Acta Derm Venereol 2010; 90: 401–405

CLINICAL REPORT

Correlation of Immunological Profile with Phenotype and Disease Outcome in Pemphigus

Josep E. HERRERO-GONZÁLEZ[1,2], Pilar IRANZO[2], Daniel BENÍTEZ[3], Francisco LOZANO[3], Carmen HERRERO[2] and José M. MASCARÓ Jr[2]

Departments of Dermatology, [1]IMIM-Hospital del Mar and [2]Hospital Clínic and [3]Department of Immunology, Hospital Clínic, Barcelona, Spain

There has been no previous clinical-immunological study of pemphigus in Spain. The aim of this study was to perform a retrospective analysis of pemphigus patients who had been followed for a period of 18 years in our centre. We characterized the autoantibody response, compared diagnostic assays and correlated the immunobiological data with phenotype and prognosis. Clinical, epidemiological and immunopathological data were collected from 40 patients. Patients sera were characterized by indirect immunofluorescence and enzyme-linked immunosorbent assay (ELISA). Epidemiological and clinical findings were comparable to other series. Mortality during follow-up was 0% and 6% in pemphigus foliaceus and vulgaris, respectively. Importantly, higher indirect immunofluorescence titres and anti-desmoglein 3 ELISA values of samples from untreated patients correlated significantly with a potentially worse clinical course. Moreover, there was a positive correlation between indirect immunofluorescence titres and anti-desmoglein 3 ELISA levels in pemphigus vulgaris patients. Based on our findings, initial high anti-desmoglein 3 antibodies in pemphigus patients correlate with a more adverse prognosis, which raises the question as to whether a more aggressive initial therapy is indicated in patients with this immunological pattern. ***Key words: autoantibodies; desmoglein; ELISA; immunofluorescence; prognosis.***

(Accepted February 12, 2010.)

Acta Derm Venereol 2010; 90: 401–405.

Josep E. Herrero-González, Institut Municipal d'Investigació Mèdica, Parc de Recerca Biomèdica de Barcelona, Doctor Aiguader, 88, ES-08003 Barcelona, Spain. E-mail: jherrero@parcdesalutmar.cat

Pemphigus is a group of organ-specific autoimmune blistering diseases, characterized by tissue-bound and circulating autoantibodies against adhesion molecules of the keratinocyte surface. Pemphigus has two major subtypes, pemphigus vulgaris (PV) and pemphigus foliaceus (PF), both showing distinct clinical and pathological findings. Patients with PV usually start with painful oral lesions, with suprabasal acantholysis by histopathological examination. In contrast, PF cases present with erosive and crusted skin lesions predominantly on seborrhoeic areas, without mucosal involvement, and subcorneal acantholysis by histopathology. Desmogleins (Dsg) are the main proteins targeted by circulating IgG autoantibodies of pemphigus patients (1). Several *in vitro* and *in vivo* studies have demonstrated the pathogenicity of anti-Dsg autoantibodies (2–8). Dsg 1 and 3 are considered the main autoantigens in pemphigus, and antibodies against these two proteins correlate with the skin and mucous membranes involvement in patients with PF and PV, respectively (9–11).

Autoantibodies to the intercellular epidermal surfaces and Dsg can be quantified by indirect immunofluorescence (IF) on monkey oesophagus sections and by enzyme-linked immunosorbent assay (ELISA) plates coated with proteins. IF titres have usually failed to correlate with clinical severity, in contrast with ELISA results (12–15). The prognostic value of these immunological features is not yet fully established.

We describe here the most relevant epidemiological, clinical and immunological findings of a large series of pemphigus patients followed in a single centre over an 18-year period. We correlated these immunological data with the long-term prognosis, the clinical phenotype (location and severity) and, finally, we analysed the correlation between indirect IF titres and anti-Dsg ELISA values.

METHODS

Patients and serum samples

Data from 88 patients with a diagnosis of pemphigus were retrieved from a computerized database of the Department of Dermatology at the Hospital Clínic, Barcelona, Spain, between 1985 and 2002. Of those, we selected 40 patients who fulfilled the following criteria: active blistering skin or mucous membrane disease, intraepithelial acantholysis and intercellular IgG deposition by direct IF. Blood was obtained at the time of diagnosis and during follow-up according to the physician's decision regarding marked changes in the clinical status (clear improvement or worsening) or special therapies needed (e.g. before and after intravenous immunoglobulins' cycles, plasma exchanges, etc.).

Collection of epidemiological and clinical data

The medical charts of all patients were examined exhaustively and the following data collected and summarized in a database: full name, age, gender, date of birth, ethnic group, pemphigus

type, date of diagnosis, date of onset of skin and/or mucous membrane disease, clinical features, medical history, previous medications, thiopurine S-methyltransferase activity level and pemphigus therapies (topical, enteral or parenteral administration, dosing, date of start and withdrawal, accumulated dose, clinical response, side-effects), date of remission and relapse, date of death (when applicable).

Clinical severity score

Since there was no agreement on a specific clinical scoring system for use during the follow-up period of these patients, disease severity was assessed with an arbitrary quantitative scoring system based on the extension of the lesions, ranging from 0 (no disease) to 5 (very severe, with extensive, confluent blisters/erosions/crusts, life-threatening), considering skin and mucous membranes separately. Briefly, scores regarding skin involvement were defined as follows: 0, no lesions; 1, hyperpigmented or erythematous patches; 2, predominant dry lesions on less than 10% of the body surface area (BSA); 3, predominant dry lesions involving ≥10% BSA; 4, predominant erosive lesions or blisters covering <20% BSA; 5, erosions or blisters on ≥ 20% BSA. Scores regarding oral involvement were based on the number of involved anatomical areas (upper gum, lower gum, lips, buccal mucosa, tongue, mouth floor, palate, pharynx): 0, no lesions; 1, epithelized erythematous patches; 2, one area; 3, two to four areas; 4, five to six areas; 5, diffuse involvement. All of the scoring values were assigned by a single clinician, who was in charge of all patients with autoimmune blistering skin diseases in the centre, during the whole study time.

Definition of prognostic groups

Each patient was assigned one of the three prognostic groups, defined as follows: 1, remittent (patients with a tendency to healing after standard treatment, without remarkable complications); 2, intermediate (patients with chronic disease and several flares, responding well to first-line therapy, and without life-threatening events associated with disease activity or drug side-effects); 3, severe (patients with severe chronic disease and several flares, refractory to standard first-line therapies, and/or with life-threatening events). Complete remission was defined as the absence of any sign of clinical activity during at least 6 months, with the patient receiving no immunosuppressive treatment.

Immunofluorescence studies

Indirect IF analysis was performed using monkey oesophagus sections, following established methods (16).

Enzyme-linked immunosorbent assay

An ELISA was performed to detect IgG antibodies against Dsg1 and Dsg3, according to the manufacturer's instructions (MBL Co., Nagoya, Japan). Highly reactive patients' sera, with index values (IV) higher than 150 U/ml, were serially diluted up to 1/1000.

Statistical analysis

All data were analysed using the STATA package (Stata Corporation, College Station, TX, USA) and SPSS 15.0 (Chicago, IL, USA) by the Units of Biostatistics at the IMIM-Hospital del Mar and the Hospital Clínic, Barcelona.

RESULTS

Epidemiological data and clinical phenotype

These data are summarized in Table I. Briefly, a final total of 40 patients was included, 33 with PV (82%) and 7 with PF (18%). A slight female predominance was observed (22/40 pemphigus cases, 55%). Mean age was 53 and 50 years in PV and PF, respectively, with a range of 20–81 years. Mean follow-up periods were 106 and 23 months in PV and PF, respectively. Isolated involvement of the oral mucosa was the most frequent initial clinical sign in PV (58%), followed by skin eruption alone (29%) and mucocutaneous disease (13%).

Disease outcome

With regard to the prognostic groups, 100% of the PF patients followed a remittent course. In contrast, 62% of PV cases were in the intermediate group, 21% in the severe group and only 17% in the remittent group. Complete remissions during the above-described observation times were recorded in 71% (5/7) and 30% (10/33) of PF and PV patients, respectively. Of those, we registered relapses in only one PF and one PV patients during clinical follow-up. Two men with severe generalized mucocutaneous PV, aged 80 and 66 years, died during the observation time (5% of all pemphigus patients). Both deaths occurred within the first 4 months after diagnosis and were attributed to respiratory failure due to pulmonary thromboembolism and pneumonia in the first patient, and gastrointestinal bleeding and nosocomial pneumonia in the second.

Anti-Dsg ELISA results at diagnosis

We analysed the reactivity of 5 PF and 14 PV patients, for whom we had serum aliquots at the time of diagnosis. 5/5 PF (100%) and 14/14 PV (100%) sera were

Table I. *Summary of the most relevant clinical and epidemiological data of the pemphigus patient series*

Disease	Gender *n*	Age (years) Mean ± SD	Follow-up period (months) Mean ± SD	Deaths *n* (%)	Initial phenotype (%)		Deaths regarding phenotype subgroups *n*	CR (%)
Pemphigus vulgaris (*n*=33)	F 18 M 15	53 ± 16	106 ± 81	2 (6)	Mucous membranes	58	0	30
					Skin	29	0	
					Mucocutaneous	13	2	
Pemphigus foliaceus (*n*=7)	F 4 M 3	50 ± 20	23 ± 22	0	–	–	0	71

F: female; M: male; SD: standard deviation; CR: complete remissions (see text).

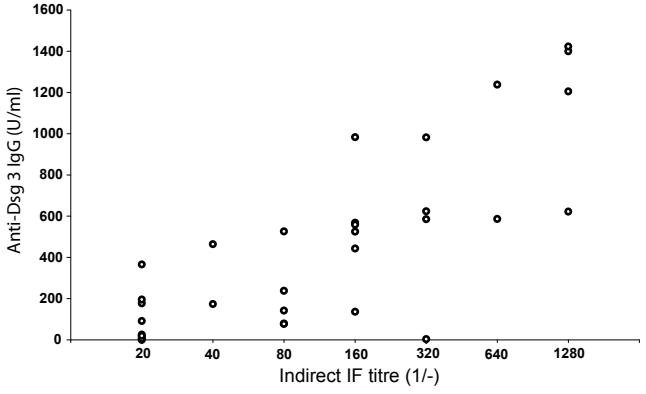

Fig. 1. Levels of anti-Dsg3 antibodies measured by enzyme-linked immunosorbent assay correlate positively with indirect immunofluorescence (IF) titres in pemphigus vulgaris, regardless of therapy ($n=33$) (Spearman's rank correlation test; $\rho=0.736$, $p<0.001$).

positive for anti-Dsg1 and anti-Dsg3 autoantibodies, respectively. In addition, 9/14 PV patients (64%) also reacted against Dsg1 only.

Indirect IF titres and anti-Dsg ELISA values in pemphigus vulgaris

For this analysis, we selected only the first serum sample of each patient ($n=33$). We observed a positive correlation between indirect IF titres and anti-Dsg3 ELISA levels (Spearman's rank correlation test; $\rho=0.736$, $p<0.001$) (Fig. 1), in contrast with anti-Dsg1 values ($\rho=0.082$, $p=0.650$).

Antigenic specificity as detected by ELISA and clinical phenotype

There was a significant correlation between the presence of anti-Dsg1 antibodies and skin involvement in both PF and PV groups (Fisher's exact test; $p=0.038$ and $p<0.001$, respectively), as well as between anti-Dsg3 antibodies and mucous membrane involvement ($p<0.001$).

Indirect IF titres or anti-Dsg1/Dsg3 ELISA values and clinical severity

No statistically significant correlation between indirect IF titres and clinical activity was found, irrespective of the pemphigus type. Anti-Dsg1 antibody levels detected by ELISA increased in parallel with the skin clinical score in PF, but without reaching statistical significance (Kruskal-Wallis test; $p=0.091$), probably due to the low size of the PF sample ($n=7$). In PV, ELISA anti-Dsg1 and anti-Dsg3 antibody levels correlated significantly with the skin and mucous membranes clinical scores, respectively (both $p<0.001$).

Correlation studies regarding disease prognosis

Only pemphigus patients with serum extractions obtained before any systemic treatment were eligible for statistical analysis ($n=16$). Moreover, we merged the intermediate and severe groups together due to the low sample size of the latter group ($n=2$). We found a significant correlation between a more adverse prognosis and higher initial indirect IF titres (Mann-Whitney U test; $p=0.007$) or anti-Dsg3 values ($p<0.001$) (Table II). In contrast, no association was observed when considering the levels of anti-Dsg1 antibodies.

DISCUSSION

Epidemiological and clinical data in our study were similar to other published series (17, 18). In contrast with other studies, where female cases almost doubled the number of affected men (18, 19), we found a female/male ratio of almost 1:1. Oral involvement was the most frequent location at disease onset in PV (71%).

Regarding the clinical course in PF, all these patients showed a tendency to rapid remission under conventional steroid or steroid plus azathioprine treatment. There was no mortality in this group and 70% of complete remissions were recorded during the follow-up period (vs. 53% in a recent Japanese series) (18).

Two-thirds of all PV patients belonged to the intermittent course subgroup. Complete remission was observed in only 30% of PV cases during the whole observation time. We recorded a 5% mortality (last death occurring in 1992), equivalent or inferior to that reported in the literature after the introduction of steroids and adjuvant steroid-sparing drugs (20). These mortal cases occurred shortly after diagnosis, both in elderly men with severe generalized mucocutaneous PV. Death causes (sepsis, cardiorespiratory disease, gastrointestinal bleeding) were related to disease extension and the use of high-dose glucocorticoids together with immunosuppressive agents, as described in other studies (19).

Table II. *In contrast to anti-Dsg1 antibodies, initial levels of antibodies against Dsg3 as detected by enzyme-linked immunosorbent assay (ELISA) correlate positively with a worse disease outcome*

	Remittent course ($n=6$)			Intermediate or severe course ($n=10$)			
	Median	p25–p75	Range	Median	p25–p75	Range	*p*-value
Dsg1 ELISA	136	7–495	3–506	50	22–78	9–196	0.792 (NS)
Dsg3 ELISA	25	2–103	1–177	623	430–1278	136–1423	<0.001

ELISA units: U/ml; p: percentile; NS: non-significant.

The association between antigen specificity and the pemphigus phenotype was described firstly by immunoblot and immunoprecipitation techniques (10) and later by ELISA studies (11, 21–23). We also found a significant correlation between the ELISA anti-Dsg1 or anti-Dsg3 response and the skin or mucous membrane phenotype, respectively. However, occasional patients show a clear discrepancy between the clinico-pathological diagnosis and the autoantibody profile (22, 24).

Although the results of different studies vary (13–15, 25, 26), to date no study has found a clear relationship between indirect IF titres and disease severity in pemphigus, and this was also the case in our study. When comparing ELISA values with clinical severity, we found a positive trend between the anti-Dsg1 response and the severity of skin involvement in PF patients, although statistical significance was not reached, probably due to the low number of serum samples available. In PV, we demonstrated a significant positive correlation between the anti-Dsg1 and Dsg3 ELISA values with skin and mucous membrane severity, respectively, as reported elsewhere (27, 28).

It is noteworthy that indirect IF titres and anti-Dsg3 ELISA levels correlated significantly in PV (Fig. 1). As mentioned previously, we found a positive correlation between clinical activity and the levels of circulating autoantibodies by ELISA, but not by indirect IF. The reason for this discrepancy is unclear; however, the reproducibility of the commercial protein-based ELISA, increases the accuracy of the results, in contrast with the potentially inaccurate indirect IF technique, which is often handmade and depends on many changeable conditions (quality of the tissue, incubation conditions, type of fluorescent-labelled antibody, etc.). We assume that, in small-size patients samples like ours, it is probably much easier to reach statistical significance when analysing ELISA values than titres obtained by indirect IF.

We attempted to identify an immunological prognostic factor, in order to define a subset of pemphigus patients who may benefit from a more powerful therapy from the very beginning of the disease, regardless of the pemphigus subtype. Therefore, we analysed serum extractions obtained before the initiation of any systemic therapy and intended to correlate these initial indirect IF titres and ELISA values with the prognostic subgroups. Interestingly, we found a positive correlation between higher levels of anti-Dsg3 and anti-epithelial circulating antibodies (by ELISA and indirect IF, respectively) and a less favourable prognostic group. Previously, Harman et al. (21) had raised the hypothesis that the presence of high anti-Dsg1 antibodies in early phases of PV may be associated with a potentially more severe disease. However, serum samples in that study were also taken from patients already under immunosuppressive therapy, and this may have affected the levels of detectable autoantibodies. To our knowledge, our study is the first to demonstrate that a higher level of anti-Dsg3 antibodies before the start of treatment may predict a potentially worse clinical outcome. The question as to whether these facts justify a more powerful initial therapy has yet to be addressed.

We conclude that indirect IF might now be replaced by ELISA as the first-choice test to detect specific circulating autoantibodies in PV and PF. Of note, in some pemphigus variants in which desmogleins are not the major autoantigens, or there is no IgG response (such as IgA pemphigus), anti-Dsg IgG ELISA systems are of no use and indirect IF study remains as the main serological test. As well as being an automatable and highly reproducible technique, ELISA has proven to be more sensitive (17), readings are objectively obtained and quantitatively expressed. In addition, ELISA results are Dsg-specific and thus help to differentiate pemphigus types. As we have shown previously, these values correlate with the pemphigus phenotype and severity, and may also help in monitoring the immunological response to therapeutic agents in daily practice (29).

ACKNOWLEDGEMENTS

This study had the financial support of the grant *Emili Letang* from the Hospital Clínic, Barcelona, Spain (to Dr Herrero). We would like to thank Mrs Maria Sala and Mrs Lucía Millán from the Immunodermatology Laboratory, Hospital Clínic, for their technical support, and Sergi Mojal from the Biostatistics Unit, IMIM-Hospital del Mar, for his invaluable help with the statistical analysis.

REFERENCES

1. Amagai M, Klaus-Kovtun V, Stanley JR. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. Cell 1991; 67: 869–877.
2. Schiltz JR, Michel B. Production of epidermal acantholysis in normal human skin in vitro by the IgG fraction from pemphigus serum. J Invest Dermatol 1976; 67: 254–260.
3. Anhalt GJ, Labib RS, Voorhees JJ, Beals TF, Diaz LA. Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease. N Engl J Med 1982; 306: 1189–1196.
4. Roscoe JT, Diaz L, Sampaio SA, Castro RM, Labib RS, Takahashi Y, et al. Brazilian pemphigus foliaceus autoantibodies are pathogenic to BALB/c mice by passive transfer. J Invest Dermatol 1985; 85: 538–541.
5. Rock B, Labib RS, Diaz LA. Monovalent Fab' immunoglobulin fragments from endemic pemphigus foliaceus autoantibodies reproduce the human disease in neonatal Balb/c mice. J Clin Invest 1990; 85: 296–299.
6. Amagai M, Hashimoto T, Shimizu N, Nishikawa T. Absorption of pathogenic autoantibodies by the extracellular domain of pemphigus vulgaris antigen (Dsg3) produced by baculovirus. J Clin Invest 1994; 94: 59–67.
7. Amagai M, Hashimoto T, Green KJ, Shimizu N, Nishikawa T. Antigen-specific immunoadsorption of pathogenic autoantibodies in pemphigus foliaceus. J Invest Dermatol 1995; 104: 895–901.
8. Amagai M, Tsunoda K, Suzuki H, Nishifuji K, Koyasu S,

Nishikawa T. Use of autoantigen-knockout mice in developing an active autoimmune disease model for pemphigus. J Clin Invest 2000; 105: 625–631.
9. Mahoney MG, Wang Z, Rothenberger K, Koch PJ, Amagai M, Stanley JR. Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris. J Clin Invest 1999; 103: 461–468.
10. Ding X, Aoki V, Mascaro JM, Jr, Lopez-Swiderski A, Diaz LA, Fairley JA. Mucosal and mucocutaneous (generalized) pemphigus vulgaris show distinct autoantibody profiles. J Invest Dermatol 1997; 109: 592–596.
11. Amagai M, Tsunoda K, Zillikens D, Nagai T, Nishikawa T. The clinical phenotype of pemphigus is defined by the anti-desmoglein autoantibody profile. J Am Acad Dermatol 1999; 40: 167–170.
12. Acosta E, Gilkes JJ, Ivanyi L. Relationship between the serum autoantibody titers and the clinical activity of pemphigus vulgaris. Oral Surg Oral Med Oral Pathol 1985; 60: 611–614.
13. Judd KP, Lever WF. Correlation of antibodies in skin and serum with disease severity in pemphigus. Arch Dermatol 1979; 115: 428–432.
14. Creswell SN, Black MM, Bhogal B, Skeete MV. Correlation of circulating intercellular antibody titres in pemphigus with disease activity. Clin Exp Dermatol 1981; 6: 477–483.
15. Fitzpatrick RE, Newcomer VD. The correlation of disease activity and antibody titers in pemphigus. Arch Dermatol 1980; 116: 285–290.
16. Harman KE, Gratian MJ, Bhogal BS, Challacombe SJ, Black MM. The use of two substrates to improve the sensitivity of indirect immunofluorescence in the diagnosis of pemphigus. Br J Dermatol 2000; 142: 1135–1139.
17. Harman KE, Gratian MJ, Seed PT, Bhogal BS, Challacombe SJ, Black MM. Diagnosis of pemphigus by ELISA: a critical evaluation of two ELISAs for the detection of antibodies to the major pemphigus antigens, desmoglein 1 and 3. Clin Exp Dermatol 2000; 25: 236–240.
18. Ishii N, Maeyama Y, Karashima T, Nakama T, Kusuhara M, Yasumoto S, et al. A clinical study of patients with pemphigus vulgaris and pemphigus foliaceous: an 11-year retrospective study (1996–2006). Clin Exp Dermatol 2008; 33: 641–643.
19. Ljubojevic S, Lipozencic J, Brenner S, Budimcic D. Pemphigus vulgaris: a review of treatment over a 19-year period. J Eur Acad Dermatol Venereol 2002; 16: 599–603.
20. Bystryn JC, Steinman NM. The adjuvant therapy of pemphigus. An update. Arch Dermatol 1996; 132: 203–212.
21. Harman KE, Gratian MJ, Bhogal BS, Challacombe SJ, Black MM. A study of desmoglein 1 autoantibodies in pemphigus vulgaris: racial differences in frequency and the association with a more severe phenotype [see comments]. Br J Dermatol 2000; 143: 343–348.
22. Herrero-Gonzalez JE, Mascaro JM, Jr, Iranzo P, Herrero C. Atypical pemphigus: discordance between clinicopathological findings and the antigenic profile in four cases. J Cutan Pathol 2006; 33: 502–507.
23. Ng PP, Thng ST. Three cases of transition from pemphigus vulgaris to pemphigus foliaceus confirmed by desmoglein ELISA. Dermatology 2005; 210: 319–321.
24. Nagasaka A, Matsue H, Miyahara A, Shimada S. Pemphigus vulgaris with no mucosal lesions showing pemphigus-foliaceus-like skin manifestations: Is there a 'cutaneous type' of pemphigus vulgaris? Dermatology 2005; 211: 372–374.
25. Judd KP, Mescon H. Comparison of different epithelial substrates useful for indirect immunofluorescence testing of sera from patients with active pemphigus. J Invest Dermatol 1979; 72: 314–316.
26. O'Loughlin S, Goldman GC, Provost TT. Fate of pemphigus antibody following successful therapy. Preliminary evaluation of pemphigus antibody determinations to regulate therapy. Arch Dermatol 1978; 114: 1769–1772.
27. Cheng SW, Kobayashi M, Kinoshita-Kuroda K, Tanikawa A, Amagai M, Nishikawa T. Monitoring disease activity in pemphigus with enzyme-linked immunosorbent assay using recombinant desmogleins 1 and 3. Br J Dermatol 2002; 147: 261–265.
28. Harman KE, Seed PT, Gratian MJ, Bhogal BS, Challacombe SJ, Black MM. The severity of cutaneous and oral pemphigus is related to desmoglein 1 and 3 antibody levels. Br J Dermatol 2001; 144: 775–780.
29. Eming R, Rech J, Barth S, Kalden JR, Schuler G, Harrer T, et al. Prolonged clinical remission of patients with severe pemphigus upon rapid removal of desmoglein-reactive autoantibodies by immunoadsorption. Dermatology 2006; 212: 177–187.