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Severity and Phenotype of Bullous Pemphigoid Relate to Autoantibody Profile Against the NH_2- and COOH-Terminal Regions of the BP180 Ectodomain

Silke C. Hofmann, Sybille Thoma-Uszynski, Thomas Hunziker,* Philippe Bernard,† Corinna Koebnick,‡ Angelika Stauber, Gerold Schuler, Luca Borradori,§ and Michael Hertl

Department of Dermatology, Friedrich-Alexander-University Erlangen-Nürnberg, Germany; *Department of Dermatology, University Hospital, Bern, Switzerland; †Department of Dermatology, University of Reims, France; ‡Department of Medical Statistics, Biometry and Epidemiology, Friedrich-Alexander-University Erlangen-Nürnberg, Germany; and §Department of Dermatology, University Hospital, Geneva, Switzerland

Bullous pemphigoid, the most common autoimmune subepidermal bullous disorder, is associated with autoantibodies targeting antigenic sites clustered within the extracellular domain of BP180. To investigate epitope and subclass specificity of autoantibodies in bullous pemphigoid, we developed an enzyme-linked immunosorbent assay utilizing baculovirus-expressed recombinant forms of the NH_2- and COOH-terminal regions of the extracellular domain of BP180 and examined sera obtained from patients with active bullous pemphigoid ($n = 116$) and controls ($n = 100$). Ninety-three (80%) and 54 (47%) of the 116 bullous pemphigoid sera recognized the NH_2- and COOH-terminal regions, respectively, of the extracellular domain of BP180. Detailed analysis demonstrates that (i) this novel enzyme-linked immunosorbent assay is highly specific (98%) and sensitive (93%) as 108 of 116 bullous pemphigoid sera reacted with at least one of the baculovirus-derived recombinants, (ii) in active bullous pemphigoid, autoantibodies against the NH_2-terminus of the extracellular domain of BP180 were predominantly of the IgG_1 class, whereas a dual IgG_1 and IgG_4 response to this region was related to a more severe skin involvement, (iii) autoreactivity against both the NH_2- and COOH-terminal regions was more frequently detected in patients with mucosal lesions, and (iv) levels of IgG (and IgG_1) against the NH_2-terminal, but not against the COOH-terminal portion of the extracellular domain of BP180, reflected disease severity indicating that autoantibodies against the NH_2-terminus are critical in the pathogenesis of bullous pemphigoid. In conclusion, this novel enzyme-linked immunosorbent assay represents a highly sensitive and specific assay for rapid diagnosis of bullous pemphigoid and related disorders and may provide predictive parameters for the management of bullous pemphigoid patients. ***Key words: adhesion molecules/autoantibodies/autoimmunity/BP180/disease activity/hemidesmosome. J Invest Dermatol 119:1065–1073, 2002***

Bullous pemphigoid (BP) is an autoimmune blistering disorder of the skin that usually affects the elderly. The disease is associated with tissue-bound and circulating IgG autoantibodies directed against components of the epidermal basement membrane zone (Jordon *et al*, 1967). Clinically, BP is characterized by generalized tense subepidermal blisters arising on apparently normal or erythematous skin with involvement of mucous membranes in 20%–30% of cases (Lever, 1953). Previous studies have demonstrated that BP autoantibodies predominantly recognize BP230 (BPAG1) and BP180 (BPAG2 or type XVII collagen) (Labib *et al*, 1986; Stanley *et al*, 1988; Giudice *et al*, 1992), two structural components of hemidesmosomes. Hemidesmosomes are multiprotein junctional complexes that promote attachment of basal keratinocytes to the underlying basement membrane zone (reviewed in Borradori and Sonnenberg, 1999). BP230, an entirely cytoplasmic protein belonging to the plakin family, is involved in the anchorage of intermediate filaments to the basal cell surface (reviewed in Ruhrberg and Watt, 1997). In contrast, BP180 is a transmembrane protein with a type II orientation. Its large extracellular portion consists of 15 interrupted collagenous subdomains (Giudice *et al*, 1992; Hopkinson *et al*, 1992). It serves as a cell surface receptor (Giudice *et al*, 1992; Hopkinson *et al*, 1992) and plays an important role in the maintenance of epidermal–stromal adhesion (reviewed in Borradori and Sonnenberg, 1999). This notion is supported by the observation that mutations in the BP180 gene (COL17A1) are the cause of a clinical variant of non-Herlitz junctional epidermolysis bullosa, a congenital disorder characterized by skin fragility and blistering (reviewed in Pulkkinen and Uitto, 1998).

Recently, strong evidence has been provided that autoantibodies to BP180 play a key role in subepidermal blister formation in BP patients. In a passive transfer model, rabbit antibody raised

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Reprint requests to: Michael Hertl, M.D., Department of Dermatology, Friedrich-Alexander-University of Erlangen-Nürnberg, Hartmannstraße 14, D-91052 Erlangen, Germany; Email: michael.hertl@derma.imed.uni-erlangen.de

Abbreviations: BP, bullous pemphigoid; BP180-N, baculoprotein containing the NC16A domain and Col15 domain of BP180; BP180-C, baculoprotein containing the COOH-terminal domains NC3–NC1 of BP180; BV13, baculoprotein containing the extracellular domain of BP180; ECD, extracellular domain; ELISA, enzyme-linked immunosorbent assay; OD, optical density; PBS, phosphate-buffered saline.

against the murine homolog of the human immunodominant region of BP180, the NC16A domain, were able to induce subepidermal blister formation, reproducing all key features of BP (Liu *et al*, 1993). Furthermore, it has been found that the serum levels of IgG autoantibodies directed against the extracellular domain (ECD) of BP180 were related to the severity of BP (Haase *et al*, 1998; Schmidt *et al*, 2000).

Autoantibodies from patients with BP and some closely related autoimmune bullous disorders, such as gestational and cicatricial pemphigoid, have been found to predominantly recognize two major antigenic regions of the ECD of BP180. Specifically, the NC16A subdomain, a region adjacent to the membrane spanning domain of BP180, harbors major epitopes recognized by almost all sera from patients with BP as well as gestational pemphigoid, a blistering disorder associated with autoimmunity against BP180 that occurs during pregnancy (Giudice *et al*, 1993; Matsumura *et al*, 1996). In these patients, reactivity with the ECD of BP180 can be almost completely abolished by pre-adsorption of IgG with recombinant NC16A protein (Zillikens *et al*, 1997a; Hata *et al*, 2000; Lin *et al*, 2000). Second, the COOH-terminal region of the ECD of BP180 has been shown to be recognized by approximately one-third of BP sera (Murakami *et al*, 1998; Nakatani *et al*, 1998) as well as by sera from a subset of patients with cicatricial pemphigoid (Balding *et al*, 1996; Bedane *et al*, 1997; Murakami *et al*, 1998; Nie and Hashimoto, 1999), a chronic autoimmune blistering disorder characterized by predominant involvement of mucosal surfaces with scarring.

The purpose of this study was to develop a sensitive and highly specific *in vitro* assay for the detection of circulating autoantibodies in BP utilizing baculovirus-derived recombinants of the NH_2- and COOH-terminal regions of the ECD of BP180. The baculovirus expression system has proved to be extremely useful for the production of recombinant proteins retaining many of the post-translational modifications of the native proteins (Kidd and Emery, 1993). Because of the putative key role of BP180 in disease initiation, we assessed the reactivity against these two BP180 recombinant proteins in serum samples obtained from BP patients and from a control group and further characterized the epitope specificity as well as the isotype distribution of the autoantibody response to BP180. Furthermore, we investigated the potential relationship between the autoantibody profile of these BP sera and the clinical phenotype and severity of BP.

MATERIALS AND METHODS

Patients and control sera Serum samples were obtained from patients with clinically active BP ($n = 116$), BP patients in remission ($n = 6$), and control donors; the latter included patients with pemphigus vulgaris ($n = 28$) and healthy volunteers (normal human sera, $n = 72$). All patients and volunteers gave written consent to participate in this study.

In the group of patients with active BP, sera were taken before immunosuppressive treatment in 99 patients, but 17 patients with chronic BP had already received systemic immunosuppressive treatment. Diagnosis of BP was based on the following criteria: (i) typical clinical presentation with tense cutaneous blisters (including 16 cases with additional involvement of mucous membranes without scarring); (ii) histopathologic evidence of subepidermal blister formation; (iii) linear IgG and/or C3 deposits at the dermo-epidermal junction of perilesional skin by direct immunofluorescence; and (iv) IgG-positive indirect immunofluorescence on saline-separated human skin. Control sera were collected from randomly chosen healthy individuals who had no history of bullous skin disorder and from 28 pemphigus vulgaris patients whose sera showed intercellular IgG reactivity on monkey esophagus or human skin and also reacted with recombinant desmoglein 3 by enzyme-linked immunosorbent assay (ELISA).

To investigate the relationship between autoantibody profiles of the BP sera and the clinical phenotype, sera from patients with active BP were compared to those of patients in remission; five of the latter patients had received systemic immunosuppressive treatment, and one patient received no therapy. A total of 114 BP patients (i.e., 108 patients with active BP and six patients in remission) were clinically well characterized with regard to disease severity and the duration of disease (**Table III**). Disease severity was classified according to the extent and number of blisters: 0, no skin lesions; 1, localized disease (few bullae or bullae at limited areas of the body); 2, generalized disease. The clinical stage of BP was defined as "acute" (duration ≤ 3 mo) or "chronic" (duration ≥ 3 mo with persisting bullae or erosions). "Remittent" was defined by the absence of skin lesions for more than 1 mo. Patient and control sera were stored at –20°C prior to analysis. Sera from 95 BP patients with IgG against the NH_2-terminus of the BP180 ectodomain (**Tables IV, V**) and 48 sera reactive to the COOH-terminus (**Table VI**) were analyzed with regard to the antibody levels and isotype distribution of BP180-reactive IgG, disease activity, duration, and mucosal involvement.

Expression and purification of recombinant BP180 proteins The recombinant proteins BP180-N (NC16A domain and the collagenous Col15 domain of BP180) and BP180-C (COOH-terminal domains NC3–NC1 of BP180) are linked to glutathione-S-transferase (GST) and 6xHis tag (**Fig 1**) and were produced in a baculovirus expression system as described below. The generation of cDNAs encoding for human BP180 has been described previously (Borradori *et al*, 1997). These cDNAs were used as a template to amplify by polymerase chain reaction (PCR) truncated variants of BP180 using Pfu DNA polymerase following the manufacturer's instructions (Stratagene, La Jolla, CA). Forward primers contained an EcoR I site and reverse primers contained either an EcoR I or a Not I site adjacent to the sequence homologous to the desired BP180 sequence. The cDNA fragments were subcloned into the pAcGHLT-A baculovirus transfer vector (Pharmingen, San Diego, CA) by ligation of double digested PCR fragments into the correspondingly cut vectors. Correctness of both constructs was verified by nucleotide sequencing. pAcGHLT-A vectors were cotransfected with BaculoGold DNA (Pharmingen) into Sf21 insect cells and recombinant baculoviruses were amplified using a previously described protocol (Amagai *et al*, 1994). The recombinant BP180 proteins and the GST/6xHis control protein (generated by cotransfection of an empty pAcGHLT-A vector) were expressed in Sf21 cells as previously described (Haase *et al*, 1998). Recombinant BP180 proteins were purified as follows: 3 d after infection, 10×10^6 baculovirus-infected insect cells were suspended in 1 ml of ice-cold lysis buffer (Pharmingen), incubated for 1 h at 4°C, and then centrifuged ($2000 \times g$, 15 min). Solubilized proteins were purified by affinity chromatography over Nickel-NTA agarose (Quiagen, Hilden, Germany) using the batch method according to the manufacturer's protocol. The BP180 recombinant BP180-N and the GST/6xHis control protein were purified under native conditions, whereas purification of BP180-C was performed under denaturing conditions by adding 6 M guanidine hydrochloride to the lysis buffer and 8 M urea to washing and elution buffers. Purified proteins were finally dialyzed against phosphate-buffered saline (PBS) (pH 7.5) at 4°C for 48 h (BP180-N, GST/6xHis) or gradually dialyzed against PBS supplemented with 8 M to 3 M urea (BP180-C). Affinity-purified BP180 proteins were stored in aliquots at −80°C. Protein concentrations were determined with a commercial kit (DC Protein Assay, Bio-Rad, Munich, Germany) according to a modified protocol by Lowry. BP180-N and BP180-C were fractionated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized

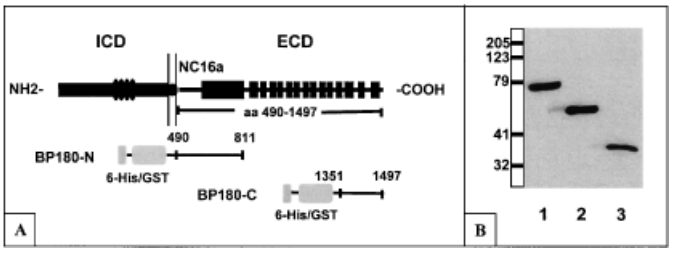

Figure 1. Scheme of the BP180 recombinants utilized in this ELISA study. (*A*) BP180 is a type II transmembrane protein containing an intracellular domain (ICD), a transmembrane domain, and an ECD with 15 interrupted collagenous domains. The extracellular NC16A domain is located adjacent to the plasma membrane. The recombinant BP180 fusion proteins are composed of a 6xHis and a GST-tag at the NH_2 end, followed by the antigenic epitopes at the COOH-terminus. BP180-N contains the NC16A domain and the collagenous domain Col15 (aa 490–811), and BP180-C encompasses the COOH-terminal domains NC3–NC1 (aa 1351–1497). (*B*) The purified BP180 recombinants were transferred to nitrocellulose membranes and detected with a monoclonal anti-GST antibody. *Lane 1*, BP180-N; *lane 2*, BP180-C; *lane 3*, GST/6xHis. Numbers shown on the left refer to molecular weight marker.

by Coomassie staining and Western blot with a monoclonal mouse anti-GST antibody (1:2000, clone GST3-4C, Zymed, San Francisco, CA) to verify molecular weight and immunoreactivity of the recombinant BP180 proteins (**Fig 1**).

ELISA utilizing BP180 recombinants Optimal conditions for the ELISA were established by various chessboard titrations following published guidelines (Kemeny, 1991). The affinity-purified recombinants of BP180 were immobilized on 96-well polystyrene plates (Maxisorb Immunoplate, Nunc, Wiesbaden, Germany) by coating each well with 0.5 μg of BP180-N, 0.5 μg of BP180-C, or a molar equivalent amount of the control protein GST/6xHis in 100 μl of 0.1 M bicarbonate buffer, pH 9.8, at 4°C for 5 h. An incubation time of 5 h was arbitrarily chosen and worked well in the laboratory routine; shorter incubation times led to a reduced sensitivity of the assay. ELISA plates were then washed five times with TBST (TBS pH 7.5 with 0.05% Tween 20; Merck, Nürnberg, Germany) and blocked for 2 h with 100 μl of 5% skimmed milk powder in TBST at room temperature. Patients' and control sera diluted 50-fold in blocking buffer were added to the wells in duplicate. After an incubation period of 2 h at room temperature and consecutive washes as above, wells were reacted with alkaline phosphatase (AP) labeled goat antihuman IgG (γ) (1:6000, Kirkegaard and Perry, Gaithersburg, MD), monoclonal biotinylated mouse antihuman IgG_1 (1:800, clone HP6069) or monoclonal biotinylated mouse antihuman IgG_4 (1:500, clone HP6025) (both Zymed, San Francisco, CA), biotinylated mouse antihuman IgE (1:500, Zymed) or peroxidase-conjugated sheep antihuman IgA (1:5000, The Binding Site, Birmingham, U.K.) for 1–2 h. After incubation of biotinylated antibody for 1 h, wells were washed as above and incubated with streptavidin–AP (ZyMAX, Zymed) diluted at 1:2000 in TBS for 1 h at room temperature. After another series of washes, 100 μl of p-nitrophenylphosphate (Kirkegaard and Perry) or the peroxidase substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, Deisenhofen, Germany) were added to the wells and optical density (OD) was measured at 405 nm when a defined patient serum (positive reference control) reached an OD of 1.0 as determined with a Wallac 1420 microplate reader (Wallac, Freiburg, Germany).

All extinction data were standardized based on the positive and negative internal standards, which were set to 1 and 0 OD units, respectively. The background reactivity of IgG to the control protein GST/6xHis was subtracted from the mean OD values obtained with BP180-N and BP180-C proteins. For the detection of IgG_1, IgG_4, IgE, and IgA reactivity to BP180, the GST/6xHis control protein was not used routinely, because a relevant total IgG reactivity against GST/6xHis had not been detected in any of the sera by ELISA. To evaluate plate-to-plate variability, each plate included identical internal controls (two positive sera, one control serum). The specificity of serum reactivity to BP180-N and BP180-C was verified by immunoadsorption of sera with these BP180 recombinants prior to ELISA ("inhibition ELISA"), which led to a significant decrease of serum reactivity to these particular BP180 recombinants. A total of 4 μg of BP180-N, BP180-C, or GST/6xHis proteins was added to nitrocellulose membranes, followed by two washes with TBST for 10 min. Subsequently, membranes were blocked with TBST containing 5% skimmed milk powder for 1 h at room temperature and then incubated with sera diluted 50-fold in blocking buffer overnight at 4°C. Patient and control sera before and after pre-adsorption were then reacted with GST/6xHis and the BP180 recombinants on ELISA and IgG reactivity was detected as described above.

Statistical analysis Statistical analysis was performed using SPSS 10.0 (SPSS, Chicago, IL). Receiver operating characteristic (ROC) curves were used to evaluate the ability of the ELISA to detect autoantibodies against the NH_2- and COOH-terminal domains of the ECD of BP180. The areas under the curves (AUC) are reported with their 95% confidence interval (95%-CI). The Youden index (J = sensitivity + specificity − 1) was used to select the best cut-off values. To assess the reproducibility of the ELISA results, coefficients of variance were calculated for a BP reference serum with an intermediate OD reading. The interassay variability of the OD_{405} readings for the different ELISAs ranged between 6.9% and 12.6% depending on the investigated BP180 recombinant and the Ig subclass.

Disease activity and disease duration were compared with ELISA values using multivariate analysis of covariance additionally consisting of age and sex. To enhance the assumption of normality, ELISA values were log-transformed. The independence of the distribution of epitope and Ig subtype specific autoantibody and the clinical phenotype was tested by Fisher's exact test. $p < 0.05$ was considered statistically significant. Values are expressed as geometric mean ± geometric standard error.

RESULTS

IgG reactivity of BP sera against the NH_2- and COOH-terminal regions of the ECD of BP180 is detected in the majority of BP sera using a novel ELISA Sera from patients with active BP ($n = 116$), from normal volunteers ($n = 72$), and from patients with pemphigus vulgaris ($n = 28$) were tested against the BP180 recombinants, BP180-N and BP180-C, that were immobilized onto ELISA microtiter plates. These BP180 recombinants presumably possessed a conformation identical or similar to the native BP180 protein (**Fig 2**). Based on the maximization of the Youden index (sensitivity + specificity − 1), the cut-off point for the ELISA with BP180-N protein was set at 0.149 OD units, which corresponds to a Youden index of 0.792. Reactivity to BP180-C was considered positive when the OD of the sera exceeded 0.065 units (Youden index = 0.436). The diagnostic accuracy of each test, as determined by ROC curves, is represented by the area under the curve (AUC (95%-CI)), which was 0.925 (0.887–0.963) for the BP180-N and 0.796 (0.737–0.855) for the BP180-C ELISA (**Fig 3**). Using the selected cut-off values, 93 (80%) and 54 (47%) of 116 BP sera exhibited specific reactivity with BP180-N and BP180-C, respectively (**Fig 3**). One (specificity 99%) and three (specificity 97%) of the control

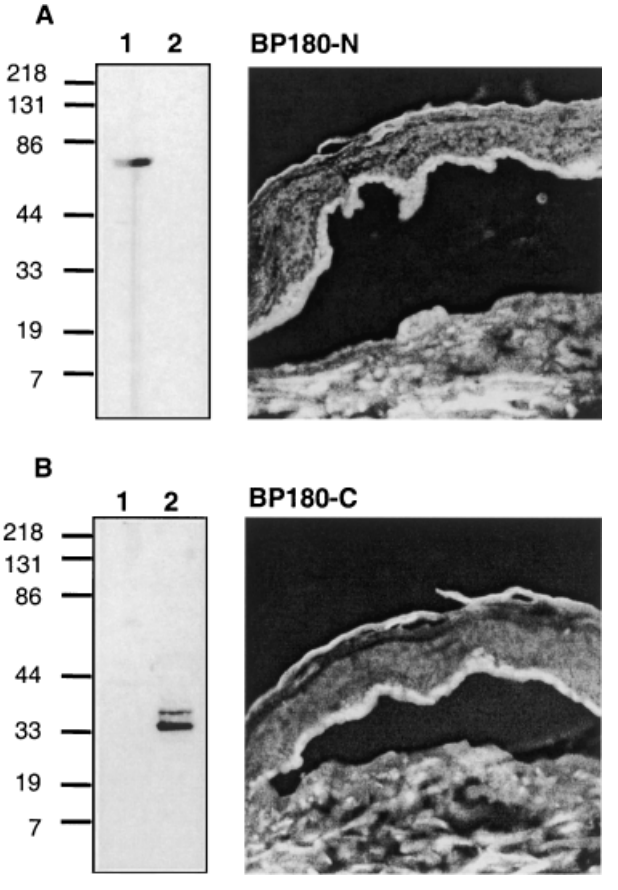

Figure 2. Rabbit sera raised against BP180-N and BP180-C bind to the epidermal site of saline-split human skin. Rabbits were immunized with BP180-N and BP180-C, respectively, and sera were collected 12 wk after immunizations. (*A*) Sera from rabbits raised against BP180-N (NH_2-terminus of the BP180 ectodomain) immunoblotted BP180-N protein and cross-reacted with the epidermal roof of saline-split human skin. (*B*) Accordingly, rabbit sera raised against BP180-C (COOH-terminus of the BP180 ectodomain) immunoblotted BP180-C protein and cross-reacted with the epidermal roof of saline-split human skin. *Lane 1*, BP180-N; *lane 2*, BP180-C. Numbers shown on the left refer to molecular weight marker.

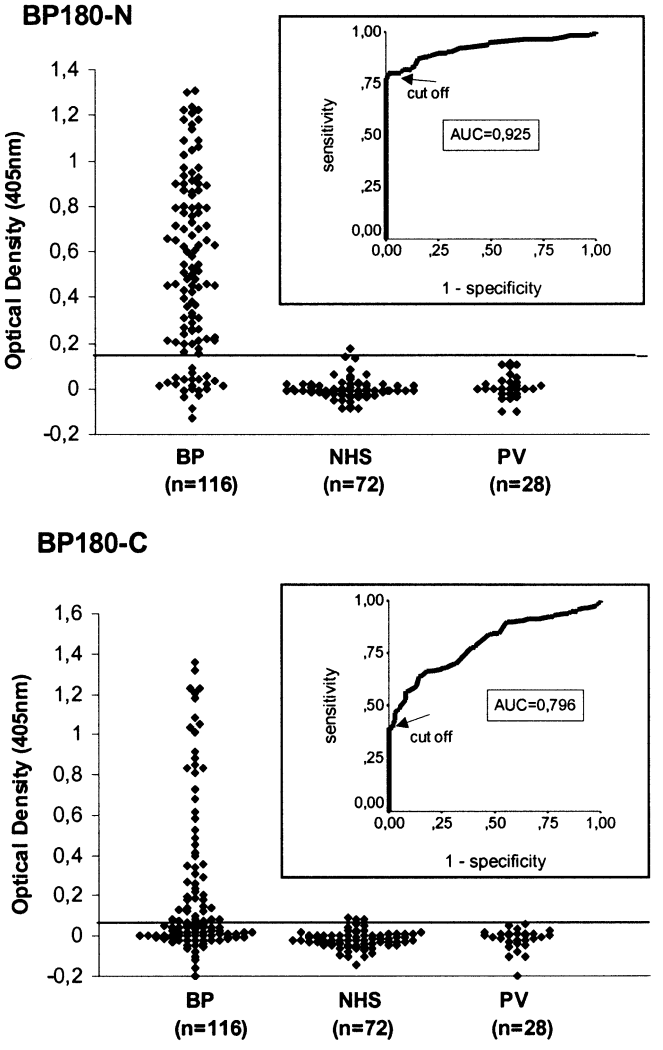

Figure 3. Detection of IgG against the NH_2-terminal and COOH-terminal regions of the BP180 ectodomain in BP sera. Detection of serum IgG against the NH_2-terminus (BP180-N, *top panel*) and the COOH-terminus (BP180-C, *bottom panel*) of the BP180 ectodomain shown as scatter plots and ROC curves (*insets*). Sera of patients with active BP ($n = 116$) and 100 control sera (NHS, normal human sera; PV, pemphigus vulgaris sera) were incubated with 0.5 μg of immobilized BP180-N and BP180-C proteins. IgG binding was detected with an AP-labeled antihuman IgG antibody. Each sample was run in duplicate and plotted dots represent the mean of the OD reading at 405 nm of a serum sample with BP180 recombinants normalized against IgG reactivity with recombinant GST/6xHis. The solid lines indicate the cut-off values as evaluated by maximization of the Youden index.

sera yielded marginal OD readings against BP180-N and BP180-C, respectively.

A total of 108 of 116 BP sera (93%) reacted with at least one of the recombinant BP180 proteins as summarized in **Table I**. One of the eight (7%) negative sera showed IgG reactivity with the entire ECD of BP180, as determined by ELISA with the BP180 recombinant BV13 (Haase *et al*, 1998). None of these eight sera reacted with the intracellular domain of BP180 as assessed by immunoblotting utilizing a recombinant form of BP180 (Perriard *et al*, 1999), whereas four other sera immunoblotted the COOH-terminal region of BP230 (Skaria *et al*, 2000) and one serum showed IgG reactivity to laminin 5. The remaining two sera were not further classified despite IgG reactivity with saline-separated human skin (**Fig 4**).

Table I. Sensitivity of IgG analysis of BP sera by the BP180 ELISA

BP180-N[a]	BP180-C[b]	BP sera[c]	%
+	−	54	46.5
+	+	39	33.5
−	+	15	13
−	−	8	7
Total		116	100

[a] NH_2-terminus (NC16A and Col15) of the ECD of BP180.
[b] COOH-terminus of the ECD of BP180.
[c] Total of 116 BP sera studied.

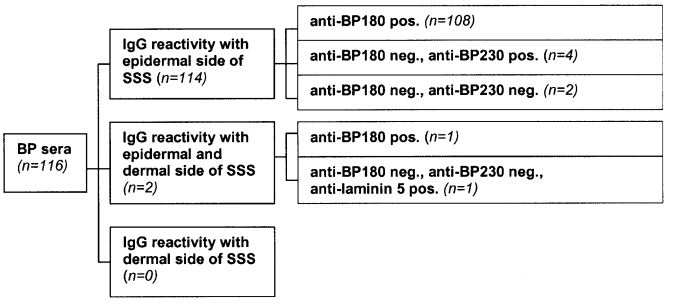

Figure 4. Serologic classification of the investigated BP sera. The sera of all 116 BP patients with clinically active disease were positive by indirect immunofluorescence on saline-split human skin. All the BP sera showed IgG reactivity with the epidermal side (and dermal side in two sera) of the saline-split human skin. Reactivity against the BP180 ectodomain (BP180-N, BP180-C, or BV13), the COOH-terminus of BP230, and laminin 5 was determined by ELISA with recombinant (BP180-N, BP180-C, BV13, BP230) and native (laminin 5) proteins, respectively.

To confirm the specificity of circulating antibody against the fusion proteins BP180-N and BP180-C, six BP sera reactive to these recombinants were examined by inhibition ELISA as described above. IgG reactivity of these sera to BP180-N, but not to BP180-C, was significantly reduced by pre-absorption with BP180-N and vice versa. Pre-absorption with GST/6xHis did not significantly alter the reactivity of these sera with BP180-N and BP180-C (data not shown).

IgG_1 and IgG_4 are the predominant Ig classes against the NH_2- and COOH-terminal regions of the ECD of BP180, whereas IgE and IgA antibodies are rarely detected Further characterization of the BP180-reactive BP sera was performed by Ig subclass analysis. Subclass specificity against the BP180 recombinants was determined in 93 BP sera reactive with BP180-N, 54 sera reactive with BP180-C, and in 100 control sera. IgG_1 reactivity to BP180-N was found in 79 of 93 (85%) and IgG_4 reactivity in 43 of 93 (46%) of the BP180-N-reactive BP sera (**Fig 5*A***). In 42 of 93 (45%) sera, antibodies of the IgG_1 class were detected exclusively, whereas six of 93 (6%) sera showed an isolated IgG_4 response to BP180-N. In contrast, the majority of BP sera reactive with the COOH-terminus (BP180-C) showed a dual IgG_1 and IgG_4 response: 41 of 54 (76%) and 37 of 54 (69%) BP sera contained IgG_1 and IgG_4 antibodies against BP180-C, respectively (**Fig 5*B***), and autoantibody reactivity to BP180-C was restricted to the IgG_1 class in 10 of 54 (19%) and to IgG_4 in seven of 54 (13%) BP patients. Two of 100 (2%) control sera reached OD values slightly above the cut-off points, resulting in a specificity of the ELISA of 98% (**Table II**). The 116 BP and 100 control sera were also analyzed with regard to IgE and IgA reactivity against the NH_2- and COOH-terminus of the ECD

of BP180. IgE antibodies against BP180-N were detected in six of 116 (5%) sera and IgA against BP180-N in four of 116 (3%) BP sera (**Fig 5**). IgE and IgA reactivity to BP180-C was detected in three of 116 (3%) and two of 116 (2%) BP sera, respectively. None of the control sera exceeded the cut-off values for IgE and IgA reactivity (**Table II**). Interestingly, one BP serum contained IgA antibody only against BP180-N in addition to IgG antibody against BP180-C, whereas another patient showed exclusive IgE autoreactivity against BP180-C in addition to IgG reactivity against BP180-N. None of the BP patients showed a dual IgE and IgA response. Seven of the nine (78%) sera containing IgE antibody against either BP180-N or BP180-C were from BP patients with extensive skin involvement.

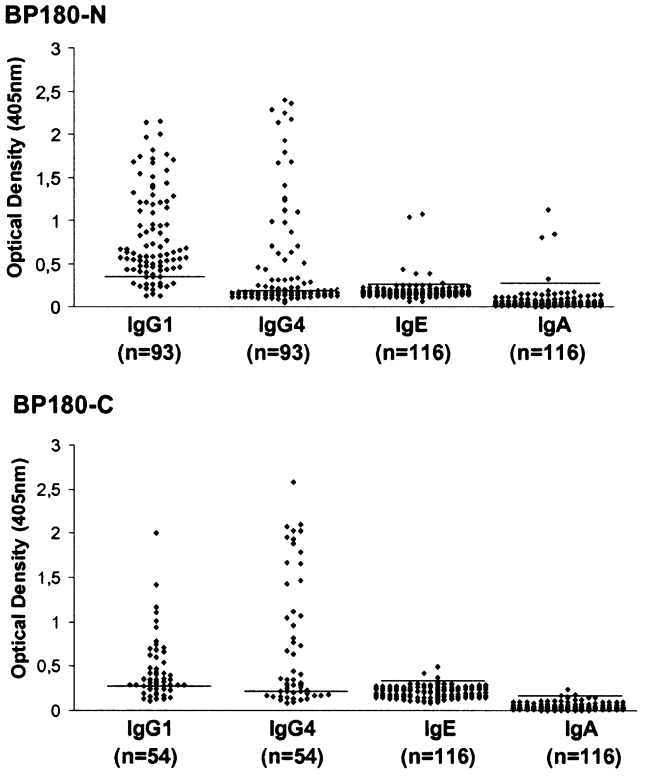

Figure 5. Distribution of IgG_1, IgG_4, IgE, and IgA reactivity of BP sera against the NH_2- and COOH-terminal regions of the BP180 ectodomain. BP sera and control sera (data not shown) were incubated with immobilized recombinant BP180-N (*top panel*) and BP180-C (*bottom panel*) and probed with one of a series of AP- or peroxidase-conjugated antibody specific for IgG_1, IgG_4, IgE, and IgA (as indicated on the horizontal axes). Each sample was run in duplicate and results are expressed as mean of OD reading at 405 nm. Solid lines indicate the cut-off values as evaluated by maximization of the Youden index (see **Table II**).

An antibody response against both the NH_2- and COOH-terminal domains of the ECD of BP180 occurs more frequently in patients with mucous membrane involvement

IgG autoreactivity to distinct regions of BP180 may be related to a particular clinical phenotype of BP. As 114 of the investigated 122 BP patients were very well characterized with regard to clinical phenotype, the sera from these 114 patients were divided into four groups according to their reactivity against (i) the NH_2-terminus (BP180-N), (ii) the COOH-terminus (BP180-C), (iii) both the NH_2- and COOH-terminus of the ECD of BP180, or (iv) none of the BP180 recombinants. Distribution of IgG reactivity to the BP180 recombinants was then compared with regard to (i) disease severity, (ii) disease duration, and (iii) mucosal involvement, using Fisher's exact probability test (**Table III**). Although the severity and duration of BP did not appear to be related to reactivity against distinct regions of the ECD of BP180, a relationship could be observed between the presence of mucosal lesions and the profile of the autoantibody response ($p = 0.021$). The majority of patients without mucosal involvement (54 of 98; 55.1%) had circulating antibodies reactive only with the NH_2-terminus, whereas more patients with mucosal involvement (nine of 16; 56.2%) had IgG autoantibodies against both the NH_2- and COOH-terminus of the ECD of BP180. In contrast, there was no association between autoantibodies against the COOH-terminus only and the presence of mucosal lesions (**Table III**).

Table II. Cut-off values for Ig subtype analysis by the BP180 ELISA

BP180 recombinant	Ig subclass	Cut-off (OD_{405})	Youden[c]
BP180-N[a]	IgG1	0.340	0.829
	IgG4	0.188	0.442
	IgE	0.262	0.052
	IgA	0.268	0.034
BP180-C[b]	IgG1	0.271	0.739
	IgG4	0.222	0.665
	IgE	0.342	0.026
	IgA	0.173	0.017

[a] NH_2-terminus of the ECD of BP180.
[b] COOH-terminus of the ECD of BP180.
[c] Youden index (sensitivity + specificity − 1).

Table III. Specificity of autoantibodies against BP180 and clinical status[a]

	Extent of disease ($n = 114$)			Disease duration ($n = 114$)			Mucosal involvement ($n = 114$)	
BP180-N[b]/C[c]	Generalized	Localized	Remittent	Acute	Chronic	Remittent	+	−
−/−	4 (5.3)	2 (6.1)	0 (0)	2 (2.6)	4 (12.9)	0 (0)	2 (12.5)	4 (4.1)
+/−	36 (48.0)	18 (54.5)	5 (83.3)	38 (49.4)	16 (51.6)	5 (83.3)	5 (31.3)	54 (55.1)
−/+	9 (12.0)	4 (12.1)	0 (0)	9 (11.7)	4 (12.9)	0 (0)	0 (0)	13 (13.3)
+/+	26 (34.7)	9 (27.3)	1 (16.7)	28 (36.4)	7 (22.6)	1 (16.7)	9 (56.2)	27 (27.6)
Total	75 (100%)	33 (100%)	6 (100%)	77 (100%)	31 (100%)	6 (100%)	16 (100%)	98 (100%)
	$p = 0.87$			$p = 0.27$			$p = 0.021$	

[a] p-values were determined by Fisher's exact probability test.
[b] NH_2-terminal domain of the ECD of BP180.
[c] COOH-terminal domain of the ECD of BP180.

IgG levels against the NH_2-terminus but not against the COOH-terminus of the ECD of BP180 reflect severity of BP Severity of BP was also compared with the OD values measured by ELISA with the BP180 recombinants, using an analysis of covariance. As IgG reactivity to either BP180-N or BP180-C was not detectable in 19 and 66 BP sera, respectively, we could not establish a significant relationship between antibody levels and disease extent when all 114 BP sera were included in the analysis. If only those sera that were reactive to the NH_2-terminus of the ECD of BP180 (BP180-N, $n=95$) were analyzed, however, IgG, IgG_1, and IgG_4 antibody levels were significantly higher in the sera of patients with extensive skin involvement ($n=62$) than in the sera of patients with moderate skin involvement ($n=27$) or remittent disease ($n=6$) (**Fig 6**, **Table IV**). Among the sera reactive to the COOH-terminus of BP180 (BP180-C, $n=48$), 35 were obtained from patients with extensive blistering whereas 13 sera were from patients with a more localized skin phenotype. Comparison of the two groups showed only marginal differences regarding the IgG response to BP180-C ($p=0.069$). A relationship between disease duration (acute *versus* chronic disease) and antibody levels to BP180-N or BP180-C by ELISA was not observed.

A dual IgG_1 and IgG_4 response to the NH_2-terminus of the ECD of BP180 is detected predominantly in patients with severe skin involvement Comparison of the IgG subclass reactivity with the clinical phenotype of the investigated BP patients did not reveal any relationship between mucosal involvement and IgG subclass reactivity against the NH_2-terminus of the ECD of BP180. In contrast, a statistically significant relationship was observed between the severity of BP and IgG isotype reactivity to BP180-N (Fisher's exact probability test, $p=0.009$; **Table V**). A dual IgG_1 and IgG_4 response to BP180-N was detected more frequently in BP patients with extensive skin involvement than in patients with localized or remittent BP (**Table V**). With regard to a potential relationship between disease duration and isotype specificity of anti-BP180-N antibody ($p=0.030$), IgG_1 reactivity decreased during the course of disease, whereas IgG_4 reactivity showed a tendency to increase with a longer disease duration. Whereas patients with acute onset BP showed predominantly IgG_1 antibody or a dual IgG_1 and IgG_4 response to the NH_2-terminus of the ECD of BP180, an exclusive IgG_4 response was detected predominantly in patients with remittent disease. Comparison of subclass specificity of autoantibodies targeting the COOH-terminus of BP180 (BP180-C) with phenotype and duration of BP did not show any relationship (**Table VI**).

DISCUSSION

The goal of this study was to analyze the relationship between subclasses and specificity of IgG autoantigenes directed against the ECD of BP180, the putative major autoantibody of BP, and the clinical phenotype. We developed a novel ELISA utilizing two eukaryotic proteins of the ECD of BP180 that consisted of either its NH_2-terminal region containing the NC16A and Col15 subdomains (residues 490–811) or its COOH-terminal domain (residues 1351–1497).

The results demonstrate that the novel ELISA is highly sensitive, as 93% of the 116 sera of patients with active BP examined

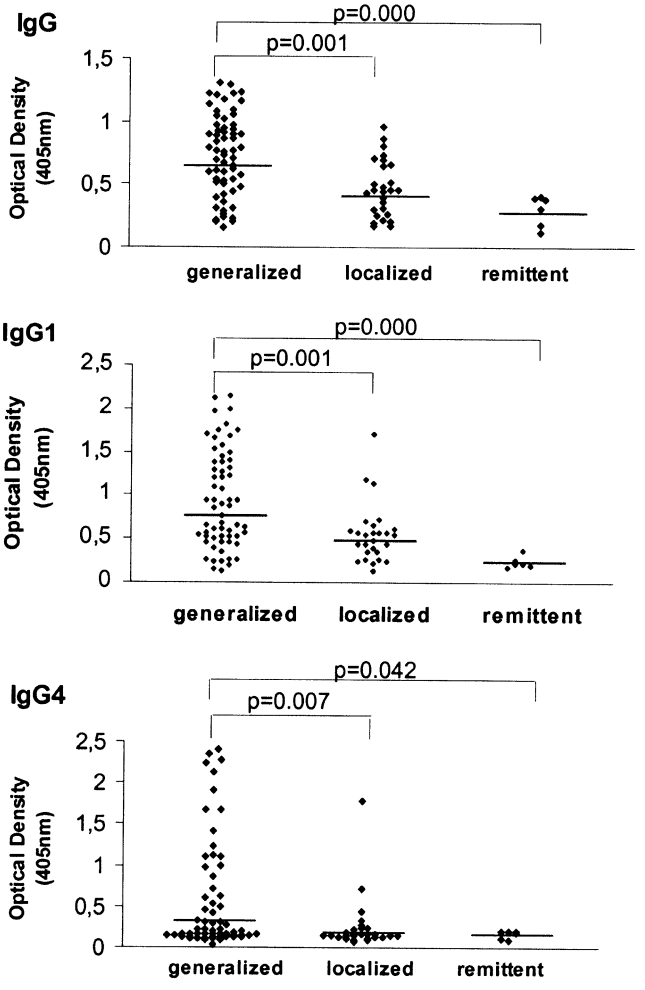

Figure 6. The titers of IgG against the NH_2-terminus of the BP180 ectodomain are related to the clinical activity of BP. Ninety-five sera from BP patients reactive to the NH_2-terminus of the ECD of BP180 (BP180-N) with generalized ($n=62$), localized ($n=27$), and remittent ($n=6$) disease were compared with regard to IgG, IgG_1, and IgG_4 reactivity against BP180-N using a multivariate analysis of covariance. Sera obtained from BP patients with extensive BP showed significantly higher IgG and IgG_1 levels than patients with localized or remittent disease. The difference was not as significant when sera were analyzed for IgG_4 reactivity. *Bars* indicate the geometric mean (see **Table IV**).

Table IV. Comparison of IgG levels[a] against the NH_2-terminal regions of the BP180 ectodomain (BP180-N) and clinical activity of BP

	Disease activity			p[b]	
Ig class	Generalized, $n=62$	Localized, $n=27$	Remittent, $n=6$	Generalized *versus* localized	Generalized *versus* remittent
IgG	0.649 (0.605–0.696)	0.413 (0.372–0.458)	0.272 (0.221–0.335)	0.001	< 0.0001
IgG_1	0.768 (0.702–0.840)	0.475 (0.425–0.529)	0.235 (0.211–0.261)	0.001	<0.0001
IgG_4	0.322 (0.281–0.368)	0.186 (0.163–0.211)	0.159 (0.137–0.185)	0.007	0.042

[a]Geometric mean ± geometric standard error.
[b]Significance of pairwise comparisons within analysis of covariance models with simultaneous adjustment for age and gender.

Table V. Comparison of IgG isotype reactivity against the NH_2-terminal region of the BP180 ectodomain (BP180-N) and the clinical status[a]

	Extent of disease ($n = 95$)			Disease duration ($n = 95$)			Mucosal involvement ($n = 95$)	
IgG_1/IgG_4 reactivity	Generalized	Localized	Remittent	Acute	Chronic	Remittent	+	−
−/−	3 (4.8)	5 (18.5)	1 (16.6)	5 (7.6)	3 (13.0)	1 (16.7)	0 (0)	9 (11.1)
+/−	26 (41.9)	14 (51.9)	1 (16.6)	32 (48.5)	8 (34.8)	1 (16.7)	3 (21.4)	38 (46.9)
−/+	5 (8.1)	1 (3.7)	3 (50.0)	3 (4.5)	3 (13.0)	3 (50.0)	3 (21.4)	6 (7.4)
+/+	28 (45.2)	7 (25.9)	1 (16.6)	26 (39.4)	9 (39.2)	1 (16.7)	8 (57.2)	28 (34.6)
Total	62 (100%)	27 (100%)	6 (100%)	66 (100%)	23 (100%)	6 (100%)	14 (100%)	81 (100%)
	$p = 0.009$			$p = 0.030$			$p = 0.063$	

[a]p-values were determined by Fisher's exact probability test.

Table VI. Comparison of IgG isotype reactivity against the COOH-terminus of the BP180 ectodomain (BP180-C) and the clinical status[a]

	Extent of disease ($n = 48$)		Disease duration ($n = 48$)		Mucosal involvement ($n = 48$)	
IgG_1/IgG_4 reactivity	Generalized	Localized	Acute	Chronic	+	−
−/−	6 (17.1)	2 (15.4)	6 (16.2)	2 (18.2)	3 (33.3)	5 (12.8)
+/−	5 (14.3)	2 (15.4)	6 (16.2)	1 (9.1)	0 (0)	7 (17.9)
−/+	2 (5.7)	0 (0)	1 (2.7)	1 (9.1)	1 (11.1)	1 (2.6)
+/+	22 (62.9)	9 (69.2)	24 (64.9)	7 (63.6)	5 (55.6)	26 (66.7)
Total	35 (100%)	13 (100%)	37 (100%)	11 (100%)	9 (100%)	49 (100%)
	$p = 1.00$		$p = 0.76$		$p = 0.14$	

[a]p-values were determined by Fisher's exact probability test.

showed IgG reactivity with one of the two recombinants of the ECD of BP180. Specifically, 80% and 47% of the investigated BP sera contained IgG autoantibodies binding to the NH_2-terminal and the COOH-terminal region of the ECD of BP180, respectively. The NC16A and Col15 domains have been identified as harboring major epitopes of BP180 (Giudice *et al*, 1993; Matsumura *et al*, 1996; Zillikens *et al*, 1997b; Schumann *et al*, 2000) whereas the COOH-terminus is a major autoantibody binding site in a subgroup of patients with bullous and cicatricial pemphigoid (Balding *et al*, 1996; Perriard *et al*, 1999; Schumann *et al*, 1999). Previously reported ELISA systems for the detection of autoantibodies specific for defined regions of the ECD of BP180 utilized prokaryotic BP180 recombinants only (Giudice *et al*, 1994; Zillikens *et al*, 1997a; Nakatani *et al*, 1998). Zillikens *et al* detected IgG reactivity against the NC16A domain of BP180 in 94% of 50 BP sera (Zillikens *et al*, 1997a). Another ELISA utilizing bacterial recombinant proteins demonstrated the presence of IgG autoantibodies against the NC16A subdomain and the COOH-extremity of BP180 in 96% and 38% of the BP sera, respectively (Nakatani *et al*, 1998). The higher frequency of IgG reactivity against the COOH-terminal domain of BP180 observed with our ELISA may depend on several factors. The BP180 recombinant (BP180-C) of this study was expressed in the eukaryotic baculovirus system that usually provides similar or identical post-translational modifications to those observed with native proteins (Kidd and Emery, 1993). Other possible explanations to be considered include differences in the selection and number of the investigated BP sera, differential sensitivities and specificities of the two ELISA systems due to different cut-off levels, etc. A direct comparison of the two ELISA systems with identical BP sera would address some of these points and the question whether BP sera recognize conformational epitopes of the C-terminal end of the BP180.

Based on thorough statistical analysis, our findings demonstrate that antibody levels against the NH_2-terminal region of the BP180 ECD relate to the severity of BP, whereas the titers of IgG autoantibodies reactive against its COOH-terminal region do not. Consistent with previous passive transfer studies with rabbit sera reactive with the murine homolog of the NC16A domain (Liu *et al*, 1993), our findings provide further support to the notion that in first line antibodies against the NH_2-terminal region of the BP180 ECD are pathogenic. Furthermore, they are in line with two recent studies suggesting a possible relationship between disease activity and autoantibody levels against the NC16A subdomain (Döpp *et al*, 2000; Schmidt *et al*, 2000).

The pathogenic relevance of antibodies against the COOH-terminus of BP180 has not yet been studied in detail. As BP180 extends from the cytoplasm of the basal keratinocyte to the lamina densa (Bedane *et al*, 1997; Masunaga *et al*, 1997; Nakatani *et al*, 1998), it has been speculated that autoantibodies against this region might be responsible for the scarring phenotype observed in cicatricial pemphigoid patients. The COOH-terminal end of BP180 may – via its binding to distinct laminin isoforms such as laminin-5, the autoantigene of a subgroup of cicatricial pemphigoid (Egan and Yancey 2000) – be critically involved in the integrity of the dermo-epidermal junction. Balding *et al* (1996) detected reactivity against the COOH-terminus of BP180 in 61% of 23 cicatricial pemphigoid sera by immunoblot analysis. None of our BP patients that exclusively reacted with the COOH-terminus of BP180 (15 of 116; 13%) had a scarring phenotype of either skin or mucous membranes, however, which is in line with the finding of a recent independent ELISA study (Nakatani *et al*, 1998). It is noteworthy that the majority (56%) of our BP patients with mucosal involvement showed IgG reactivity against both the NH_2- and COOH-terminal regions of the ECD of BP180, whereas more BP patients without mucosal involvement showed an exclusive IgG reactivity against the NH_2-terminal portion (**Table III**). Autoantibodies against both the NH_2- and COOH-terminal region of the ECD of BP180 thus may act synergistically in the development of mucosal lesions.

The findings of this study clearly demonstrate that autoantibodies against the NH_2-terminus of BP180 are predominantly of the IgG_1 class and that their titers relate to the clinical activity of BP. Previous studies reported a preferential detection of IgG_1 and

IgG_4 autoantibodies in BP, whereas BP180-specific IgG_2 and IgG_3 antibodies were detected in a minority of BP sera (Bernard *et al*, 1990; Döpp *et al*, 2000; Laffitte *et al*, 2001). As the various IgG subclasses have distinct functional properties and their secretion is differentially regulated, we have also assessed the relative isotype distribution among the major IgG subclasses. The results demonstrate that 79 of 93 (85%) and 43 of 93 (46%) of the sera contained IgG_1 and IgG_4 antibodies against the NH_2-terminal region, in contrast to 41 of 54 (76%) and 37 of 54 (69%) of sera containing IgG_1 and IgG_4 against the COOH-terminus of the ECD of BP180, respectively. Our results indicate that a dual IgG_1 and IgG_4 response to the NH_2-terminus of the ECD of BP180 is associated with a more severe skin involvement in BP (**Table V**). IgG_1 against the NH_2-terminal portion of the BP180 ECD was predominantly detected in sera of BP patients with acute onset BP, whereas IgG_4 autoantibodies predominated in sera of BP patients in remission (**Table V**). In contrast, IgG_1 and IgG_4 reactivity to the COOH-terminus of BP180 was independent of the clinical activity of BP. Previous studies aiming at the identification of IgG subclass reactivity to BP180 showed controversial results. Whereas Bernard *et al* (1990) found a predominance of BP180-reactive IgG_1 (90% *vs* 80% IgG_4) in 10 BP sera by Western blot of epidermal extracts, Döpp *et al* (2000) detected IgG_4 and IgG_1 reactivity with the NC16A subdomain in 66% and 50% of 18 BP sera, respectively. In line with our findings, Laffitte *et al* (2001) recently reported a predominance of IgG_1 autoantibodies, as 100% and 77%, respectively, of 27 BP180-reactive BP sera had IgG_1 and IgG_4 autoantibodies to the ECD of BP180 by immunoblot. The concept that the complement-fixing IgG_1 as the predominant IgG isotype plays a key role in the initiation of BP is supported by the clinical finding that, in addition to IgG, C3 is frequently detected at the dermo-epidermal junction in the perilesional skin of patients with BP (Jordon *et al*, 1967). Furthermore, in the murine passive transfer model of BP, subepidermal blister formation has been found to depend on a functional complement system (Liu *et al*, 1995). A recent study in our laboratory has identified in BP patients the presence of autoreactive Th1 and Th2 cells, which presumably regulate the secretion of anti-BP180 autoantibodies of the IgG_1 and IgG_4 subclasses (Büdinger *et al*, 1998).

IgE reactivity to the NH_2- and COOH-terminal regions of the ECD of BP180 was detected in 8% of the BP sera and was clearly associated with a more severe phenotype of BP as 78% of the IgE-reactive BP patients had a generalized skin involvement. This is in line with a previous report by Delaporte *et al* who detected IgE antibodies against BP230 in patients with severe BP (Delaporte *et al*, 1996). A recent ELISA study with a bacterial NC16A protein detected BP180-reactive IgE in 55% (10 of 18) of selected BP sera (Döpp *et al*, 2000). In addition, this ELISA study demonstrates that only 5% of a large cohort of 116 BP sera contained IgA reactive with these BP180 recombinants; none of these patients presented with a particular clinical phenotype. Previous ELISA studies did not detect circulating IgA against bacterial proteins representing the NC16A and/or COOH-terminal domains of BP180 in BP sera (Giudice *et al*, 1994; Murakami *et al*, 1998), whereas in one study IgA reactivity with a bacterial recombinant form of NC16A was detected in 65% of 40 BP sera (Kromminga *et al*, 2000). This variable prevalence of IgE and IgA autoantibodies in BP sera may largely depend on the applied BP180 recombinants, the selected BP patients, and the utilized test systems (i.e., immunoblot, ELISA).

In summary, our novel ELISA using eukaryotic recombinants of the NH_2- and COOH-terminal regions of the ECD of BP180 is highly useful for rapid and sensitive analysis of the autoantibody profile, which reflects distinct clinical phenotypes of BP. By this ELISA, we demonstrated that distinct clinical phenotypes of BP are associated with differential autoantibody responses to BP180: (i) IgG_1 antibodies against the NH_2-terminal region of the ECD of BP180 are predominantly detected in sera of BP patients with acute BP, whereas IgG_4 autoantibodies predominate in sera of BP patients in remission; (ii) a dual IgG_1 and IgG_4 response against the NH_2-terminal region of the ECD of BP180 is associated with a more severe phenotype of BP; (iii) IgG autoantibodies from BP patients with mucosal involvement appear to target more frequently both the NH_2- and COOH-terminal regions of the ECD of BP180; and (iv) IgG autoantibody titers against the NH_2-terminal region but not against the COOH-terminus of the ECD of BP180 reflect the extent of skin involvement, supporting the hypothesis that autoantibodies against the NC16A subdomain of BP180 are critical in the pathogenesis of BP.

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