Desmoglein 3-ELISA
A Pemphigus Vulgaris–Specific Diagnostic Tool

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Background: Pemphigus vulgaris (PV) is an autoimmune-blistering disease of the skin and mucous membranes caused by autoantibodies against desmoglein 3 (Dsg3), an epidermal desmosomal adhesion protein of the cadherin family. Cloning of the Dsg3 gene and expression of the protein in a native conformation enabled the recent development of a specific and sensitive enzyme-linked immunosorbent assay (ELISA) for the detection of PV autoantibodies.

Objectives: To evaluate serum samples from patients with PV and other dermatologic diseases for anti-Dsg3 antibodies. To compare ELISA values with autoantibody titers obtained by classic indirect immunofluorescence (IIF).

Design: Serum samples from patients with PV and various other bullous and nonbullous skin diseases were tested for anti-Dsg3 reactivity by ELISA.

Setting: Ambulatory and hospitalized patients from a university hospital.

Patients: Fifty-two serum samples from 11 patients with PV, and serum samples from 11 patients with bullous pemphigoid, 12 patients with other bullous diseases, 22 patients with various nonbullous skin disorders, and 10 healthy individuals were tested.

Results: Forty-seven (98%) of 48 serum samples from patients with PV that were positive by IIF on monkey esophagus were also reactive by Dsg3-ELISA, whereas 4 of 4 IIF-negative PV serum samples showed no reactivity by ELISA. In addition, negative ELISA results were obtained from 11 of 11 serum samples from patients with bullous pemphigoid, 10 of 12 serum samples from patients with other bullous skin disorders, 7 of 9 serum samples from patients with autoimmune-connective tissue diseases, and 13 of 13 serum samples from patients with other nonbullous skin diseases. Interestingly, 1 patient with paraneoplastic pemphigus had positive ELISA results. There was a positive correlation ($r = 0.654$) between ELISA values and IIF titers within the whole population with PV. In addition, when multiple serum samples from 1 patient with PV sampled over a 2-year period were tested, ELISA reactivity paralleled both the IIF titers and the clinical course.

Conclusion: The Dsg3-ELISA is a sensitive, objective, and PV-specific test that should be considered as an adjunct test for the management of patients with PV.

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Pemphigus vulgaris (PV) is an autoimmune-blistering disease of the skin and mucous membranes. Histologically, PV is characterized by suprabasal acantholysis and intraepidermal blister formation, and clinically by flaccid blisters and erosions that usually arise on normal-appearing skin and/or mucosa.¹ Immunologically, antibodies directed against the keratinocyte cell surface are detected in the skin and in the circulation; they recognize the extracellular domain of Dsg3.²³ Desmoglein 3, a 130-kd transmembrane glycoprotein of the cadherin family, mediates weak calcium-dependent homophilic cell-to-cell adhesion of epidermal keratinocytes.³⁴ In animal studies⁵ and in vitro⁶ experiments, anti-Dsg3 antibodies have been shown to induce loss of cell adhesion, indicating an important role in disease pathogenesis.

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Pemphigus antibodies are routinely detected by direct or indirect immunofluorescence (IIF) testing using monkey esophagus as a substrate to establish the diagnosis, and for monitoring disease activity that frequently follows antibody titers.¹⁰¹³ However, these methods are subjective, require an experienced examiner, and often fail to clearly distinguish...
PATIENTS, MATERIALS, AND METHODS

PATIENTS AND CONTROLS

A total of 52 serum samples (stored at −20°C until assayed) from 11 patients with PV (6 women, 5 men; mean age, 36.8 years; age range, 34-85 years) treated in the Department of Dermatology at the University of Vienna, Vienna, Austria, over 2 years (September 1995-November 1997) were tested for anti-Dsg3 reactivity. The diagnosis of PV was established clinically and by the typical histologic and immunopathologic findings. Forty-eight of these PV serum samples obtained from 2 patients with PV were negative by IIF at the time of serum sampling (Table 1). In addition, serum samples from 45 patients with a variety of other bullous and nonbullous skin diseases were included in the study. Of these patients, 11 were diagnosed as having bullous pemphigoid; 12 had other bullous diseases including pemphigus foliaceus (1 patient), PNP (2 patients), epidermolysis bullosa acquisita (1 patient), porphyria cutanea tarda (1 patient), and unclassified bullous diseases (4 patients); 9 had autoimmune–connective tissue diseases and high-titer antibodies against nuclear antigens, and 13 had other nonbullous skin diseases including eczema (2 patients), lichen ruber planus (3 patients), thermal burn (1 patient), and sexually transmitted diseases (4 patients). To establish a cutoff value for the ELISA we tested serum samples from 10 healthy individuals.

IIF ANALYSIS

Serum samples were tested at serial dilutions of 1:10 to 1:320 for the presence of antibodies against keratinocyte cell surface proteins by IIF analysis with cryosections of monkey esophagus as a substrate using 1:30 dilution of fluorescein isothiocyanate–conjugated anti–human IgG antibodies (INOVA Diagnostics, San Diego, Calif) as a second antibody.

PRODUCTION AND PURIFICATION OF rDsg BACULOVIRUS PROTEIN

Desmoglein 1 and Dsg3 were expressed and purified as described previously. Recombinant baculovirus encoding the entire extracellular domain of Dsg1 or Dsg3 fused with complementary DNA encoding for an E-tag and a histidine-tag was used to infect “high-five” insect cells (Invitrogen, Grand Island, NY) cultured under serum-free conditions (Sf 900 II SFM; Gibco BRL, Grand Island, NY) for 3 days at 27°C, resulting in the secretion of rDsg into the culture supernatant. Cell debris was removed by centrifugation, and supernatants were immediately affinity purified or stored at −70°C. For purification of rDsg, culture supernatants were applied to nickel nitrilotriacetic acid–agarose (Qiagen Inc, Chatsworth, Calif) columns allowing binding of the protein via the histidine-tag. The protein was eluted with sequential 1-mL volumes of 200 mmol/L of imidazole in a 20-mmol/L sodium phosphate and 500-mmol/L sodium chloride solution (pH 6.3) containing 1 mmol of calcium chloride per liter, dialyzed against 50-mmol/L Tris-buffered saline (TBS)–calcium, analyzed by Western blot and used for coating of the ELISA plates. The 1-mmol/L calcium chloride was added to all purification steps containing native rDsg3, rDsg1, or denatured (at 96°C) rDsg3 protein and reevaluated by IIF analysis.

ELISA

Immunomicrotiter plates (Nunc; Roskilde, Denmark) were coated with 50 µL of eluate and incubated overnight at 40°C. After extensive washing in ice-cold TBS-Ca/T and added for 1 hour at room temperature. In initial experiments the most specific optical density (OD) value with the least background was achieved at a serum dilution of 1:10 000 and subsequently serum samples were tested at this dilution only. Plates were then incubated with 2000-fold diluted peroxidase–conjugated rabbit anti–human IgG antibodies (monoclonal antibody against the k-light chain of all immunoglobulin classes) for 1 hour at room temperature. Color development was achieved by adding 100 µL of ABTS (Boehinger Mannheim, Mannheim, Germany) for 20 minutes, and OD at 405 nm was determined using an ELISA reader (Dynatech, Guernery, Channel Island). All assays were performed in duplicate and background values determined for each test serum sample in uncoated wells were subtracted. Each plate contained at least 1 negative control serum sample and a positive PV reference serum sample (IIF titer, 160). The OD of the tested serum samples obtained for each plate were normalized relative to the mean OD obtained for the PV reference serum sample (dilution, 1:10 000) tested on the same 96-well plate. The mean of the normalized OD was used in the analysis. A test serum sample was considered positive by ELISA when the mean OD was 3 SDs above the mean OD of serum samples from 10 healthy volunteers (OD, 0.09).

STATISTICS

To determine the correlation between ELISA OD and IIF titer, we applied the Spearman correlation for nonparametric variables. A correlation coefficient (r) of +1 indicates a perfect positive, of −1 a perfect negative correlation.
between PV, pemphigus foliaceus, and paraneoplastic pemphigus (PNP) since these diseases share the lace-like squamous intercellular substance (ICS)–binding pattern. Although the differentiation is possible by immunoprecipitation and immunoblotting of epidermal extracts with patient serum samples, both methods are time consuming and laborious. A significant advance in pemphigus diagnostics was the recent cloning of the Dsg3 gene. This allowed the in vitro production of recombinant (r) Dsg3 antigen in a native conformation and, subsequently, the development of a sensitive and PV-specific ELISA.

To further validate the suitability and reliability of ELISA for the diagnosis of PV and to expand the published results, we produced rDsg3 protein via the baculovirus expression system and tested serum samples from patients with PV and patients with a variety of other bullous and nonbullous dermatologic disorders for anti-Dsg3 reactivity.

### RESULTS

The extracellular domain of Dsg3 was produced as a secreted recombinant protein by a baculovirus expression system and purified by affinity chromatography. The 80-kd rDsg3 was detected by Western blot analysis using a monoclonal antibody against the E-tag that was fused to the C-terminal end of the protein. Anti-Dsg3 antibodies in the serum samples of patients with PV recognize predominantly conformational epitopes of the extracellular Dsg3 domain. To test if the purified rDsg3 retained the native protein conformation containing these relevant epitopes, the antigenicity of the rDsg3 baculoprotein was tested in immunoabsorption assays. A PV serum sample (IIF titer, 320) was diluted 1:20 with rDsg3 and subsequently reevaluated by IIF analysis. The rDsg3 protein completely removed the binding activity of the PV serum sample, indicating that the purified rDsg3 protein was suitable for use as an ELISA antigen. To rule out the possibility that the serum samples reacted nonspecifically with epitopes of the linear E-tag or histidine-tag of the rDsg3-fusion protein, the PV serum sample was absorbed with similar amounts of control proteins: (1) an rDsg1-, E-tag, and histidine-tag fusion protein and (2) the Dsg3 fusion protein denatured by high-temperature treatment that destroys the conformational Dsg3 epitopes while leaving the linear epitopes of Dsg3, the E-tag, and histidine-tag intact. Following absorption with rDsg1 or denatured rDsg3, the PV serum sample showed an ICS-binding pattern indistinguishable from the nonabsorbed serum sample, indicating that the reactivity was specific for conformational Dsg3 epitopes.

Of 48 IIF positive serum samples from patients with PV, 45 (98%) were positive by ELISA, whereas the 4 PV serum samples that were negative by IIF were also negative by ELISA (Table, Figure 2). To examine the specificity of ELISA, we investigated a total of 45 additional serum samples from patients with several other bullous or nonbullous skin diseases (Figure 2), including 11 with bullous pemphigoid, 10 with various bullous skin diseases, 9 with connective tissue diseases and high titers of antibodies against nuclear antigens, and 13 with various nonbullous skin disorders.

While all 11 serum samples from patients with bullous pemphigoid tested negative in the ELISA, 2 serum samples from patients with other bullous disorders showed ELISA reactivity—1 with a PNP and 1 with an unspecified bullous disease. In addition, 7 of 9 serum samples of patients with autoimmune–connective tissue diseases, 13 of 13 serum samples of patients with other nonbullous skin diseases, and 10 of 10 serum samples from healthy individuals were negative by ELISA. Thus, 4 of 55 control serum samples were positive by ELISA, demonstrating a disease specificity of 93% for the PV ELISA.

The Dsg3 autoantibody titers may follow disease activity and thus, antibody levels detected by IIF are used clinically for patient management. Statistical analysis of our data revealed a positive correlation (r = 0.65) between Dsg3-ELISA OD and IIF titer. Furthermore, when multiple serum samples from 1 individual patient sampled...
over a 2-year period were analyzed, ELISA OD values paralleled both the IIF titer and the clinical course (Figure 3).

**COMMENT**

Recently, a Dsg3-ELISA was shown to represent a sensitive and disease-specific test for the diagnosis of PV.\textsuperscript{16} We produced rDsg3 protein in our laboratory and established ELISA to validate the test by screening serum samples from patients with PV seen in our department. Furthermore, we expanded the published results by testing additional disease groups, including other bullous dermatoses or autoimmune-connective tissue diseases.

Forty-seven of 52 PV serum samples obtained from 11 patients were positive by Dsg3-ELISA, indicating a sensitivity of 93%. Interestingly, the sensitivity of ELISA increased to 98% when IIF-negative PV serum samples were excluded, indicating that both tests have a similar sensitivity. In addition, 4 of 55 non-PV serum samples were positive by ELISA, resulting in a specificity of 93%. These results obtained in a population of mostly Austrian dermatologic patients are comparable with those obtained from Japanese patients as previously published by Ishii et al.\textsuperscript{16}

To determine whether ELISA was prone to false-positive results in patients with other autoimmune diseases who often have high levels of autoantibodies that might cross-react with Dsg3, we tested serum samples from patients with non-PV bullous diseases and autoimmune–connective tissue diseases. The high specificity of the test became obvious from the fact that 11 of 11 serum samples from patients with bullous pemphigoid were negative by ELISA as were 10 of 12 serum samples from patients with various other bullous diseases and 7 of 9 serum samples from patients with autoimmune–connective tissue diseases. A patient with pemphigus foliaceus, a subtype of pemphigus characterized by antibodies against the 160-kd glycoprotein Dsg1, was included in the controls.\textsuperscript{19,21} As expected, this patient with PF had no detectable reactivity to Dsg3 by ELISA, supporting the previously established diagnosis.

Among the 4 ELISA-positive serum samples from patients without PV, 1 was obtained from a patient with PNP. The positive Dsg3-ELISA result was surprising, as the autoantigens identified in PNP are commonly directed against components of the hemidesmosomes and the cytoplasmic plaque of desmosomes with molecular weights of 250 kd (desmoplakin I), 230 kd (bullous pemphigoid antigen), 210 kd (desmoplakin II, envoplakin),\textsuperscript{22} 190 kd (periplakin); (G. Anhalt, oral communication, May 1998), and 170 kd (not yet identified).\textsuperscript{23,24} However, recent reports\textsuperscript{25,26} provide evidence that in PNP, autoantibodies may also be directed against components of the extracellular matrix including Dsg3, suggesting the possibility of overlapping autoantibody specificities in the diseases of the pemphigus group. Recently, Amagai et al\textsuperscript{26} found that 25 of 25 patients with PNP were reactive in the Dsg3-ELISA and half of them in the Dsg1-ELISA, indicating that desmoglein antibodies may play a pathogenic role in disease expression. Our patient with PNP demonstrated weak bands of 250, 230, 210, and 190 kd in the immunoprecipitation, but no 130-kd band, suggesting that the Dsg3-ELISA allows detection of low levels of anti-Dsg3 antibodies that may be missed by conventional detection methods.

The second ELISA-positive non-PV serum sample was derived from a patient with an unspecified bullous disorder that showed a nondiagnostic histopathologic condition and was negative by IIF for anti-ICS and basement membrane antibodies. It remains unclear whether this patient had PV with low levels of pathogenic anti-
Dsg3 antibodies or whether the detected antibodies are irrelevant for disease expression.

Furthermore, serum samples from 2 patients with high-titer antibodies against nuclear antigens showed ELISA reactivity, 1 with a high OD. Since these patients completely lacked blisters or erosions, the positive ELISA values may represent nonspecific binding of circulating autoantibodies as frequently observed for serologic testing in patients with connective tissue diseases, although cross-reactivity or the presence of nonpathogenic Dsg3 antibodies cannot formally be excluded. Similar to the Dsg3-ELISA, IIF assays may detect anti-ICS reactivity in serum samples of patients with bullous disorders other than PV, including cicatricial pemphigoid, bullous pemphigoid, dermatitis herpetiformis, erosive lichen planus, recurrent oral ulcers, and toxic epidermal necrolysis.27

A major advantage of the Dsg3-ELISA is the selective detection of antibodies against extracellular and conformational epitopes of Dsg3, which include most pathogenic antibodies in patients with PV.2,3,16 In contrast, conventional IIF testing may also identify a variety of antibodies against proteins of the extracellular matrix.3,16,28-30 Indirect immunofluorescence also may fail to distinguish between antibodies directed against the intracellular portion of Dsg3, antibodies against Dsg1, or desmocollins, or other ICS constituents. Antibodies against Dsg1 are commonly present in PV serum samples. Injection studies in neonatal mice suggested that they do not play a major role in the pathogenesis of PV.3 However, in humans, both Dsg3 and Dsg1 antibodies seem to be required to induce skin blisters, whereas patients with PV with Dsg3 antibodies in the absence of detectable Dsg1 antibodies will only develop blisters orally. This has been proposed as a possible explanation for the differential requirements of Dsg1 and Dsg3 antibodies in blister formation of mucosa vs skin. Therefore, testing patient serum samples with a Dsg3-ELISA alone or in combination with a similar Dsg1-ELISA may be valuable for the characterization of autoantibodies in patients with bullous diseases.

Determination of antibody titers by IIF analysis requires serial dilutions of serum samples, is subjective, and depends on an experienced examiner. A potential advantage of the PV ELISA over IIF is that the process can be fully automated and that an objective OD value is obtained at a single dilution of 1:10,000. Similar to IIF, the PV ELISA allows the estimation of pemphigus antibody levels. This aspect may be of importance for patient management, because the levels of antibody can parallel the clinical course.10,11,16,31,22 When we compared antibody titers by IIF with ELISA OD values, we found a positive correlation (r = 0.65) between the 2 tests. Furthermore, ELISA values paralleled IIF titers and, even more important, the clinical course, as shown for 1 patient with a long-lasting history of PV involving the oral mucosa and the skin (Figure 4), in which multiple serum samples collected over a period of 2 years were available. Although statistical analysis was not performed because of the small sample size, ELISA values and IIF titers increased at the time of disease relapse and decreased after treatment-induced remission. Interestingly, the increase in ELISA values occurred earlier and were more pronounced compared with IIF titers. This may reflect a higher sensitivity of ELISA in specifically detecting pathogenic anti-Dsg3 antibodies, which may be helpful in monitoring the clinical course and for adjustment or discontinuation of the immunosuppressive therapy. Larger and prospective studies are required to determine the exact relationship between Dsg3-ELISA values, IIF titers, and the clinical course.

Thus far, the most reliable indicator for PV remission seems to be a negative direct immunofluorescence result,33 requiring repeated skin biopsy specimens. Further investigations will determine whether a negative Dsg3-ELISA result obtained during clinical remission is a sensitive indicator to predict if the disease will stay in remission following cessation of therapy.

Our data confirm that the Dsg3-ELISA is a highly sensitive and specific test that allows objective and quantitative detection of pathogenic Dsg3 antibodies. Thus, Dsg3-ELISA should be considered an additional diagnostic tool for the routine evaluation and monitoring of patients with PV.

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