A Subset of Pemphigus Foliaceus Patients Exhibits Pathogenic Autoantibodies Against Both Desmoglein-1 and Desmoglein-3

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In pemphigus vulgaris the major pathogenic antibody binds desmoglein-3, and mediates mucosal disease. Development of cutaneous disease is associated with acquisition of antibodies to desmoglein-1. In pemphigus foliaceus, and its endemic form, fogo selvagern by contrast, the major pathogenic antibody recognizes desmoglein-1 and mediates cutaneous disease only. In this study, we sought to determine the prevalence of antibodies to desmoglein-3 in patients with pemphigus foliaceus and fogo selvagem. We produced recombinant desmoglein-1 and desmoglein-3, and used them in highly sensitive and specific enzyme-linked immunosorbent assays, as well as immunoprecipitation assays. We detected antibodies to desmoglein-3 in 19 of 276 patients with pemphigus foliaceus and fogo selvagem, who had cutaneous disease only. We showed that these antibodies to desmoglein-3 could be absorbed in a concentration-dependent manner by desmoglein-3 but not by desmoglein-1. Also antibodies to desmoglein-1 could be absorbed in a concentration-dependent manner by desmoglein-1 but not desmoglein-3. This suggests that two separate species of antibody are present rather than one antibody capable of cross-reacting with both desmoglein-1 and desmoglein-3. Finally, it was shown that affinity-purified antibodies to desmoglein-3 from patients with pemphigus foliaceus and fogo selvagem induced a pemphigus vulgaris-like skin disease in mice by passive transfer. These results suggest that a subset of patients with pemphigus foliaceus and fogo selvagem have antibodies to desmoglein-3 that may be involved in the pathogenesis of their cutaneous disease. Key words: autoantibodies/autoimmunity/desmoglein/fogo selvagem/ pemphigus. J Invest Dermatol 118:806–811, 2002

P
emphigus vulgaris (PV) is characterized by the gradual onset of blisters and erosions involving not only the skin but also mucous membranes (Lever, 1965). Approximately one-third of PV patients may have mucosal lesions months before the diagnosis of PV is established. The vesicles are intraepidermal and located above the epidermal basal cell layer (suprabasal blisters). In contrast, pemphigus foliaceus (PF) and fogo selvagem (FS) are characterized by cutaneous blisters and erosions only, without mucosal involvement (Lever, 1965; Diaz et al, 1989). The vesicles in PF and FS are located just below the stratum corneum. Blister formation in PV, PF, and FS results from a progressive epidermal cell detachment also known as acantholysis. Electron microscopic examination of lesional epidermis in these patients shows separation of desmosomes with widening of the epidermal intercellular spaces (ICS). Keratinocytes round up and exhibit retraction of intermediate filaments around the nucleus (Lever, 1965). Patients with PV, PF, and FS produce anti-epidermal autoantibodies, which can be detected in the ICS of perilesional epidermis and circulating in the patients’ serum using immunofluorescence (IF) techniques (Beutner and Jordon, 1964; Beutner et al, 1968; Diaz et al, 1989). The serum titers of autoantibodies by indirect IF roughly correlate with disease activity and extent of the skin involvement. The IgG fractions of PV (Anhalt et al, 1982) and PF (Roscoe et al, 1985) sera are pathogenic in passive transfer experiments using neonatal mice. The injected animals display the key clinical, histologic, and immunologic features of each disease.

The epidermal autoantigens recognized by antibodies and T cells in PF/FS and PV have been identified as desmoglein-1 (dsg1) and desmoglein-3 (dsg3), respectively (Stanley et al, 1984; Labib et al, 1990; Amagai et al, 1991; Olague-Alcala et al, 1994; Emery et al, 1995; Wucherpfennig et al, 1995; Lin et al, 1997a, b, 2000). Dsg1 and dsg3 are transmembrane desmosomal glycoproteins that belong to the cadherin family of proteins (Buxton et al, 1993). These molecules exhibit extensive amino acid homology, especially in their extracellular domains (Fig 1). Recombinant dsg1 and dsg3, generated in the baculovirus expression system, are capable of absorbing pathogenic antibodies from the sera of PF or PV patients (Amagai et al, 1994, 1995). It appears that genetic predisposition is a common feature in patients developing PV or PF, as each disease exhibits unique DR alleles. FS shows a strong association with the HLA-DRB1*0102, 0404, 1402, 1602 alleles (Cerna et al, 1993; Moraes et al, 1997),
whereas PV is linked to the HLA-DRB1*0402 and 1401 alleles (Scharf et al, 1988; Sinha et al, 1988; Szafer et al, 1988; Ahmed et al, 1990, 1991).

Recent reports have documented the existence of two clinical and serologic variants of PV (Ding et al, 1997; Amagai et al, 1999; Miyagawa et al, 1999; Harman et al, 2000a, b). A mucosal variant exhibits a predominantly anti-dsg3 antibody response and a mucocutaneous form, comprising approximately 50% of the patients, is characterized by a mixed anti-dsg3 and anti-dsg1 antibody response and a T cell response to these antigens (Lin et al, 1997b). It has been demonstrated that both affinity-purified anti-dsg3 and anti-dsg1 autoantibodies from these PV sera are pathogenic in the mouse model (Ding et al, 1999). In these studies, each antibody system was shown to reproduce the phenotype of each disease, i.e., antibodies to dsg3 induced PV-like lesions and antibodies to dsg1 induced PF-like lesions. There was no immunologic cross-reactivity between these autoantibody systems. Interestingly, it has been reported recently that patients with classic cutaneous and mucocutaneous forms, comprising approximately 50% of the patients, is characterized by a mixed anti-dsg3 and anti-dsg1 antibody response and a T cell response to these antigens (Lin et al, 1997b). It has been demonstrated that both affinity-purified anti-dsg3 and anti-dsg1 autoantibodies from these PV sera are pathogenic in the mouse model (Ding et al, 1999). In these studies, each antibody system was shown to reproduce the phenotype of each disease, i.e., antibodies to dsg3 induced PV-like lesions and antibodies to dsg1 induced PF-like lesions. There was no immunologic cross-reactivity between these autoantibody systems. Interestingly, it has been reported recently that patients with classic cutaneous, histologic, and immunologic findings of PV may evolve into PF (Iwatsuki et al, 1991; Kawana et al, 1994; Chorzelski et al, 1995; Chang et al, 1997). The evolution of PV into PF is associated with a serologic switch from antibodies against dsg3, typical of PV, to antibodies against dsg1, which are a marker of PF.

The transition from PF into PV has also been described, however, this progression appears to be extremely rare (Ishii et al, 2000). It is well established that the sera of the majority of PF and FS patients possess a predominantly anti-dsg1 IgG response (Stanley et al, 1986; Warren et al, 2000). The frequency of anti-dsg3 autoantibodies in PF and FS patients, however, is unknown.

In this communication, using specific enzyme linked immunosorbent assays (ELISA) methods, we found anti-dsg3 autoantibodies in 19 of 276 patients with PF and FS. The reactivity of these autoantibodies with dsg3 was further confirmed by immunoprecipitation using purified recombinant desmoglein-3 (rdsg3). The specific binding of anti-dsg3 autoantibodies detected by ELISA was inhibited in a concentration-dependent manner by purified rdsg3, but not by purified rdsg1. Under the same experimental conditions, antibodies to dsg1 were inhibited by rdsg1 but not by rdsg3. Furthermore, using the passive transfer mouse model, we found that purified anti-dsg3 antibodies from PF and FS patients are capable of inducing PV-like lesions in neonatal mice. These results suggest that a subset of patients with PF and FS have antibodies to dsg3 that may be involved in the pathogenesis of their disease.

MATERIALS AND METHODS

Source of sera We tested sera from 35 PF patients (25 from the U.S.A. and 10 from Japan) and 241 FS patients from Brazil (40 from Campo Grande, five from Sao Paulo, 44 from Belo Horizonte, 62 from Goiania, 57 from Brazilia, and 33 from Limao Verde). The diagnosis of PF and FS was established clinically, histologically, and immunologically, as reported previously (Diaz et al, 1989). These patients had active disease and none had mucosal lesions. We also tested sera from patients with other cutaneous diseases such as bullous pemphigoid (n = 20), lupus erythematosus (n = 19), herpes gestationis (n = 23), and PV (n = 83). Patients with PV included 53 from the U.S.A., seven from Spain, 13 from Brazil, and 10 from Japan. We also tested normal donors from the U.S.A. (n = 50) as controls. No pooled sera were used in this study.

Production and purification of rdsg1 and rdsg3 The expression of the extracellular domain of dsg3 using the baculovirus system has been described previously (Ding et al, 1999). In order to facilitate the purification of this protein, a six histidine stretch was engineered immediately downstream of the dsg3 extracellular domain. The rdsg3 was purified using a Ni²⁺ NTA column following procedures suggested by the manufacturer (Qiagen, Valencia, CA). The extracellular domain of dsg1 (rdsg1) was produced by similar methods (Ding et al, 1999). The purity of each recombinant protein was assured by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblot analysis.

Establishment of dsg1 and dsg3 ELISA The rdsg1 and rdsg3 ELISA were established following procedures described by Warren et al (2000) except that mouse monoclonal anti-human IgG conjugated to horseradish-peroxidase (Zymed Laboratories, San Francisco, CA) was used as a secondary antibody. Each ELISA plate contained three dilutions of a standard positive serum sample that were used to correct for plate-to-plate variability by linear regression analysis. Logarithmic transformation was used to analyze the assay results. The cut-off values were set at the mean plus 3 SD of values of the normal subjects from the U.S.A. This resulted in a cut-off for the dsg1 ELISA of 0.3 OD units and for the dsg3 ELISA of 0.48 OD units.
The inhibition of the ELISA assays was performed by incubating a fixed dilution of PF or FS serum in Tris-buffered saline (TBS), with calcium 5 mM, pH 7.3 (TBS Ca²⁺), with different amounts of rdsg3 or rdsg1 (0–8 μg) at room temperature for 1 h. Following a centrifugation step at 10,000g at 4°C for 10 min, the supernatant was tested by rdsg3 ELISA (or rdsg1 ELISA).

Immunoprecipitation assay
The immunoprecipitation of rdsg3 was carried out as described previously (Ding et al., 1999) with the following modifications. Three micrograms of rdsg3 (or rdsg1) was first incubated with 3 ml of serum from PF and FS patients for 1 h at room temperature. Subsequently, 30 ml of a 50% suspension of immobilized recombinant Protein G (Zymed) was added and incubated for 90 min. The protein G immune complexes were washed three times with TBS-Ca²⁺/0.05% Tween buffer (pH 7.3) and then extracted using 30 ml of reducing buffer (4% sodium dodecyl sulfate, 10% 2-mercaptoethanol from Sigma) and boiled for 5 min. The extracts were then fractionated using 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the protein bands analyzed by western blotting using rabbit anti-dsg3 (or rabbit anti-dsg1) as the primary antibody and horseradish peroxidase-labeled goat anti-rabbit IgG (Zymed) as the second antibody at a dilution of 1:10,000. The specific rdsg3 bands (or dsg1 bands) were visualized by enzymic chemiluminescence western blotting and autoradiography (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Affinity purification of anti-dsg1 and anti-dsg3 autoantibodies from PF/FS sera
The anti-dsg1 and anti-dsg3 autoantibodies from PF and FS sera were purified using rdsg1 or rdsg3 Ni-NTA columns as previously described (Ding et al., 1999). The affinity-purified autoantibodies were dialyzed against phosphate-buffered saline,

### Table I. Anti-dsg1 and anti-dsg3 autoantibodies in a subset of 19 patients with FS and PF

<table>
<thead>
<tr>
<th>Patient code</th>
<th>ELISA⁺</th>
<th>dsg1²</th>
<th>dsg3²</th>
<th>Indirect IF titre</th>
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<tr>
<td>BH18</td>
<td>5.683</td>
<td>1.536</td>
<td></td>
<td>1:160</td>
</tr>
<tr>
<td>BH26</td>
<td>9.964</td>
<td>2.034</td>
<td></td>
<td>1:2560</td>
</tr>
<tr>
<td>BH28</td>
<td>9.974</td>
<td>1.416</td>
<td></td>
<td>1:160</td>
</tr>
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<td>BH30</td>
<td>8.332</td>
<td>2.113</td>
<td></td>
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<td>1:320</td>
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<td>9.982</td>
<td>5.197</td>
<td></td>
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<td>Bra48</td>
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<td></td>
<td>1:640</td>
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<td>Bra49</td>
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<td>1.899</td>
<td>1.495</td>
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</tr>
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<td>Bra52</td>
<td>0.435</td>
<td>3.831</td>
<td></td>
<td>1:320</td>
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<td>Bra55</td>
<td>3.078</td>
<td>2.167</td>
<td></td>
<td>1:20</td>
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<td>Bra61</td>
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<td>CG14</td>
<td>9.977</td>
<td>9.980</td>
<td></td>
<td>1:1280</td>
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<td>CG44</td>
<td>9.859</td>
<td>4.788</td>
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<tr>
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<tr>
<td>PH</td>
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<td>3.246</td>
<td></td>
<td>1:640</td>
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*The cut-off value of the ELISA was set at the mean of the 50 normal sera plus 3 SD.

*The cut-off for the dsg1 ELISA was 0.30 OD units.

*The cut-off for the dsg3 ELISA was 0.48 OD units.

### Figure 3. All 19 patients with antibodies to dsg3 by ELISA were also positive by immunoprecipitation. The autoradiogram shows immunoprecipitates analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Lane P: PV patient included as a positive dsg3 control. Lane C: Normal human serum included as a negative control. Lanes 1–19: All 19 patients with PF or FS with antibodies to dsg3 by ELISA.

### Table II. Pathogenicity of affinity purified anti-dsg1 and anti-dsg3 autoantibodies from patients with PF and FS

<table>
<thead>
<tr>
<th>Patient IgG</th>
<th>No. of mice tested</th>
<th>Disease activity</th>
<th>Lesional site (H/E)</th>
<th>Direct IF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-dsg1 (FS12 serum)</td>
<td>5</td>
<td>3 +</td>
<td>suprabasal (+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Anti-dsg3 (FS12 serum)</td>
<td>5</td>
<td>3 +</td>
<td>subcorneal (+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Anti-dsg1 (FS26 serum)</td>
<td>4</td>
<td>3 +</td>
<td>subcorneal (+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Anti-dsg3 (FS26 serum)</td>
<td>4</td>
<td>3 +</td>
<td>suprabasal (+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Anti-dsg1 (PH serum)</td>
<td>5</td>
<td>3 +</td>
<td>subcorneal (+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Anti-dsg3 (PH serum)</td>
<td>5</td>
<td>3 +</td>
<td>suprabasal (+)</td>
<td>(+)</td>
</tr>
</tbody>
</table>

*Affinity purified anti-dsg1 and anti-dsg3 autoantibodies from patients with PF and FS were passively transferred into neonatal mice. The dose of anti-dsg1 IgG was 200 μg per g of body weight, whereas the dose of anti-dsg3 IgG was 130 μg per g of body weight.

Direct IF is reported as positive (+), if FITC-labeled anti-human IgG antibodies stained the epidermal intercellular spaces.
concentrated, and filter sterilized. They were then characterized by ELISA assays. These antibodies were then used in passive transfer experiments in neonatal mice as described below.

**Induction of experimental pemphigus by passive transfer experiments** Institutional approval from UNC-Chapel Hill IRB was obtained for the animal studies. Approximately 200 μg per g body weight of affinity-purified anti-dsg1 autoantibodies (A–C) or anti-dsg3 autoantibodies (D–F), respectively, were administered intradermally to neonatal BALB/C mice following established procedures (Anhalt et al., 1982; Roscoe et al., 1985; Ding et al., 1999). The skin of neonatal mice was examined 18 h after the IgG injection and any signs of skin disease recorded. The animals were then killed. Specimens were obtained from lesional skin for histologic examination by light microscopy. Perilesional skin samples were also obtained for direct IF analysis, which employed monospecific fluorescein isothiocyanate-conjugated goat anti-human IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

**RESULTS**

**Sensitivity and specificity of the dsg3 ELISA** The rdsg3 ELISA was highly sensitive, and detected antibodies to dsg3 in 82 of 83 patients with PV (sensitivity: 98.8%). All 50 normal subjects were negative, as well as all 20 patients with bullous pemphigoid, all 19 patients with lupus erythematosus and all 23 patients with herpes gestationis, giving an overall specificity of 100% (Fig 2).

**A subset of patients with PF and FS produce antibodies to dsg3** Employing the dsg3 ELISA described above we tested a
large number of serum samples from PF and FS patients. These samples were obtained from several regions of Brazil (n = 241), Japan (n = 25), and the U.S.A. (n = 10). We detected antibodies to dsg3 in 28% of the nonendemic PF samples (one of 35), and in 7.5% of the FS patients (18 of 241). This difference was not statistically significant. (Using Fisher’s exact test and one-sided p-value, p = 0.28. The two-sided p-value is 0.48.)

The corrected OD495 readings ranged from 1.3 to 9.98 (Table I). The sera was also assayed by immunoprecipitation (Fig 3). Antibodies to dsg3 were detected in all 19 patients with PF and FS by immunoprecipitation. Sera of these patients also immunoprecipitated rdsg1 (not shown). Serum samples from rdsg3 ELISA negative PF and FS patients, as well as sera from bullous pemphigoid, herpes gestationis, and lupus erythematosus failed to react with rdsg3 by immunoprecipitation (not shown).

**Preincubation with rdsg3 inhibits only reactivity with dsg3, and preincubation with rdsg1 inhibits only reactivity with dsg1** Using serum from patients with PF and FS who had detectable antibodies to dsg3, we added a preincubation step with rdsg3, and then tested the sera in the ELISA assay. As shown in Fig 4A preincubation with purified rdsg3 removes antibodies to dsg3 in a concentration-dependent manner, but has minimal effect on antibodies to dsg1. Pre-incubation with rdsg1 (Fig 4B) removes antibodies to dsg1 in a concentration-dependent manner but has minimal effect on antibodies to dsg3. These data therefore provide strong evidence that these 19 PF/FS patients exhibit two independent antigen–antibody systems, i.e., anti-dsg3 and anti-dsg1.

**Antibodies to dsg3 and dsg1 from PF and FS patients are pathogenic** Antibodies from two patients with FS and one with PF were affinity purified on dsg1 and dsg3 affinity columns and the purity of the preparations confirmed by dsg1 and dsg3 ELISA. Affinity-purified antibodies were passively transferred into neonatal mice (Table II). Mice injected with antibodies to dsg1 reproduced the clinical (Fig 5A), histologic (Fig 5B), and immunologic (Fig 5C) features of PF and FS, i.e., subcorneal blisters and antibodies bound to the ICS of lesional skin. Mice that received affinity-purified antibodies to dsg3 developed clinical skin lesions (Fig 5D) that showed suprabasilar acantholysis on histologic examination (Fig 5E), and positive direct IF staining of the epidermal ICS (Fig 5F). These clinical, histologic, and immunologic features are identical to those seen in mice injected with anti-dsg3 IgG from PV patients. The IgG fraction of all three sera (one PF and two FS) when injected into neonatal mice induced clinical disease (Fig 6A) and subcorneal acantholysis (Fig 6B) identical to that found previously in other patients with PF.

**DISCUSSION**

In PV the presence of mucosal disease alone correlates with antibodies to dsg3 (Miyagawa et al, 1999; Harman et al, 2000b). Development of additional cutaneous disease has been correlated with acquisition of antibodies to dsg1 (Miyagawa et al, 1999; Harman et al, 2000b). An attractive hypothesis to explain these and other findings has been proposed (Udey and Stanley, 1999). In mucosal epithelia, antibodies to dsg3 would cause disease due to lack of compensation by dsg1. Antibodies to dsg3 alone would not be able to mediate cutaneous disease because of “compensation” by dsg1 in the skin. In a PV patient who acquires antibodies to dsg1, compensation by dsg1 is no longer possible, resulting in cutaneous as well as mucosal disease. In PF by contrast, antibodies to dsg1 cannot cause mucosal disease as dsg3 is present at high levels throughout the mucosa. They do cause cutaneous disease, however, which is limited to the upper epidermis where dsg3 is at very low levels.

We detected antibodies to dsg3 in 19 of 276 patients with PF and FS, in addition to the previously recognized antibodies to dsg1, using highly sensitive and specific ELISA assays. We confirmed our results using immunoprecipitation. Using preincubation assays we showed that reactivity with dsg3 was depleted by preincubation with dsg3 but not dsg1. This suggests that there may be two separate groups of antibodies in these patients, one group reactive with dsg1 and one group reactive with dsg3. In addition we saw a small drop in reactivity to dsg1 using preincubation with dsg3, and a small drop in reactivity to dsg3 using preincubation with dsg1. As there is a high degree of homology (see Fig 1) in the ectodomains of dsg1 and dsg3, it is likely that a subpopulation of antibodies may cross-react with both molecules. These results would need to be developed using epitope mapping studies. Finally, we showed that the antibodies to dsg3 from these patients are indeed pathogenic in the mouse model, and reproduce the clinical, histologic, and immunologic features of PV.

All 19 patients with PF and FS who had antibodies to dsg3 had clinical and histologic features of PF, with no mucosal lesions. It is possible that in these 19 patients with PF and FS, antibodies may contribute to disease pathogenesis by blocking compensation by dsg3 in the skin. As the total IgG fraction from three patients produced only subcorneal acantholysis in the mouse model, the levels of antibodies to dsg3 in these 19 may be too low to cause mucosal disease in these 19. Alternatively these patients may subsequently develop mucosal disease as has been previously reported in nonendemic PF.
in Japan (Ishii et al, 2000). Additional support for this suggestion comes from the identification of more clear-cut cases of an endemic form of PV in the same regions of Brazil where FS is endemic. These authors collected a small group of young patients with a severe mucocutaneous disease that on histologic and immunologic grounds overlapped with PV. These patients need to be further characterized in terms of antigenic targets.

In conclusion, these results show that the line between PF and PV is becoming somewhat blurred, with the possibility of transition between the two clinical forms existing, presumably by epitope spreading (Lehman et al, 1992, 1993; Kaufman et al, 1993).

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