The Clinical Phenotype of Pemphigus Vulgaris Correlates to the Anti-Desmoglein Antibody Levels: The First Study Performed in Greece

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Purpose: The aim of the present study was to evaluate the levels of circulating antibodies against desmoglein (Dgs) 1 and Dgs3 in serum samples from patients with pemphigus vulgaris (PV) and correlate these values to the clinical phenotype of the disease.

Materials and Methods: Serum samples were obtained from 25 patients with oral mucosal dominant PV (group II), 23 patients with mucocutaneous PV (group III), 15 PV patients free of clinical symptoms (group I) and 30 healthy donors. Antibody titres against Dgs1 and Dgs3 were measured with enzyme-linked immunoabsorbent assay (ELISA) using recombinant Dsg1 and Dsg3. The relationship between the clinical phenotype and Dgs1 or Dgs3 levels was examined using ordinal logistic regression.

Results: Serum samples from groups I and II were positive for antibodies against Dgs3. Positive anti-Dsg3 and anti-Dgs1 antibodies were detected in the mucocutaneous PV group. Antibody titres against Dsg3 correlated with the serum circulating antibodies and the disease progression. Dgs1 levels and clinical phenotype were not significantly associated.

Conclusions: The clinical phenotype of the disease is defined by the anti-Dsg autoantibody profile whereas the measurement of the anti-Dsg3 autoantibody levels should be considered as a reliable indicator of disease progression.

Key words: pemphigus vulgaris, desmoglein 1, desmoglein 3, clinical phenotype

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INTRODUCTION

Pemphigus vulgaris (PV) is either an acute or a chronic autoimmune blistering mucocutaneous disease, which presents with blisters and erosions of the skin and/or mucous membranes (Robinson et al, 1997; Sirois et al, 2000a; Anhalt and Diaz, 2001). Virtually >50% of all patients with PV develop painful oral erosions prior to the development of cutaneous lesions (Robinson et al, 1997; Sirois et al, 2000a; Fassmann et al, 2003).

Patients with PV present a classic triad of diagnostic features in the absence of any of which, the diagnosis should be questioned. Histological evaluation of a biopsy specimen obtained from seemingly healthy perilesional skin or mucosa, will reveal the development of intraepithelial acantholytic blisters. Secondly the use of direct immunofluorescence (DIF) will show the presence of IgG and often C3 antibodies that bind to the cell surfaces of perilesional skin or mucosa and thirdly, indirect immunofluorescence (IIF) will demonstrate autoantibodies in the patient's serum (Sirois et al, 2000b; Fassmann et al, 2003).

The autoantigens for PV have been identified as Dsg1 and Dsg3. These transmembrane glycoproteins are members of the cadherin family and they mediate weak calcium-dependent homophilic cell-to-cell adhesion of keratinocytes in stratified squamous epithelia (Stanley, 1993; Amagai, 1996; Koch et al, 1997; Anhalt and Diaz, 2001). It has been previously reported that serum samples from patients with mucosal dominant PV are positive against Dsg3 but negative against Dsg1, while serum samples from patients with mucocuta-

neous PV are posistive against both Dsg1 and Dsg3 (Amagai et al, 1999b; Harman et al, 2001).

The severity of the disease process in relation to serum autoantibody levels has been investigated in many studies using IIF (Katz et al, 1969;, Sams and Jordan, 1971; Beutner et al, 1985). Harman et al (2001) have recently demonstrated that IIF titres vary according to both the epithelial substrate used and the relative quantities of Dsg1 and Dsg3 antibodies in the test serum, thus complicating correlations with the disease severity. In addition, IIF is a semiquantitative and subjective technique depending on an experienced examiner and may fail to distinguish between antibodies directed against desmogleins, desmocollins or other intercellular substance constituents (Lenz et al, 1999; Harman et al, 2001).

In the past few years, researchers have managed to produce recombinant proteins (rDsg1 and rDsg3), which represent epitopes of the native antigens, and developed a sensitive and highly specific enzyme-linked immunoabsorbent assay (ELISA) system for the detection of autoantibodies against Dsg1 and Dsg3 (Amagai et al, 1999a; Cheng et al, 2002). ELISA provides a highly sensitive and disease specific technique that allows objective and quantitative detection of pathogenic Dsg1 and Dsg3 antibodies separately. Since then, ELISA has been a useful tool for establishing a proper diagnosis and monitoring disease activity in patients with PV (Amagai et al, 1999a; Amagai et al, 1999b; Lenz et al, 1999; Cheng et al, 2002).

The aim of the present study was to evaluate, using ELISA, the levels of circulating antibodies against Dgs1 and Dgs3 in patients with PV and correlate those values to the clinical phenotype of the disease.

MATERIALS AND METHODS

Patients

Serum samples were obtained from 63 (16 men, 47 women) ambulatory and hospitalized patients with PV, from the Oral Pathology and Dermatology Clinics of "Evagelismos" General Hospital in Athens, and 30 healthy donors (18 men, 12 women). The patients were subdivided into three clinical groups according to the phenotype of the disease. Twenty-five patients (8 men, 17 women) had oral mucosa disease only (group-II, mucosal dominant type), 23 (4 men, 19 women) mucosal and minor skin disease (group-III, mucocutaneous type), while the remaining 15 PV patients (4 men, 11 women) were free of clinical signs (group-I), on a stable dose of medication and low antibody titres. Mucosal dominant type showed extensive oral erosions or blisters with no skin involvement. Mucocutaneous type showed extensive oral erosions or blisters and minor skin involvement (extensive confluent erosions were not seen). Diagnosis was based on clinical and histological findings as well as on positive DIF and IIF. The mean age was 57,1 years for the female patients (age range: 40-78 years), 57,8 years for the male patients (age range: 44-68 years) and 48,1 years for the healthy donors (age range: 35-55 years) (Table 1).

Table 1 Subdivision of PV patients according to sex, age and clinical phenotype of the disease

	Number of subjects	Female	Male	Mean age (years)
Absence of clinical signs (group I)	15	11	4	61.7
Oral mucosal dominant PV (group II)	25	17	8	55.9
Mucocutaneous PV (group III)	23	19	4	53.7
Healthy donors	30	12	18	48.1

Antibody Studies

Autoantibodies against intracellular substances were detected by IIF using, as antigen, cryosections of primate oesophagus and fluorescin isothiocyanate conjucated goat antihuman IgG (INOVA). A titer ≥ 1: 40 was considered to be positive.

Antibody titer against Dsg1 and Dsg3 were measured with ELISA using recombinant antigen Dsg1 and Dsg3 (Rhi Gene Inc).

Briefly, ELISA plates coated with recombinant purified proteins of Dsg1 and Dsg3 were incubated for 60±5 minutes at room temperature with calibrators and patient sera diluted 100-fold with dilution buffer (Tris-HCl, NaCl, CaCl2, bovine serum, high five cell extract and sodium azide, pH= 7,5). Each sample was run in duplicate. After washing to remove any unbound serum protein, the plates were incubated with 100-fold diluted horsedish peroxidase conjucated mouse monoclonal antihuman IgG (Fab) antibody for 60±5 minutes (1 hour). Following another wash step, the peroxidase substrate [3, 3', 5, 5'- tetramethyl- benzidine dihydrochloride/hydrogen peroxide (TMB/H2O2)] is added and allowed to incubate for 30±5 minutes. Stop solution (1,0N sulfuric acid) is then added to each well to terminate the enzyme reaction and to stabilize the color development. The absorbance of each well was readed on a platereading spectrophotometer at wavelength

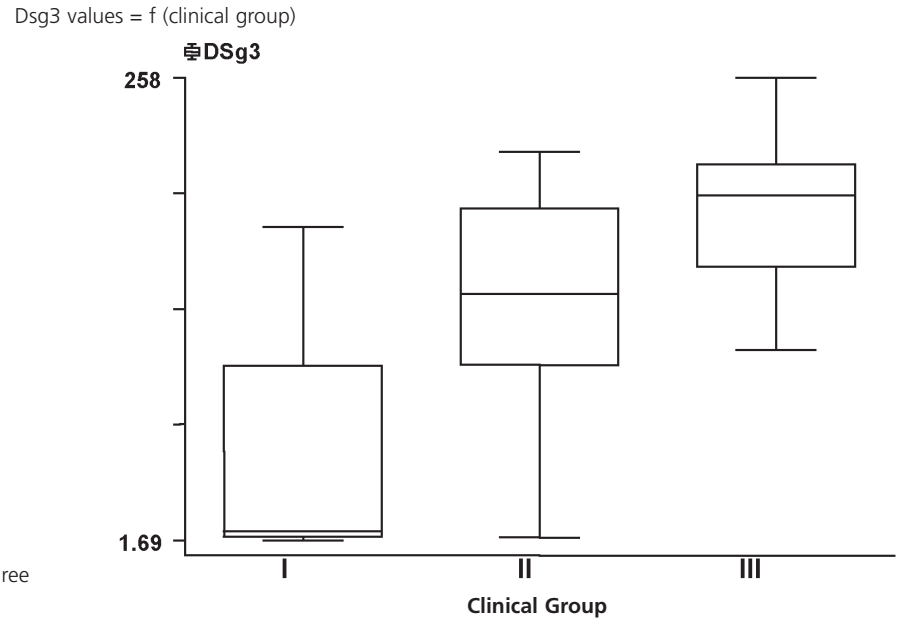

Fig. 1 Mean values of Dsg3 in the three groups of patients.

450nm and the reference at 630nm. The index value was calculated by the following formula:

Index value= (optical density [OD] of tested serum sample–OD of negative control) / (OD of positive control-OD of negative control) X100.

A cut-off value was defined to be 14,0 for Dsg1 and 9,0 for Dsg3 ELISA. An index value above 20,0 was considered to be positive. Index value between the cutoff value and 20,0 was defined to be a gray zone (indeterminate).

Statistical Analysis

In order to compare Dgs1 and Dgs3 levels among the three clinical groups, the Kruskal Wallis non-parametric test was used. Multivariate ordinal logistic regression was also used to determine the relationship between Dgs1, Dgs3 levels and the clinical phenotype of the disease. Model fitting was performed using a backward elimination procedure. Maximum likelihood estimators were produced using this type of analysis. Logistic regression analysis was performed to produce estimates using as outcome the presence or not of skin lesions and the development or not of oral lesions as well. Then lroc graphs were drawn. Analyses were performed using the statistical package Stata.

RESULTS

None of the 30 healthy donors' serum samples exceeded the index value of 20.0, above which a sample was considered to have a positive reaction against Dsg1 or Dsg3.

All the patients with mucocutaneous PV as well as 92% of the patients with mucosal dominant PV were positive for antibodies against Dgs3. It is of interest to note that 40% of the patients with no clinical signs of disease were also positive for antibodies against Dgs3 (Table 2). Dsg3 levels were increased in groups II and III compared to group I and the differences were statistically significant among the three groups (Fig 1).

No significant difference in Dsg1 levels among the three clinical groups was found. It is noteworthy however that the mean value of Dsg1 levels in the group of patients with oral and skin lesions was two times greater than Dsg1 levels in the other two groups (Table 2, Fig 2).

Table 2 Mean values of Dsg1 and Dsg3 in the three groups of patients

	Obs	Mean	Std.Dev.	Min	Max
Group I					
dsg1	15	10.55467	16.26873	0.15	56.5
dsg3	15	46.32267	63.65831	1.69	175.4
Group II					
dsg1	25	10.9184	20.14188	0.3	77
dsg3	25	127.372	74.46384	3.1	216.8
Group III					
dsg1	23	20.56826	37.88693	0.15	141.4
dsg3	23	183.3609	34.77061	107.1	258

Dsg1 levels were not significantly associated with the clinical phenotype of the disease (β:0,0117, p-value: 0,214, 95% confidence interval (CI): -0.006 to 0.03). In

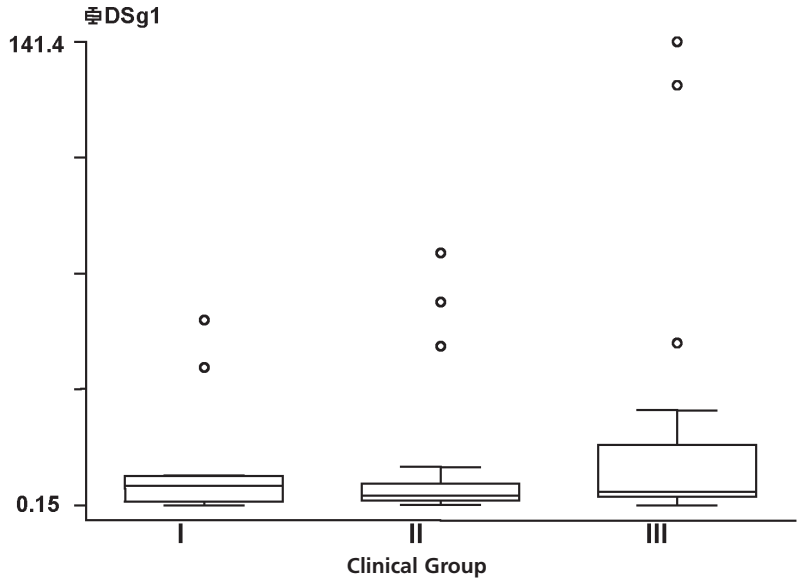

Fig. 2 Mean values of Dsg1 in the three groups of patients.

contrast clinical phenotype of PV and reactivity to Dsg3 were significantly associated. One unit increase in Dsg3 levels lead to 2% more odds of disease progression (β: 0,0204 , p-value: <0,001 , 95% CI: 0,012 to 0,028).

Lroc graphs were produced, using simple logistic regression, in order to estimate the cut-off value that best predicts clinical progression according to Dsg3 levels. Cut-off value of 149 for Dgs3 (sensitivity 87%, specificity 80%) best predicts the initiation of the disease and the development of at least one oral mucosal lesion (Fig 3), whereas the cut-off value of 182 (sensitivity 87%, specificity 70%) underlines the progression of the disease with the development of at least one cutaneous lesion (Fig 4).

Cut-off points were not produced for Dsg1 because no significant association with the clinical phenotype of the disease was noted. It should be also emphasized that autoantibody levels as detected by IIF paralled ELISA values for both Dsg1 and Dsg3 in all three clinical groups of patients.

DISCUSSION

Ever since the cloning of Dsg3 gene and expression of the protein in a native formation, researchers have been able to develop a highly specific and sensitive ELISA method for the detection of PV autoantibodies, which has been a very useful additional diagnostic tool for the evaluation and monitoring of PV patients with varying clinical severity (Amagai et al, 1999a; Cheng et al, 2002). In the present study we evaluated the autoantibody responses to Dgs1 and Dgs3 by the recently established ELISA method, in a large number of serum samples obtained from patients with various clinical PV phenotypes and healthy individuals.

In previous studies Amagai et al (1999b) showed a clear association between the clinical phenotype of pemphigus and the Dsg antibody profile. Their findings indicated that patients with oral mucosal dominant PV are positive against Dsg3 but negative against Dsg1 while patients with mucocutaneous PV are positive against both Dsg1 and 3. On the other hand, Harman et al (2001) related Dsg1 antibodies to skin severity and Dsg3 antibodies to oral severity. In particular, the balance of skin and oral disease was determined principally by the quantities of Dsg1 and 3 autoantibodies respectively. They were unable to demonstrate a relationship between Dsg1 antibodies and oral severity or Dsg3 antibodies and skin severity.

Our data are also suggestive of a strong relationship between Dsg3 autoantibodies and both oral and skin involvement, since all patients (100%) with mucocutaneous PV as well as 92% of the patients with mucosal dominant PV were positive for antibodies against Dgs3. We were also able to demonstrate that a high percentage of controlled PV patients with no obvious clinical signs of disease (40%) had also detectable Dsg3 antibodies. These observations suggest that Dsg3 autoantibodies are directly pathogenic and should therefore be very useful in monitoring PV patients regardless of the clinical phenotype of the disease.

We were also able to determine that the cut-off value of 149 for Dgs3 (sensitivity 87%, specificity 80%) seems to trigger the initiation of the disease with the development of at least one oral mucosal lesion, whereas the cut-off value of 182 (sensitivity 87%, specificity 70%) initiates and/or predicts the disease progression to the skin with the development of at least one cutaneous lesion. Researchers have previously reported that a 10-unit increase in the Dsg3 ELISA value was associated

Cut off point for DSG3: 149
SENSITIVITY: 87%
SPECIFICITY: 80%

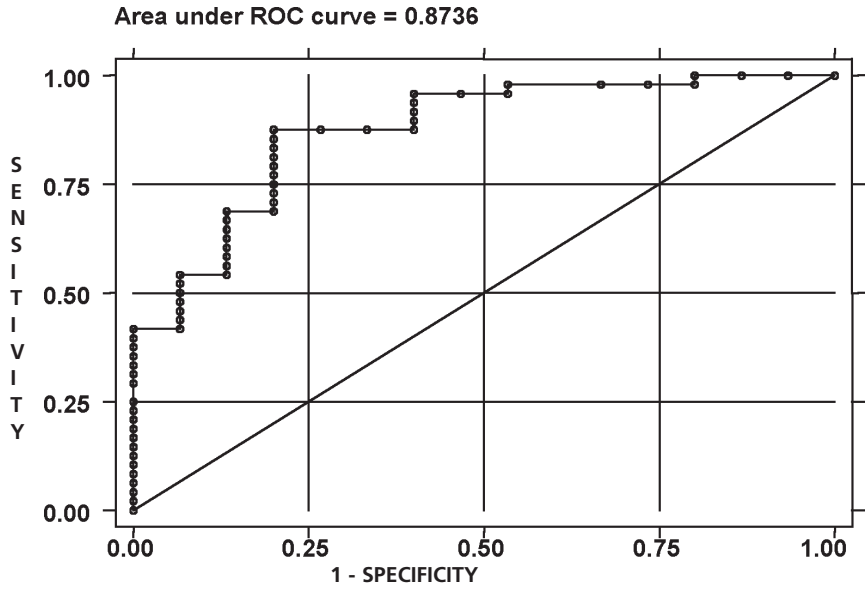

Fig. 3 Roc curve for Dsg3-Oral mucosal dominant PV (group II).

Cut off point for DSG3: 182
SENSITIVITY: 87%
SPECIFICITY: 70%

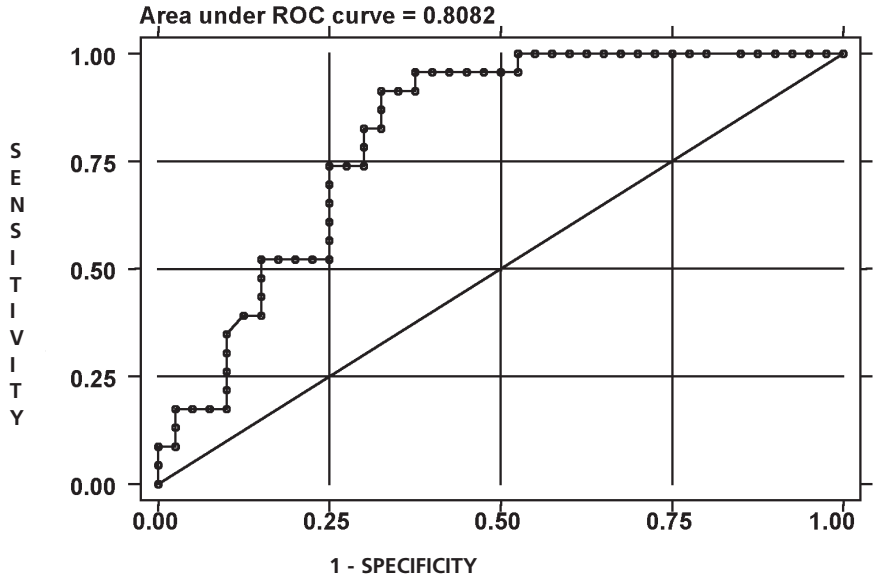

Fig. 4 Roc curve for Dsg3-Mucocutaneous PV (group III).

with a 25% chance of a higher oral severity score (Harman et al, 2001). We have also observed that an increase of one unit in the Dsg3 ELISA value was significantly associated with 2,04% increased odds of disease progression which demonstrates that Dsg3 autoantibodies are not only directly pathogenic but they are also useful markers of the disease activity.

It has been experimentally confirmed that the development of both Dsg1 and Dsg3 autoantibodies is necessary for the induction of gross skin blisters and more specifically that anti-Dsg3 antibodies alone are unable to cause dermal involvement (Ding et al, 1997). Ding et al (1997) found that Dsg1 antibodies were detected in patients with mucocutaneous PV but not in mucosal PV. A few years later several investigators confirmed these findings (Ishii et al, 1997; Amagai et al, 1999b; Harman et al, 2000). While other studies have shown that there is a late development of anti-Dsg1 antibodies in PV, which correlates with the disease progression (Miyagawa et al, 1999), Harman et al (2000), in a follow-up study, detected Dsg1 autoantibodies early in the course of the disease which did not relate to the use of any systemic therapy and suggested that these antibodies are predictive of a potentially more severe disease with extensive cutaneous ulcerations. On the other hand they reported that Dsg3+/Dsg1- patients would not necessarily develop Dsg1 antibodies. The same authors also suggested

that genetic factors may play an important role in determining the Dsg1 antibody profile since a statistically significant difference in Dsg1 reactivity was observed in various racial groups of patients (Harman et al, 2000).

In the present study, we were unable to demonstrate a statistically significant correlation between detectable reactivity against Dgs1 and clinical group of PV patients. Since the presence of Dsg1 is indicative of a severe disease process with extensive cutaneous involvement (Harman et al, 2000), these results can be presumably explained taking under consideration that in our group of mucocutaneous PV, skin involvement was minor compared to the severity of oral lesions.

It was of great interest to note however, that the mean value of Dgs1 in the group of patients with mucocutaneous PV (group III) was two times greater compared both with the group of patients with mucosal dominant PV (group II) and group of patients without clinical signs. Further studies using a larger number of patients with mucocutaneous disease and varying degree of cutaneous involvement, will eventually enable us to subdivide them into major and minor skin disease, and may disclose significant correlation with Dsg1 antibodies.

In summary, the clinical phenotype of PV is related to the anti-Dgs3 antibody levels. Dgs3 in contrast to Dgs1 had a significantly quantitative and qualitative association with the development of oral and cutaneous PV lesions. A high percentage of patients free of clinical signs also had detectable serum antibodies against Dgs3. In our series Dgs1 did not correlate with the development of cutaneous lesions but its values seemed to be increased in the group of patients with mucocutaneous disease (group III). The ELISA method, which can separately detect Dgs1 and 3 antibodies, despite its minor limitations (Bystryn et al, 2002) is a reliable and helpful guide for the diagnosis and evaluation of patients with PV.

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