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INVESTIGATIVE REPORT

Autoantibody Levels and Clinical Disease Severity in Patients with Pemphigus: Comparison of Aggregated Anti-desmoglein ELISA Values and Indirect Immunofluorescence Titres

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Detecting serum-autoantibodies by anti-Desmoglein-1 (anti-Dsg1) and anti-Dsg3 ELISAs as well as indirect immunofluorescence (IIF) are established complementary methods to diagnose pemphigus. Whether autoantibody levels also reflect clinical disease activity is still a matter of debate, as head-to-head comparisons of ELISA values and IIF titres with clinical activity over a longer treatment period are scarce. In our retrospective study, we compared aggregated repetitive intra-patient ELISA values and IIF titres with grades of clinical disease (1 = remission, 2 = moderate activity, 3 = exacerbation) in 47 patients suffering from pemphigus vulgaris (PV, *n* = 36) and pemphigus foliaceus (PF, *n* = 11). We found that anti-Dsg1 ELISA values in PF and mucocutaneous PV as well as anti-Dsg3 ELISA values in PV best reflect disease activity. IIF titres, by contrast, did not show a significant association with disease severity. From these data we conclude that ELISA index values can be a valuable tool to monitor disease in patients with pemphigus, whereas IIF titres reflect clinical activity only insufficiently. ***Key words: pemphigus vulgaris; pemphigus foliaceus; anti-Dsg1; anti-Dsg-3; indirect immunofluorescence.***

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Pemphigus includes a group of autoimmune blistering skin diseases, which are mediated by autoantibodies against epithelial adhesion molecules. Both the mucous membranes and the skin can be affected, leading to blister formation and erosions. These can be extensive and, thus, be accompanied by high morbidity and, rarely, even mortality. For this reason, prompt initiation of immunosuppressive therapy and close clinical disease monitoring is mandatory (1–3).

The two major pemphigus subtypes, i.e. pemphigus vulgaris (PV) and pemphigus foliaceus (PF), are typically characterised by circulating as well as tissue-bound autoantibodies against desmoglein (Dsg) 3 (mucosal PV) and Dsg1 (PF), or both (mucocutaneous PV) (4, 5).

The pathogenicity of anti-Dsg1 and anti-Dsg3 antibodies has been demonstrated by a number of independent studies (6). After the description of IgG autoantibodies in the serum of pemphigus patients by Beutner & Jordon (7), indirect immunofluorescence (IIF) on monkey oesophagus has become a standard tool for the diagnosis of disease (8). In the late 1990s, enzyme-linked immunosorbent assays (ELISA) were introduced, using recombinant human Dsg (Dsg1 and Dsg3). These test systems demonstrated higher sensitivity and specificity than IIF for the detection of IgG serum autoantibodies (9, 10). Yet, IIF still has its role in detecting autoantibodies which are outside of the epitopes used in the Dsg ELISA systems or directed against non-Dsg targets such as desmocollins (11). Moreover, there are studies claiming that even in anti-Dsg positive pemphigus, intraepidermal blistering cannot be attributed exclusively to the presence of anti-Dsg 1 and 3 antibodies (12).

Whether ELISA index values and, especially, IIF titres are useful surrogates of clinical disease activity, is still a matter of debate (2, 12–15). A correlation between ELISA scores and disease activity has been assessed in several studies and it was proposed that ELISA values tend to correlate with the severity of the disease (2, 10, 13, 14, 16–24). More precisely, skin disease severity was initially suggested to be related to Dsg1 antibody levels, whereas Dsg3 antibody levels correlate directly with the involvement of mucous membranes (4, 21).

Some studies show that clinical improvement of pemphigus lesions is not always accompanied by a decrease in ELISA autoantibody values (2, 14), and even patients in complete clinical remission with persistently high anti-Dsg antibody levels have been described (10, 25–27). Studies on the usefulness of IIF titres to monitor disease are even more contradictory (10, 13, 20, 28–34).

Previous studies are often limited by small sample size and/or the lack of evaluating multiple timepoints over a longer period of time.

For this reason, we performed a head-to-head comparison of ELISA index values and IIF serum titres with

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different grades of clinical disease activity in patients suffering from PV or PF in an Austrian cohort by aggregating all repetitive measurements available for each patient over the entire treatment phase.

METHODS

Patients

In this retrospective study, we analysed data from 47 pemphigus patients (36 PV, 11 PF) treated between the years 2000 and 2012 at the Department of Dermatology, Medical University of Vienna, Austria, after approval by the Ethics Committee of the Medical University of Vienna (EK No. 637/2011) according to the Declaration of Helsinki.

All patients fulfilled the following inclusion criteria: (*i*) characteristic clinical and histological (including direct immunofluorescence) features of pemphigus; (*ii*) positive serological tests in PV (IIF, anti-Dsg3) and PF (IIF, anti-Dsg1) at least once during the course of the disease, combined with (*iii*) sufficient documentation of skin and mucous membrane involvement to allow a retrospective evaluation of disease activity. Paraneoplastic pemphigus was excluded by IIF on rat bladder, immunoblot and/or immunoprecipitation. Medical records were retrieved from patient charts and samples were collected from treated as well as untreated individuals. Due to inconsistent documentation of disease activity, we developed our own grading system for the retrospective assessment of disease severity (Table I). All of the grading values were assigned by a single investigator throughout the entire study period to guarantee consistency of results.

Immunological assays

Circulating IgG antibodies against Dsg1 and Dsg3 were detected using the MESACUP TEST by Medical Biologicals Laboratories Co. Ltd., Nagoya, Japan, according to the manufacturer's instructions. As the assay range of the Desmoglein ELISA kits is from 5 U/ml to 150 U/ml, initial ELISA index values > 150 were further diluted 1:16, and final results were multiplied with this dilution factor as previously described (14). IIF was performed on monkey oesophagus sections as previously described (8). Briefly, patient sera were incubated with an anti-AB neutralising reagent for 10 min at room temperature. Serial dilutions from 1:10 to 1:2,560 were performed in phosphate-buffered saline and incubated on monkey oesophagus slides for 30 min at room temperature. Antibody binding was eventually visualised by incubation with a FITC-labelled goat anti-human IgG antibody (all INOVA Diagnostics, San Diego, CA).

Statistical analysis

The parameters clinical activity, IIF titre, anti-Dsg1 and anti-Dsg3 ELISA index were assessed from the patients' charts for each visit. In order to prevent the results from being dominated by a few patients with a high number of repetitive observations, repeated measurements on the same patient and the same disease grade were aggregated by calculating the mean value. This aggregation resulted in a single mean value per patient and grade (if observations on that grade were available), equally weighing the information at the level of this observational unit. All further calculations were performed on the aggregated data.

Due to their highly skewed distribution, IIF titres and anti-Dsg index values were transformed by taking the logarithm to the base 2 for all further calculations. The resulting distribution was symmetric, allowing for a more robust estimation of mean values.

Linear mixed models were calculated to explain the mean differences of dependent variables between the 3 grades. In these models, the most general case of an unstructured error covariance matrix was estimated, allowing for different variances at each stage and different correlations for each pairwise combination of clinical grades. In a first step, the null hypothesis that the mean values in all 3 clinical grades are identical was tested by a global F-test. Only when this test was significant (i.e. $p \leq 0.05$), pairwise tests were calculated (i.e. grade 1 vs. 2, grade 1 vs. 3 and grade 2 vs. 3). The method of Kenward & Roger (35) was applied to calculate modified standard errors for the model coefficients and denominator degrees of freedom for the resulting test statistics. The calculations were performed using the MIXED procedure in SAS 9.3.

RESULTS

Baseline characteristics

Forty-seven patients with pemphigus (PV=36, PF=11) were enrolled in our study. Thirty-three (70.2%) of them were female, 14 (29.8%) were male. Their age ranged from 12 to 84 years with a mean ± SD of 55.3 ± 16.3. During the course of the disease, the following treatment regimens were introduced: Glucocorticoids 93.6%, mycophenolate mofetil 42.6%, azathioprine 31.9%, rituximab 17%, dapsone 4.3%, immunoapheresis 2%, and 6.4% received no systemic treatment at the visits documented. On average, patients were followed over 2.7 years and were tested for autoantibodies 6.2 times (range from 1 to 24). For 79.3% of individual ELISA tests ($n = 291$), concomitant IIF titres were available, and there was no IIF titre without corresponding ELISA value.

In the subgroup of PV, all patients tested positive at least once for autoantibodies in IIF and the anti-Dsg3 ELISA, and mucosal involvement was seen in 35 individuals. There was one atypical case of a $Dsg1^{-}/Dsg3^{+}$ PV patient with erosive plaques on the scalp, but lacking oral lesions. In 18 patients, the disease was limited to the mucosa, the rest showed mucocutaneous involvement. Anti-Dsg1 was positive in 41.7% of all 36 PV patients,

Table I. *Grading system for the retrospective assessment of disease severity*

Grade 1	Reflects a current period of remission and includes (*i*) patients with complete absence of pemphigus lesions as well as (*ii*) patients with single sporadic blisters/erosions during an otherwise quiescent course of the disease.
Grade 2	Comprises moderate disease activity, including patients with (*i*) cumulative discrete blisters/erosions and/or (*ii*) distinctively regressed lesions after an exacerbation (Grade 3), of which some, but not all, may be showing a tendency to heal.
Grade 3	Severe disease, including a significant worsening of the patient's clinical appearance with newly developed confluent blisters/erosions since the last visit.

namely in 11 (out of 17) patients with mucocutaneous and 4 (out of 18) patients with mucosal-limited disease.

In the subgroup of PF patients, all individuals tested positive at least once for autoantibodies in IIF and the anti-Dsg1 ELISA, and presented with characteristic skin involvement, lacking oral lesions. Nine patients (81.8%) were $Dsg1^+/Dsg3^-$ and 2 patients showed additional autoantibodies against Dsg3 ($Dsg1^+/Dsg3^+$) slightly above the cut-off level.

In PV, anti-Dsg3 and anti-Dsg1 autoantibodies but not IIF titres show a significant association with disease activity

In our PV patients, we assessed differences of anti-Dsg3 levels among our 3 predefined grades of disease activity. We found a constant increase in mean ELISA values with worsening disease (Fig. 1A). In the linear mixed model (global F-test $p = 0.019$), differences between grades 1 and 3 were found to be highly significant ($p = 0.004$). In comparison, IIF titres did not show significant differences (global F-test $p = 0.165$) (Fig. 1B). In parallel, we calculated anti-Dsg1 levels in patients suffering from mucocutaneous PV, as only these PV patients frequently show a positive anti-Dsg1 profile. We found the mean level of anti-Dsg1 autoantibodies to increase with worsening clinical disease (global F-test $p = 0.003$), and differences between grades 1 and 3 ($p = 0.003$) as well as between grades 2 and 3 ($p = 0.003$) were found to be highly significant (Fig. 1C). IIF titres showed a slight tendency of increase with worsening disease activity, but no significant differences could be detected (global F-test $p = 0.125$) (Fig. 1D).

In PF, anti-Dsg1 autoantibodies but not IIF titres show a significant association with disease activity

In the cohort of PF patients, anti-Dsg1 ELISA levels were significantly increased between grades 1 and 2 ($p = 0.019$), and even more so between grades 1 and 3 ($p < 0.001$; global F-test $p < 0.001$) (Fig. 2A). In contrast, IIF titres did not differ significantly among clinical grades (global F-test $p = 0.606$) (Fig. 2B).

DISCUSSION

We performed a retrospective analysis of 47 pemphigus patients (PV=36, PF=11) who had undergone

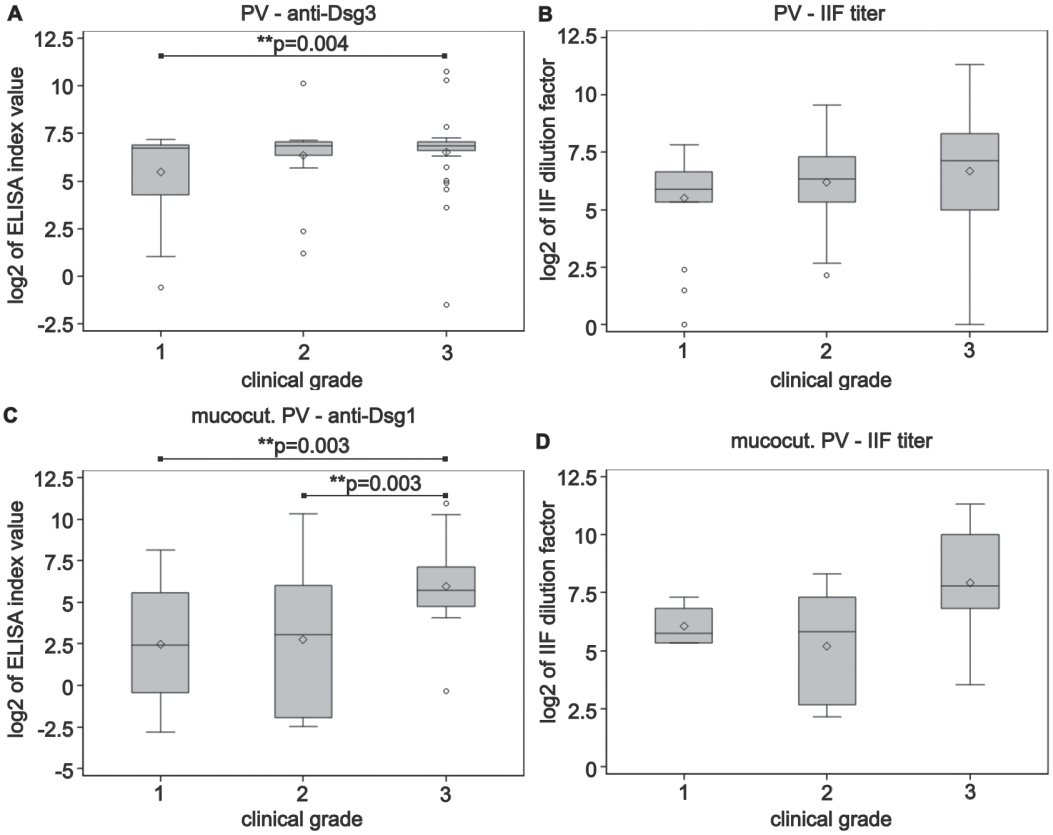

Fig. 1. Pemphigus vulgaris. The distribution of aggregated ELISA index or indirect immunofluorescence (IIF) values in each disease grade is depicted by box-and-whisker plots for all patients suffering from pemphigus vulgaris (PV) (A, B) or mucocutaneous PV (C, D). A diamond indicates the mean value. The lower end of the box, the horizontal bar within the box and the upper end of the box represent the first quartile, the median and the third quartile, respectively. The whiskers are drawn unto the smallest or largest observed value that is still within 1.5 times the interquartile range below the first quartile or above the third quartile, respectively. All observations not contained within these limits are shown as open circles. Significant differences ($p \leq 0.05$) are indicated with their *p*-values.

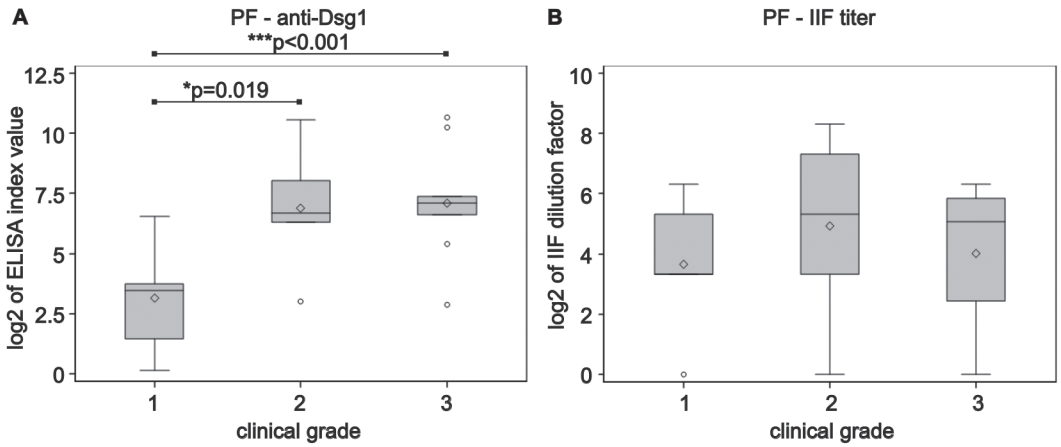

Fig. 2. Pemphigus foliaceus. The distribution of aggregated ELISA index (A) or indirect immunofluorescence (IIF) values (B) in each disease grade is depicted as box-and-whisker plots for patients suffering from pemphigus foliaceus (PF). A diamond indicates the mean value. The lower end of the box, the horizontal bar within the box and the upper end of the box represent the first quartile, the median and the third quartile, respectively. The whiskers are drawn unto the smallest or largest observed value that is still within 1.5 times the interquartile range below the first quartile or above the third quartile, respectively. All observations not contained within these limits are shown as open circles. Significant differences ($p \leq 0.05$) are indicated with their p-values.

treatment at the Department of Dermatology, Medical University of Vienna, Austria. Only patients with a "classical" antibody profile (IIF$^+$ and anti-Dsg3$^+$ in PV, IIF$^+$ and anti-Dsg1$^+$ in PF, at least once during the course of the disease) were included in our study. Epidemiological data and antibody profiles of our patients were comparable to previous reports (2, 22, 36–40), except for one female patient who presented with erosive, crusted, vegetating plaques exclusively on her scalp, but negative anti-Dsg1 values. Based on histological findings (suprabasal acantholysis), ELISA values (Dsg1$^-$/Dsg3$^+$), and immunoprecipitation (single band at 130 kDa, consistent with Dsg3) we favoured the diagnosis of a cutaneous type PV (22, 41), reminiscent of a previously published case described as Dsg1$^-$/Dsg3$^+$ pemphigus vegetans of the scalp (42), but lacking histological changes typical for this variant of PV such as papillomatosis, acanthosis and an abundance of eosinophils (43).

While anti-Dsg1/3 ELISAs and IIF have an established complementary role in the diagnosis of pemphigus (10, 19, 44), their usefulness for disease monitoring, based on a possible correlation of autoantibody levels with disease activity, is still controversial. Especially with IIF titres, many studies have been performed with relatively small sample sizes, resulting in contradictory data (12–15, 21, 28, 31, 32, 45, 46). To our knowledge, a comprehensive head-to-head comparison of anti-Dsg ELISA values and IIF titres with clinical disease activity in a PV/PF patient population with a sufficient sample size to obtain robust statistical analyses is lacking. In our model, we compared the mean of aggregated ELISA and IIF values between clinical grades (grades 1 vs. 2, 2 vs. 3, and 1 vs. 3), taking into account intraindividual dependencies of measurements. Consequently, our method does not rely on single observations, and is more robust against outliers. As we compare paired samples within a patient whenever available, we reduce variability in estimating differences between disease grades. One major limitation of our study was its retrospective nature, which precluded a statistically meaningful analysis of the impact of the various treatment modalities on autoantibody levels and clinical disease activity, or the separate assessment of oral and skin disease activity. Nevertheless, it was possible to grade global disease severity.

Among all parameters investigated, we observed the largest difference in autoantibody levels for anti-Dsg1 in PF patients, significantly increasing with worsening of disease. In PV patients, anti-Dsg3 antibody levels were significantly associated with clinical disease severity, and in the subgroup of mucocutaneous PV, anti-Dsg1 levels showed a significant increase with higher disease activity. In all populations investigated, IIF titres did not show any significant differences between clinical grades. Apart from a previously reported poorer sensitivity of IIF to detect autoantibodies compared to the ELISAs (9, 10) as well as a possible inter-observer variability when reading the IIF slides, this effect might be due to the visualisation of pathogenetically irrelevant autoantibodies by IIF (10, 47). In fact, over 50 human proteins have been claimed to specifically react with pemphigus IgG. Some of these putative autoantigens have been suggested to mediate skin blister formation even in anti-Dsg-positive patients (12). The fact that in our study population, anti-Dsg autoantibody levels, but not IIF titres, significantly reflect clinical disease severity, challenges the notion of autoantigens other than desmogleins as being key elicitors of disease, at least in anti-Dsg$^+$ patients.

In summary, we feel that our statistical method, which was used in this field for the first time, quite reliably clarifies that IIF titres – in contrast to anti-Dsg ELISA index values – do not show a relevant correlation with

disease activity in a representative cohort of PV as well as PF patients.

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