

Enzyme-Linked Immunosorbent Assay for the Combination of Bullous Pemphigoid Antigens 1 and 2 in the Diagnosis of Bullous Pemphigoid

Aude Roussel, MD; Jacques Benichou, MD, PhD; Zely Arivelo Randriamanantany, MD; Danièle Gilbert, PhD; Kossara Drenovska, MD, PhD; Estelle Houivet, PhD; François Tron, MD, PhD; Pascal Joly, MD, PhD

Objective: To assess the usefulness of enzyme-linked immunosorbent assay (ELISA) assessment of the combination of bullous pemphigoid antigen 1 (BPAG1) and BPAG2 in the diagnosis of bullous pemphigoid (BP).

Design: Retrospective study of serum samples from patients with BP.

Setting: Tertiary care center.

Patients: A total of 190 patients with newly diagnosed BP and 78 controls with other autoimmune bullous diseases.

Intervention: Serum samples were tested using commercialized BPAG1 and BPAG2 ELISA and indirect immunofluorescence (IIF).

Main Outcome Measures: The sensitivity and specificity of ELISA for the combination of BPAG1 and BPAG2 in the diagnosis of BP were contrasted with ELISA for each of the antigens alone and with IIF.

Results: The sensitivity and specificity of ELISA for the combination of BPAG1 and BPAG2 were 87% and 88%,

respectively, compared with 79% and 90% for BPAG2 ELISA, 61% and 96% for BPAG1 ELISA, and 81% and 63% for IIF. The combination of BPAG1 ELISA and BPAG2 ELISA permitted 8% and 16% gains in sensitivity compared with each of BPAG2 ELISA and BPAG1 ELISA alone, respectively. Anti-BPAG1 antibodies were detected in 15 of 40 BP serum samples with no anti-BPAG2 antibodies (38%) and in 8 of 13 serum samples from patients with BP and mucosal involvement (62%) compared with 2 of 22 samples of cicatricial pemphigoid ($P = .002$) and 0 of 16 epidermolysis bullosa acquisita serum samples ($P < .001$). The BPAG2 ELISA values were more closely correlated with initial extent of BP lesions ($r = 0.44$, $P < .001$) than BPAG1 ELISA values ($r = 0.16$, $P = .03$).

Conclusion: Since the combination of BPAG1 and BPAG2 ELISA only slightly increases the sensitivity of BP diagnosis over BPAG2 ELISA alone, BPAG1 ELISA could be adequately proposed in a minority of BP cases with mucosal involvement and in those with no circulating anti-BPAG2 antibodies.

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Author Affiliations: Department of Dermatology (Drs Roussel, Drenovska, and Joly), Department of Biostatistics and Inserm U657 (Drs Benichou and Houivet), and Inserm U905 (Drs Randriamanantany, Gilbert, Tron, and Joly), Rouen University Hospital, University of Rouen, Rouen, France.

BULLOUS PEMPHIGOID (BP) IS the most frequent autoimmune blistering disease of the skin.¹⁻⁵ It is characterized by circulating and tissue-bound autoantibodies directed against 2 structural components of the hemidesmosomes: a 230-kDa protein of the plakin family located in the inner plaque of hemidesmosomes called BP antigen 1 (BPAG1),⁶⁻⁹ and a 180-kDa transmembrane protein of the collagen family called BPAG2.⁹⁻¹² The pathogenicity of human anti-BPAG2 antibodies has been demonstrated in different humanized murine models.¹³⁻¹⁵ In addition, serum levels of autoantibodies directed against BPAG2-NC16A detected by enzyme-linked immunosorbent assay (ELISA) have been shown to parallel disease activity in patients with BP.¹⁶⁻²²

Currently, BPAG2 ELISA is considered a useful tool for the diagnosis of BP. The role of anti-BPAG1 antibodies in the pathogenesis of BP remains controversial. Hall et al²³ showed in a rabbit model that injection of BPAG1-derived peptides

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led to an enhanced inflammatory response to UV-B irradiation, which was associated with a neutrophilic and eosinophilic infiltrate and linear deposits of IgG and C3 at the dermoepidermal junction but with no clinical evidence of BP lesions. Guo et al²⁴ generated a mouse that was deficient for

BPAG1 gene expression and that developed skin fragility but with no evidence of bullous lesions. Kiss et al²⁵ showed that the injection of rabbit antibodies directed against an antigenic sequence of human BPAG1 into neonatal mice induced clinical, histologic, and immunopathologic features rather similar to human BP. An ELISA using a recombinant protein covering the N and C domains of BPAG1 has been recently commercialized.²⁶ The usefulness of the combination BPAG1 and BPAG2 ELISA in the diagnosis of BP has only been evaluated in few studies.^{26,27}

The aim of the present study was to assess the usefulness of the routine combination of BPAG1 and BPAG2 ELISA in the diagnosis of BP. In addition, we evaluated the correlation between BPAG1 and BPAG2 ELISA values with initial extent of BP lesions.

METHODS

PATIENTS

Serum samples from 190 patients with a newly diagnosed BP who were seen between 1996 and 2004 in 23 dermatologic centers in France were used in this study. All patients fulfilled the following inclusion criteria: (1) clinical features suggestive of BP^{28,29}; (2) subepidermal blister on skin biopsy³⁰; (3) linear deposits of IgG and C3 along the basement membrane zone (BMZ) by direct immunofluorescence³¹; and (4) serum samples tested for measurement of anti-BPAG1 and anti-BPAG2 antibody values by ELISA and for the detection of anti-BMZ antibodies by IIF on normal and sodium chloride (NaCl)-split human skin. In addition, all patients had been previously included in 2 clinical trials,^{32,33} and patients' clinical data were available on standardized forms. The initial extent of cutaneous lesions was evaluated by the number of daily new blisters for 3 consecutive days at diagnosis before the start of therapy.^{32,33} Control serum samples were obtained from 40 patients with pemphigus, 22 patients with cicatricial pemphigoid (CP) and 16 patients with epidermolysis bullosa acquisita (EBA). In patients with no circulating anti-BMZ antibodies, the diagnosis of CP and EBA was confirmed using direct immunoelectron microscopy. According to French law, this type of non-interventional study on serum samples used for the routine diagnosis of BP does not require the approval of an ethics committee.

BPAG1 AND BPAG2 ELISA ASSAYS

Serum samples were collected for routine diagnosis before initial treatment. The ELISA procedures were all performed in 1 center. To determine anti-BPAG1 and anti-BPAG2 antibody ELISA values, BPAG1 and BPAG2-NC16A ELISA tests (MBL, Nagoya, Japan) were performed with 1:100 diluted serum, according to the manufacturer's instructions.^{26,34} Data were expressed in units per milliliter of serum. For the evaluation of sensitivity and specificity of ELISA assays, we used the cutoff value proposed by the manufacturer (ie, 9 U/mL).

IIF ANALYSIS

We performed IIF serum analysis using normal and NaCl-split human skin and anti-human IgG (The Binding Site, Birmingham, England), as previously described.³⁵

STATISTICAL ANALYSIS

Continuous and dichotomous characteristics were compared between subgroups of patients using the Mann-Whitney non-

parametric test and the Fisher exact test, respectively. Correlations of BPAG1 and BPAG2 ELISA values with the number of daily new blisters were assessed using the Spearman rank correlation coefficient. Sensitivity and specificity of BPAG1 and BPAG2 ELISA findings were assessed in the usual manner. The combination of BPAG1 and BPAG2 ELISA was considered positive when BPAG1 or BPAG2 ELISA assays were positive alone or in combination. No valid statistical comparisons of sensitivities or specificities (eg, using the McNemar test) between BPAG2 ELISA alone and the combination of BPAG1 and BPAG2 ELISA could be performed. Indeed, while findings from any given sample might be positive for the combination and negative for BPAG2 ELISA (ie, positive for BPAG1 ELISA, thus being positive for the combination, but negative for BPAG2 ELISA), the reverse was not possible because positive results of BPAG2 ELISA implies being positive to the combination. Comparisons of sensitivity and specificity between ELISA and IIF assays were performed using the McNemar test.

A *P* value less than .05 was considered statistically significant. We used SAS statistical software, version 9 (SAS Institute, Cary, North Carolina).

RESULTS

BASELINE CLINICAL CHARACTERISTICS OF PATIENTS WITH BP

A total of 190 patients with BP were included in the study (115 women and 75 men), mean (SD) age, 81 (9) years. The mean (SD) number of daily new blisters at diagnosis was 27 (39). Ninety-seven patients had moderate BP (≤ 10 new blisters/d), and 93 had extensive disease (> 10 new blisters/d). Thirteen patients had mucosal involvement at presentation.

SENSITIVITY AND SPECIFICITY OF THE DETECTION OF ANTI-BPAG1 AND ANTI-BPAG2 ANTIBODIES BY ELISA

When using the cutoff value proposed by the manufacturer ie, 9 U/mL, anti-BPAG1 and anti-BPAG2 antibodies were detected in 115 (61%) and 150 (79%) of the serum samples, respectively (**Table 1**). Mean (SD) ELISA values of anti-BPAG1 and anti-BPAG2 antibodies were 43 (44) and 71 (58) U/mL, respectively. Among the 40 BP samples that did not contain anti-BPAG2 antibodies, anti-BPAG1 antibodies were detected in 15 (38%), with a mean ELISA value of 44.9 U/mL. Anti-BPAG2 antibodies were detected in 50 of the 75 serum samples without anti-BPAG1 antibodies (67%), with a mean ELISA value of 104.3 U/mL.

Finally, anti-BPAG1 antibodies were detected in 1 pemphigus, 2 CP, and none of the EBA control serum samples, while anti-BPAG2 antibodies were detected in 6 pemphigus, 2 CP, and none of the EBA control samples. The sensitivity and specificity of BPAG1 ELISA alone were 61% and 96%, respectively, and those of BPAG2 ELISA alone were 79% and 90%, respectively. The combination of BPAG1 and BPAG2 ELISA increased the sensitivity from 61% with BPAG1 ELISA alone and 79% with BPAG2 ELISA alone up to 87%, with a slightly decreased specificity to 88%. The combination of BPAG1 and BPAG2 ELISA assays was also particularly useful to distinguish patients with BP who had mucosal involvement from those with CP. Indeed, among the

Table 1. Results of BPAG1 and BPAG2 ELISA in the 190 BP Serum Samples^a

BPAG2 ELISA Finding	BPAG1 ELISA Finding		Total
	Positive	Negative	
Positive	100 (53)	50 (26)	150 (79)
Negative	15 (8)	25 (13)	40 (21)
Total	115 (61)	75 (39)	190 (100)

Abbreviations: BP, bullous pemphigoid; BPAG, BP antigen; ELISA, enzyme-linked immunosorbent assay.

^aAll data are reported as number (percentage) of serum samples. A positive finding is defined as higher than 9 U/mL; negative finding, 9 U/mL or lower.

13 patients with BP from this series who had mucosal involvement at presentation, anti-BPAG1 antibodies were present in 8 cases (62%); anti-BPAG1 antibodies were detected at low ELISA values (18 U/mL and 28 U/mL) in only 2 of the 22 control samples from patients with CP (9%) ($P=.002$) and in none of the 16 samples from patients with EBA ($P<.001$).

CORRELATION BETWEEN BPAG1 AND BPAG2 ELISA VALUES AND INITIAL EXTENT OF BP LESIONS

A mild (Spearman $r=0.16$) albeit significant ($P=.03$) correlation was estimated between BPAG1 ELISA values and the number of daily new blisters. Since a preferential association between the presence of anti-BPAG1 antibodies and localized types of BP has previously been reported,^{26,27,36} we compared the frequency of anti-BPAG1 antibodies between patients with moderate BP (≤ 10 new blisters/d) and those with extensive disease (>10 new blisters/d). Anti-BPAG1 antibodies were detected in 59% of samples from patients with moderate BP and in 62% of samples from those with extensive disease ($P=.66$), with median ELISA values of 24 and 47 U/mL, respectively ($P=.052$).

A stronger correlation was found between BPAG2 ELISA values and the number of daily new blisters (Spearman $r=0.44$, $P<.001$). Anti-BPAG2 antibodies were less frequently detected in samples from patients with moderate BP (70%) than in those with extensive BP (88%) ($P=.002$), with median ELISA values of 22 and 97 U/mL, respectively ($P<.001$).

COMPARISON OF THE SENSITIVITY AND SPECIFICITY OF BPAG1 AND BPAG2 ELISA AND IIF

Anti-BMZ antibodies were detected by standard IIF in 153 of the 190 BP samples (81%), including 13 of 15 samples with only anti-BPAG1 antibodies in ELISA (87%), 33 of 50 samples with only anti-BPAG2 antibodies (66%), 96 of 100 samples with both anti-BPAG1 and anti-BPAG2 antibodies (96%), and 11 of 25 samples with no anti-BPAG1 nor anti-BPAG2 antibodies (44%). Among the 78 control serum samples from patients with various autoimmune bullous diseases other than BP, anti-BMZ antibodies were detected by standard IIF in 1 of the 40 pem-

Table 2. Results of BPAG1 and BPAG2 ELISA and IIF on NaCl-Split Skin in Patients With BP With Mucosal Involvement, CP, or EBA^a

Assay	BP With Mucosal Involvement (n=13)	CP (n=22)	EBA (n=16)
Positive ELISA			
BPAG1	8	2	0
BPAG2	11	2	0
NaCl-split skin IIF			
Positive			
Epidermal side	10	6	0
Dermal side	1	5	16
Both sides	0	0	0
Negative	2	11	0

Abbreviations: BP, bullous pemphigoid; BPAG, BP antigen; CP, cicatricial pemphigoid; EBA, epidermolysis bullosa acquisita; ELISA, enzyme-linked immunosorbent assay; IIF, indirect immunofluorescence; NaCl, sodium chloride.

^aAll data are reported as number of serum samples.

phigus samples (2.5%), 10 of the 22 CP samples (45%), and 6 of the 16 EBA samples (38%). The sensitivity and specificity of IIF were 81% and 63%, respectively.

Comparison of the sensitivity of ELISA and IIF showed a nonsignificantly higher sensitivity of the combination of BPAG1 and BPAG2 ELISA compared with IIF (87% vs 81%) ($P=.06$), whereas no difference was evidenced between the sensitivity of IIF and that of BPAG2 ELISA alone (81% vs 79%) ($P=.77$). The sensitivity of IIF was higher than that of BPAG1 ELISA alone (81% vs 61%) ($P<.001$).

Finally, to compare the usefulness of ELISA and IIF on NaCl-split skin in the diagnosis of patients with autoimmune bullous disease and mucosal involvement, we analyzed the samples from 13 patients with BP with mucosal involvement, 22 with CP, and 16 with EBA using ELISA and IIF on NaCl-split human skin (**Table 2**). Circulating antibodies directed against the epidermal side of the detachment were detected in 10 of 13 (77%), 6 of 22 (27%), and 0 of 16 samples from patients with BP, CP, and EBA, respectively. Antibodies against the dermal side of the detachment were observed in 16 EBA samples (100%), 5 of the 22 CP samples (23%), and 1 of the 13 samples from patients with BP with mucosal involvement (8%). Finally, a staining of both the dermal and epidermal sides of the detachment was observed in none of the tested samples. Anti-BPAG1 and anti-BPAG2 antibodies, respectively, were detected by ELISA in 8 and 11 of the 13 BP samples, 2 and 2 of the 22 CP samples, and none of the 16 EBA samples.

COMMENT

In the present study, the combination of BPAG1 and BPAG2 ELISA permitted an 8% gain in sensitivity from 79% with BPAG2 ELISA alone to 87%. Yoshida et al²⁶ using the same BPAG1 and BPAG2 ELISA reported a 27% gain in sensitivity from 70% with BPAG2 ELISA alone to 97% with the combination of BPAG1 and BPAG2 ELISA. It is likely that the lower benefit from the use of the combination ELISA in our study was mainly owing

to the recruitment of cases of newly diagnosed active disease, whereas in the study by Yoshida et al,²⁶ patients in both active stage and remission were included. If only patients with BP in active stage alone were considered from the study reported by Yoshida et al,²⁶ the sensitivity of BPAG1 and BPAG2 ELISA, as well as the gain in sensitivity permitted by the combination of BPAG1 and BPAG2 ELISA procedures, were close to those in the present study: BPAG1 ELISA sensitivity of 58% vs 61% in the present study; BPAG2 ELISA sensitivity of 84% vs 79%; and sensitivity gain with combination of BPAG1 and BPAG2 ELISA relative to BPAG2 ELISA alone: 10% (from 84% to 94%) vs 8% (from 79% to 87%). The 79% sensitivity of the BPAG2-NC16A ELISA used in the present study was also close to the 84% sensitivity reported by Kobayashi et al³⁴ using the same BPAG2-NC16A ELISA. Higher sensitivity values up to 94% have been reported with BPAG2 ELISA using the entire ectodomain of BPAG2,²⁷ resulting in an even lower gain in sensitivity from the combination of BPAG1 and BPAG2 ELISA: 6% (from 94% to 100%) in the study by Di Zenzo et al.²⁷

The rather low gain in sensitivity (8% to 10%) of combined BPAG1 and BPAG2 ELISA for the diagnosis of BP in our study and in that of Yoshida et al,²⁶ which used the same ELISA assays, raises the question of the cost-effectiveness ratio of the routine use of the combination of these 2 assays. The combination assay leads to a 100% increased cost for the diagnostic procedure (€24.00, or \$32.00), which is 2-fold higher than the cost of IIF on human skin (€10.80, or \$14.00) but quite similar to that of the addition of standard IIF and NaCl-split skin IIF. Indeed, the sensitivity of the combination of BPAG1 and BPAG2 ELISA was only slightly higher than that of BPAG2 ELISA alone (87% vs 79%) and that of standard IIF (87% vs 81%) ($P=.06$).

Nevertheless, the combination of BPAG1 and BPAG2 ELISA was a useful tool in 2 limited groups of patients. The first group consisted of patients with no circulating anti-BPAG2 antibodies, since anti-BPAG1 antibodies were detected in 15 of 40 (38%) and in 6 of 10 (60%) samples with no anti-BPAG2 antibodies from patients with active BP in the present study and in that by Yoshida et al,²⁶ respectively. Second, the combination of BPAG1 and BPAG2 ELISA could also be useful to distinguish between patients with BP with mucosal involvement and patients with CP, since anti-BPAG1 antibodies were present in 62% of samples from mucosally involved BP (8 of 13) and in 2 of the 22 patients from the CP group (9%) ($P=.002$), with low ELISA values (18 and 28 U/mL, respectively). Low levels of anti-BPAG1 antibodies have previously been reported in a few samples from patients with CP using immunoblot analysis.³⁷⁻³⁹

In the present study, the specificity of the combination of BPAG1 and BPAG2 ELISA was rather good: 88%. However, low ELISA values of anti-BPAG1 and anti-BPAG2 antibodies have occasionally been reported in patients with various conditions identified from a "waste blood list," as well as in elderly patients with various pruritic dermatoses not corresponding to autoimmune bullous diseases.^{40,41}

Although the specificity of BPAG1 and BPAG2 ELISA depends on the proportion of serum samples from patients with pemphigus and subepidermal autoimmune

bullous disease in the control group, the combination of BPAG1 and BPAG2 ELISA had a higher specificity than standard IIF in the diagnosis of BP (88% vs 63%) ($P=.006$). This difference was even higher (88% vs 58%) ($P=.002$) when only the group of subepidermal autoimmune bullous diseases was analyzed (ie, after pemphigus samples from the control group were excluded), although low ELISA values of anti-BPAG2 antibodies were occasionally detected in pemphigus foliaceus samples, in association with anti-desmoglein 1 antibodies.⁴² Indirect immunofluorescence on NaCl-split skin had a rather good value in the diagnosis of EBA and BP: antibodies directed against the dermal side of NaCl-split skin were detected in 16 of 16 EBA samples and in only 1 of 13 BP samples, whereas antibodies directed against the epidermal side of NaCl-split skin were detected in 10 of 13 BP samples and in none of the 16 EBA samples. On the contrary, the different labelings observed with CP samples (epidermal or dermal side) did not allow a clear identification of CP samples with this technique.^{39,43-46} Therefore, both ELISA and NaCl-split skin IIF had a good value in the diagnosis of BP and EBA and a poor value in the diagnosis of CP.

In the present study, a rather strong correlation between BPAG2 ELISA values and initial extent of BP lesions was found ($r=0.44$, $P<.001$), whereas we only observed a rather low correlation between BPAG1 ELISA values and disease extent ($r=0.16$, $P=.03$). Anti-BPAG2 antibodies have been previously demonstrated to be strongly associated with disease activity in BP,¹⁶⁻²² whereas the relationship between BPAG1 ELISA values and disease activity remains controversial.^{26,27,36} Yoshida et al²⁶ evidenced a correlation between BPAG1 ELISA values and BP activity in only 5 of the 10 patients they studied, whereas 2 other studies suggested that anti-BPAG1 antibodies could be preferentially associated with localized types of BP.^{27,36} Our results did not confirm these data, since anti-BPAG1 antibodies were detected in 59% and 62% of patients with moderate and extensive BP, respectively ($P=.66$).

In conclusion, the present study does not argue for a routine use of the combination of BPAG1 and BPAG2 ELISA assays in the diagnosis of BP. The use of BPAG1 ELISA could be adequately proposed in a minority of BP cases with no circulating anti-BPAG2 antibodies and in patients with mucosal involvement at presentation.

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Correspondence: Pascal Joly, MD, PhD, Department of Dermatology, Hôpital Charles Nicolle, 1 rue de Germont, 76031 Rouen CEDEX, France (Pascal.Joly@chu-rouen.fr).

Author Contributions: Drs Roussel and Joly had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Roussel and Joly. *Acquisition of data:* Roussel, Randriamanantany, Gilbert, Drenovska, and Joly. *Analysis and interpretation of data:* Roussel, Benichou, Drenovska, Houivet, Tron, and Joly. *Drafting of the manuscript:* Roussel, Benichou, and Joly. *Critical revision of the manuscript for important intellectual content:* Roussel, Randriamanantany, Gilbert,

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Notable Notes

Articles of Faith: Bruiser

Although it was never my intent with “Articles of Faith” to mock or belittle folk, home, or alternative remedies, it would surely be hard to do so, when modern medicine can’t offer a legitimate option of its own. I don’t know how many times a day I, as a dermatologist, have to admit to the frail, the anticoagulated, or the just plain clumsy that there is really nothing I can do for their bruising. Well, here’s Bruiser to illustrate some of the things that have been suggested to make those ugly boo-boos go away faster (**Figure**).

Mark S. Bernhardt, MD

Contact Dr Bernhardt at
1601 E Broward Blvd, Ft
Lauderdale, FL 33301
(cheesedb@aol.com).



Figure. Body, olive oil; legs, blackstrap molasses or honey; tail, hemorrhoid ointment; wattle, hot water bottle; neck, ice; head, petroleum jelly; snout, mustard; eyes, quail eggs; and ears, keys (chilled).