

Clinical and Laboratory Investigations

Evaluation of a BP180-NC16a enzyme-linked immunosorbent assay in the initial diagnosis of bullous pemphigoid

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Accepted for publication 8 March 2004

Summary

Background Bullous pemphigoid (BP) is the most common subepidermal immunobullous disease, characterized by circulating IgG autoantibodies targeting BP180 and BP230 hemidesmosomal proteins. Several immunological studies have demonstrated that the membrane proximal noncollagenous domain NC16a of BP180 is the immunodominant region targeted by BP autoantibodies. Recently, a commercial BP180 NC16a-specific enzyme-linked immunosorbent assay (ELISA) has become available for detecting pathogenic anti-BP180 autoantibodies in BP sera. However, it remains unclear whether the diagnostic potential of the ELISA is equivalent to that of the 'gold-standard' diagnostic technique of immunofluorescence (IF).

Objectives To examine the usefulness of a commercially available BP180-NC16a ELISA in the initial serodiagnosis of BP.

Methods Sera from a large cohort of patients with BP ($n = 102$) and control subjects (age- and sex-matched normal volunteers, $n = 60$; pemphigus foliaceus, $n = 18$; pemphigus vulgaris, $n = 16$) were assayed by BP180-NC16a ELISA. All BP sera were obtained at presentation before initiation of systemic immunosuppressive therapy. The values of IgG antibody levels measured by ELISA were compared with those measured by indirect IF on salt-split skin.

Results Receiver operating characteristic analysis was used to calculate the cut-off value for the ELISA in the diagnosis of BP which maximizes both sensitivity and specificity, and to estimate the diagnostic accuracy of the ELISA as represented by the area under the curve ($AUC = 0.965$). A cut-off value of 9 was associated with a sensitivity of 89% (91 of 102 BP sera showed a positive result) and a specificity of 98%. Fifty-eight of 60 normal controls and all the pemphigus sera showed a negative result. There was a correlation between the mean ELISA values and indirect IF titres (Spearman rank correlation 0.286; $P = 0.004$).

Conclusions Our results suggest that the BP180-NC16a ELISA is a useful tool for the detection of pathogenic anti-BP180 IgG autoantibodies at the initial disease stage of BP. Because it is not only highly sensitive and specific, but is also easy to perform, is objective, and semiquantitative, the ELISA may provide valuable information for the accurate and reliable serodiagnosis of BP.

Key words: adhesion molecule, autoantigen, autoimmune response, bullous pemphigoid, hemidesmosome, type XVII collagen

Bullous pemphigoid (BP) is an acquired autoimmune subepidermal blistering disorder that usually affects an elderly population. BP autoantibodies predominantly target two hemidesmosomal components, BP180 (type XVII collagen) and BP230.¹ BP230 is a cytoplasmic

protein which mediates the interaction between intermediate filaments and the basal cell surface.² In contrast, BP180 is a transmembrane protein component of hemidesmosomes, consisting of 15 interrupted collagenous domains and 16 noncollagenous (NC) domains in the extracellular region.³ The membrane proximal NC16a domain has been identified to contain the antigenic epitopes most frequently recognized by BP sera.⁴ The pathogenic significance of antibodies against the NC16a domain in BP has been demonstrated both *in vivo* and *in vitro*; passive transfer of antibodies against a murine homologue of the human NC16a induced skin blister formation in neonatal mice, and antibodies to the NC16a domain caused epidermal-dermal separation on cryosections of human skin.^{5,6}

Previous studies on the use of enzyme-linked immunosorbent assays (ELISAs) in BP have employed various recombinant proteins encoding antigenic epitopes of BP180, with varying results.⁷⁻¹³ The aim of a new commercially available BP180-NC16a ELISA is to standardize the sensitivity and reproducibility of this assay. To our knowledge, however, there are no available data to estimate the usefulness of the commercially available BP180 ELISA in the initial accurate diagnosis of BP.

In this study therefore we focused on the potential usefulness of a BP180-NC16a ELISA for the initial serodiagnosis of BP. We also analysed the ELISA values in comparison with the 'gold standard' diagnostic technique of indirect immunofluorescence (IF).

Materials and methods

Serum samples

Serum samples from 102 patients with BP (mean age 78 years; 46 men and 56 women), and 94 control donors were used in this study: the latter included 60 healthy individuals with no history of autoimmune disorders (mean age 82 years; 24 men and 36 women), 16 with pemphigus vulgaris (mean age 52 years; seven men and nine women) and 18 with pemphigus foliaceus (mean age 49 years; 12 men and six women). The normal control subjects were age- and sex-matched to the population with BP. More than 90% of the BP group were caucasian. BP sera were taken at the initial disease stage prior to systemic immunosuppressive therapy, and the diagnosis was made on the basis of the following clinicopathological criteria: (i) typical tense cutaneous blister with or without oral mucosal lesion; (ii) linear IgG deposition at the

epidermal side of salt-split skin on indirect IF; and (iii) the histopathological finding of subepidermal blister formation.

The diagnosis of pemphigus was made on the basis of clinical and immunohistological features. All the sera from pemphigus patients showed intercellular IgG reactivity on normal human skin and/or monkey oesophagus at titres ranging from 1 : 10 to 1 : 3200. Approval from the local ethics committees was obtained for all parts of the study.

Indirect immunofluorescence

Antibasement membrane zone (anti-BMZ) IgG autoantibody titres were measured by indirect IF using normal human split skin as substrate, as described previously.¹⁴ In brief, BP sera were serially diluted at 1 : 10 to 1 : 3200 with phosphate-buffered saline, and incubated on salt-split skin section at 37 °C for 1 h, followed by incubation with fluorescein isothiocyanate-conjugated IgG secondary antibodies at 37 °C for 1 h (Dako, Glostrup, Denmark).

BP180 enzyme-linked immunosorbent assay and quantification

Ninety-six-well microtitre plates were provided pre-coated with recombinant baculoproteins encompassing the NC16a portion of BP180 (Medical and Biological Laboratories Co., Ltd, Nagoya, Japan). Following the manufacturer's instructions, sera were diluted 1 : 101 and tested in duplicate. Anti-NC16a autoantibodies were detected by a standard sandwich method using horseradish peroxidase (HRP)-conjugated antihuman IgG antibodies, followed by tetramethyl benzidine as a substrate of HRP. The substrate is oxidized in the presence of HRP and results in a detectable colour change which is related to the amount of HRP and thus indirectly to the quantity of anti-BP180 NC16a antibodies. The colour development was stopped by addition of 1 mol L⁻¹ sulphuric acid. The absorbance or optical density (OD) of each well was then read at 450 nm by an automated plate reader (MRT Microplate Reader®; Dynex Technologies, Chantilly, VA, U.S.A.). The mean OD of duplicate test sera was calculated in each sample.

To allow comparison of results from different plates, test sample ODs were adjusted relative to positive and negative control samples supplied in the ELISA kit, which were set to 100 and 0, respectively. The index value was defined by the following formula:

Index = {(mean OD of test sera – mean OD of negative control)/(mean OD of positive control – mean OD of negative control)} $\times$ 100.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 (SPSS, Chicago, IL, U.S.A.). Receiver operating characteristic (ROC) curves were used to evaluate the ability of the ELISA to detect specific autoantibodies directed against the NC16a, and to determine a cut-off value for the ELISA in differentiating BP from the control groups. To estimate the diagnostic accuracy, the area under the curve (AUC) was reported with a 95% confidence interval (CI). The Youden index ($J = \text{sensitivity} + \text{specificity} - 1$) was used to select the best cut-off value, which maximizes the sum of sensitivity and specificity.

The Spearman rank test was performed to assess the significance of the correlation between ELISA values and indirect IF titres of the investigated sera; $P < 0.01$ was considered statistically significant.

Results

Detection of anti-BP180 NC16a IgG antibodies by enzyme-linked immunosorbent assay

Sera from patients with BP ($n = 102$) prior to systemic immunosuppressive therapy, normal volunteers ($n = 60$) and patients with pemphigus ($n = 34$) were tested for IgG reactivity by using the BP180-NC16a ELISA. The results were expressed as index values relative to positive and negative reference sera. As shown in Table 1, the ELISA gave index values ranging from 0 to 205 (mean $\pm$ SD, 69 ± 51) in BP, from 0 to 15 (2 ± 3) in normal volunteers, and from 0 to 9 (4 ± 4) in pemphigus groups. The mean coefficient of variation for all the investigated sera was 3.2%. To assess the reproducibility of the ELISA,

Table 1. Summary of BP180-NC16a enzyme-linked immunosorbent assay (ELISA) data

Diagnosis	<i>n</i>	Age (mean $\pm$ SD), years	Mean ELISA value ^a (range)
Bullous pemphigoid	102	78 ± 11.2	69 (0–205)
Normal controls	60	82 ± 12.6	2 (0–15)
Pemphigus	34	50 ± 17.9	4 (0–9)

^aELISA value was calculated by using the formula supplied by the company.

inter- and intraplate variability was tested using two BP reference sera, and was calculated to be 2.7% and 3.2%, respectively.

To determine a cut-off value of the ELISA in discriminating BP from the control groups, the ELISA values from all the BP sera were compared with those from a pooled cohort of normal control sera plus pemphigus sera. By maximizing the Youden index ($J = \text{sensitivity} + \text{specificity} - 1$), the cut-off value was established at 9, which corresponds to a Youden index (J) of 0.88. This value is identical to the manufacturer's suggested cut-off, suggesting a high reproducibility of the ELISA. The ROC analysis confirmed the diagnostic accuracy of the BP180-NC16a ELISA, represented by an AUC of 0.965 (95% CI 0.940–0.989) (Fig. 1).

Using the selected cut-off value, 91 of 102 BP sera (89%) exhibited IgG reactivity to the NC16a of BP180; only two normal control sera, but none of 34 pemphigus sera, exceeded the cut-off value (sensitivity and specificity of 89% and 98%, respectively) (Fig. 2). The two false-positive normal control sera did not show any reactivity on indirect IF and immunoblotting using human epidermal extracts (data not shown). Therefore, these sera were not further characterized for BP180 reactivity.

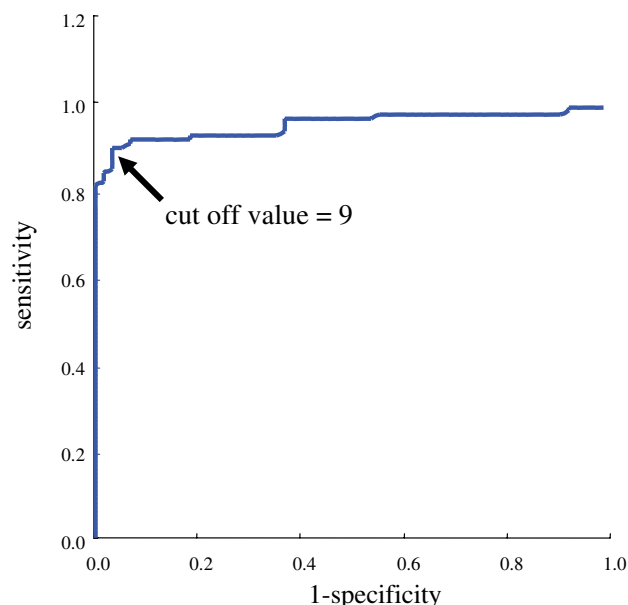


Figure 1. Receiver operating characteristic curve to determine a cut-off value for BP180-NC16a enzyme-linked immunosorbent assay (ELISA). The ELISA value of 9 was determined by maximization of the Youden index.

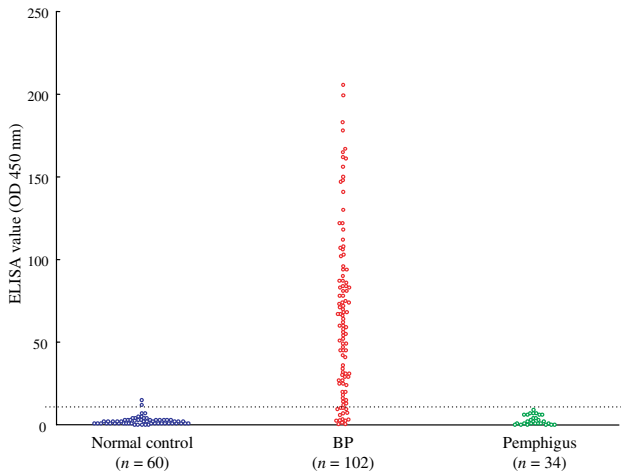


Figure 2. Enzyme-linked immunosorbent assay (ELISA) values for each diagnostic variant. The line indicates the cut-off value at 9 determined by receiver operating characteristic analysis. OD, optical density; BP, bullous pemphigoid.

Correlation between enzyme-linked immunosorbent assay value and indirect immunofluorescence titre

We next examined the correlation between the ELISA values of 102 BP sera and their IgG reactivity to the BMZ on indirect IF (Fig. 3). The IF titres of BP sera ranged from 1 : 100 to 1 : 3200. A correlation was observed between the two assays (Spearman rank correlation 0.286; $P = 0.004$).

Discussion

The aim of this study was to explore the diagnostic value of a commercially available BP180-NC16a ELISA in BP at disease presentation. Using this assay, we examined the initial sera of 102 patients with BP before systemic immunosuppressive therapy, and as comparative controls, 60 sera from normal volunteers and 34 sera from pemphigus patients. Our results clearly demonstrated that most pretreatment BP sera ($\approx 90\%$) contain detectable levels of IgG autoantibodies against the NC16a domain of BP180. This assay was highly specific (98%) at the cut-off value of 9 in discriminating BP and normal/disease control groups. Among all the controls ($n = 94$), only two normal sera showed ELISA values slightly exceeding the cut-off. One of the two sera remained positive when we reassessed with an alternative formula to determine a cut-off value (mean ELISA value of control group + 3 SD; cut-off value = 12), but these two false-positive sera did not show any immunoreactivity on indirect IF and immunoblots with

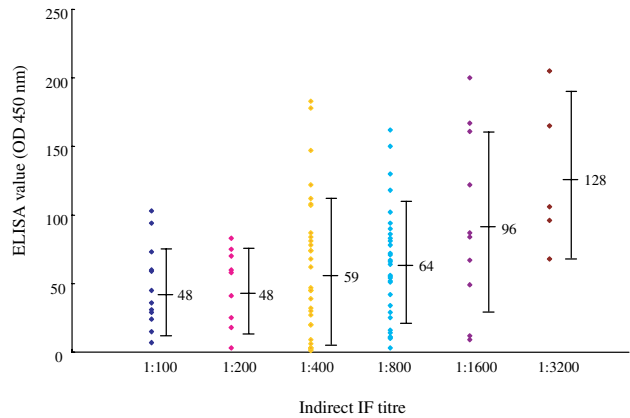


Figure 3. A comparison of the BP180-NC16a enzyme-linked immunosorbent assay (ELISA) values against the indirect immunofluorescence (IF) titre for bullous pemphigoid sera. The ELISA values (vertical axis) and indirect IF titre (horizontal axis) for each patient's serum were plotted. Error bars indicate the mean $\pm$ SD. OD, optical density.

epidermal extract. The discrepancy might be due to minor contamination or differences in techniques (e.g. differences in antigen preparation and reagents).

The cut-off value of 9 which we established in our study was identical to that suggested by the manufacturers of the ELISA kit. This value had been determined by using a large cohort of sera from the Japanese population.⁷ These results suggest that the ELISA is highly reproducible without regard to the ethnicity of the patients.

Although several ELISAs using different portions of the BP180 extracellular domain have previously been developed and tested, their detection sensitivities varied. An early study, using as antigen a prokaryotic recombinant protein that covered a partial sequence within the NC16a domain, exhibited a low sensitivity (53%).⁸ Nakatani *et al.* improved the sensitivity to 96% with the same length of the NC16a domain recombinant, following studies demonstrating that 94% of BP sera react to the full length of the NC16a recombinant protein.^{9,10} The series of ELISA studies has been further extended by the development of baculoprotein expression systems, which allow the use of longer recombinant BP180 peptides as antigen in this assay.^{11–13} One advantage of this system is that the baculoproteins retain their native conformational epitopes, which contributes to a proper antigen–antibody reaction, and would therefore be expected to increase the potential sensitivity of the assay.¹⁵ One of these studies demonstrated a high immunoreactivity of BP sera (92%) against the BP180 extracellular domain devoid of 68 amino acids at the

C-terminus, while more recent studies revealed a sensitivity of 80% using a BP180 antigen covering the NC16a plus Coll 5, which is adjacent to the NC16a domain and is considered to be another target epitope recognized by BP sera.^{12,13} Comparing the results of the ELISA systems thus far reported, there is no significant improvement in sensitivity between bacterial and baculoviral BP180 antigens. The varying sensitivities among the ELISA studies may be due to the following reasons: (i) different methods of determining a cut-off value; (ii) differences in the selection and number of the investigated BP sera (e.g. the phase of the disease course, or whether or not treatment is ongoing); and (iii) different lengths and sources of recombinant proteins. These variables might not allow a direct comparison among the ELISAs. Overall, it is notable that ELISAs using full-length NC16a peptide as antigen have consistently been associated with a high level of sensitivity at discriminating BP from control subjects, indicating a principal immunoreactivity harbouring this epitope in BP sera. This concept is supported by *in vitro* and *in vivo* studies.^{5,6} For example, antibodies to the NC16a domain caused epidermal-dermal separation on cryosections of human skin, and passive transfer of IgG antibodies targeting a murine homologue of human NC16a induced subepidermal (clinicopathologically similar to BP) blistering in neonatal mice. Further studies suggest that in BP the level of NC16a-specific antibody as detected by ELISA correlates with disease severity. A well-standardized NC16a ELISA is valuable for detecting pathogenic autoantibodies in BP sera.^{7,16,17}

When comparing the ELISA values with indirect IF titres of BP sera, we found a correlation between the two assays (Spearman rank correlation 0.286; $P < 0.01$). These results conflict with previous observations showing less correlation between ELISA and indirect IF studies for BP.^{8,9} The lack of correlation in the previous studies may result from the fact that indirect IF staining reflects not only the presence of anti-BP180 antibodies but also antibodies to another BMZ antigen, BP230, which plays a role in hemidesmosomal assembly and formation. In fact, our preliminary immunoblotting using human epidermal extracts revealed that eight of 10 ELISA-negative BP sera showed IgG reactivity against BP230 (data not shown). Unlike BP180, however, antibodies to BP230 are thought to be of less pathogenic relevance in BP; rabbits immunized with a BP230 polypeptide did not develop skin blisters, but showed an intense inflammatory response after additional injury, implying that the development of anti-BP230 antibodies might be a secondary event, perhaps associated with chronic skin

damage.¹⁸ In our study, all the BP sera were taken at initial disease presentation, before confounding factors such as systemic immunosuppressive therapy were introduced and presumably also after a short disease duration. It is thus clear that detecting anti-NC16a antibodies contributes to the definitive diagnosis of BP at initial disease stage. Because the BP180-NC16a ELISA is easy to perform (it takes less than 3 h), is highly sensitive and specific, and is objective, semiquantitative and reproducible, it provides useful information in the accurate diagnosis at the initial disease stage of BP.

References

- 1 Stanley JR. Cell adhesion molecules as targets of autoantibodies in pemphigus and pemphigoid, bullous diseases due to defective epidermal cell adhesion. *Adv Immunol* 1993; **53**: 291–325.
- 2 Ishiko A, Shimizu H, Kikuchi A *et al.* Human autoantibodies against the 230-kD bullous pemphigoid antigen (BPAG1) bind only to the intracellular domain of the hemidesmosome, whereas those against the 180-kD bullous pemphigoid antigen (BPAG2) bind along the plasma membrane of the hemidesmosome in normal human and swine skin. *J Clin Invest* 1993; **91**: 1608–15.
- 3 Giudice GJ, Emery DJ, Diaz LA. Cloning and primary structural analysis of the bullous pemphigoid autoantigen BP180. *J Invest Dermatol* 1992; **99**: 243–50.
- 4 Matsumura K, Amagai M, Nishikawa T, Hashimoto T. The majority of bullous pemphigoid and herpes gestationis serum samples react with the NC16a domain of the 180-kDa bullous pemphigoid antigen. *Arch Dermatol Res* 1996; **288**: 507–9.
- 5 Liu Z, Diaz LA, Troy JL *et al.* A passive transfer model of the organ-specific autoimmune disease, bullous pemphigoid, using antibodies generated against the hemidesmosomal antigen, BP180. *J Clin Invest* 1993; **92**: 2480–8.
- 6 Sitaru C, Schmidt E, Petermann S *et al.* Autoantibodies to bullous pemphigoid antigen 180 induce dermal-epidermal separation in cryosections of human skin. *J Invest Dermatol* 2002; **118**: 664–71.
- 7 Kobayashi M, Amagai M, Kuroda-Kinoshita K *et al.* BP180 ELISA using bacterial recombinant NC16a protein as a diagnostic and monitoring tool for bullous pemphigoid. *J Dermatol Sci* 2002; **30**: 224–32.
- 8 Giudice GJ, Wilske KC, Anhalt GJ *et al.* Development of an ELISA to detect anti-BP180 autoantibodies in bullous pemphigoid and herpes gestationis. *J Invest Dermatol* 1994; **102**: 878–81.
- 9 Nakatani C, Muramatsu T, Shirai T. Immunoreactivity of bullous pemphigoid (BP) autoantibodies against the NC16A and C-terminal domains of the 180 kDa BP antigen (BP180): immunoblot analysis and enzyme-linked immunosorbent assay using BP180 recombinant proteins. *Br J Dermatol* 1998; **139**: 365–70.
- 10 Zillikens D, Mascaro JM, Rose PA *et al.* A highly sensitive enzyme-linked immunosorbent assay for the detection of circulating anti-BP180 autoantibodies in patients with bullous pemphigoid. *J Invest Dermatol* 1997; **109**: 679–83.
- 11 Hata Y, Fujii Y, Tsunoda K, Amagai M. Production of the entire extracellular domain of BP180 (type XVII collagen) by baculovirus expression. *J Dermatol Sci* 2000; **23**: 183–90.
- 12 Haase C, Budinger L, Borradori L *et al.* Detection of IgG autoantibodies in the sera of patients with bullous and gestational

- pemphigoid: ELISA studies utilizing a baculovirus-encoded form of bullous pemphigoid antigen 2. *J Invest Dermatol* 1998; **110**: 282–6.
- 13 Hofmann S, Thoma-Uszynski S, Hunziker T *et al*. Severity and phenotype of bullous pemphigoid relate to autoantibody profile against the NH₂- and COOH-terminal regions of the BP180 ectodomain. *J Invest Dermatol* 2002; **119**: 1065–73.
 - 14 Bhogal BS, Black MM. Diagnosis, diagnostic and research techniques. In: *Management of Blistering Diseases* (Wojnorowska F, Briggaman RA, eds). London: Chapman & Hall Ltd, 1990: 15–34.
 - 15 Kidd IM, Emery VC. The use of baculoviruses as expression vectors. *Appl Biochem Biotechnol* 1993; **42**: 137–59.
 - 16 Amo Y, Ohkawa T, Tatsuta M *et al*. Clinical significance of enzyme-linked immunosorbent assay for the detection of circulating anti-BP180 autoantibodies in patients with bullous pemphigoid. *J Dermatol Sci* 2001; **26**: 14–18.
 - 17 Schmidt E, Obe K, Bröcker EB, Zillikens D. Serum levels of autoantibodies to BP180 correlate with disease activity in patients with bullous pemphigoid. *Arch Dermatol* 2000; **136**: 174–8.
 - 18 Hall RP III, Murray JC, McCord MM *et al*. Rabbits immunized with a peptide encoded for by the 230-kD bullous pemphigoid antigen cDNA develop an enhanced inflammatory response to UVB irradiation: a potential animal model for bullous pemphigoid. *J Invest Dermatol* 1993; **101**: 9–14.