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*J Immunol* 2003; 170:2170-2178; doi: 10.4049/jimmunol.170.4.2170

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Induction of Pemphigus Phenotype by a Mouse Monoclonal Antibody Against the Amino-Terminal Adhesive Interface of Desmoglein 3

Kazuyuki Tsunoda,*† Takayuki Ota,† Miyo Aoki,* Taketo Yamada,‡ Tetsuo Nagai,† Taneaki Nakagawa,† Shigeo Koyasu,§ Takeji Nishikawa,* and Masayuki Amagai**

Pemphigus vulgaris (PV) is a life-threatening autoimmune blistering disease that is caused by IgG autoantibodies against the cadherin-type adhesion molecule desmoglein (Dsg)3, which is a desmosomal transmembrane glycoprotein that belongs to the cadherin superfamily of cell-cell adhesion molecules (2–4). Compelling evidence exists to indicate that IgG autoantibodies against Dsg3 play a pathogenic role in blister formation in PV. When whole IgG from patients’ sera or IgG affinity purified on human recombinant Dsg (hDsg)3 were injected into neonatal mice, the mice developed intraperiodal blister formation with the typical histological features of PV (5–7). In addition, PV patients’ sera, from which the anti-Dsg3 IgG was depleted by immunoabsorption with hDsg3, no longer induced blistering in neonatal mice (8).

The titers of serum anti-Dsg3 IgG autoantibodies, as measured by indirect immunofluorescence or ELISA, generally correlate with disease activity when monitored in individual patients (9–12). However, these titers are not absolute indicators for the severity of the disease among groups of patients, and it is sometimes the case that patients with low titers of anti-Dsg3 IgG autoantibodies show severe phenotypes, while patients with high titers show mild phenotypes (10, 11). It is not known whether all IgG autoantibodies that bind in vivo to the native Dsg3 are equally pathogenic, or whether each anti-Dsg3 IgG has a distinct potency for the induction of blister formation. Furthermore, if different autoantibodies have different pathogenic activities, what defines pathogenic strength at the molecular level? The major obstacle to addressing this question is that patients’ sera contain polyclonal autoantibodies that react with different parts of the Dsg3 molecule (13, 14). Therefore, it is not feasible to compare the pathogenic activities of individual anti-Dsg3 IgGs using patients’ sera. Although several reports describe the generation of human mAbs from patients (15–17), the isolation of a comprehensive set addressing this question is that patients’ sera contain polyclonal autoantibodies that react with different parts of the Dsg3 molecule (13, 14). Therefore, it is not feasible to compare the pathogenic activities of individual anti-Dsg3 IgGs using patients’ sera. Although several reports describe the generation of human mAbs from patients (15–17), the isolation of a comprehensive set of human mAbs to compare differences in activities has proven difficult.

Recently, we developed an active disease mouse model for PV by using a novel approach, which involves autoantigen-knockout mice that do not acquire tolerance to the defective gene product (18). The adoptive transfer of splenocytes from mDsg3-immunized Dsg3−/− mice to Dsg3-expressing Rag2−/− recipient mice resulted in the stable production of anti-Dsg3 IgG and the development of PV phenotypes, which included oral erosions with typical PV histology. Thus, the PV model mice produced pathogenic anti-Dsg3 IgG Abs. To address the issue mentioned above, we used the PV model mice to generate a panel of anti-Dsg3 IgG mAbs that could bind in vivo to different parts of the native Dsg3. We then examined the pathogenic activities of these mAbs using a well-established passive transfer assay, as well as an ascites formation assay in adult mice, and we characterized the mAb-binding epitopes on Dsg3. In this study, we demonstrate that the in vivo binding of IgG to Dsg3 is not sufficient to cause blistering, and that
affinity metal resin (Clontech, Palo Alto, CA), according to the manufacturer’s recommendation.

**ELISA and live keratinocyte staining**

The reactivities of acantholytic keratinocyte (AK) mAbs with hDsg3, mDsg1, hDsg3, and hDsg1 were measured by ELISA using the respective Dsg molecules, as described previously (10, 11, 18). Live keratinocyte staining was performed using the mouse keratinocyte cell line PM2212, as described previously (18).

**Passive transfer assays using neonatal mice**

To evaluate the pathogenic activities of the AK mAbs, we performed a passive transfer study with neonatal mice, as described previously (5, 7, 8). We injected AK mAbs (75–200 μg/mouse) alone or together with a small amount of total IgG that was prepared by ammonium sulfate precipitation of pemphigus foliaceus (PF) serum or a small amount of exfoliative toxin A (ETA), which specifically cleaves Dsg1 (22). The dosages of PF IgG and ETA were set at 50% of the minimum dose needed to induce gross blisters, i.e., 1 mg/mouse of PF IgG and 1 μg/mouse of ETA. Neonatal ICR mice at 12–24 h of age (body weight of 1.5–2.0 g) were used (Japan SLC, Shizuoka, Japan), and the skin was evaluated grossly and microscopically 18–24 h after injection. To evaluate microscopic blisters, the entire body skin was sectioned into six strips of ~3 mm in width. Blister formation was assessed as positive when two or more sites with suprabasilar acantholysis were noted in whole sections.

**Ascites formation assay**

We developed an assay using hybridoma cells to evaluate the pathogenic activities of AK mAbs in adult mice. We inoculated i.p. 5 × 10^7–10^8 hybridoma cells of each AK mAb into Rag2^−/− mice that were primed with 2.6,10,14-tetramethyl-pentadecane (Wako Pure Chemical Industries, Osaka, Japan). The inoculated mice were monitored for ascites formation as well as the appearance of the PV phenotype, which was manifested by weight loss and patchy hair loss. Biopsies of the oral mucous membranes

### Table I. Primers used for PCR amplification of the domain-swapped hDsg molecules

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Primers hDsg1</th>
<th>Primers hDsg3</th>
</tr>
</thead>
<tbody>
<tr>
<td>hDsg31–34/hDsg135–496</td>
<td>primer 1, primer 2</td>
<td>primer 3, primer 4</td>
</tr>
<tr>
<td>hDsg31–10/hDsg111–146</td>
<td>primer 5, primer 2</td>
<td>primer 3, primer 6</td>
</tr>
</tbody>
</table>

* Primer 1, 5′-ccagcaaccagaacaatcatacactcaggtga-3′; primer 2, 5′-tctgctc atctggtcagtcga-3′; primer 3, 5′-gacaagctctggctctgctgctcagggctg-3′; primer 4, 5′-tctggagcaccgggtctgctgctcagggctg-3′; primer 5, 5′-ctggagcaccgggtctgctgctcagggctg-3′; primer 6, 5′-tctggagcaccgggtctgctgctcagggctg-3′.

### Table II. Primers used for PCR amplification of the point-mutated hDsg1/hDsg3 molecules

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Primers</th>
<th>Amino Acid Change</th>
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</thead>
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<td>7, 9, 11, 12</td>
<td>T 25 H, Y 28 C, Q 29 A, T 31 N, K 33 Q, I 34 V</td>
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<tr>
<td>hDsg1-M1-2</td>
<td>8, 10, 15, 16</td>
<td>H 25 T, C 28 Y, A 29 Q, N 31 T, Q 33 K, V 34 I</td>
</tr>
<tr>
<td>hDsg1-M1-2-3</td>
<td>8, 10, 17, 18</td>
<td>Y 48 F, I 53 V, N 54 D, Q 55 K, K 56 N</td>
</tr>
<tr>
<td>hDsg3-M7</td>
<td>7, 9, 19, 20</td>
<td>V 3 I, K 7 A, P 8 A</td>
</tr>
<tr>
<td>hDsg3-M8</td>
<td>7, 9, 21, 22</td>
<td>D 59 E</td>
</tr>
<tr>
<td>hDsg1-M7</td>
<td>8, 9, 10, 23</td>
<td>I 3 V, A 7 K, A 8 P</td>
</tr>
<tr>
<td>hDsg1-M7-8</td>
<td>8, 10, 23, 24, 25, 26</td>
<td>I 3 V, A 7 K, A 8 P, E 59 D</td>
</tr>
</tbody>
</table>

* Primer 7, 5′-gcaacagctggtctgctcaggtgtaaag-3′; primer 8, 5′-gcaacagctggtctgctcaggtgtaaag-3′; primer 9, 5′-gcaacagctggtctgctcaggtgtaaag-3′; primer 10, 5′-tctggagcaccgggtctgctgctcagggctg-3′; primer 11, 5′-ctggagcaccgggtctgctgctcagggctg-3′; primer 12, 5′-gacaacagctggtctgctcaggtgtaaag-3′; primer 13, 5′-tctggagcaccgggtctgctgctcagggctg-3′; primer 14, 5′-tctggagcaccgggtctgctgctcagggctg-3′; primer 15, 5′-tctggagcaccgggtctgctgctcagggctg-3′; primer 16, 5′-gacaacagctggtctgctcaggtgtaaag-3′; primer 17, 5′-gacaacagctggtctgctcaggtgtaaag-3′; primer 18, 5′-gacaacagctggtctgctcaggtgtaaag-3′; primer 19, 5′-ctggagcaccgggtctgctgctcagggctg-3′; primer 20, 5′-ctggagcaccgggtctgctgctcagggctg-3′; primer 21, 5′-tctggagcaccgggtctgctgctcagggctg-3′; primer 22, 5′-tctggagcaccgggtctgctgctcagggctg-3′; primer 23, 5′-tctggagcaccgggtctgctgctcagggctg-3′; primer 24, 5′-tctggagcaccgggtctgctgctcagggctg-3′; primer 25, 5′-tctggagcaccgggtctgctgctcagggctg-3′; primer 26, 5′-tctggagcaccgggtctgctgctcagggctg-3′.

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**Materials and Methods**

**Mice**

PV model mice were produced, as previously described (18). In brief, 6- to 10-wk-old Dsg3−/− mice were immunized s.c. with 5 μg of purified mouse recombinant Dsg (mDsg3 in CFA), boosted twice for 2 wk i.p. with mDsg3 in IFA, and boosted twice for 2 wk with soluble mDsg3. Splenocytes from the immunized mice were adoptively transferred into C57BL/6 Rag2−/− mice (Central Institute for Experimental Animals, Tokyo, Japan) via the tail vein. Recipient mice with PV phenotype were fed with moistened food (Oriental Yeast, Tokyo, Japan) to improve food intake. Anti-Dsg3 Ab production was examined by ELISA and live keratinocyte staining. All mouse studies were approved by the animal ethics review board of Keio University.

**Production of mAbs**

Splenocytes were isolated from mice that had the active PV phenotype, and fused with P3 mouse myeloma cells (a kind gift from S. Tsukita (Department of Cell Biology, Kyoto University Faculty of Medicine)) at a ratio of 5:1 with PEG 4000 (Merck, Darmstadt, Germany), followed by selection with hypoxanthine aminopterin thymidine in the presence of 10% hybridoma cloning factor (IGEN, Gaithersburg, MD). Hybridoma cells were screened initially using mDsg3 ELISA, and positive clones were tested subsequently by live keratinocyte staining. Of 220 ELISA-positive clones, 11 clones were positive by live keratinocyte staining and 8 of them were further analyzed. Each clone was obtained by three replicates of the limiting dilution method. The isotypes of the mAbs were determined using the Isotyping Kit (Roche Diagnostics, Mannheim, Germany). The mAbs were purified from culture supernatants using the HitTrap Protein A FF column (Amersham Bioscience, Piscataway, NJ).

**Production of recombinant proteins**

Some of the recombinant proteins used in this study have been described previously (10, 14). In this study, two domain-swapped and eight point-mutated hDsg1/hDsg3 molecules were produced using the baculovirus expression system. Four domain-swapped mDsg1/mDsg3 molecules were also produced (H. Anzai, Y. Fujii, T. Nishikawa, and M. Amagai, unpublished results). To generate the domain-swapped molecules, various regions of the hDsg1 and hDsg3 were PCR amplified with the appropriate primers (Table I), digested with either NcoI/XhoI for the hDsg1 fragments or NcoI/EcoRI for the hDsg3 fragments, and subcloned into the pQE-Tri-System vector (Qiagen, Hilden, Germany). To generate the point-mutated molecules, mutations were introduced by two-step PCR using the appropriate primers (Table II), as described previously (19). The PCR products were digested with either NcoI/XhoI for the Dsg1 fragments or NcoI/EcoRI for the Dsg3 fragments, and subcloned into the pQE vector. Recombinant proteins were obtained, as previously described (7, 20, 21). In brief, recombinant baculoviruses were obtained by cotransfection of each construct with Baculo Saffhire DNA (Oribigen, San Diego, CA) into cultured insect Sf9 cells. High titer cells (Invitrogen, San Diego, CA) cultured in serum-free EX cell medium (IRH Bioscience, Lenexa, KS) were infected with the recombinant viruses, and incubated at 27°C for 3–4 days. Recombinant proteins produced in the culture supernatants were purified on TALON

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The AK23 epitope is conserved between the mouse and human Dsg3 proteins. Human skin were used as the substrates. Splenocytes of PV model mice to produce anti-Dsg3 IgG mAbs. With resultant blister formation. We used hybridoma cells from the IgGs that can induce the loss of cell-cell adhesion of keratinocytes. Results

5 mutated Dsg1/Dsg3 molecules to determine the mAb epitopes. Typically, ascites formation was observed after day 14. (c) and AK23 (c) bind to keratinocyte cell surfaces in similar patterns to that of polyclonal sera from PV model mice (a; inset, normal mouse serum), when added to cultures of mouse keratinocyte PAM212 cells. The reactivity of AK19 and AK23 mAbs was removed when those mAbs were immunoadsorbed with mDsg3-His (insets in b and c). AK23 mAb (e) as well as mouse sera from PV model mice (d) stain the lower parts of the human epidermis in which Dsg3 is expressed. AK23 did not stain the cell surfaces of epithelial cells in mouse heart (f) and liver (g) in which Dsg3 is not expressed. Bar = 50 μm.

and skin were taken when mice developed the PV phenotype or when ascites formation was observed after day 14.

**Epitope mapping by immunoprecipitation**

Immunoprecipitation was performed using the domain-swapped or point-mutated Dsg1/Dsg3 molecules to determine the mAb epitopes. Typically, 5 μg of purified mAb and 300 μl of culture supernatant that contained the recombinant proteins were mixed and incubated at room temperature for 30 min. Anti-E-tag mAb (Amersham Bioscience) was used as a positive control. The proteins were immunoprecipitated with protein G-Sepharose (Amersham Bioscience) at 4°C overnight. The immunoprecipitants were applied to SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The recombinant proteins were visualized with an anti-6× histidine Ab (R&D Systems, Minneapolis, MN). For the EDTA treatment, culture supernatants that contained the recombinant proteins were incubated with 5 mM EDTA for 30 min at room temperature, and subjected to immunoprecipitation, as described above.

**Results**

**Generation of anti-Dsg3 IgG mAbs from PV model mice**

PV model mice with active disease have circulating anti-Dsg3 IgGs that can induce the loss of cell-cell adhesion of keratinocytes with resultant blister formation. We used hybridoma cells from the splenocytes of PV model mice to produce anti-Dsg3 IgG mAbs. Initially, we screened the hybridomas by ELISA using mDsg3; positive clones were further screened by live staining of mouse keratinocyte PAM212 cells (Fig. 1, a–c). The second screening selected mAbs that could bind to the native Dsg3 on keratinocyte cell surfaces in vivo. Eight independent clones were isolated and designated as AK mAbs (Table III).

All of the mAbs had the IgG1 isotype for the H chain and the κ isotype for the L chain. Using indirect immunofluorescence with different tissue substrates, we found that all of these mAbs reacted with the cell surfaces of stratified squamous epithelia, such as those in the hard palate and skin, but not with simple epithelia, such as found in the liver, heart, and intestine (Table III; Fig. 1, f and g). Some mAbs (AK1, 15, 18, 19, 20, and 23) cross-reacted with human skin or mucosa. These mAbs stained entire layers in the mucosa and the lower layers of the human epidermis in which Dsg3 was expressed (23, 24) (Fig. 1, d and e). All of the mAbs reacted exclusively, as assessed by ELISA, with the recombinant extracellular domain of the mDsg3, except AK1, which cross-reacted with mDsg1 (Table III). The mAbs that had detectable cross-reactivity by immunofluorescence with human tissues also reacted with hDsg3 in the ELISA (Table III).

**Pathogenic activities of AK mAbs following passive transfer to neonatal mice**

To determine whether the AK mAbs could induce blistering, we performed a passive transfer assay using neonatal mice, which is a well-established assay for pemphigus (5). Purified IgG from the culture supernatant of hybridoma cells was injected s.c. into neonatal mice, and the mice were observed for 18–24 h after injection (Table IV). The neonatal mice that were injected with AK19 or AK23, but not those that received the other mAbs, developed microscopic blisters with suprabasilar acantholysis in histology, although none of these mice developed apparent gross blisters (Fig. 2, a and b). The failure to induce gross blisters was not unexpected, because Dsg1 coexpression in the skin of neonatal mice compensates for the impaired adhesive function of Dsg3 (25, 26).

To overcome this problem, we used IgG preparations from PF sera that contained anti-Dsg1 IgG. We titrated the pathogenic strength of the PF IgG, and determined the minimum dose that induced gross blisters. Neonatal mice that were cojected with AK19 and one-half of the minimum dose of PF IgG developed extensive gross blistering with suprabasilar acantholysis at 18–24 h (Fig. 2e), although mice that received PF IgG alone did not show blister formation (Fig. 2c). The neonatal mice that were cojected

<p>| Table III. Characterization of the AK mAbs |
| --- | --- | --- | --- | --- | --- | --- |</p>
<table>
<thead>
<tr>
<th>AK</th>
<th>Isotype</th>
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<th>Human</th>
<th>Mouse</th>
<th>Human</th>
<th>Live</th>
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<th>Ascites formation</th>
<th>Pathogenic Activity</th>
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<tr>
<td>1</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>V3, K7, P8, D59</td>
</tr>
</tbody>
</table>

Indirect immunofluorescence staining. Mouse oral mucous membrane (hard palate), mouse liver (the same negative staining was observed with heart and intestine), and human skin were used as the substrates.

The reactivity of AK mAbs against mDsg3 was determined in the presence and absence of EDTA treatment. +, Indicates the abolition of activity by EDTA.

The epitopes of AK7, AK9, AK15, AK18, and AK20 are indicated by the residue numbers for mDsg3. The AK19 epitope is indicated by the residue numbers for hDsg3. The AK23 epitope is conserved between the mouse and human Dsg3 proteins.
with AK23 and the same dose of PF IgG also had extensive blistering (Fig. 2f). No apparent blisters appeared following coinfection of mice with PF IgG and any of the remaining mAbs (data not shown).

We also took another approach to inactivate the adhesive function of Dsg1 in this neonatal mouse assay. Recently, it was shown that ETA, which is a serine protease that is produced by Staphylococcus aureus, specifically digests Dsg1 (22, 27). We titrated the activity of ETA in the manner described above. When either AK19 or AK23 was coinjected with one-half of the minimum effective dose of ETA, the mice demonstrated extensive blistering, although mice that were injected with ETA alone did not have any apparent blisters (Fig. 2d, g, and h). Judging from the number of mice with gross blisters, AK23 appeared to be more potent than AK19 (Table IV). Blister formation did not develop in mice that were coinjected with ETA and any of the remaining mAbs (data not shown).

These findings indicate that the AK19 and AK23 mAbs cause the loss of cell-cell adhesion of keratinocytes in neonatal mice, while the other AK mAbs do not have apparent blister-inducing activities.

Pathogenic activities of AK mAbs, as assessed by ascites formation in adult mice

We developed an assay using hybridoma cells to further determine whether AK mAbs showed pathogenic activity in inducing blisters in adult mice. We i.p. inoculated hybridoma cells into Rag2−/− immunodeficient mice, and evaluated the appearance of the PV phenotype, which was manifested by weight loss, patchy hair loss, and mucosal erosions (Table IV).

Mice that received AK23 hybridoma cells showed patchy hair loss and sudden mortality between days 7 and 9, which preceded ascites formation (Fig. 3a–c). In these mice, in vivo IgG deposition was found on keratinocyte cell surfaces of stratified squamous epithelia, which included the oral and esophageal mucosal membranes and the skin, as was seen in both PV model mice and patients with PV (Fig. 3e). Histological examination of these mice revealed suprabasilar acantholysis in the hard palate (Fig. 3g) and in the skin around the snout. The development of oral erosions probably inhibited food and water intake, which led to dehydration and eventual death. Skin biopsies around the area of patchy hair loss showed intense IgG depositions on the cell surfaces of keratinocytes that surrounded the telogen hair club (data not shown), and cleft formation between the cells that surrounded the telogen club and the basal layer of the outer root sheath epithelium (Fig. 3h). The bald skin contained empty dilated telogen hair follicles. These gross, histologic, and immunopathologic findings were virtually identical with those observed in PV model mice, which indicates that AK23 is pathogenic and capable of regenerating the phenotype of the PV model mouse.

When hybridoma cells of the other AK mAbs (including AK19) were used, the murine PV phenotype did not develop, even after obvious ascites fluid formation at day 14 (Fig. 3d). Although all of the mice showed clear in vivo IgG deposition on keratinocyte cell surfaces in the stratified squamous epithelia, no blister formation was observed in the oral mucosa at the histological level (Fig. 3f; Table IV). The titers of circulating AK mAbs were measured by mDsg3 ELISA in mice that had apparent ascites formation (Table IV). For the mice that received AK23, the titers were measured when they showed signs of hair loss at days 7–9. The mAb titers were virtually identical with those observed in PV model mice.

![FIGURE 2](http://www.jimmunol.org/) AK19 and AK23 induce PV blisters in the passive transfer assay. Neonatal mice that were injected with AK19 (a) or AK23 (b) developed microscopic blisters with suprabasilar acantholysis without apparent gross blistering. Neonatal mice that were coinjected with either AK19 (e) or AK23 (f) and PF IgG (c) in which the individual dosages were insufficient to induce blisters developed extensive blistering (arrows) with suprabasilar acantholysis in histology. Neonatal mice that were coinjected with either AK19 (g) or AK23 (h) and ETA (d; in which the individual dosages were insufficient to induce blisters) developed extensive blistering (arrows) with the typical histology of PV. Bars = 50 μm.
of mice with AK15, 19, and 20 were at least 4-fold higher than those of mice with AK23, which excludes the possibility that the failure to induce blister formation was due to insufficient mAb production. These findings indicate that AK1, 7, 9, 15, 18, 19, and 20 are not sufficiently potent to induce the loss of cell-cell adhesion of keratinocytes in adult mice that have ascites formation.

Taken together, our findings indicate that AK23 induces blister formation with typical PV phenotype in both assays, whereas AK19 induces PV blisters in the passive transfer assay, but not in the ascites formation assay. The remaining mAbs lacked apparent pathogenic activities in either assay. Therefore, AK23 has the most potent pathogenic activity among the AK mAbs used in this study. AK19 also has pathogenic activity, but is weaker than AK23. The other AK mAbs have no apparent blister-inducing activities per se.

The pathogenic AK23 mAb recognizes a calcium-dependent conformational epitope consisted of V3, K7, P8, and D59 at the N terminus of Dsg3

To characterize the epitopes of the AK mAbs, we first determined whether mAb binding to Dsg3 was dependent on calcium. mDsg3-His was left untreated or treated with EDTA, and subjected to immunoprecipitation with the AK mAbs (Table III; Fig. 4a). EDTA treatment abolished the reactivities of the AK19 and AK23 mAbs, but not those of the other AK mAbs. When a mDsg3-coated ELISA plate was treated with EDTA, the binding of AK19 and AK23 was also abolished, while the binding of the other AK mAbs was not affected significantly (data not shown). Therefore, the AK19 and AK23 mAbs recognize calcium-dependent epitopes, while the other AK mAbs recognize calcium-independent epitopes.

Recombinant Dsg3-His is produced by baculoviruses as a doublet protein, in which the lower and upper bands are the mature and immature forms, respectively (8, 10, 27). When the hDsg3-His was immunoprecipitated with the AK mAbs as well as with anti-E-tag Abs, Dsg3 forms, respectively (8, 10, 27). When the hDsg3-His was immunoprecipitated with the AK mAbs, lane 1, mDsg31–162/mDsg3163–512; lane 2, mDsg31–402/mDsg3163–512; lane 3, mDsg31–194/mDsg3195–565; lane 4, mDsg31–402/mDsg3195–565; lane 5, hDsg31–24/hDsg353–566; lane 6, hDsg31–64/hDsg353–566; lane 7, hDsg31–87/hDsg353–566; lane 8, hDsg31–103/hDsg353–566; lane 9, hDsg31–118/hDsg353–566; lane 10, hDsg31–139/hDsg353–566; lane 11, hDsg31–162/hDsg353–566; lane 12, hDsg31–182/hDsg353–566. d, Immunoprecipitation of point-mutated Dsg1/Dsg3 proteins with AK23 mAb (upper panel). Anti-E-tag mAb was used as a positive control (lower panel). Lane 1, hDsg1-M3; lane 2, hDsg1-M2; lane 3, hDsg1-M3; lane 4, hDsg3-M4; lane 5, hDsg3-M3; lane 6, hDsg3-M6; lane 7, hDsg3-M7; lane 8, hDsg3-M4; lane 9, hDsg1-M1–2; lane 10, hDsg1-M1–2-3; lane 11, hDsg1-M7; lane 12, hDsg1-M7–8.

We then attempted to map the epitopes of the AK mAbs by immunoprecipitation of domain-swapped Dsg1/Dsg3 recombinant molecules. It is believed that these domain-swapped Dsg1/Dsg3 molecules maintain the proper conformation, at least in terms of the binding of IgG autoantibodies, as determined previously (13, 14). Initially, we conducted gross mapping of the epitopes on Dsg3 using four domain-swapped molecules that contained the mDsg3 residues 1–162 (mDsg31–162/mDsg3163–512), 1–402 (mDsg31–402/mDsg3195–565), and 1–565 (mDsg31–565/mDsg3195–565).
403–565 (mDsg1\(^{1–402}\)/mDsg3\(^{403–565}\)) (Figs. 4c and 5a). The AK1 epitope could not be determined using these domain-swapped molecules, because AK1 cross-reacted with both Dsg3 and Dsg1 (Table III). The AK7, AK9, and AK20 mAbs precipitated residues 195–565 and 403–565, but not residues 1–162 and 1–402. Therefore, the epitopes of AK7, AK9, and AK20 appear to reside in residues 403–565 of the mDsg3, which represents the C-terminal portion of the extracellular domain. The AK15 and AK18 mAbs reacted with residues 1–402 and 195–565, but not with residues 1–162 and 403–565. Therefore, the epitopes of AK15 and AK18 appear to be present in residues 195–402 of the mDsg3, which represents the middle portion of the extracellular domain. The AK19 and AK23 mAbs recognized residues 1–162 and 1–402, but not residues 195–565 and 403–565. Therefore, the epitopes of AK19 and AK23 appear to be present in residues 1–162 of the mDsg3, which represents the amino-terminal portion of the extracellular domain. The mAbs that cross-reacted with hDsg3 (AK15, 18, 19, 20, and 23) gave compatible mapping results with domain-swapped molecules using hDsg3 and hDsg1 (data not shown).

We identified more precisely the epitopes of AK19 and AK23, because these mAbs showed blister-induction activities. Because both of these mAbs cross-reacted with hDsg3, but not with hDsg1, and because their epitopes were at the amino terminus, we used eight additional domain-swapped hDsg3/hDsg1 molecules that contained the hDsg3 residues 25–566 (hDsg1\(^{1–24}/\text{hDsg3}^{25–566}\)), 65–566 (hDsg1\(^{1–64}/\text{hDsg3}^{65–566}\)), 87–566 (hDsg1\(^{1–87}/\text{hDsg3}^{88–566}\)), 1–88 (hDsg3\(^{1–88}/\text{hDsg1}^{89–496}\)), 1–63 (hDsg3\(^{1–63}/\text{hDsg1}^{64–496}\)), 1–34 (hDsg3\(^{1–34}/\text{hDsg1}^{35–496}\)), 1–26 (hDsg3\(^{1–26}/\text{hDsg1}^{27–496}\)), and 1–10 (hDsg3\(^{1–10}/\text{hDsg1}^{11–496}\)) (Fig. 5b). AK19 precipitated residues 87–566, but not residues 1–88 (Fig. 4c). Therefore, the AK19 epitope appears to be located in residues 87–161 of hDsg3. AK23 immunoprecipitated residues 1–88 and 1–63, which indicates that the AK23

![Figure 5](http://www.jimmunol.org/DownloadedFrom.png)

**FIGURE 5.** Molecular structures of the domain-swapped and point-mutated Dsg3/Dsg3 recombinant proteins, and their reactivities with the AK mAbs. Each of these constructs has a His-tag at the C terminus. The amino acid residues are numbered from the N terminus of the mature form of Dsg1 and Dsg3. The results of reactivity assays with AK mAbs are listed in the right panel. a, Domain-swapped mDsg1/mDsg3 molecules used in gross epitope mapping. b, Domain-swapped hDsg1/hDsg3 molecules used in fine epitope mapping. c, Point-mutated Dsg1/Dsg3 molecules used for epitope mapping of AK23. The 22 aa residues that are not conserved between hDsg1 and hDsg3 are indicated. Those nonconserved residues were switched between Dsg1 and Dsg3 to generate point-mutated molecules. The hatched boxes at the top indicate the residues that are predicted to form the adhesive interface of the W2 donor side (E1 to P8, P20, K23, T25, S26, D27, and D59) (31).
epitope resides in residues 1–63 of hDsg3 (Fig. 5b). However, because AK23 failed to react with residues 25–566, 1–34, 1–26, or 1–10, overlapping epitopes for AK23 could not be verified.

The amino-terminal sequences were highly conserved between Dsg3 and Dsg1. Only 22 aa residues of amino-terminal residues 1–87 were not conserved between the hDsg3 and hDsg1 proteins (Fig. 5c). We constructed a series of point-mutated hDsg3 molecules, in which the Dsg3-specific residues were replaced with the corresponding Dsg1-specific residues. We used six point-mutated Dsg3 (hDsg3-M1 to M6) constructs, which covered most of the Dsg3-specific residues between 25 and 84. However, none of these mutations led to loss of reactivity with AK23 (Figs. 4d and 5c). We then combined the M1 and M2 mutations to construct Dsg3-M1–2; however, AK23 still bound to Dsg3-M1–2. We replaced the Dsg3-specific residues 25–56 with the corresponding Dsg1-specific M1, M2, M3, and F48 residues to construct Dsg3-M1–2–3. However, AK23 still bound to Dsg3-M1–2–3. In contrast, AK23 did not bind to hDsg1-M1–2 or hDsg1-M1–2-3, which have the corresponding Dsg3-specific residues on the Dsg1 backbone. We then focused on the N-terminal residues, and point mutated the V3, K7, and P8 residues of Dsg3 to the I3, A7, and A8 residues of Dsg1 (hDsg3-M7). The hDsg3-M7 construct lacked reactivity with the AK23 mAb. However, the introduction of V3, K7, and P8 from Dsg3 onto the Dsg1 backbone (hDsg1-M7) was not sufficient to restore AK23 binding, which suggests that additional Dsg3-specific residues are necessary to form the AK23 epitope. When D59 was introduced into hDsg1-M7 (hDsg1-M7–8), hDsg1-M7–8 showed strong reactivity with AK23 (Fig. 4d). The substitution of D59 with E59 (hDsg3-M8) did not abolish reactivity with AK23. Thus, V3, K7, and P8 are essential residues for AK23 binding, and the combination of V3, K7, P8, and D59 is sufficient to generate the AK23 epitope on the Dsg1 backbone. The observed requirement for D59 in the formation of the AK23 epitope explains why the domain-swapped molecules that contained the residues 1–34, 1–36, and 1–10 of Dsg3 failed to react with AK23 (Figs. 4c and 5b). Thus, the epitope of AK23 maps to the V3, K7, P8, and D59 residues of Dsg3. All four of these Dsg3 residues are conserved between mice and humans.

Taken together, the epitope mapping of AK mAbs shows that the most potent mAb (AK23) recognizes an amino-terminal, calcium-dependent conformational epitope that is constituted by the V3, K7, P8, and D59 residues. The AK23 epitope becomes available only after cleavage of the prosequence. AK19, which shows weaker pathogenic activity, recognizes a calcium-dependent conformational epitope at residues 87–161 of Dsg3. The other AK mAbs, which lacked apparent pathogenic activities, recognize epitopes in the middle or carboxyl-terminal portions of the extracellular domain of Dsg3.

Discussion

It is well accepted that IgG autoantibodies to Dsg play a primary pathogenic role in blister formation on the skin and mucous membranes of pemphigus patients. The anti-Dsg3 IgG autoantibody titers correlate with disease activity when monitored in a single patient (9–12). However, when compared among different patients, the titers of anti-Dsg3 IgG autoantibodies do not necessarily reflect disease severity. In this study, we attempted to determine whether anti-Dsg3 IgG Abs had different pathogenic activities, and to define the factors that influenced pathogenic potency. To address this question, we isolated eight AK series mAbs against Dsg3 from active PV model mice (18). All of the AK mAbs were of the IgG1k isotype and reacted in vivo with the cell surfaces of stratified squamous epithelia, but not with the surfaces of simple epithelia. AK7 and AK9 reacted with only mDsg3, while the others cross-reacted with hDsg3. The binding of AK19 and AK23 was calcium dependent, while that of the others was calcium independent.

We used two methods to evaluate the pathogenic activities of these mAbs. The first method involved passive transfer of mAbs into neonatal mice (5), and the second method assayed ascites formation in adult mice. The passive transfer assay may be more sensitive than the ascites formation assay because highly concentrated IgG can be applied in passive transfer, while the amount of IgG that can be used in the ascites formation assay is dependent on the production rate of each hybridoma. AK19 and AK23 induced blister formation after passive transfer, while the remaining mAbs failed to display pathogenic activities. In the ascites formation assay, only AK23 induced blisters, and the phenotypes of the mice that received AK23 hybridoma cells were virtually identical with those of PV model mice and Dsg3−/− mice. AK19 failed to induce blisters in the ascites formation assay, although the titers of circulating AK19 were more than 5-fold those of mice that were administered with the AK23 hybridoma cells. These findings indicate that AK23 and AK19 are capable of inducing both the loss of cell-cell adhesion of keratinocytes and blister formation with different potencies, while the other AK mAbs apparently lack pathogenic activities. This is the first demonstration of pathogenic heterogeneity among anti-Dsg3 IgG Abs that bind in vivo to the native Dsg3.

The epitopes of the AK mAbs were characterized by immunoprecipitation using domain-swapped as well as point-mutated Dsg1/Dsg3 molecules that were produced by baculovirus expression. Following immunoprecipitation with the domain-swapped molecules, the epitopes of AK7, AK9, AK15, AK18, and AK20, which lacked pathogenic activities, were mapped to the middle or carboxyl-terminal regions (between residues 195 and 565) of the extracellular domain of mDsg3. The epitopes of AK19 and AK23, which possessed pathogenic activities, were calcium dependent and located in residues 89–161 and 1–63, respectively. Subsequent extensive studies with point-mutated Dsg1/Dsg3 molecules revealed that AK23 recognizes a conformational epitope that consists of the V3, K7, P8, and D59 residues of Dsg3.

Cadherins are a family of calcium-dependent cell-cell adhesion molecules that play important roles in the formation and maintenance of complex tissues (28). Cadherins are classified into two major subgroups based on sequence similarities: classic cadherins (e.g., E-, P-, and N-cadherins) and desmosomal cadherins (Dsg and desmolins). These cadherins share a common domain organization, which consists of five tandem extracellular cadherin domains (EC1 to EC5), a single transmembrane segment, and a highly conserved cytoplasmic domain (3, 4). Recent high resolution crystal structure analyses of classic cadherins have provided a mechanistic basis for intermolecular cadherin interactions (29, 30), and most recently, the crystal structure of the entire extracellular domain of C-cadherin, which is a member of the classic cadherin family, has been deduced with a resolution of 3.1 angstroms (31). This structure provides a new framework for understanding both the cis (same cell) and trans (juxtaposed cell) interactions of cadherin. The trans adhesive interface is a 2-fold symmetrical interaction that is defined by a conserved tryptophan (W2) side chain at the amino-terminal, membrane-distal end of the cadherin molecule from one cell, which inserts into the hydrophobic pocket at the amino-terminal end of a cadherin molecule on an opposing cell. This simple 2-fold symmetry provides a rationale for the generally observed homophilic specificity of cadherins, and reveals the molecular determinants of cadherin specificity.

When the amino acid sequences of Dsg3 were superimposed on the predicted structure of C-cadherin, the predicted residues for the adhesive interface of the W2 donor side were E1 to P8, P20, K23,
T25, S26, D27, and D59 (hatched boxes in Fig. 5c) (31). Among these residues, the ones that are not conserved between Dsg3 and Dsg1 and, therefore, probably involved in the determination of binding specificity of Dsg are V3, K7, P8, T25, and D59. Surprisingly, residues V3, K7, P8, and D59 of the epitope of AK23, which is the most potent pathogenic mAb tested, were located precisely on the Dsg3-specific residues of the adhesive interface. This structural prediction of the AK23 epitope is also supported by the observation that AK23 recognizes preferentially the mature form of Dsg3 (Fig. 4b). The consequence of Dsg3 blocks the N-terminal adhesive region of cadherins to prevent self-aggregation during protein synthesis in the Golgi or endoplasmic reticulum (32). In contrast, the AK mAbs, which lacked apparent pathogenic activities, recognize the middle to C-terminal portion of the extracellular domain, a region in which no direct molecular interaction is predicted. Taken together, these findings indicate that the pathogenic heterogeneity among anti-Dsg3 IgG Abs in terms of blister formation is, at least in part, explained by their epitopes, and that the amino-terminal trans adhesive interface may represent a critical location for blister formation by IgG Abs in PV. Our findings also provide biological evidence for the predicted three-dimensional structure of cadherins (31).

Our findings do not necessarily exclude the possibility that Abs against the middle to carboxyl-terminal region of the extracellular domain of Dsg3 play some pathogenic role in blister formation. It is possible that the AK mAbs that lack apparent pathogenic activities per se may induce blisters when administered in combinations. When the epitopes of the anti-Dsg3 IgG autoantibodies were characterized using PV patients’ sera, a minor proportion of the PV patients contained only IgG autoantibodies that were directed against the middle regions of Dsg3 (14). The clinical profiles of these patients were similar to those who had IgG autoantibodies against the N-terminal region of Dsg3. Therefore, polyclonal Abs against the middle portion of Dsg3 may also induce blistering.

The mechanism through which the binding of IgG Abs to Dsg3 leads to the loss of keratinocyte cell-cell adhesion is a matter of some controversy. In this respect, two hypotheses are currently favored: 1) direct inhibition of the adhesive function of Dsg3; and 2) the involvement of signal transduction. When IgG from PV sera was added to cultured keratinocytes, it caused a transient increase in intracellular calcium and inositol 1,4,5-triphosphate, which was followed by the activation of protein kinase C in the squamous cell carcinoma cell line DJM-1 (33). The addition of IgG from PV sera to cultured keratinocytes induced phosphorylation of Dsg3 and the dissociation of Dsg3 from plakoglobin (34). More convincingly, keratin retraction from cell-cell contact sites was induced by IgG from PV sera and required plakoglobin-mediated signaling in cultured mouse keratinocytes (35). These observations support the signal transduction hypothesis, although all of these observations were in vitro ones. In contrast, we demonstrated in this study that the most potent pathogenic mAb (AK23) recognized the functionally important N-terminal adhesive interface, while the other mAbs lacked apparent pathogenic activities bound to the functionally less important middle to C-terminal domain, showing that the pathogenic mAb does not simply cross-link the molecule or interfere with the desmosome through steric hindrance, but interferes with the actual function of Dsg3. In addition, the dominant epitopes in human PV were localized to the N-terminal residues 1–161 of Dsg3, which include the adhesive region (13, 14). The phenotype of mice with genetic disruption of Dsg3 is essentially identical with that of PV model mice (18, 36, 37). These findings indicate that the direct inhibition of the trans interaction of Dsg3 by IgG Abs is an initial molecular event of blister formation in pemphigus. Combining these two hypotheses, it is also possible to speculate that signal transduction may occur subsequent to the functional disruption of Dsg3 by IgG binding.

The importance of conformational epitopes was previously demonstrated in the production of pathogenic Abs by mouse immunization (38, 39) as well as epitope characterization with human PV sera (13, 14). The requirement of D59 residues in addition to V3, K7, and P8 residues to form the epitope of the pathogenic mouse mAb AK23 on Dsg1 backbone has ensured the importance of conformational epitopes in the pathogenesis of pemphigus. It was recently suggested that anti-Dsg Abs alone do not cause blistering, but require additional Abs to cholinergic receptors, and that pemphigus lesions can be caused by non-Dsg Abs (40–42). In addition to the extensive evidence that has been summarized previously (43), our findings that PV blisters could be induced by a single mAb (AK23) confirm that Dsg3 and anti-Dsg3 Abs are directly involved in causing blistering in PV, in the absence of any additional non-Dsg Abs.

The definition of the pathogenic hot spot on the molecule has provided an important framework to understanding the molecular mechanism of blister formation in pemphigus, as well as cell-cell adhesion in desmosomes. The availability of a series of mAbs against Dsg3 that serve as pathogenic and nonpathogenic Abs will provide a valuable tool to investigate the role of Dsg and desmosomes in keratinocyte differentiation and proliferation. Furthermore, from the diagnostic point of view, we should be able to develop an ELISA against the identified single epitope, which may provide absolute scores of disease severity. From the therapeutic point of view, the identification of pathogenic hot spot on Dsg3 will lead us to develop epitope-specific plasmapheresis or epitope-specific B cell elimination as targeted therapies for pemphigus.

Acknowledgments

We thank Dr. Shojiro Tsukita for the mouse myeloma P3 cells and technical advice on the development of mAbs. Thanks also goes to Central Institute for Experimental Animals for Dsg3 mice and Rag2–/– mice. We also thank Yoshiko Fujii for the preparation of recombinant proteins, and Minea Suzuki for the immunofluorescence staining.

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