

Mapping the Binding Sites of Anti-BP180 Immunoglobulin E Autoantibodies in Bullous Pemphigoid

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Bullous pemphigoid (BP) is a subepidermal blistering disease characterized by autoantibodies against the hemidesmosomal protein BP180 (BPAg2, type XVII collagen). NC16A, a non-collagenous stretch of the BP180 ectodomain, is the primary target of pathogenic immunoglobulin (Ig)G autoantibodies and IgE class autoantibodies. This study further characterized the IgE-reactive regions of BP180. Of the ten sera from untreated BP patients, eight contained IgE reactive with the entire BP180 ectodomain. The IgE in four of these eight sera reacted with NC16A, whereas in the remaining four sera IgE immunoreactivity was restricted to sites downstream of NC16A. In contrast, IgG reactivity to NC16A was detected in nine of the ten BP sera, and in the remaining serum, IgG, as well as IgE, reacted exclusively with non-NC16A sites on the BP180 ectodomain. Fine mapping of the antigenic sites within NC16A revealed very similar reactivity patterns for IgE and IgG, with NC16A subregion-2 being the major site recognized by both isotypes. Eight of the untreated BP patients were tested for histamine release from their basophils in response to NC16A. Antigen-specific histamine release was observed only in those patients with detectable circulating IgE directed against NC16A (three of eight). Future studies will investigate the pathogenic relevance of anti-BP180 IgE.

Key words: autoimmunity/bullous pemphigoid/collagen XVII/IgE
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Immunoglobulin (Ig)E has classically been thought to be important in allergic diseases and in response to parasitic infections. Recently, however, a role for IgE and mast cells has been proposed in the regulation of immune responses and the development of autoimmune disease (reviewed in Frossi *et al*, 2004). The concept of IgE as an autoantibody is a relatively new one. Although IgE class anti-thyroid autoantibodies have been described in patients with autoimmune thyroid disease, their pathogenic relevance has yet to be studied (Guo *et al*, 1997; Sato *et al*, 1999). Elevated levels of IgE have also been demonstrated in anti-SSA-positive women who experience fetal loss, but again the specificity of the IgE and any pathogenic role it plays have not been determined (Sekigawa *et al*, 2004). In contrast, there is a growing body of data regarding the specificity and potential pathogenic role of IgE class autoantibodies in bullous pemphigoid (BP) (Provost and Tomasi, 1974; Delaporte *et al*, 1996; Christophoridis *et al*, 2000; Dopp *et al*, 2000; Chen *et al*, 2002; Dimson *et al*, 2003).

BP is an acquired blistering skin disease that is characterized by the development of autoantibodies directed against two hemidesmosomal proteins: BP180 (BPAg2, type XVII collagen) and BP230 (BPAg1) (Mutasim *et al*, 1985; Labib *et al*, 1986; Stanley *et al*, 1988; Diaz *et al*, 1990). BP180, a transmembrane protein of the collagen family (Giudice *et al*, 1993; reviewed by Van den Bergh and Giu-

dice, 2003), has been shown to be the primary target of pathogenic IgG class BP autoantibodies (Liu *et al*, 1993, 1995).

BP is unusual among the autoimmune bullous diseases in that circulating IgE levels are elevated in these patients (Arbesman *et al*, 1974; Bowszyc-Dmochowska *et al*, 2000; Dimson *et al*, 2003). Subepidermal vesiculation in BP is often preceded by the development of urticarial plaques, and it has been speculated that IgE might play a role in this early phase of the disease. Recently we and others have demonstrated that in addition to the IgG autoantibodies, BP patients also produce anti-BP180 autoantibodies of the IgE class (Christophoridis *et al*, 2000; Dopp *et al*, 2000; Merk *et al*, 2000; Dimson *et al*, 2003). A functional role for IgE autoantibodies is suggested by our recent finding that exposure to recombinant NC16A can induce degranulation of circulating basophils from BP patients with active disease (Dimson *et al*, 2003).

The major disease-associated IgG epitopes of BP180 have been mapped to the NC16A domain, a membrane-proximal non-collagenous stretch of its ectodomain (Zillikens *et al*, 1997). Previous studies to map the antigenic regions targeted by the IgE class anti-BP180 autoantibodies have however yielded inconsistent results. Several reports suggested that the IgE autoantibodies primarily target BP230 or the intracellular domain of BP180, both sites of which would be less likely to be directly involved in disease pathogenesis (Delaporte *et al*, 1996; Ghohestani *et al*, 1998), whereas others reported that BP IgE targets the

Abbreviations: BP, bullous pemphigoid; Ig, immunoglobulin

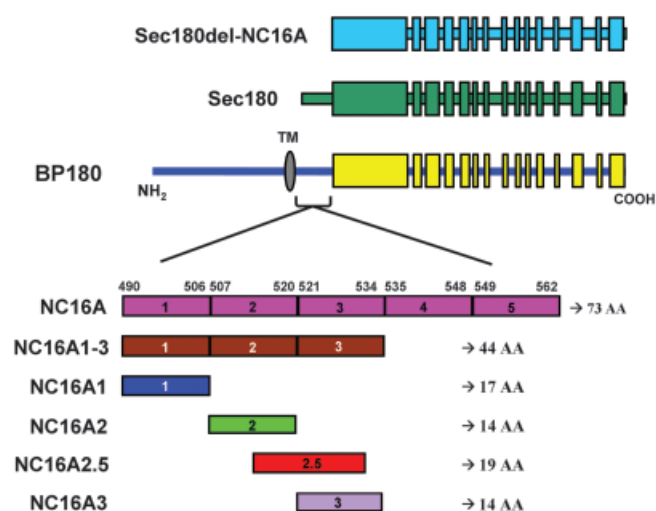


Figure 1
Schematic representation of recombinant forms of bullous pemphigoid (BP)180 used in this study. Sec180 contains the entire BP180 ectodomain, whereas sec180ΔNC16A is the extracellular domain with the NC16A segment deleted. The 73 amino acid non-collagenous domain, NC16A has been divided into five subregions as indicated. NC16A1, NC16A2, and NC16A3 have no overlap. NC16A2.5 overlaps NC16A2 and NC16A3. NC16A1-3 encompasses the contiguous subregions 1-3, and was constructed in order to detect epitopes that might be located in the fusion protein junctions.

NC16A region of BP180 (Christophoridis *et al*, 2000; Dopp *et al*, 2000; Dimson *et al*, 2003). In order to further characterize the IgE autoantibodies that are present in BP patients we mapped the binding sites of IgE autoantibodies utilizing recombinant domains of BP180 (Figure 1). The IgE and IgG binding sites were compared and the relationship between NC16A immunoblot reactivity and NC16A-induced histamine release was determined.

Results

IgE reactivity of BP patient's sera with the extracellular domain of BP180 correlates with IgG reactivity In our preliminary study of 30 patients, 27 had IgE directed against the extracellular domain of BP180 and 50% (15 of 30) had IgE against NC16A (data not shown). Ten untreated BP patients were further characterized in this study. Nine of the ten patients had IgE that reacted with at least one region of the extracellular domain of BP180. All ten had detectable anti-BP180 IgG. Eight of the ten patients included in this study had IgE reactivity with the entire extracellular domain of BP180, expressed as sec180. These same eight also had IgG reactivity to sec180. Both patients with no detectable IgG sec180 reactivity (BP-La, BP-Pl) did show IgG reactivity to NC16A as well as smaller segments of NC16A. IgE reactivity with these smaller subdomains was seen in one of these two patients (BP-Pl). It should be noted that the immunoblot assays for detecting immunoreactivity to NC16A and its fragments are more sensitive than those for detecting reactivity to sec180 or sec180ΔNC16A, because of the difference in the amount of antigen that can be loaded on the gel. These sensitivity differences are the likely explanation for the observation that some sera show immunoreactivity to NC16A but not to sec180 (which con-

Table I. Patients' immunoblot reactivity with regions of the extracellular domain of BP180 (N = 10)

Region	IgG	IgE
BP180	10	9
Sec180	8	8
Sec180ΔNC16A	5	4
NC16A	9	5
NC16A1	4	3
NC16A2	8	5
NC16A2.5	5	3
NC16A3	2	2
NC16A1-3	8	5

BP, bullous pemphigoid; Ig, immunoglobulin.

tains NC16A). A summary of the IgE and IgG reactivity with the extracellular regions of BP180 is shown in Table I.

Five of the patients had IgE reactivity with NC16A. In contrast, nine of ten had IgG reactivity with NC16A. Of the five patients that did not have IgE reactive against NC16A, four of them showed IgE reactivity with sec180ΔNC16A, indicating reactivity outside of NC16A. One patient with IgE against NC16A also had IgE directed against sec180ΔNC16A. One had no detectable IgE reactivity with any segment of the BP180 ectodomain. IgG reactivity outside of NC16A (sec180ΔNC16A) was seen in five of the patients.

IgE reactivity within the NC16A domain is predominantly in the NC16A2 subdomain The subdomain recognized most frequently by IgE was NC16A2 (five of ten). Eight of the ten patients had IgG reactivity with the NC16A2 subdomain. These same patients also recognized NC16A1-3 with IgE autoantibodies. Three patients had IgE autoantibodies directed against the NC16A1 domain, three with NC16A2.5 that spans the junction between NC16A2 and NC16A3, and two with NC16A3. A representative blot from a BP patient (BP-An) is shown in Fig 2. The reactivity of all ten patients with the larger constructs and the subdomains of BP180 are summarized in Fig 3.

Histamine release by basophils in response to NC16A corresponds with IgE immunoreactivity Basophils from eight of the ten patients were tested in the histamine release assay. All samples showed spontaneous release of less than 5% of their total histamine content. Of the eight, three patients had IgE immunoreactivity with the NC16A domain of BP180 and five did not. The results of the histamine assay correlated with the IgE immunoreactivity. The three patients who had immunoreactivity to NC16A by immunoblot also released histamine from their basophils in response to this region. In contrast, the five patients who did not have IgE reactivity with NC16A did not release histamine above baseline levels in response to this region (Table II). No control patient showed release above baseline (not shown).

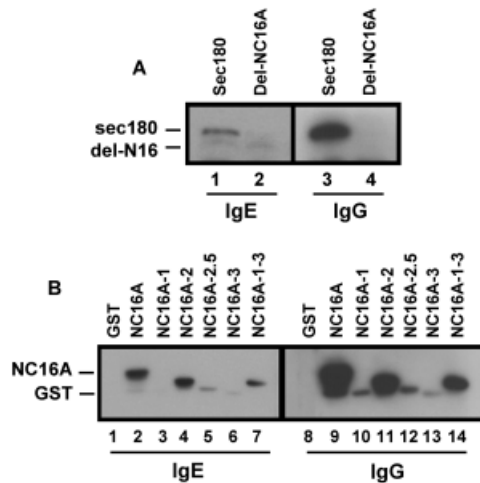


Figure 2
Mapping of bullous pemphigoid (BP) autoantibody-reactive sites: Representative western blot. The serum of patient BP-An was diluted 1:25 for immunoglobulin (Ig)E detection and 1:50 for IgG detection. (A) The patient's IgE and IgG reactivity with the entire extracellular domain of BP180 (sec180) and the portion of the extracellular domain excluding NC16A (Del-NC16A) are shown. As seen, this patient exhibited both IgE and IgG reactivity with sec180 but little or no reactivity with Del-NC16A, indicating that the autoantibody reactivity is largely restricted to the NC16A domain. (B) Reactivity of the patient's serum with smaller segments of NC16A are shown. Lanes 2, 4, and 7 show strong IgE reactivity with NC16A, NC16A2, and NC16A1-3, respectively. These same recombinant proteins were ones most strongly recognized by the patient's IgG (lanes 9, 11, and 14). These results indicate that, for patient BP-An, both IgE and IgG anti-BP180 autoantibodies are predominantly directed against subregion 2 of the NC16A domain.

Discussion

IgE class autoantibodies associated with BP and directed specifically against BP180 have been reported by several authors (Christophoridis *et al*, 2000; Dopp *et al*, 2000; Merk and Hertl, 2000; Dimson *et al*, 2003). In this study, nine of ten untreated BP patients were shown to have IgE class autoantibodies against the extracellular domain of BP180. IgE from five of these ten patients recognized the longest non-collagenous stretch of the BP180 ectodomain, NC16A. Multiple IgE-reactive sites were uncovered within NC16A, but only one—subregion-2—was targeted by IgE from all five of these patients. An additional 20 BP patients' sera were partially characterized as part of this study, and in all, 15 of the 30 BP sera (50%) had IgE reactivity to NC16A.

It is well documented that NC16A is an immunodominant region for IgG autoantibodies in BP (Giudice *et al*, 1993; Zillikens *et al*, 1997). Consistent with this, nine of ten patients in our study exhibited anti-NC16A IgG immunoreactivity. It has previously been reported that NC16A subregion-2 is an immunodominant site for BP IgG (Zillikens *et al*, 1997), and our present results provide further support for this conclusion. We further showed that for six of the ten patients in our study, the fine specificity profiles of the IgE and IgG autoantibodies were quite similar. It should be noted that with our current methodology, IgG and IgE are likely to be competing for binding sites on the recombinant peptides. It is possible the IgG is outcompeting the IgE for some sites on BP180 and this may account for the obser-

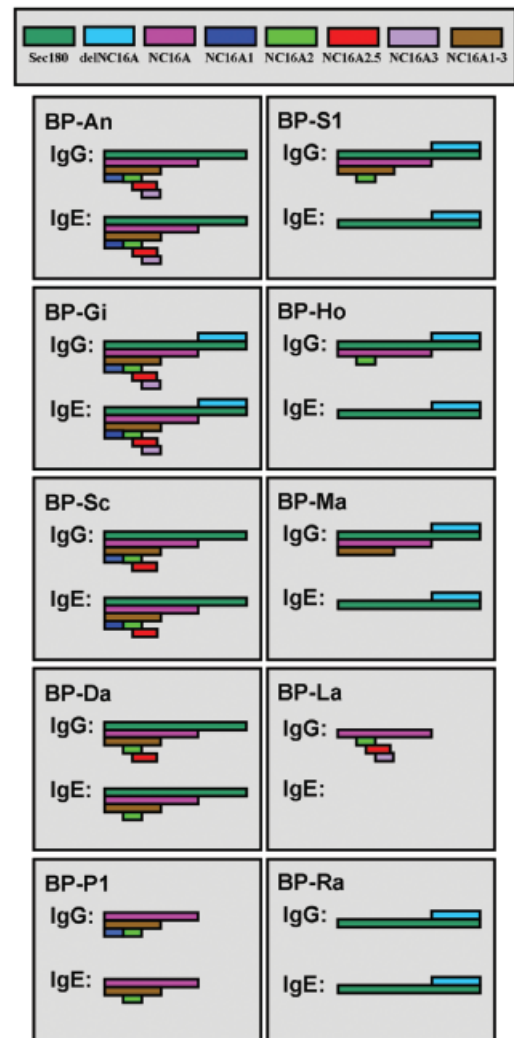


Figure 3
Schematic representation of epitope mapping results. Recombinant forms of bullous pemphigoid (BP)180 were assayed by western blotting for reactivity with immunoglobulin (Ig)G and IgE in ten BP sera. The color-code key at the top of the figure correlates with construct map in Fig 1. The presence of a color-coded bar indicates a positive reaction by immunoblotting. The relative sizes of the recombinant peptides are not drawn to scale.

vation of IgG reactivity at certain sites without corresponding IgE reactivity.

The similarity of the BP180 reactivity patterns exhibited by the IgG and IgE class autoantibodies suggests that these two isotypes might have arisen via clonal selection. In clonal selection the initial response of B cells to the antigen is typically production of IgM. Through specific signals that include cytokines and CD40 binding to the CD40 ligand (CD40L), isotype switching occurs, allowing DNA recombination of switch regions and the production of different class antibodies, including IgG, IgE, or IgA. Since this isotype switching does not affect the complementarity determining region (CDR), the specificity of these different classes of autoantibodies would likely be the same. But, somatic hypermutation of the CDR during isotype switching in some cases could lead to subtle differences in the binding affinity to the target antigen (Chaudhuri and Alt, 2004). Production of IgE requires the cytokines interleukin

Table II. Correlation of IgE reactivity with NC16A by immunoblot and basophil histamine release in response to NC16A

BP patient	IgE immunoblot reactivity with NC16A	Histamine release % above spontaneous release [(stimulated-spontaneous/spontaneous) × 100]
Sc	+	4054
Da	+	446
P1	+	4468
S1	—	0*
Ho	—	7
Ma	—	18
La	—	17
Ra	—	0*

*Samples for which the stimulated histamine release was less than spontaneous were reported as zero.

BP, bullous pemphigoid; Ig, immunoglobulin.

(IL)-4 and IL-13 as well as ligation of CD40 to CD40L on B cells (reviewed in Prussin and Metcalfe, 2003).

There is a large body of evidence supporting the hypothesis that IgG autoantibodies that react with sites on NC16A play a major role in the development of BP (Liu *et al*, 1993, 1995; Sitaru *et al*, 2002); however, it remains to be determined whether anti-BP180 IgE autoantibodies also play a role in the pathology of this disease. One possible scenario is that anti-BP180 IgE binds to the IgE receptor on the surface of mast cells and basophils. Subsequent binding of a BP180 fragment (e.g., the 120 kDa shed form of BP180) to the mast cell-bound IgE would result in activation and degranulation of the mast cells, setting off an inflammatory cascade. Animal model studies have demonstrated the key role played by various elements of the inflammatory response in BP (reviewed by Liu, 2004).

To begin to address this issue, we carried out the histamine release experiments. We observed an antigen-specific release of histamine from basophils of all BP patients whose sera were IgE immunoblot positive for recombinant NC16A. Conversely, basophils from BP patients who lacked circulating IgE autoantibodies against NC16A failed to release histamine in response to NC16A exposure. In other words, the IgE immunoreactivity of our patients' sera predicted their response in the histamine release assay. Furthermore, for those patients exhibiting a positive histamine release result, we can infer that anti-NC16A IgE autoantibodies were bound to the IgE surface receptors of their basophils (and presumably mast cells as well). These findings provide support for the hypothesis that anti-BP180 IgE autoantibodies are involved in the pathogenesis of BP, and that antigen-specific degranulation of mast cells is one plausible mechanism.

Another possible scenario for the role of IgE in BP involves the binding of IgE to BP180 on the surface of basal keratinocytes. One might speculate that anti-BP180 IgG levels are much higher than those of IgE, and therefore the IgG would "outcompete" for the available binding sites on BP180, diminishing the importance of IgE autoantibodies.

Our research group has recently initiated a study directed at the quantification and comparison of BP180-specific IgE and IgG levels in BP. In the one BP patient for which we have data, the concentrations of circulating BP180-specific IgE and IgG were very similar (unpublished data), although the relative avidities were not measured. Much more work is needed to determine the plausibility of this type of role for IgE in the pathogenesis of BP.

Of particular interest are the data regarding autoantibody-reactive sites located on the BP180 ectodomain, but outside of the NC16A region. Circulating IgE autoantibodies with such a reactivity profile were found in half of our BP patients (five of ten). Furthermore, in four of these cases no IgE reactivity to NC16A was detected. In contrast, only one of the ten patients exhibited IgG reactivity that was restricted to sites on BP180 outside of NC16A. It is noteworthy that in this last patient, IgE autoantibody reactivity was also restricted to non-NC16A sites on BP180, and this patient's basophils did not release histamine in response to NC16A. All other clinical parameters in this patient, including histology, indirect and direct immunofluorescence (IF) and disease course, were typical of BP. Immunoblotting against an extract of normal human skin revealed reactivity to full-length BP180 but not to BP230. These findings suggest that there may be pathogenic relevance to regions of BP180 other than NC16A in at least a subset of patients.

Although we did not study the BP230 antigen in detail, we found IgE reactivity against this protein in only one of the ten BP patients. In a previous study we found IgE reactivity against the 230 kDa antigen in five of 30 patients (Dimson *et al*, 2003). The patients that participated in these studies were early in their course and untreated at the time we mapped their autoantibodies and tested for histamine release. Although untested, it remains possible that the natural course of BP or particular treatments might affect the antigens targeted or the specific regions of the antigens recognized by these autoantibodies.

In summary, this study further characterizes the IgE response in BP. With the advent of new therapeutic agents that can block the activity of IgE, further studies on the pathogenic role of IgE in BP are warranted. Better understanding the role of IgE in BP may lead to a better understanding of the development of this disorder and may also suggest new therapeutic approaches.

Materials and Methods

Patient material Thirty patients with well-characterized BP were initially tested by ELISA and immunoblotting for IgE reactivity with BP180. All patients had clinical and histologic features characteristic of BP. All patients had direct IF demonstrating IgG and/or C3 at the basement membrane zone and 90% had initial positive indirect IF that showed IgG binding the roof of sodium chloride-split skin (Gammon *et al*, 1984). Ten untreated patients from whom adequate amounts of serum were available took part in more detailed immunologic studies. This study was approved by the Human Research Review Committee of the Medical College of Wisconsin Affiliated Hospitals (#230-01) and was performed in adherence to the Declaration of Helsinki Guidelines. Participants gave their written informed consent.

BP180 constructs A recombinant protein comprising the entire BP180 ectodomain (designated sec180) was generated using the pCEP4 eukaryotic expression system (Invitrogen, Carlsbad, California), as previously reported (Olague-Marchan *et al*, 2000). Briefly, the cDNA encoding the amino-terminal signal sequence and precursor peptide from human desmoglein-1 was inserted into the multiple cloning region of the pCEP4 vector. The cDNA for the entire BP180 ectodomain (nucleotides 1573–4596; numbering taken from GenBank #M91669; Giudice *et al*, 1992) was subsequently inserted downstream of the desmoglein-1 sequence to generate pCEP4-sec180. The desmoglein-1 sequence was included to ensure that the recombinant BP180 protein would be transported through the secretory pathway of the transfected cell. The portion of BP180 encoded by pCEP4-sec180 extends from Glu-490 to the C-terminus, Pro-1497 (*note*: amino acid numbering begins with Met-36 from GenBank M91669). The pCEP4-180ΔN16A construct was generated via a standard PCR-based mutagenesis protocol (Olague-Marchan *et al*, 2000) using pCEP4-sec180 as the template. This truncated construct encodes all but the NC16A region of the BP180 ectodomain (Gly-567 through Pro-1497).

DNA segments encoding the entire NC16A domain, or segments thereof, were obtained by PCR amplification using a previously cloned human BP180 cDNA as template (Giudice *et al*, 1992). The amplification products were subcloned into the pGEX-2T vector (Pharmacia, Piscataway, New Jersey) and these fusion proteins were purified by glutathione-agarose affinity chromatography, as described previously (Zillikens *et al*, 1997). The NC16A fusion proteins that were used in this study were NC16A1, NC16A2, NC16A2.5, NC16A3, and NC16A1–3. The relative positions of these BP180 domains are shown in Fig 3.

Immunoblotting of recombinant proteins and skin extracts An ELISA analysis was performed in order to ensure that there was no cross reactivity between the secondary antibodies used in this study to detect human IgE and IgG. Briefly, 50 ng of IgG, IgA, IgM, and IgE standards were adsorbed onto an ELISA plate. After a series of washes with phosphate-buffered saline (PBS) containing 0.05% Tween-20, each well was incubated for 1 h with one of the peroxidase-labeled monoclonal antibodies directed against the γ , α , ϵ , μ Ig chain (Zymed, South San Francisco, California) diluted 1:250 in PBS with 1% BSA and 0.05% Tween-20. Following another series of washes, 50 μ L of substrate solution (*o*-phenylenediamine in 0.1% H_2O_2) was added to each well. Ten minutes after substrate addition, the reaction was stopped by the addition of 50 μ L 2 N H_2SO_4 , and the optical density (OD)₄₉₂ was measured using a microplate reader (model 550, Biorad, Richmond, California). OD readings were not significantly above baseline except when the standard was tested with the anti-human antibody directed against that isotype.

The epitope mapping studies were carried out using a standard immunoblotting protocol, as previously described (Zillikens *et al*, 1997). Briefly, affinity purified recombinant proteins or human epidermal extracts were electrophoresed on sodium dodecyl sulfate polyacrylamide gels and electrotransferred to nitrocellulose. Sera of patients and controls were preadsorbed with a bacterial cell lysate containing recombinant glutathione-S-transferase (GST) to eliminate reactivity with the GST moiety of the fusion proteins. The blots were blocked with a carrier protein and incubated for 18 h with the diluted sera (for IgE and IgG detection, the sera were diluted 1:25 and 1:50, respectively). After washing and reblocking, the blots were incubated with horseradish peroxidase-conjugated anti-human IgG (γ chain specific) or IgE (ϵ chain specific). The bound antibodies were detected by the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, New Jersey).

Histamine release by BP patient basophils Histamine release from peripheral blood basophils in response to the NC16A domain of BP180 was measured using a commercially available compet-

itive immunoassay (Immunotech, Fullerton, California) as previously described (Botulinska *et al*, 1999; Dimson *et al*, 2003). Peripheral blood was available from eight untreated subjects and controls. Controls were 16 patients with other skin diseases including pemphigus, systemic lupus erythematosus, and psoriasis. The blood was exposed to the NC16A domain of BP180, 10 μ g per mL, and incubated for 30 min at 37°C. Aliquots of the samples were also treated with ionomycin (non-immunologic release), buffer only (spontaneous histamine release) or lysed in distilled water (total histamine content). The NC1 region of collagen VII (gift of Drs David Woodley and Mei Chen, University of Southern California) or GST alone served as additional controls. The histamine samples and controls were acylated and the histamine content was measured by competitive histamine immunoassay. After incubation, the wells were washed and bound tracer was detected with the chromogen para-nitrophenylphosphate. Histamine concentration in the experimental samples was determined by comparison with a standard curve utilizing standards supplied with the kit. The samples were corrected for spontaneous release. The threshold for elimination from further analysis was spontaneous release of more than 5% of the total histamine content.

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