Correlation of Antibodies against Desmogleins 1 and 3 with Indirect Immunofluorescence and Disease Activity in 72 Patients with Pemphigus Vulgaris

Dubravka Živanović[1], Ljiljana Medenica[1], Ivan Soldatović[2], Dušan Popadić[3]

[1]Clinic of Dermatovenereology, Clinical Center of Serbia, School of Medicine, University of Belgrade, Belgrade, Serbia; [2]Institute of Medical Statistics and Informatics, School of Medicine, University of Belgrade, Belgrade, Serbia; [3]Institute of Microbiology and Immunology, School of Medicine, University of Belgrade, Belgrade, Serbia

Corresponding author:

Dubravka Živanovic, MD, PhD

Clinic of Dermatovenereology

School of Medicine, University of Belgrade

Pasterova 2

11000 Belgrade

Serbia

dubravkazivanovic@yahoo.com

ABSTRACT The enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IIF) have both been used for testing of antibodies to desmogleins 1 and 3 (anti-Dsg1 and anti-Dsg3) and for the serologic diagnosis of pemphigus. IIF values and antibody concentrations and profile do not always correlate with a specific clinical phenotype and with the disease activity. The purpose of the present study was to correlate the clinical phenotype of patients with pemphigus vulgaris (PV) and the disease activity with anti-Dsg1 and anti-Dsg3 antibodies and IIF titers. A total of 72 patients with PV underwent ELISA serum testing for the presence and titers of anti-Dsg1 and anti-Dsg3 and IIF which were correlated with the severity of the disease (evaluated using the Pemphigus Disease Area Index, PDAI), clinical phenotype, and clinical course. In 79.2% patients there was a perfect correlation between the clinical phenotype and antibody profiles; in 20.8% patients, clinical features and antigenic findings were discordant. A statistically significant correlation was found between disease activity and a) anti-Dsg3 and anti-Dsg1 concentrations (Rho=0.679, $P<0.001$ and Rho=0.363, $P=0.02$, respectively) and b) IIF titers (Rho=0.426, $P<0.01$), as well between IIF titers and anti-Dsg3 and anti-Dsg1 antibodies (Rho=0.742, $P<0.01$ and Rho=0.372, $P=0.02$, respectively). This study supports the previous observations that the disease severity in most patients with pemphigus correlates with IIF titers, which in turn is determined by the quantities of Dsg1 and Dsg3 antibodies, as well as the previous observation that the clinical phenotype and antibody profile are not always in correlation.

KEY WORDS: pemphigus vulgaris, desmogleins, antibodies, ELISA, IIF

Abbreviations:

PV: pemphigus vulgaris

PF: pemphigus foliaceus

Dsg: desmogleins

ELISA: enzyme-linked immunosorbent assay

IIF: Indirect immunofluorescence

M PV: mucosal dominant pemphigus vulgaris

MC PV: mucocutaneous pemphigus vulgaris

PDAI: Pemphigus Disease Area Index

INTRODUCTION

Pemphigus vulgaris (PV) is a rare and serious autoimmune blistering disease of the skin and mucous membranes, with autoantibodies directed against two major antigens on the surface of keratinocytes – desmoglein 1 (Dsg1) and desmoglein 3 (Dsg3) (1). Studies using indirect immunofluorescence (IIF) suggested that antibody titers correlate with disease activity (2). Development of enzyme-linked immunosorbent assays (ELISAs) for detection of anti-Dsg1 and anti-Dsg3 confirmed the significance of those autoantibodies in the pathogenesis of pemphigus and demonstrated higher sensitivity and specificity than IIF (3-5). It is generally believed that predominantly oral PV is characterized by the presence of anti-Dsg3 alone, whereas mucocutaneous PV is characterized by the presence of both anti-Dsgs (4-6) and that the alterations in the autoantibody profile in the sera of patients with PV correlate with the disease activity (7-10).

In this study, correlations were made between antibody profiles in patients with different subtypes of PV and between anti-Dsg1, anti-Dsg3, and IIF values which were also assessed in relation to disease severity and stage.

PATIENTS AND METHODS

This study was conducted as a cross-sectional study. From January 2012 to December 2013, seventy-two patients (46 women and 26 men) aged 20-88 years (mean age 53.5 years), with diagnosed PV based on clinical examination, direct immunofluorescence, and histopathology, were included in the study. Patients were classified into two groups (mucocutaneous and mucosal dominant type) based on the distribution of lesions. Different stages of the disease were defined following the consensus statement on pemphigus (11) and disease severity was evaluated using the Pemphigus Disease Area Index (PDAI) (12).

A single blood sample for autoantibodies detection was obtained from all patients (N=72). Newly-diagnosed patients were sampled at the time of admission, before starting the treatment. Patients in relapse were sampled after exacerbation, and patients in remission at routine check-ups. Before sampling, written consent was obtained from each subject. This study is in accordance with the standards of the Helsinki Declaration and has been approved by the Ethical Committee of the Clinical Center of Serbia and the Ethical Committee of the University of Belgrade.

Table 1. Indirect immunofluorescence (IIF) titers, antibody profile and disease severity of patients with pemphigus vulgaris (PV)

Pemphigus type	Dsg1-/Dsg3-	Dsg1+/Dsg3-	Dsg1-/Dsg3+	Dsg1+/Dsg3+	Total
MC PV (n=64)	5 (7.8%)	7 (10.9%)	20 (31.2%)	32 (50.0%)	64 (100%)
Negative IIF	3	3	3	3	12 (18.75%)
IIF: 1/20, 1/40	2	3	7	8	20 (31.25%)
IIF: 1/80	0	1	2	9	12 (18.75%)
IIF: ≥1/160	0	0	8	12	20 (31.25%)
PDAI, median (IQR)	9 (1.5-24.0)	32 (11-36)	10.5 (1.75-46.0)	**53.5 (37.5-65.7)**[†,‡]	38 (11.25-54.0)
M PV (n=8)	3 (37.5%)	0	5 (62.5%)	0	8 (100.0%)
Negative IIF	2	0	2	0	4 (50.0%)
IIF: 1/20, 1/40	1	0	0	0	1 (12.5%)
IIF: 1/80	0	0	2	0	2 (25.0%)
IIF: ≥1/160	0	0	1	0	1 (12.5%)
PDAI, median (IQR)	3	0	21 (8-26.5)	0	10 (4-23.25)

*IQR: interquartile range; MC PV: mucocutaneous pemphigus vulgaris; M PV: mucosal dominant pemphigus vulgaris; PDAI: Pemphigus Disease Area Index

PDAI was significantly higher in MC PV patients with a Dsg1+/Dsg3+ profile (bold) compared with MC PV patients with a [†]Dsg1-/Dsg3+ (p<0.001) or with [‡]Dsg1+/Dsg3- profile (*P*=0.016).

IIF titers were significantly higher in MC PV patients with a Dsg1+/Dsg3+ profile compared with MC PV patients with [†]Dsg1-/Dsg3+ (*P*=0.029)

Indirect immunofluorescent tests and autoantibody determination

Serum specimens for IIF were serially diluted with phosphate-buffered saline and incubated on monkey esophagus as the epithelial substrate. IIF titers were classified into four groups, 1) negative, 2) positive, titers at 1/20 and 1/40, 3) positive, titer at 1/80, and 4) positive at 1/160 or higher dilution.

ELISA was performed using commercially available Dsg1 and Dsg3 ELISA tests (EUROIMMUN Medizinische LabordiagnostikaAG, Lübeck, Germany) according to the manufacturer's instructions. All sera were initially diluted 100-fold; if necessary, sera were diluted at 1:400 as instructed by manufacturer to obtain an index value (IV) under 200 RU/mL. True IVs were obtained by multiplying the original IVs with the dilution factor (×4). An ELISA index value >20.0 was considered positive for both anti-Dsg1 and anti-Dsg3.

Obtained Dsg IV and IIF values were correlated with disease activity expressed as a PDAI score.

Statistical analysis

Data are presented as number (percent) and or median (along with 1st-3rd quartile), depending on data distribution. The Kruskal-Wallis test and Mann-Whitney U-test were used to compare groups. Spearman correlation analysis was used to analyze correlation between variables. *P* values were corrected for multiple comparisons according to the Bonferroni method. All data were analyzed using SPSS 20.0 (IBM corp.). All *P* values lower than 0.05 were considered significant.

RESULTS

Disease severity, antibody profile, and IIF titers

The disease severity (PDAI), presented as the median value with interquartile range (IQR), was stratified in Table 1 according anti Dsg profile and IIF values.

There was a correlation between the clinical phenotype and antibody profiles in 79.2% of patients (52/64 with MC PV and 5/8 with M PV); in 20.8% patients the clinical phenotype and the antigenic profile were discordant (Table 1).

The IIF test was positive in 56/72 (77.8%) patients and negative in 16/72 (22.2%) patients (12 with MC PV, 4 with M PV) (Table 1). Of 8 patients with negative anti-Dsg antibodies 5 had a negative IIF test as well; the other 3 had lower IIF values (1/20 or 1/40). The IIF test was negative in 9 MC PV cases with either positive anti-Dsg3 or anti-Dsg1 or with an anti-Dsg3+/anti-Dsg1+ profile, as well in two M PV patients with positive anti-Dsg3 antibodies (Table 1).

Concentrations of anti-Dsg antibodies, IIF titers, and disease severity

A significant correlation was found between the severity of PV (PDAI) and IIF titers (Rho=0.426, p<0.001) and between IIF titers and anti-Dsg3 and anti-Dsg1 antibodies (Rho=0.742, p<0.001 and Rho=0.372, p=0.02, respectively) (Table 2).

Concordance was observed between IIF and anti-Dsg3 values since the kappa coefficient was high (κ=0.469) but not between IIF and anti-Dsg1 values (κ=0.178) (data not shown).

A significant correlation was detected between PDAI and levels of both anti-Dsg1 and anti-Dsg3 antibodies (Rho=0.679; p<0.001 and Rho=0.363; p=0.020, respectively) (Table 2).

In patients with MC PV, PDAI was significantly higher in those with an anti-Dsg1+/anti-Dsg3+ profile compared with those with anti-Dsg1-/anti-Dsg3+ or with anti-Dsg1+/anti-Dsg3- profiles (p<0.001 and p=0.016, respectively, data not shown). IIF titers were significantly higher in MC PV patients with an anti-Dsg1+/anti-Dsg3+ profile compared with those with anti-Dsg1-/anti-Dsg3+ (p=0.029, data not shown).

Concentrations of anti-Dsg1 and anti-Dsg3 antibodies in different stages of PV

Concentrations of anti-Dsg1 and anti-Dsg3 anti-

Table 2. Correlation of disease severity (PDAI), anti-desmoglein (Dsg) antibody concentration, and indirect immunofluorescence (IIF) values

	PDAI Correlation Coefficient	*P*	IIF Correlation Coefficient	*P*
Anti-Dsg3	0.363*	0.020	0.742**	p<0.001
Anti-Dsg1	0.679**	p<0.001	0.372*	0.020
IIF values	0.426**	p<0.001		

** Correlation is significant at the 0.01 level
* Correlation is significant at the 0.05 level
PDAI: Pemphigus Disease Area Index

bodies and IIF values in patients in various stages of the disease are summarized in Table 3.

The statistical significance of differences in anti-Dsg1 titers was calculated comparing new patients and 1) patients in relapse, 2) patients in complete remission, on and off therapy, as there was statistically significant difference in anti-Dsg3 titers between patients in complete remission on therapy and 1) new patients and 2) patients in relapse. After correction, *P* values showed a statistically significant difference only between anti-Dsg1 levels in new patients and a) patients in complete remission on and b) off therapy (*P* adj= 0.01 and *P* adj= 0.011, respectively).

The statistical significance of IIF values was calculated comparing patients in complete remission on therapy and 1) patients in disease relapse and 2) new patients. Corrected *P* values showed a statistically significant difference only between new patients and patients in complete remission on therapy (*P*=0.016).

The statisticaly significant difference in IIF titers and anti-Dsg3 levels between MC and M PV in new patients and patients in relapse was not recorded due to the small sample of M PV patients.

In all patients in complete remission and off therapy (n=7), anti-Dsg1 antibodies were negative, while anti-Dsg3 antibodies were detectable in 6/7 patients although they were disease-free and without any treatment ≥6 months; IIF titers were detectable or in a high titer in 5/7 patients (Table 4). In 9/13 patients in complete remission on therapy, anti-Dsg1 antibodies were negative; anti-Dsg3 antibodies were negative in 8/13 and detectable in 5/13 patients, with IIF values either negative or positive at lower dilutions, with the exception of two patients with highly positive anti-Dsg3 antibodies (Table 4).

DISCUSSION

For many years, IIF has been the standard test for detection of pemphigus autoantibodies and is still the standard assay to detect the intercellular antibodies associated with pemphigus (13). In patients with PV, IIF sensitivity has been reported at 70% to 90%, depending partially on the substrate used (2,13). To increase IIF sensitivity, a combination of substrates is used; with this modification IIF could still be used as an alternative test especially when ELISA is not available (2). ELISA is regarded as a specific tool for the diagnosis of pemphigus (14). Positive correlation between IIF titers and ELISA values has been recorded (6).

We found positive IIF tests in 77.8% patients; among 16 patients with negative IIF, 5 had negative anti-Dsg3 and anti-Dsg1 antibodies, 3 had negative anti-Dsg3 antibodies, and the other 8 patients had positive anti-Dsg antibodies (anti-Dsg3 or anti-Dsg1 or both) which could suggest ELISA as a useful diagnostic tool in patients with PV that have negative IIF. The significance of both assays is indicated by the recorded correlation of ELISA and IIF with the disease activity as well as the high correlation rate between IIF values and anti-Dsg3 (κ=0.469). This correlation rate was not observed between anti-Dsg1 values and IIF titers.

Table 3. Values of anti desmoglein (Dsg) 1 and 3 antibodies levels and total Pemphigus Disease Area Index (PDAI) in patients with pemphigus vulgaris (PV) in various stages of the disease

Stage of disease and PV subtype	Dsg1 (RU/mL)*	Dsg3 (RU/mL)*	IIF*	PDAI*
New patients (n=40) (M=4, MC=36)	131.20 (20.45-183.40)[†,‡]	172.70 (93.78-281.90)	1/80 (0-1/1280)[†]	50.50 (36.00-59.75)
Previously treated, relapse (n=12) (M=2, MC=10)	3.0 (1.25-157.78)	155.25 (122.13-249.53)	1/80 (0-1/640)	26.50 (18.75-45.25)
Complete remission, on therapy (n=13) (M=1, MC=12)	2.45 (0.67-22.80)[a]	21.70 (2.15-203.73)	1/20 (0- 1/320)[c]	8.50 (3.75-11.25)
Complete remission, off therapy (n=7) (M=1, MC=6)	2.50 (0.83-5.90)[b]	152.15 (33.43-800.0)	1/80 (0- 1/640)	0.50 (0.00-1.00)
Total (n=72)	26.65 (2.03-163.33)	153.60 (30.63-254.05)	1/40 (0-1/1280)	32.50 (10.25-53.75)

M: mucosal dominant type PV, MC: mucocutaneous PV
*Median (interquartile range)
†compared with patients in complete remission, on therapy
‡compared with patients in complete remission, off therapy
After Bonferroni correction, statistical significance was recorded in anti Dsg1 levels values in patients during complete remission, on therapy, compared with new patients with PV (*P* adj=[a]0.01 and [b]0.011), and for IIF titers in patients during complete remission, on therapy, compared with new patients (*P* adj=[c]0.016) with PV.

Several studies have shown that antibody specificities and titer do not always correlate to the clinical phenotype (7,9,15). In the majority of our patients (79.2%), the antibody profile was in correlation with the clinical phenotype, whereas this was not the case in the other 20.8% patients. In seven (10.9%) patients with MCP, only anti-Dsg1 antibodies were present (with IIF titers ranging from negative up to 1/80 in one patient). Contrary to Mortazavi *et al.*, who reported an antibody profile compatible with PF in one patient with the clinicopathological setting of PV and suggested that the anti-Dsg3 autoantibody level might be too low to cause mucosal lesions (16), all our patients with only anti-Dsg1 antibodies had mucosal lesions. The IIF test was 1/80 in one patient and ranged from negative to 1/40 in other anti-Dsg3 negative patients. The clinicopathological settings of our Dsg1+/Dsg3- MC PV patients were not consistent with PF. The patients received further follow-up due to a possible shift to PF.

It has been suggested that the presence of anti-Dsg1 antibodies predicts a potentially more severe disease subgroup (7). Our observations are similar. A statistically significance difference was recorded between the disease severity of patients with MC PV who were Dsg1+/Dsg3- vs. Dsg1+/Dsg3+.

We recorded 5/64 (7.8%) MC PV patients who were Dsg3-/Dsg1-; IIF test was negative in three of them. They were in complete remission while on therapy. Although it could be argued that immunosuppressive therapy may influence the antigenic profiles, this is presently unknown (17,18).

All our patients with M PV were anti-Dsg1 negative. In other studies, anti-Dsg1 autoantibodies were recorded in various percentages in patients with M PV (6,7,17,19). Mucosal-limited PV may rarely lack anti-Dsg3 (6,20). In our study, 3/8 patients with M PV were anti-Dsg3 negative (one new patient, with a low IIF titer of 1/20, the other two patients being in complete remission on or off therapy, with negative IIF). We can only speculate that our results (both in patients with MC and M PV) could be similar to other reports where negative ELISA values were explained by the presence of pathogenic antibodies to non-desmoglein molecules or to the intracellular domain of Dsg1/Dsg3, undetectable by ELISA (4,20,21). Alternatively, following Avgerinou *et al*. who attributed their findings to a possibly particular feature of PV in that geographic area (6), we could make a similar attribution.

We found anti-Dsg3+ and anti-Dsg1+ in a lower percentage than other reports, 81.2% and 60.9% of patients with MC PV, respectively (6,16,17).

Interestingly, anti-Dsg1 antibodies were negative in 7/12 patients with MC PV in disease relapse (two of the patients had negative IIF, while the other five had IIF titers ranging from 1/20-1/80). In the other three patients with MC PV with disease relapse that were anti-Dsg1 negative, IIF titer was 1/80.

Table 4. Anti-desmoglein (Dsg) 1 antibodies, anti-Dsg3 antibodies, and indirect immunofluorescence (IIF) values in patients in complete remission (on and off therapy)

Patients in complete remission, on therapy	Pemphigus type	antiDsg1 (RU/mL)	antiDsg3 (RU/mL)	IIF	Treatment	Patients in complete remission, off therapy	Pemphigus type	Remission (months)	antiDsg1 (RU/mL)	antiDsg3 (RU/mL)	IIF
N1	MC	0.5	1.5	1/20	CS, A	N 1	MC	27	8.3	780.0	1/640
N2	MC	162.2	3.7	1/20	CS, A	N 2	MC	48	5.1	780.0	1/80
N3	MC	21.8	28.1	1/40	CS	N3	MC	6	7.7	800.0	1/320
N4	MC	25.8	2.0	0	CP	N4	MC	88	1.5	91.3	1/20
N5	MC	0.6	4.3	0	CP	N5	MC	102	3.5	213.0	1/80
N6	MC	7.5	233.8	1/40	CS, A	N6	MC	6	7.7	780.0	1/80
N7	MC	0.9	2.0	0	CS, A	N7	M	8	0.3	3.8	0
N8	MC	1.8	150.0	1/20	CS, A						
N9	MC	85.5	62.4	1/40	A						
N10	MC	3.5	213.0	1/80	CS						
N11	MC	2	287.2	1/320	CS						
N12	MC	2.9	193.7	1/20	CP						
N13	M	0.7	15.3	0	CS						

M: mucosal dominant; MC: mucocutaneous; CS: corticosteroids (prednisone equivalent of 10-30 mg daily); A: Azathioprine (25-50 mg daily); CP: Cyclophosphamide (50 mg daily)

Unexpectedly, there was no statistically significant difference between anti-Dsg3 levels in patients in various stages of PV nor between patients with active disease and either MC or M PV. We might explain the former by the presence of extremely high anti-Dsg3 levels in the majority of patients in complete remission, while the latter is probably due to the small number of patients with M PV in various stages of disease.

We conclude that, despite a rough correlation between antibody profile and clinical phenotype, the former cannot be regarded as an absolute marker for predicting the latter. This is in accord with other reports (6,7,9). In several studies, disappearance of Dsg3 antibodies was steadier and delayed compared with anti-Dsg1 antibodies (7,16,18,21,22). Our observations were similar to previously published data.

Major study limitations included a lack of initial ELISA evaluation and IIF testing and serial sampling in various stages of the disease in all patients. A detailed prospective study for evaluating the ELISA levels of a given patient for prolonged periods would be helpful in determining actual correlation with disease severity and changes that may occur in the spectrum of the disease.

CONCLUSIONS

Our findings suggest that the clinical phenotype is related to the antibody profile in the majority of patients, with a discordant clinical phenotype and antibody profile in occasional cases. The antibody concentrations and IIF titers correlate with disease severity in most cases. Anti-Dsg3 antibodies and IIF values are reduced upon treatment but may persist in higher concentrations for long periods in patients in complete clinical remission.

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