Evaluation of Recombinant Antigen-Based Assays for Diagnosis of Bullous Autoimmune Diseases

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The diagnosis of autoimmune bullous diseases is based on clinical observation and on the presence of autoantibodies directed to molecules involved in the adhesion systems of the skin. Immunofluorescence assays are the currently accepted method for detection of autoantibodies; such assays depend greatly on the skill of operators and are difficult to standardize. Recombinant desmoglein-1 (Dsg1), Dsg3, and BP180 peptides, the main autoantigens in pemphigus or bullous pemphigoid, have been used to develop new quantitative enzyme immunoassays (EIA) for the detection of specific antibodies. The present study was undertaken to evaluate the sensitivity and specificity of these immunoassays and to determine the correlation between the results and the clinical aspects of diseases. Serum samples from patients with pemphigus vulgaris, pemphigus foliaceus, bullous pemphigoid, or mucous membrane pemphigoid, from healthy individuals, and from patients with unrelated autoimmune conditions were tested. Anti-desmoglein reactivity was detected in all the patients with pemphigus and in none of the controls. Patients with the more benign form of cutaneous disease had anti-Dsg1 antibodies, while patients with deeper cutaneous lesions or with mucosal involvement had anti-Dsg3 reactivity also, or exclusively. The BP180-based assay was positive for 66.6% of patients with bullous pemphigoid and for none of the patients with mucous membrane pemphigoid, and no reactivity was detected in the control sera. In conclusion, the anti-Dsg1 and anti-Dsg3 assays are useful in the diagnosis of pemphigus and provide information on the clinical phenotype of the disease. However, the sensitivity of EIA for detection of autoantibodies in bullous pemphigoid should be improved by the use of additional antigens or epitopes.

Bullous autoimmune diseases are mediated by autoantibodies directed to the main adhesion systems of the skin (17). These diseases are classified into two major groups according to the level at which the skin blister occurs: pemphigus, characterized by epidermal blistering which is caused by loss of epidermal cell adhesion, and pemphigoid, characterized by subepidermal blistering which is caused by loss of adhesion between the basal keratinocytes and dermis (18). The detection of circulating or tissue-bound autoantibodies by immunofluorescence assay (IFA) is an important criterion for diagnosis in both clinical conditions (14). Recently, enzyme immunoassays (EIAs) based on recombinant desmoglein-1 (Dsg1) or Dsg3, the main autoantigens in pemphigus (1), and on recombinant BP180, the hemidesmosomal autoantigen involved in the pathogenesis of bullous pemphigoid (BP) (12, 19), have been commercialized. In the present study we have analyzed retrospectively the levels of anti-Dsg1 and anti-Dsg3 in the sera of patients with diagnoses of pemphigus and the levels of anti-BP180 in the sera of patients with pemphigoid in order to establish the correlation between the serum reactivity and clinical features of the patients. In fact, EIAs, compared to IFAs, have several advantages: they provide information on antibody specificity in the diseases, are easier to perform, give quantitative results, and can be executed by automatically operating systems, thus helping to standardize laboratory procedures.

MATERIALS AND METHODS

Patients. Sera from 89 patients with autoimmune bullous diseases (42 with pemphigus vulgaris [PV], 7 with pemphigus foliaceus [PF], 36 with BP, and 4 with mucous membrane pemphigoid [MMP]), 30 patients with inflammatory skin diseases (10 with plaque type psoriasis, 10 with atopic dermatitis, 5 with progressive systemic scleroderma, and 5 with lupus erythematosus), and 20 healthy controls were included in the present study. All the patient sera were collected at the time of diagnosis (T0) and after 12 months (T1), from individuals who have been under observation in our institute during the past 3 years; these sera were stored frozen at −80°C in small aliquots. The diagnosis of bullous diseases was based on clinical, histologic, and immunopathologic findings in biopsy specimens (epidermal blister with acantholysis, and immunoglobulin G [IgG] and C3 deposition in the intercellular space [Fig. 1A and B] or dermal-epidermal junction blister with IgG and C3 deposition in the basement membrane zone [BMZ] [Fig. 1C and D]). The same patients had circulating autoantibodies detected by IFA on primate and guinea pig esophagus as substrates (IMMCO Diagnostics, Buffalo, N.Y.) or on human skin split by 1 M NaCl (14). For 35 of 89 patients, repeated samples were collected and tested during the 12-month clinical assessment.

EIAs. Seventy-four serum samples from 49 patients with PV or PF and 46 serum samples from 36 patients with BP and 4 with MMP were tested for the presence of autoantibodies anti-Dsg1 and anti-Dsg3 or anti-BP180 by using EIAs based on recombinant antigens (Medical and Biological Laboratories Co. Ltd., Nagoya, Japan). Serum samples were tested in the same session for each antigen; results were calculated as a percentage of the reactivity of a positive control, included in the commercial kit, which was arbitrary considered to contain 100 U of anti-Dsg or anti-BP180 antibodies. Results were expressed as “index.”

Samples were considered to have positive reactivities when the index anti-Dsg1 was >14, the index anti-Dsg3 was >7, or the index anti-BP180 was >15.

Statistical analysis. Statistical analysis was performed by nonparametric tests: the Mann-Whitney test, to compare EIA mean index values; the chi-square test, to determine whether the clinical phenotype and antibody specificity were related; and the rho Spearman rank correlation coefficient, to analyze the relationship between the results of IFAs (expressed as the reciprocal of the highest positive dilution) and EIAs (expressed as indexes).
RESULTS

In pemphigus patients, anti-intercellular substance of epidermis (anti-ICS) reactivities were detected at serum dilutions between 1:40 and 1:2,560 (Table 1); in BP patients, serum titers of anti-BMZ reactivity ranged between 1:20 and 1:5,120; and in MMP patients, serum anti-BMZ reactivity was detected in 2 of 4 patients with titers between 1:40 and 1:2,560 (Table 2).

Anti-Dsg1 and Anti-Dsg3 assays. All the sera of patients with pemphigus gave positive results in the assay for detection of anti-Dsg1 (PF mean index, 186.71 ± 66.44; PV mean index, 65.65 ± 74.43; P < 0.0001) and/or anti-Dsg3 (PF mean index, 2.45 ± 0.72; PV mean index, 99.27 ± 60.37; P < 0.0001) reactivities (means are given with standard deviations), while the values of all control sera from healthy individuals were lower than the cutoff value (anti-Dsg1 mean index, 3.53 ± 3.44; anti-Dsg3 mean index, 3.43 ± 1.56). Sera from patients with inflammatory skin diseases did not significantly differ from healthy-control sera (anti-Dsg1 mean index, 3.34 ± 3.27 [P = 0.84]; anti-Dsg3 mean index, 2.60 ± 1.79 [P = 0.10]). Notably, PF patient sera showed values significantly higher than controls for anti-Dsg1 (mean index, 186.71 ± 66.44; P < 0.0001) rather than anti-Dsg3 (mean index, 2.45 ± 0.72; P = 0.22) reactivity, while in sera of patients with PV, both reactivities were significantly high (anti-Dsg1 mean index, 65.65 ± 74.43 [P < 0.0001]; anti-Dsg3 mean index, 99.27 ± 60.37 [P < 0.0001]). The different anti-Dsg1 and/or anti-Dsg3 reactivities were found to be significantly associated with the clinical expression of the disease: patients with only anti-Dsg1 reactivity had the more benign form of disease, PF, characterized by acantholysis of the superficial epidermal layers (Table 3) and had lesions limited to the cutaneous compartment (Table 4). Patients with both anti-Dsg3 and anti-Dsg1 reactivities, or with only anti-Dsg3 reactivity, had the more severe form of disease, PV, characterized by acantholysis of the suprabasal keratinocytes and mucocutaneous lesions. Interestingly, among the 16 patients with single reactivity against Dsg3, 10 were newly diagnosed, confirming that PF and PV have different behaviors from the early stages of disease. Subsequently, patients with PF main-
Diagnosed. Obtained the single anti-Dsg 1 reactivity (out of seven patients were repeatedly analyzed [Table 1]), while patients with single anti-Dsg 3 reactivity also developed anti-Dsg1 antibodies in the first year of follow up (three out of eight tested [Table 1]). Moreover, the presence of anti-Dsg3 antibodies was significantly associated with mucosal lesions, as shown in Table 2.

**TABLE 2. Antibody specificity in BP and MMP**

| Patient | $T_0$ | BP180 index | $T_1$ | BP180 index |
|---------|-------|------------|-------|-------------|
| BP1     | 1:40  | 26.7       | 1:40  | 19          |
| BP2     | 1:320 | 53         |       |             |
| BP3     | 1:1,280 | 5     | 1:640 | 4          |
| BP4     | 1:40  | 39.7       |       |             |
| BP5     | 1:20  | 104        | 1:20  | 82          |
| BP6     | 1:640 | 19.3       | 1:640 | 20.2        |
| BP7     | 1:20  | 44.2       | 1:20  | 36          |
| BP8     | 1:20  | 65         |       |             |
| BP9     | 1:5,120 | 25.5    | 1:2,560 | 32        |
| BP10    | 1:40  | 17.4       |       |             |
| BP11    | 1:2,560 | 4.9   | 1:1,280 | 6        |
| BP12    | 1:10,240 | 25.7  |       |             |
| BP13    | 1:640 | 16.6       |       |             |
| BP14    | 1:10,240 | 135  | 1:5,120 | 110       |
| BP15    | 1:640 | 2.4        |       |             |
| BP16    | 1:20  | 99         | 1:20  | 82          |
| BP17    | 1:2,560 | 11.4  |       |             |
| BP18    | 1:20  | 79         |       |             |
| BP19    | 1:20  | 128        |       |             |
| BP20    | 1:1,280 | 55     |       |             |
| BP21    | 1:40  | 106        |       |             |
| BP22    | 1:40  | 11         |       |             |
| BP26    | 1:80  | 93         |       |             |
| BP24    | 1:10,240 | 162  | 1:5,120 | 158       |
| BP25    | 1:40  | 5          |       |             |
| BP26    | 1:5,120 | 8      |       |             |
| BP27    | 1:20  | 3          |       |             |
| BP28    | 1:640 | 30         |       |             |
| BP29    | 1:320 | 2          |       |             |
| BP30    | 1:2,560 | 38.7  |       |             |
| BP31    | 1:20  | 5.3        |       |             |
| BP32    | 1:40  | 65         |       |             |
| BP33    | 1:20  | 11         |       |             |
| BP34    | 1:80  | 34.3       |       |             |
| BP35    | 1:40  | 1.8        |       |             |
| BP36    | 1:40  | 41         |       |             |
| MMP1    | Neg   | 15         |       |             |
| MMP2    | 1:320 | 8.8        |       |             |
| MMP3    | 1:640 | 1.5        |       |             |
| MMP4    | Neg   | 8          |       |             |

$^a$ Neg, negative.

$^b$ A BP180 index greater than 15 is considered positive. Positive values are boldfaced.

TABLE 3. Association between antibody specificity and clinical diagnosis in pemphigus

| Diagnosis | No. of samples with the indicated reactivity/no. tested |
|-----------|------------------------------------------------------|
|           | Anti-Dsg1 | Anti-Dsg3 | Anti-Dsg1 + anti-Dsg3 |
| PF        | 7/7       | 0/7       | 0/7                   |
| PV        | 0/42      | 16/42*    | 26/42                 |

$^a$ $P < 0.0001$. Ten of the 16 patients with single anti-Dsg3 reactivity were newly diagnosed.

Statistical analysis of EIA indexes and IFA serum titers obtained for all pemphigus patients showed that serum anti-Dsg 3 levels and anti-ICS titers are significantly correlated ($p = 0.49; P < 0.001$).

**Anti-BP180 assay.** None of the sera from the healthy-control or inflammatory-skin-disease group were found positive in the assay for anti-BP180 reactivity (mean indexes, 5.74±1.56 and 5.64±1.73, respectively; $P = 0.79$). Among the sera from pemphigoid patients, 24 of 36 with BP (66.66%) and none of 4 with MMP were found positive. In BP sera, mean reactivities were significantly higher than in controls (mean index, 43.70±43.24; $P < 0.0001$) but were inversely but not significantly correlated with the anti-BMZ antibody titers by IFA (Spearman's $r = -128; P > 0.05$).

**DISCUSSION**

It has been demonstrated extensively that the desmosome-associated cadherins Dsg1 and Dsg3 are the main autoantigens in PF and PV, respectively. The availability of the recombinant molecules has allowed new assays to be developed to detect specific antibodies in patient sera (9).

Pemphigoid includes a wide spectrum of subepidermal blistering diseases that can affect the skin and all stratified squamous epithelial mucous membranes. In BP, autoantibodies are directed against the normal components of hemidesmosomes, BP180 and BP230. The pathogenetic role of BP180 has been demonstrated to be localized on the noncollagenous portion (NC16A) of its extracellular domain (12, 19), so the NC16A between BP180 and the recombinant peptide has been used to develop the EIA that was used in this study.

In this paper we retrospectively analyzed the antibody specificities in patients with a diagnosis of PV, PF, BP, or MMP, based on clinical, histological, and immunopathological findings (15), through tests based on recombinant antigens.

Autoantibodies directed to the specific cutaneous compartments were present in all the patient sera, with the exception of two of four MMP patients. In fact, in this group of subepidermal blistering diseases, which affect multiple mucous membrane sites, detectable circulating autoantibodies to BMZ have not always been revealed (6) or heterogeneous patterns of antibody specificity have been described. As far as detection of anti-Dsg1 and/or anti-Dsg3 antibodies by EIAs is concerned, our data showed a prevalence of 100% in pemphigus patients and a lack of reactivity in healthy controls and patients with inflammatory or autoimmune dermatosis, confirming the excellent sensitivity and specificity of both assays (8, 10). The results were completely comparable to those obtained by IFA,
based on primate and guinea pig esophagus as substrates, which has been considered the “gold standard” for anti-hemidesmosomal autoantibody detection in these diseases. Moreover, statistical analysis of IFA titers and EIA indexes for anti-Dsg3 showed that the two measures are significantly correlated. This finding is particularly relevant considering the established correlation of anti-ICS titers, disease severity, and levels of additional biological indicators of disease activity in PV (3). We could also confirm the already proposed association between antibody specificity and clinical phenotype of pemphigus disease: all the sera of patients with PF reacted exclusively with Dsg1, and this reactivity did not shift toward Dsg3 over time. These data confirm that PF is different from PV and remains a more benign disease. In patients with PV-associated skin or mucocutaneous lesions, both reactivities were present; only at the onset of disease did patients have single anti-Dsg3 reactivity, associated with mucous involvement. These features sustain the pathogenetic model described by Amagai, Stanley, and colleagues, based on the different distributions of Dsg1 and Dsg3 in skin or mucous epithelia (13). According to this model, based on the fact that Dsg1 is expressed in the entire epidermis and is minimally expressed in mucosal epithelia while Dsg3 expression is restricted to deeper epidermal layers and is widely expressed in mucosal epithelia, anti-Dsg1 autoantibodies should cause only skin lesions and affect the more superficial epidermal layers, since Dsg3 can compensate for the antibody-induced loss of Dsg1 function. On the other hand, the presence of anti-Dsg3 should cause erosions in mucous epithelia, where this antigen is expressed more widely, and acantholysis of deep cutaneous layers can induce exposure of Dsg1 epitopes to the immune system, with subsequent development of severe cutaneous lesions. These conditions are well represented by the significant association between anti-Dsg1 and/or anti-Dsg3 reactivity and the clinical diagnosis or site of lesions, described in the present study. However, additional biological mediators probably play a role in the development of lesions (7).

As far as measurement of anti-BP180 reactivity was concerned, the specificity of the EIA, comparable to that of the IFA, was demonstrated by the lack of positive sera among healthy controls or patients with a different dermatosis; the sensitivity of the assay was 66.6% for BP patients, significantly lower than that of the IFA, by which all the sera were found positive. Moreover, statistical analysis of EIA and IFA results showed that the two measures are correlated, with a negative correlation coefficient inversely. This finding could be explained by the possibility that antigens different from BP180, or different epitopes of the same molecule, could elicit the autoimmune reaction in BP patients. In fact, intracellular antigens such as BP230 or laminin have been described in BP or MMP, respectively (20). Consequently, the recombinant-protein-based assay cannot at present be proposed as a satisfactory diagnostic laboratory tool, although anti-BP180 reactivity has been described as correlated to disease activity in previously described home-made EIAs (1, 4, 16). In conclusion, the new EIAs based on recombinant Dsg1 and Dsg3 can be used in the diagnosis of pemphigus and in the monitoring of disease, while the sensitivity of the EIA based on BP180 needs to be improved in order for it to be used in the diagnosis and monitoring of BP. The IFA should still be considered the reference test for laboratory diagnosis of autoimmune subepidermal blistering diseases. Nevertheless, besides their usefulness as diagnostic tools, the availability of assays detecting specific antibodies in patient sera opens up the possibility of designing innovative therapies based on antigen-specific plasmapheresis (2, 5, 11).

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