsg autoantibodies by immobilization of chimeric Dsg proteins using Talon dynabeads (Invitrogen) as previously reported for Dsg 1 (24). A total of 50 μg of each Dsg 1 and Dsg 3 were incubated with 5-mg beads in 200 μl of HBSS for 0.5 h at RT and slow overhead rotation. The supernatant was discharged and proteins were bound to beads were washed three times with HBSS (200 μl) using a magnetic tube holder. To absorb Dsg autoantibodies, PV-IgG 3 (200 μl containing 0.6 mg IgG) were applied to the beads and incubated for 0.5 h at RT and slow overhead rotation. The supernatant containing the Dsg IgG-depleted IgG fraction (PV-IgG 3 Abs) was finally collected and used for additional experiments. Generation of PF-IgG 1 Abs depleted of Dsg 1 autoantibodies was achieved similarly by incubation of PF-IgG 1 with Dsg 1 immobilized on Talon beads. PV- and PF-IgG Abs were used at 200 μg/ml.

Cytchemistry

HaCaT cells were grown on coverslips to confluence (7 days) and incubated with pemphigus IgG or mAbs for 24 h at 37°C. After incubation with autoantibodies, culture medium was removed and monolayers were fixed for 10 min in RT with 2% polyvinylalcohol (PVA)-containing paraformaldehyde in PBS. Afterward, monolayers were treated with 0.1% Triton X-100 in PBS for 5 min at RT. After rinsing with PBS at RT, HaCaT cells were preincubated for 30 min with 10% normal goat serum and 1% BSA in PBS at RT and incubated for 16 h at 4°C with mouse mAb directed to Dsg 3 (Zytomed) (dilution 1/100 in PBS). For experiments using mouse mAbs, AK 23, AK 18, AK 9, or Abg 1, a rabbit polyclonal Dsg 3 Ab was used (dilution 1/100 in PBS). After washing 3× with PBS at RT and a 5-min rinse with PBS (three washes for 5 min each), monolayers were incubated for 60 min at RT with Cy3-labeled goat anti-mouse or goat-anti-rabbit IgG (Dianova). Cells were then rinsed with PBS (three washes for 5 min each). Finally, coverslips were mounted on glass slides with 60% glycerol in PBS, containing 1.5% N-propyl gallate (Serva) as antifading compound. Monolayers were examined using a LSM 510 (Zeiss). Images were processed using Adobe Photoshop 7.0 software (Adobe Systems).

AFM measurements

Homophilic transinteractions of Dsg 1 and Dsg 3 were characterized by force-distance measurements of Dsg 1 or Dsg 3 coupled via flexible linkers to the tip and substrate of a Biora AFM driven by a Nanoscope III controller (Digital Instruments). Dsg 1 or 3 was linked covalently to the Si₃N₄ tip of the cantilever (Veeco Instruments) and freshly cleaved mica (Sigma-Aldrich) was incubated with Dsg 1 or 3 solution (100 μM) in 0.1 M HEPES, 5 mM CaCl₂, by force-distance cycles at amplitudes of 500 nm and at 2 Hz frequency. “Ca²⁺-free conditions” (no CaCl₂) were defined as buffer A without addition of CaCl₂. Force-distance cycles were performed at constant lateral positions and analyzed as described previously in detail (27). Binding activity was normalized to experiments using a cantilever not labeled with Dsg 1 or Dsg 3 to eliminate the contribution of unspecific interactions. For Dsg 1/3 heterophilic-binding analysis, heterophilic-binding activities of VE-cadherin-Fc with Dsg 1 or 3 were measured. These values were subtracted from heterophilic Dsg-binding activities before the latter were normalized to homophilic-binding activities of Dsg 1 or Dsg 3, respectively. For some experiments, both Dsg 1 and Dsg 3 at equal amounts were coated on mica or AFM tips.

Laser tweezers

Coating of polystyrene beads and the working principle of the laser tweezer set-up were described previously in detail (24). Coated beads (10 μl of stock solution) were suspended in 200 μl of culture medium and allowed to interact with HaCaT monolayers for 30 min at 37°C before measuring the number of bound beads (=control values). Beads were considered tightly bound when resisting laser displacement at 42 mW setting. For every condition, 100 beads were counted. Afterward, PV-IgG or PF-IgG were applied for 30 min. Percentage of beads resisting laser displacement under various experimental conditions was normalized to control values.

Electrophoresis and Western blotting

HaCaT cells grown for 1 or 7 days were dissolved in sample buffer, sonicated, heated at 95°C for 5 min, and finally subjected to SDS 7.5% PAGE.
and immunoblotting to Hybond nitrocellulose membranes (Amersham). Membranes were blocked with 5% low fat milk for 1 h at RT and incubated with anti-Dsg 1 (1/200; Progen) or anti-actin (1/3000; Sigma-Aldrich) primary Ab overnight at 4°C. As secondary Ab, HRP-labeled goat anti-mouse Ab (Dianova) was used. Visualization was achieved using the ECL technique (Amersham).

Statistics
Differences in bead adhesion or single-molecule transinteraction between different protocols have been assessed using the two-tailed Student t test. Values throughout are expressed as mean ± SEM. Statistical significance is assumed for p < 0.05.

Results
PV-IgG as well as PF-IgG induced cell dissociation in cultured human keratinocytes (HaCaT)

IgG fractions of six different patients with clinically, histologically, and serologically verified pemphigus were used (Table I). PV-IgG 1 and 2 contained autoantibodies against Dsg 1 and Dsg 3, whereas PV-IgG 3 and 4 contained Dsg 3-specific autoantibodies only. Abs to Dsg 1 but not to Dsg 3 were present in PF-IgG 1 and 2.

First, we studied the pathogenic effect of pemphigus IgG on cultured human keratinocytes (HaCaT; Fig. 1). Under control conditions or following treatment with IgG from a healthy volunteer (control IgG), Dsg 3 was continuously distributed along cell borders (Fig. 1, A and B). In contrast, PV-IgG treatment resulted in disruption of Dsg 3 staining. Cell dissociation leading to formation of intercellular gaps (indicated by arrows in Fig. 1D) was observed and further substantiated using F-actin staining for all PV-IgG fractions used (data not shown)—comparable to our previous studies (28, 29) (Fig. 1, C–E). It is noteworthy that the effects of PV-IgG were similar independent of whether Dsg 1 Abs were present (PV-IgG 1) or not (PV-IgG 3 and 4). Gap formation was also observed (arrows) when Fab of PV-IgG were used, whereas the profound disruption of Dsg 3 staining was abolished (Fig. 1F).

However, all pathogenic effects were eliminated by depletion of autoantibodies against Dsg 1 and Dsg 3 from PV-IgG using rDsg for immunoabsorption (Fig. 1G).

To further examine the effect of Dsg 3-specific PV Abs, we used three different mouse monoclonal PV Abs directed to well-characterized epitopes on the Dsg 3 extracellular domain (20). It has been shown that AK 23, which is directed against the Dsg 3 N-terminal EC 1, is pathogenic, whereas AK 9 directed against the middle part or AK 18 directed against the juxtamembrane part of the Dsg 3 extracellular domain are not. Consistent with these findings, when applied to HaCaT cells, AK 23 (75 μg/ml) disturbed continuous localization of Dsg 3 at cell borders whereas AK 9 and AK 18 had no effect (H–J). PF-IgG 1 and 2 as well as PF-IgG Fab caused intercellular gap formation (arrows in K–M), which where not seen in PF-IgG depleted of Dsg 1 IgG (PF-IgG 1 Abs, N). Note that the mouse mAb against Dsg 1 (aDsg 1) had no effect (O). Scale bar, 20 μm for all panels (n = 5).
shown). AK 9 and AK 18 had no effect (Fig. 1, H–J), even when applied at higher concentrations (data not shown). PF-IgG 1 and 2 as well as PF-IgG Fab induced keratinocyte dissociation leading to formation of intercellular gaps (arrows in Fig. 1, K–M). However, in contrast to PV-IgG, Dsg 3 staining was only missing at gap margins indicating that Dsg 1 Abs were sufficient to cause keratinocyte dissociation whereas Dsg 3 Abs in PV-IgG were responsible for profound fragmentation of Dsg 3 immunostaining. These effects were abolished by depletion of Dsg 1 Abs from PF-IgG (Fig. 1N). A mouse mAb directed against the extracellular domain of Dsg 1 (aDsg 1) had no effect (Fig. 1O).

**FIGURE 2.** Determination of single-molecule binding activities by AFM studies and characterization of chimeric Dsg proteins. Binding activities of Dsg fusion proteins covalently coupled to the tip and plate of the AFM via flexible PEG-linker were monitored by force-distance cycles (A). Molecules were brought into contact by repeated downward (approach) and upward movement (retrace) of the AFM tip (B). During upward movement, a downward deflection of the cantilever occurred if plate- and tip-bound Dsg molecules underwent specific transinteractions (C). After reaching a critical force, the bond broke and the cantilever jumped back to the neutral position. In a sample force-distance plot, several unbinding events of Dsg-Fc molecules are shown (D). Determination of binding activity was done by subtracting approach from retrace curves and integrating the area between the resulting curves. Chimeric proteins Dsg 1 and Dsg 3 used in this study but not BSA bound Ca$^{2+}$ as demonstrated with fluorescent Ca$^{2+}$ indicator quin-2 (E). Compared with homophilic transinteraction (Dsg 3/3 and Dsg 1/1), no specific binding activity was detected for heterophilic transinteraction of Dsg 3 and Dsg 1 in AFM experiments, no matter if Dsg 3 was coupled to the tip (Dsg 3/1) or the plate (Dsg 1/3) of the setup (F).
proteins, which we used in the following to study Dsg transinterac-
tion, retained the correct conformation during purification pro-
cessure (31, 32). This was supported by the ability of AK 23 to
immunoprecipitate rDsg 3 (data not shown). In addition, Ca\(^{2+}\)
binding of rDsg 1 and rDsg 3 was confirmed using the fluorescent
Ca\(^{2+}\) indicator quin-2 (Fig. 2E).

To investigate whether rDsg 1-Fc and rDsg 3-Fc, in addition to
homophilic, also undergo heterophilic transinteraction, we studied
the transinteraction of Dsg 3-Fc coupled to the tip of the cantilever
to Dsg 1-Fc molecules coupled to the plate (Dsg 3/1) and vice
versa (Dsg 1/3) (Fig. 2F). As negative controls, we used experi-
ments where transinteraction of Dsg 1 and Dsg 3 to VE-cadherin
was probed. When these values were subtracted and heterophilic-
binding activities were normalized to the levels of homophilic Dsg
3 and Dsg 1 transinteraction, heterophilic transinteraction of these
two molecules was negligible. Therefore, in the following exper-
iments, we focused on the effect of pemphigus IgG on homophilic
Dsg 1 and 3 interactions.

**PV-IgG and AK 23 blocked Dsg 3 transinteraction in cell-free
AFM experiments**

Dsg 3 transinteraction has so far not been probed by AFM single-
molecule experiments. As illustrated from the single unbinding
events in Fig. 2D, the resulting unit unbinding force of two transin-
teracting Dsg 3 molecules was in the range of 50 pN, which is
comparable to the unbinding force of Dsg 1 (24) and other cad-
herins probed under same conditions (27). Moreover, we observed
higher order unbinding events indicating additional mechanisms of
interaction. Dsg 3 transinteractions were strongly Ca\(^{2+}\) dependent;
Ca\(^{2+}\) depletion reduced Dsg 3-binding activity to 23 ± 3% (Fig.
3A). Interestingly, for all Dsg 3 autoantibody-containing PV-IgG
(PV-IgG 1, 3, and 4) a reduction of Dsg 3 transinteraction was
detected to 31 ± 7%, 38 ± 11%, and 44 ± 10% of controls,
respectively (Fig. 3A). To investigate whether PV-IgG cross-
linked Dsg 3 at the tip or the plate of the AFM setup and thereby
prevented Dsg 3 transinteraction, PV-IgG Fab were used. How-
ever, PV-IgG Fab still reduced Dsg 3 transinteraction to 51 ± 7%.
Together with the finding that PV-IgG depleted of Dsg 3 Abs
(PV-IgG 3 Abs) did not reduce Dsg 3 transinteraction (110 ± 19%,
Fig. 3A), these data indicate that Dsg 3-specific autoantibodies in
PV-IgG directly interfered with Dsg 3 transinteraction. Because in
previous studies we showed that PF-IgG did not block Dsg 1
transinteraction (24), we further analyzed the effect of PF-IgG on
Dsg 3 transinteraction. Not surprisingly, PF-IgG 1, which only
included Dsg 1 autoantibodies, did not interfere with Dsg 3
transinteraction (97 ± 14%, Fig. 3A).

Next, to investigate whether Dsg 3-specific PV Abs directly
hinder Dsg 3 transinteraction, mouse monoclonal PV Abs AK 23,
AK 18, and AK 9 were tested in Dsg 3 AFM experiments (Fig.
3B). Consistent with the pathogenicity of AK 23 in the mouse
model, only AK 23 (75 µg/ml) blocked Dsg 3 transinteraction in
AFM experiments to a comparable extent like Ca\(^{2+}\) depletion
(21 ± 9%). Higher concentrations of AK 23 up to 160 µg/ml
did not yield significantly different results (22 ± 5%). AK 23 Fab were
equally effective in blocking Dsg 3 transinteraction (15 ± 4%, Fig.
3B), whereas AK 18 (86 ± 2%) and AK 9 (107 ± 1%) had no
effect, even when applied at higher concentrations (data not
shown). Taken together, these data demonstrate that both PV-IgG
containing Dsg 3 autoantibodies as well as a monoclonal PV Ab
binding to the N-terminal EC 1 domain of Dsg 3 directly interfered
with Dsg 3 transinteraction.

**Dsg 1 transinteraction was not blocked by pemphigus IgG
in cell-free AFM experiments**

Because PV-IgG 1 also contained Dsg 1 autoantibodies, we next
examined the effect of PV-IgG on Dsg 1 transinteraction by AFM
force measurements. As illustrated in Fig. 4A, specific reduction of
Dsg 1-binding activity could be achieved by Ca\(^{2+}\) depletion or
addition of a mAb directed against the extracellular domain of Dsg
1 (aDsg 1). In these experiments, Dsg 1-binding activity was re-
duced to 20 ± 3% and 29 ± 7%, respectively. Surprisingly,
although including Dsg 1 autoantibodies, treatment with PV-IgG 1
did not change Dsg 1-binding activity (95 ± 5%). As to be ex-
pected, PV-IgG 3, only including Dsg 3 autoantibodies, PV-IgG
Fab and AK 23 also did not interfere with Dsg 1 transinteraction
(94 ± 13%, 96 ± 9%, and 101 ± 3%, respectively). In line with

![FIGURE 3.](http://www.jimmunol.org/)

**FIGURE 3.** PV-IgG and AK 23 blocked Dsg 3 transinteraction in AFM experiments. A, Binding activities of Dsg 3 in the presence or absence of different pemphigus IgG were probed by AFM. Depletion of Ca\(^{2+}\) by using a Ca\(^{2+}\)-free buffer (no Ca\(^{2+}\)) significantly reduced Dsg 3-binding activity to 20%. PV-IgG 1, 3, and 4 reduced binding activity of Dsg 3 to 31, 38, and 44% of control values, respectively. PV-IgG Fab also significantly blocked transinteraction to 51%, whereas Dsg autoantibody-depleted PV-IgG had no effect. Moreover, PF-IgG 1 did not interfere with Dsg 3 transinteraction. (n = 3–4 for each condition). B, AK 23, directed against the N-terminal domain of Dsg 3, blocked Dsg 3 transinteraction when applied at 75 µg/ml, up to 160 µg/ml or as monovalent Fab (21, 22, and 15% of control activity, respectively). AK 18 directed against the middle or AK 9 directed against the C terminus of the Dsg 3 extracellular domain had no effect (n = 3–4 for each condition).
and 95/H11006

IgG fractions blocked Dsg 1 transinteraction (97 treated four times), PF-IgG Fab, nor a pool of three additional PF-

observed: neither PF-IgG 1 at normal or higher doses (concen-

3 but not Dsg 1 transinteraction, whereas PF-IgG did not interfere with Dsg-mediated transinteraction in the cell-free system.

PV-IgG did not block Dsg 1 transinteraction in AFM experiments. A, Comparable to Fig. 3, binding activities of Dsg 1 were probed by AFM in the presence or absence of different pemphigus IgG. Depletion of Ca$^{2+}$ by using a Ca$^{2+}$-free buffer (no Ca$^{2+}$) as well as incubation with a mAb directed against the extracellular part of Dsg 1 (aDsg 1) significantly reduced Dsg 1-binding activity to 20 and 29% of controls, respectively. Although containing Dsg 1 autoantibodies, PV-IgG 1 as well as PV-IgG 3 and PV-IgG Fab did not block Dsg 1 transinteraction in this cell-free assay. Similarly, AK 23 directed against Dsg 3 did not affect Dsg 1 transinteraction. PF-IgG 1 and 2 as well as PF-IgG Fab did not interfere with Dsg 1 transinteraction. (n = 3–4 for each condition). B, Dsg 1 AFM experiments were repeated using rDsg 1 purified by Ni-NTA-column imidazole elution. This protein showed an identical Ca$^{2+}$ dependency of homophilic transinteraction compared with Dsg 1 purified via protein A column. Similarly, mouse monoclonal Dsg 1 Ab (aDsg 1) reduced Dsg 1-binding activity to prove the specificity of Dsg 1 interaction. Neither PV-IgG 1 and 2, both containing Dsg 1 and 3 autoantibodies, nor PV-IgG Fab reduced Dsg 1-binding activity. Moreover, PF autoantibodies also did not interfere with Dsg 1 transinteraction because neither PF-IgG 1 at normal or high (concentrated four times) doses, nor PF-IgG Fab or a pool of three additional PF-IgG fractions blocked Dsg 1 transinteraction (n = 3–4 for each condition).

DETECTED. CA$^{2+}$/H11006

B

Binding activity (% of control)

PV-IgG 1
PV-IgG 3
PV-IgG Fab
PV-IgG 1

Control

PV-IgG 1

PV-IgG 3

PV-IgG Fab

FIGURE 4. PV- and PF-IgG did not block Dsg 1 transinteraction in AFM experiments. A, Comparable to Fig. 3, binding activities of Dsg 1 were probed by AFM in the presence or absence of different pemphigus IgG. Depletion of Ca$^{2+}$ by using a Ca$^{2+}$-free buffer (no Ca$^{2+}$) as well as incubation with a mAb directed against the extracellular part of Dsg 1 (aDsg 1) significantly reduced Dsg 1-binding activity to 20 and 29% of controls, respectively. Although containing Dsg 1 autoantibodies, PV-IgG 1 as well as PV-IgG 3 and PV-IgG Fab did not block Dsg 1 transinteraction in this cell-free assay. Similarly, AK 23 directed against Dsg 3 did not affect Dsg 1 transinteraction. PF-IgG 1 and 2 as well as PF-IgG Fab did not interfere with Dsg 1 transinteraction. (n = 3–4 for each condition). B, Dsg 1 AFM experiments were repeated using rDsg 1 purified by Ni-NTA-column imidazole elution. This protein showed an identical Ca$^{2+}$ dependency of homophilic transinteraction compared with Dsg 1 purified via protein A column. Similarly, mouse monoclonal Dsg 1 Ab (aDsg 1) reduced Dsg 1-binding activity to prove the specificity of Dsg 1 interaction. Neither PV-IgG 1 and 2, both containing Dsg 1 and 3 autoantibodies, nor PV-IgG Fab reduced Dsg 1-binding activity. Moreover, PF autoantibodies also did not interfere with Dsg 1 transinteraction because neither PF-IgG 1 at normal or high (concentrated four times) doses, nor PF-IgG Fab or a pool of three additional PF-IgG fractions blocked Dsg 1 transinteraction (n = 3–4 for each condition).

our previous observation (24), Dsg 1-binding activity was not altered by treatment with PV-IgG 1 and 2 as well as PF-IgG Fab (84 ± 7%, 102 ± 14%, and 85 ± 5%, respectively).

The rDsg 1 used for this study was capable of depleting all pathogenic Dsg 1 Abs from PV- and PF-IgG fractions (Fig. 1, G and N), which is a strong indication that the correct conformation of Dsg 1 was retained (31). Nevertheless, this does not completely rule out the possibility that the pH shift during purification by protein A affinity chromatography induced minimal conformational changes of the Dsg 1 extracellular domain that inhibited binding of a small fraction of autoantibodies being capable of inducing direct inhibition of Dsg 1 transinteraction. Therefore, essentially all experiments were repeated using Dsg 1 purified via its his-tag by Ni-NTA columns and imidazole elution (Fig. 4B). This Dsg 1 protein also proved to be cleaved by ETA (data not shown). ETA cleavage is known to be strictly dependent on the proper folding of Dsg 1 (32). In AFM experiments, Ni-NTA-column-purified Dsg 1 displayed strong Ca$^{2+}$ dependency of homophilic Dsg 1 transinteraction and was blocked by the monoclonal Dsg 1 Ab (aDsg 1) (24 ± 3% and 27 ± 6% of control binding, respectively), similar to what was detected using Dsg 1 purified by the protein A column. Nevertheless, neither PV-IgG 1 and 2 (both containing Dsg 1 and 3 autoantibodies) nor PV-IgG Fab reduced Dsg 1-binding activity (106 ± 4%, 93 ± 3%, and 95 ± 3%, respectively). Similar to our previous investigations (24), no direct inhibition of Dsg 1 transinteraction by PF-IgG was observed: neither PF-IgG 1 at normal or higher doses (concentrated four times), PF-IgG Fab, nor a pool of three additional PF-IgG fractions blocked Dsg 1 transinteraction (97 ± 4%, 102 ± 3%, 96 ± 6%, and 101 ± 7%, respectively). Taken together, the AFM studies revealed that PV-IgG and AK 23 selectively blocked Dsg 3 but not Dsg 1 transinteraction, whereas PF-IgG did not interfere with Dsg-mediated transinteraction in the cell-free system.

Pemphigus IgG caused loss of binding of Dsg 1- and Dsg 3-coated microbeads to the surface of cultured human keratinocytes

To study the role of PV- and PF-IgG in the presence of cellular signaling mechanisms, we used the laser tweezer technique (Fig. 5). For this purpose, microbeads coated with human Dsg 1 or Dsg 3 were allowed to settle on the surface of HaCaT cells for 30 min. Afterward, we counted the number of bound beads resisting the separating forces of the laser beam (Fig. 5A). Fig. 5B summarizes all laser tweezer experiments using pemphigus IgG. Under control conditions, 77 ± 5% of Dsg 3 and 82 ± 2% of Dsg 1 beads could not be displaced by the laser beam focus and were taken as tightly bound (100%). Following incubation with 5 mM EGTA for 30 min to deplete extracellular Ca$^{2+}$, the number of bound Dsg 3- or Dsg 1-coated beads dropped to 22 ± 5% and 29 ± 6%, respectively, again documenting the strong Ca$^{2+}$ dependency of Dsg adhesion. When HaCaT cells with surface-bound beads were incubated with PV- or PF-IgG fractions for 30 min, the number of both Dsg 3- and Dsg 1-coated beads was significantly reduced. PV-IgG 1, containing Dsg 1 and 3 autoantibodies, reduced the number of Dsg 3 and Dsg 1 beads to 52 ± 8% and 45 ± 4%, respectively. PV-IgG 4, including Dsg 3 autoantibodies alone, reduced both Dsg 3- and Dsg 1-mediated binding as well (83 ± 6% and 71 ± 14%, respectively). Ab-mediated cross-linking was not required for loss of Dsg 3 binding because PV-IgG Fab also blocked Dsg 3 and Dsg 1 binding (48 ± 3% and 62 ± 5%, respectively). PV-IgG depleted of Dsg 1 and 3 Abs did not reduce the number of bound Dsg 3- or Dsg 1-coated beads (97 ± 3% and 101 ± 2%, respectively) indicating that autoantibodies specific for Dsg 1 and 3 mediate blocking of Dsg transinteraction in this assay.

In addition, following incubation with PF-IgG 1, the number of bound Dsg 3 and Dsg 1 beads dropped to 31 ± 8% and 50 ± 7%,
respectively. PF-IgG Fab had similar effects and reduced Dsg 3- and Dsg 1-mediated binding to 53% and 66%, respectively, whereas Dsg 1 autoantibody-depleted PF-IgG had no effects on Dsg 3 and Dsg 1 binding (101% and 104%, respectively). Incubation of monolayers with attached beads for 30 min with IgG fractions from PV patients (PV-IgG 1 containing autoantibodies to both Dsg 1 and Dsg 3 and PV-IgG 4, autoantibodies to Dsg 3 only) as well as with PV-IgG Fab significantly reduced the number of bound Dsg 3- and Dsg 1-coated beads. Depletion of Dsg IgG from pemphigus IgG abolished the reduction in Dsg 1- and Dsg 3-mediated bead binding. PF-IgG 1 (only containing autoantibodies to Dsg 1) or PF-IgG Fab also led to a loss in Dsg 1 and Dsg 3 bead binding, whereas Dsg 1 Ab-depleted PF-IgG had no effect (n = 6 for each condition).

We also examined the effect of mouse monoclonal PV Abs on binding of Dsg 3- and Dsg 1-coated beads to HaCaT cells (Fig. 5C). In contrast to pemphigus patients' IgG, monoclonal AK 23 Ab selectively reduced the number of bound Dsg 3 beads to 46% whereas the number of bound Dsg 1 beads was not affected (101%). Moreover, AK 23 Fab also reduced Dsg inhibiting binding of Dsg 1- and Dsg 3-coated beads to cultured keratinocytes via Dsg-specific autoantibodies.

FIGURE 5. Pemphigus IgG blocked Dsg 1 and Dsg 3 bead binding in laser tweezer experiments. A, Principle of laser tweezer experiments. On HaCaT cells, Dsg-coated microbeads could be trapped in a laser beam focus to distinguish bound from unbound beads. Dsg 3- (■) and Dsg 1-coated beads (□) were allowed to settle on the surface of HaCaT cells for 30 min (control) and bound beads were counted. B, Pemphigus IgG caused loss of binding of Dsg 1- and Dsg 3-coated microbeads to the surface of cultured human keratinocytes. The number of bound beads resisting laser beam displacement at 42 mW was reduced by simultaneous incubation with EGTA (5 mM, 30 min) to 22 and 29% for Dsg 3 and Dsg 1, respectively. Incubation of monolayers with attached beads for 30 min with IgG fractions from PV patients (PV-IgG 1 containing autoantibodies to both Dsg 1 and Dsg 3 and PV-IgG 4, autoantibodies to Dsg 3 only) as well as with PV-IgG Fab significantly reduced the number of bound Dsg 3- and Dsg 1-coated beads. Depletion of Dsg IgG from pemphigus IgG abolished the reduction in Dsg 1- and Dsg 3-mediated bead binding. PF-IgG 1 (only containing autoantibodies to Dsg 1) or PF-IgG Fab also led to a loss in Dsg 1 and Dsg 3 bead binding, whereas Dsg 1 Ab-depleted PF-IgG had no effect (n = 6 for each condition).

FIGURE 6. Presence of Dsg 3 did not alter PV- or PF-IgG-induced effects on cell-free Dsg 1 transinteraction but absence of Dsg 1 blocked PF-IgG-mediated effects on Dsg 3 bead binding. A, AFM tips were either coated with Dsg 1 alone (■) or in combination with Dsg 3 (□) and probed on substrates covered with an equal mixture of Dsg 1 and Dsg 3. PV-IgG 1 did not alter Dsg 1 transinteraction when tips were coated with Dsg 1 alone but was efficient at reducing binding activity when Dsg 3 was also present at AFM tips. In contrast, PF-IgG 1 had no effects under these experimental conditions (n = 3–4 for each condition). B, Dsg 1 Western blotting of HaCaT cells cultivated for either 1 or 7 days demonstrated that Dsg 1 was not present after 1 but after 7 days. β-actin was used to show equal loading of cell lysates (n = 3). C, In laser tweezer experiments with HaCaT cells cultivated for 1 day, PV-IgG 1 were effective at reducing the number of bound Dsg 3 beads. In contrast, PF-IgG 1 and 2 had no effect (n = 6 for each condition).
3-mediated binding to 62 ± 5%. In contrast, AK 18 and AK 9 did not alter the number of bound Dsg 3-coated beads on HaCaT cells (99 ± 2% and 100 ± 1%, respectively).

In other studies it has been shown that presence of Dsg 1 prevents PV-IgG from disrupting intercellular adhesion (33). Similarly, PF-IgG might require the presence of coexpressed Dsg 3 to disrupt homophilic transinteraction of Dsg 1. Therefore, we tested this hypothesis using AFM and laser tweezer experiments (Fig. 6). As a first step, AFM tips were either coated with Dsg 1 alone (Fig. 6A, ■) or in combination with Dsg 3 (Fig. 6A, □) and probed on substrates covered with an equal mixture of Dsg 1 and Dsg 3. PV-IgG 1, though containing Dsg 1 autoantibodies, did not alter Dsg 1 transinteraction when tips were coated with Dsg 1 alone (109 ± 26%) but was efficient at reducing binding activity when Dsg 3 was also present at AFM tips (reduction to 31 ± 8% of controls). In contrast, PF-IgG 1 had no effects under the two experimental conditions (110 ± 21% and 100 ± 8%, respectively). This indicated that the presence of Dsg 3 did not alter PV- or PF-IgG-induced effects on cell-free Dsg 1 transinteraction. We further tested the effects of PF-IgG on Dsg 3 bead binding on keratinocytes under conditions where Dsg 1 was absent. Western blotting (Fig. 6B) showed that Dsg 1 was not present when HaCaT cells were cultivated for 1 day after passaging but was detected after 7 days – the condition used in previous experiments (Fig. 5). In laser tweezer experiments with HaCaT cells cultivated for 1 day, PV-IgG 1 reduced the number of bound Dsg 3 beads to 62 ± 6% of controls (Fig. 6C). However, PF-IgG 1 and 2 had no effects (Dsg 3 bead binding 93 ± 5% and 99 ± 3%, respectively), in contrast to experiments with HaCaT cells cultured for 7 days and expressing Dsg 1 (Fig. 5). These data demonstrated that Dsg 1 is required to mediate the effects of PF-IgG on Dsg 3 binding in keratinocytes.

**Discussion**

We provide first evidence that Dsg 3 autoantibodies in PV directly inhibit Dsg 3 transinteraction, whereas in contrast Dsg 1 autoantibodies in PV- and PF-IgG reduce Dsg 1 transinteraction not directly but rather indirectly via cellular mechanisms. Using a combined approach of cell-free AFM studies, together with laser tweezer trapping of Dsg-coated microbeads on the surface of human keratinocytes, these conclusions are based on the following observations (Fig. 7): 1) PV-IgG containing Abs to both Dsg 3 and Dsg 1 as well as PV-IgG Fab selectively blocked Dsg 3 but not Dsg 1 transinteraction in single-molecule AFM studies. 2) PF-IgG and PF-IgG Fab did neither interfere with Dsg 1 nor with Dsg 3 transinteraction in AFM studies. 3) PV-IgG and PV-IgG Fab reduced binding of both Dsg 3- and Dsg 1-coated beads to the keratinocyte cell surface. 4) PF-IgG containing Dsg 1 but not Dsg 3 Abs as well as PF-IgG Fab also reduced binding of Dsg 3- and Dsg 1-coated beads. 5) The mouse monoclonal PV Ab AK 23 directed against the putative Dsg 3-binding site and AK 23 Fab specifically blocked Dsg 3 transinteraction in the presence and absence of cells. 6) mAbs against the middle and C-terminal parts of the extracellular Dsg 3 domain did not interfere with Dsg 3 transinteraction.

In a previous study, we showed that PF-IgG reduced transinteraction of Dsg 1 molecules only when assayed in keratinocyte cultures but not in the cell-free AFM setup (24). In the recent study, we confirmed these results and found in addition that autoantibodies to Dsg 1 in PV-IgG also did not directly interfere with Dsg 1 transinteraction. These negative findings seem not to be caused by a loss of proper conformation of Dsg 1, because Dsg 1 was able to bind Ca2+, to deplete all pathogenic Abs from PF-IgG (31) and also was cleaved by ETA (32). However, we cannot completely rule out that in vivo conformational changes of the Dsg 1 structure occur in response to autoantibody binding which are not equally present in our experiments. Nevertheless, a commercial mouse mAb directed against Dsg 1 blocked Dsg 1 transinteraction demonstrating that this EC 2-directed Ab (manufacturer’s specifications and our observations) may cause allosteric conformational changes which impair Dsg 1 adhesion. In clear contrast, we found that PV-IgG and PV-IgG Fab inhibited Dsg 3 transinteraction both in AFM studies and on keratinocytes. The use of Fab allows the conclusion that the loss of binding activity was not due to Ab cross-linking of molecules on the cantilever or the plate of the AFM. Abs to Dsg 3 in PV-IgG were equally effective at inhibiting Dsg 3 transinteraction in AFM studies like the monoclonal Dsg 3 Ab AK 23, which binds to the N-terminal EC 1 domain of Dsg 3 where the predicted binding interface is located and which is targeted by most of Dsg 3 Abs in PV patients (23). These data indicate that direct inhibition of Dsg 3 transinteraction was most likely caused by steric hindrance (34). However, because ~20% of PV Abs have been shown to bind to other parts of the Dsg 3 extracellular domain (23), at this stage we cannot completely rule out the possibility that some autoantibodies in PV-IgG fractions also interfered with Dsg 3 transinteraction by allosteric effects. Nevertheless, we found that AK 9 and AK 18, which target different parts of the extracellular domain, had no effect on Dsg 3 transinteraction. Finally, depletion of Dsg 3-specific Abs by immunoadsorption using rDsg 3 demonstrated that inhibition of Dsg 3 transinteraction was mediated by Dsg 3-specific Abs. Together with the findings that depletion of Dsg-specific Abs from PV-IgG and PF-IgG completely abolished keratinocyte dissociation and loss of Dsg 3 and Dsg 1 bead binding, these data further support the hypothesis that Dsg-specific Abs are required for pemphigus pathogenesis (13). This is also supported by the observation that, in the absence of Dsg 1 in HaCaT cells, PF-IgG-mediated loss of Dsg 3 bead binding was abolished indicating that Dsg 1 is the major autoantigen required for autoantibody-mediated outside-in signaling.

Because heterophilic transinteraction of desmosomal cadherins is thought to be important (35, 36), we also sought to address this issue in our study. However, because we did not observe heterophilic interactions of Dsg 3 and Dsg 1, we were only in the position to probe direct inhibition of homophilic transinteraction. Nevertheless, we can rule out the possibility that the presence of both Dsg 1 and Dsg 3 is needed for PV- or PF-IgG to alter Dsg transinteraction in cell-free single-molecule experiments. It has to be emphasized that this does not exclude the possibility that PV- or PF-IgG also directly interfere with the transinteraction of Dsg 3 or...
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Disclosures

The authors have no financial conflict of interest.

References


